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Preferential Binding of Yeast Rad4·Rad23 Complex to Damaged DNA*

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The yeast Rad4 and Rad23 proteins form a complex that is involved in nucleotide excision repair (NER). Their function in this process is not known yet, but genetic data suggest that they act in an early step in NER. We have purified an epitope-tagged Rad4·Rad23 (tRad4·Rad23) complex from yeast cells, using a clone overproducing Rad4 with a hemagglutinin-tag at its C terminus. tRad4·Rad23 complex purified by both conventional and immuno-affinity chromatography complements the *in vitro* repair defect of *rad4* and *rad23* mutant extracts, demonstrating that these proteins are functional in NER. Using electrophoretic mobility shift assays, we show preferential binding of the tRad4·Rad23 complex to damaged DNA *in vitro*. UV-irradiated, as well as *N*-acetoxy-2-(acetylamino)fluorene-treated DNA, is efficiently bound by the protein complex. These data suggest that Rad4·Rad23 interacts with DNA damage during NER and may play a role in recognition of the damage.

Nucleotide excision repair (NER)[1] is the main mechanism responsible for the error-free removal of many distinct types of DNA damage. This process is strongly conserved in eukaryotes ranging from yeast to man and involves several proteins. NER consists of the following basic steps: (i) damage recognition, (ii) DNA unwinding around the lesion, (iii) dual incision on either side of the lesion, and (iv) template-dependent DNA synthesis followed by ligation of the remaining nick (1). The factors involved in the NER reaction have been identified, and biochemical activities have been assigned to most of them.

A detailed model for NER has been proposed, but the exact mechanism of DNA damage recognition is still poorly understood. Identification of the factors implicated in damage recognition is a prerequisite to gain insight in this first step of NER. Although some yeast proteins that bind damaged DNA have been identified, notably the Rad14 and Rad7·Rad16 proteins (2, 3), other proteins may also be involved in DNA damage recognition. In *Saccharomyces cerevisiae*, the Rad4 and Rad23 proteins might have a function in this step. These proteins are associated (4–6), as are their human homologs XPC and hHR23B (7). The Rad4·Rad23 complex is essential in a reconstituted NER reaction using purified yeast proteins (4). Although biochemical experiments suggest a role for Rad4 and Rad23 in the assembly and disassembly of NER complexes, their exact function is unknown (6, 8, 9). Genetic studies have suggested a role for Rad4/XPC in damage recognition. In a *rad4*Δ strain, the genome overall is not repaired (10); however, repair of photoproducts in the transcribed strand of rDNA genes is still observed (11). Likewise, in human cells, XPC is not required for the repair of photolesions in RNA polymerase II-transcribed strands (12). Apparently, in yeast and in humans, transcription can lead to Rad4 or XPC-independent repair, respectively. In addition, *in vitro* assays using human NER proteins have shown that repair of certain types of lesions, such as thymine dimers in locally unwound DNA as well as specific cholesterol moieties, can take place in the absence of XPC (13–15). All these observations suggest that the requirement for Rad4 or XPC in NER depends on the structure of the lesion, implicating a function in damage recognition by these proteins (see also Ref. 16).

This prompted us to investigate a possible interaction of yeast Rad4 with DNA damage. We purified a tagged Rad4·Rad23 (tRad4·Rad23) complex and assayed its DNA damage binding properties using electrophoretic mobility shift experiments. We found a marked increase in DNA binding when tRad4·Rad23 complex was incubated with UV-irradiated or NA-AAF-treated DNA compared with untreated DNA. We therefore conclude that Rad4·Rad23 complex preferentially binds to damaged DNA and suggest that this complex may have a role in damage recognition.

EXPERIMENTAL PROCEDURES

Purification of tRad4·Rad23 Complex from Yeast—A clone containing full-length *RAD4* behind a *CUP1* promoter was obtained via homologous recombination after co-transformation of linearized pCS28, containing the N-terminal portion of Rad4, together with a fragment of pCS31, containing the C-terminal part of Rad4 fused to a HA-epitope (both plasmids kindly provided by Dr. K. Madura, University of Medicine and Dentistry of New Jersey), into a *rad4*Δ strain. Complementation of the UV-sensitivity of this strain was used to verify functional tRad4 production. Plasmids (pRad4HA) were isolated from this yeast strain and transformed to the protease-deficient yeast strain JEL1 (genotype: *MAT*α *leu2 trp1 ura3–52 prb1–1122 pep4–3* Δ*his3::PGAL10-GAL4*, a gift of Dr. J. C. Wang, Harvard University). JEL1 containing pRad4HA was grown in selective medium overnight, diluted in yeast extract-peptone-dextrose medium containing 0.1 mM $CuSO_4$ and grown overnight at 30 °C, harvested by centrifugation, washed, pelleted, and stored at −80 °C. Extract was prepared from 200 g of these pellets in E-buffer (200 mM Tris, pH 7.5, 0.39 M $(NH_4)_2SO_4$, 10 mM $MgSO_4$, 20% (v/v) glycerol, 1 mM EDTA, 1 mM DTT) containing 500 mM NaCl and protease inhibitors (17), using a bead beater (Biospec Products, Inc.) according to the instructions of the manufacturer. The extract was clarified by centrifugation at 100000 × *g* for 2 h, and protein was precipitated by addition of 301 mg/ml $(NH_4)_2SO_4$. The ammonium sulfate pellet was dissolved in buffer A (25 mM Tris, pH 7.5, 10% (v/v) glycerol, 5 mM $MgCl_2$, 0.5 mM EDTA, 10 mM β-mercaptoethanol, 300 mg/ml benzamidine) containing 100 mM NaCl and dialyzed overnight against buffer A containing 100 mM NaCl. The dialysate was fractionated by sequential chromatography on the following columns in buffer A with linear gradients of NaCl (the concentration of salt around which the peak of tRad4 eluted is indicated between brackets): P11 phosphocellulose (Whatman) [500 mM NaCl], Bio-Gel

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[1] The abbreviations used are: NER, nucleotide excision repair; XP, xeroderma pigmentosum; hHR23B, human homolog of Rad23 B; NA-AAF, *N*-acetoxy-2-(acetylamino)fluorene; HA, hemagglutinin; bp, base pair(s); kb, kilobase pairs; TFIIH, transcription factor IIH; DTT, dithiothreitol.

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HTP hydroxylapatite (in this case, a linear gradient of KPO_4 pH7.4 in buffer A containing 50 mM NaCl was used; tRad4 peak around 240 mM KPO_4), single-stranded DNA-cellulose (Sigma) [410 mM NaCl], Hi-Trap SP-Sepharose (1 ml) [500 mM NaCl], and finally either Resource Q (1 ml) or Mono Q (HR5/5) [350 mM NaCl], which both gave comparable results. Alternatively, 10 ml (7 mg protein) of the tRad4·Rad23 peak fractions from P11 was incubated overnight with 160 μg of 12CA5 monoclonal antibody (anti-HA) and 160 μl of protein G beads (Amersham Pharmacia Biotech) in the presence of 0.1% Nonidet P-40 under constant agitation. After extensive washing, tRad4·Rad23 was eluted in buffer A containing 100 mM NaCl and 0.01% Nonidet P-40, supplemented with 2 mg/ml synthetic HA peptide (sequence YPYDVPDYA) overnight with constant agitation. Proteins were stored in small portions at −80 °C. During the purification, the presence of tRad4 was followed by immunoblot analysis using the 12CA5 antibody. The identity of Rad23 in the final fraction was confirmed using a Rad23-specific antiserum kindly provided by Dr. K. Madura.

In Vitro NER Complementation—Activity of the tRad4·Rad23 complex was analyzed by *in vitro* NER assays. Yeast cell free extracts were prepared as described by He *et al.* (18). NER reactions were performed essentially as described (17). Approximately 10 ng of purified tRad4·Rad23 was added for complementation.

Construction of DNA Substrates—The DNA probe used in electrophoretic mobility shift analysis was a 200-bp polymerase chain reaction-amplified fragment from the yeast *URA3* gene (251–451 bp downstream of the transcription start site). After amplification, the fragment was purified from acrylamide gel and was 5′-terminal labeled using T4 polynucleotide kinase (Amersham Pharmacia Biotech) and [γ-^{32}P]ATP (ICN) in 25 μl of kinase buffer (70 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 5 mM DTT) for 1.5 h at 37 °C. DNA was irradiated with UV in 15-μl droplets of water at 0–40 kJ/m^2 at 254 nm. NA-AAF treatment of DNA was essentially performed as described (19). Briefly, 50 ng of DNA was incubated with indicated concentrations of NA-AAF in 10 μl reactions containing 2.5 mM citrate and 19% ethanol for 3.5 h at 37 °C in the dark. DNA was purified by phenol-chloroform extraction, precipitated twice with ethanol, and redissolved in water.

Linear DNA fragments containing a single AAF adduct were constructed using a protocol based on Refs. 15 and 20. The DNA substrate consists of six oligonucleotides comprising a 146-bp fragment, −92 to +54 bp relative to the transcription start site from the *Lytechinus variegatus* 5S rDNA gene (21). Each strand is derived from three oligonucleotides (from 5′ to 3′: top strand, 71, 19, and 56 bp; bottom strand, 48, 39, and 59 bp). The central 19-mer oligonucleotide (5′-CTTAACTTCGAACAACCAA) in the top strand was treated with NA-AAF as described (19) to yield near complete adduct formation at the single guanine residue. After purification via phenol-chloroform extraction and ethanol precipitation of the damaged or mock treated 19-mer, 200–400 pmol of each oligonucleotide was 5′-phosphorylated in 200 μl of kinase buffer (see above) by using 40 units of T4 polynucleotide kinase in the presence of 2 mM ATP for 2 h at 37 °C. The six phosphorylated oligonucleotides were mixed together, NaCl was added to 100 mM, and the sample was heated to 95 °C for 5 min and allowed to cool slowly to room temperature to anneal the oligonucleotides. The DNA was ethanol precipitated, redissolved, and ligated in 50 μl containing 50 mM Tris-HCl, pH 7.5, 10 mM NaCl, 10 mM $MgCl_2$, 5 mM DTT, 50 μg/ml bovine serum albumin, 1 mM ATP, and 10 units T4-ligase (Fermentas) for 16 h at 14 °C. DNA was extensively purified by elution of full-length fragments from two subsequent 10% acrylamide-urea gels to remove unligated oligonucleotides. Purified fragments consist of two 146-mer strands, one of which contains an AAF adduct at position 81 from the 5′ terminus. These strands were rehybridized, and double-stranded fragments were purified on a native 6% polyacrylamide gel. After elution, the DNA was precipitated twice with ethanol and redissolved in water. Typical yield of full-length double-stranded substrate containing a single AAF lesion was about 1 pmol. The presence and position of the AAF lesion was verified by incubation with UvrABC endonuclease (20) and by restriction analysis (a *Taq*I recognition site is lost because of the presence of the damage) (data not shown). Samples of approximately 100 fmol of AAF substrate and its undamaged counterpart were 5′-terminal labeled in 25 μl of kinase buffer (see above) using 10 units of polynucleotide kinase and 4 pmol of [γ-^{32}P]ATP (ICN; 7000 Ci/mmol) for 2 h at 37 °C. Unincorporated label was removed by a Sephadex G-50 spin column. DNA was ethanol precipitated and redissolved in water.

Electrophoretic Mobility Shift Analysis—1 ng (7.5 fmol of double-stranded fragment) of a damaged or undamaged 200-bp fragment was mixed with 10 ng of tRad4·Rad23 complex in a 15-μl reaction containing 30 mM Tris-HCl, pH 8.0, 50 mM NaCl, 1.5% glycerol, 0.5 mM $MgCl_2$, 1 mM DTT, 100 μg/ml bovine serum albumin, and 10–100 ng of poly(dI-dC)·poly(dI-dC) (Amersham Pharmacia Biotech) or plasmid DNA (pIC20R linearized with *Bam*HI) as carrier. Binding reactions on the 146-bp single lesion substrate were performed using 100 pg of DNA (1 fmol), 30 ng of tRad4·Rad23, and 10 ng of poly(dI-dC)·poly(dI-dC) carrier DNA. Addition of ATP was not required. Reaction mixtures were mixed on ice and incubated at 28 °C for 30 min. For supershift experiments, 2.4 μg of purified 12CA5 antibody was added, and the mixture was incubated for another 15 min at 28 °C. After incubation, 3 μl of loading buffer (100 mM Tris-HCl, 50% glycerol, 0.05% bromphenol blue) was added. Samples were loaded on 3.5% polyacrylamide (acrylamide:*N*,*N*′-methylenebisacrylamide, 37.5:1) gel and run in 25 mM Tris-Glycine, pH 8.5, 1 mM EDTA at 1 mA/cm for 30 min at 4 °C. Gels were dried and exposed to Fuji RX film.

RESULTS

Purification of the tRad4·Rad23 Complex—To characterize the Rad4·Rad23 complex, we purified these proteins from yeast cells overproducing Rad4 containing a HA epitope at its C terminus, here referred to as t(agged)Rad4. Full-length *RAD4* sequences cannot be propagated in *Escherichia coli* (22). Therefore, two partially overlapping clones bearing either the N-terminal or the C-terminal part of the *RAD4* gene (kind gifts from Dr. K. Madura) were co-transformed to yeast to obtain a full-length clone via homologous recombination. This clone complements the UV sensitivity of a *rad4*Δ strain, indicating that a functional protein is produced (data not shown). The gene is under the control of a *CUP1* promoter, which is inducible by Cu^{2+} ions.

We have purified tRad4 in two distinct ways: first by sequential chromatography on phosphocellulose, hydroxylapatite, single-stranded DNA-cellulose, SP-Sepharose, and Resource Q columns; second, we used a two-step immuno-affinity purification protocol in which phosphocellulose fractions were immunopurified using 12CA5 monoclonal antibodies (anti-HA) and elution with synthetic HA-peptide.

Both procedures yield a complex of two proteins, with some minor contaminants as shown by silver staining of SDS-polyacrylamide electrophoresed gels (Fig. 1*A*). The band corresponding to a protein of apparent molecular mass of around 120 kDa (4) was shown to be tRad4 by Western blotting using anti-HA monoclonal antibodies (data not shown). A protein of 57 kDa co-purifies with tRad4, and in agreement with previous reports (4, 6), this protein was shown to be Rad23 using anti-Rad23 antiserum and immunoblotting (not shown). Rad23 is not overproduced in these cells, but the endogenous level of Rad23 exceeds natural Rad4 levels (4). As immuno-affinity purification of tRad4·Rad23 proceeds via the HA-epitope of tRad4, Rad23 is co-purified via direct binding to tRad4, once more confirming a direct interaction between these proteins. To determine whether our purified tRad4·Rad23 preparations are still functional in NER, we attempted to rescue the defective repair activity of cell-free extracts of *rad4*, *rad23*, and *rad4rad23* deletion strains. *In vitro* NER was assayed by means of measuring DNA synthesis in AAF-damaged plasmids incubated with cell-free extracts (23, 24). The *in vitro* NER deficiency of cell free extracts from *rad4*, *rad23*, and *rad4rad23* disruption mutants (see also Refs. 5 and 18) is rescued when they are supplemented with tRad4·Rad23 (Fig. 1*B*), purified by either of the two approaches described above. This indicates that the complex is functional in NER.

DNA Damage Binding by tRad4·Rad23—Using the purified functional tRad4·Rad23 complex, we have characterized its DNA damage binding properties. We conducted electrophoretic mobility shift assays using a ^{32}P-labeled 200-bp linear DNA fragment that runs as a single band when no protein was added (not shown). DNA binding by tRad4·Rad23 protein is observed as the appearance of slower migrating forms of DNA. When the DNA is irradiated with UV, tRad4·Rad23-DNA complex formation is markedly increased (Fig. 2). Binding of tRad4·Rad23

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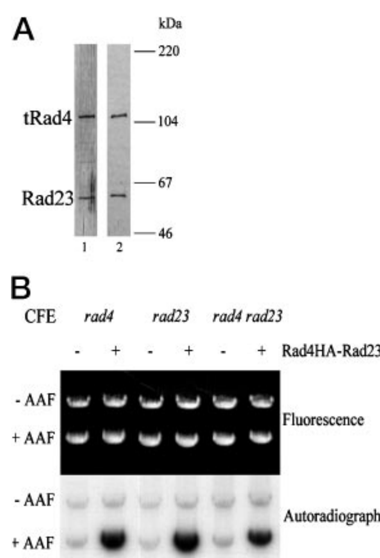

FIG. 1. **Purification of active tRad4·Rad23 complex.** *A*, purified tRad4·Rad23 complex was subjected to denaturing polyacrylamide gel electrophoresis (7.5% acrylamide) and visualized by silver staining. *Lane 1* contains a Resource Q peak fraction; *lane 2* shows tRad4·Rad23 isolated by immuno-affinity purification. Molecular mass in kDa is indicated on the *right*. *B*, tRad4·Rad23 complex is active in NER. 250 μg of indicated cell-free extracts (*CFE*) were incubated with 300 ng of each undamaged pNP81 (4.4 kb) and pUC18 containing AAF damage (2.7 kb) in the presence of [α-^{32}P]dCTP, without (−) or with (+) addition of 10 ng of purified tRad4·Rad23 as indicated. Reactions in *lanes 2* and *4* contain Resource Q peak fractions. To the reaction in *lane 6*, immuno-affinity purified tRad4·Rad23 was added. DNA was deproteinized, linearized, and electrophoresed in 1% agarose gels. *Top* shows an ethidium bromide stain of the gel (fluorescence); *bottom* shows autoradiograph of the dried gel, indicating the level of damage-dependent DNA synthesis.

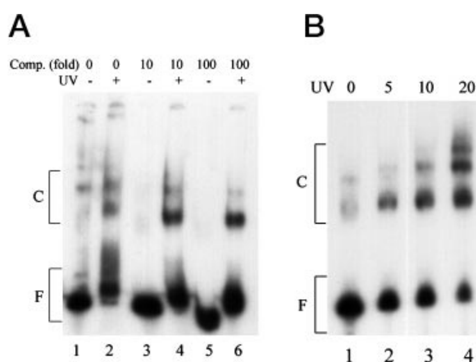

FIG. 2. **Preferential binding of tRad4·Rad23 to UV-irradiated DNA.** *A*, 1 ng of a 200-bp DNA fragment with (+) or without (−) prior treatment with UV light (40 kJ/m^2) was incubated with 10 ng of tRad4·Rad23. Excess unlabeled unirradiated competitor DNA (*comp.*) was included in the reactions as indicated. After subjecting the reaction mixtures to electrophoresis in a polyacrylamide gel, an autoradiograph was prepared to visualize the nucleoprotein complexes (labeled as *C*) and free DNA (labeled as *F*). *B*, binding of Rad4·Rad23 to UV-irradiated DNA depends on UV dose. tRad4·Rad23 was incubated with DNA irradiated with indicated UV doses (in kJ/m^2) in the presence of excess competitor.

complex is specific for damaged DNA because complexes persist in the presence of a large excess of competitor DNA, whereas binding to undamaged DNA is strongly decreased (Fig. 2*A*). Complex formation increases with increasing UV dose (Fig. 2*B*) and was observed using independent protein preparations. In addition, we conducted a similar experiment using a DNA-probe containing adducts induced by NA-AAF. Also using this substrate, we found preferential binding by tRad4·Rad23 to damaged DNA in a dose-dependent manner (Fig. 3*A*), showing that damage-induced binding of Rad4·Rad23

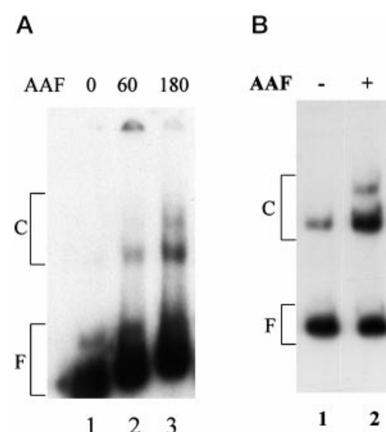

FIG. 3. **tRad4·Rad23 binding to NA-AAF-treated DNA.** *A*, dose-dependent binding of NA-AAF-treated DNA. 10 ng of tRad4·Rad23 is incubated with DNA treated with increasing doses of NA-AAF as indicated (in μM). Reaction mixtures contained excess competitor. After polyacrylamide electrophoresis of the reaction mixtures, the gel was dried and autoradiographed to visualize protein·DNA complexes (labeled as *C*) and the free unbound DNA (labeled as *F*). *B*, tRad4·Rad23 complex binds to DNA containing a single AAF lesion. 30 ng of tRad4·Rad23 was incubated with a 146-bp fragment with (+) or without (−) a single positioned AAF adduct in the presence of excess competitor.

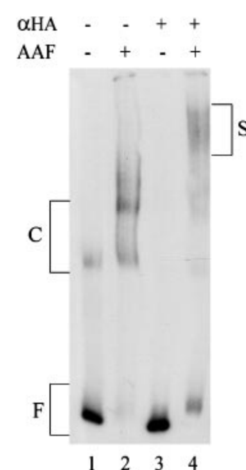

FIG. 4. **Protein·DNA complexes contain tRad4.** Supershift experiment showing binding of anti-HA antibodies to protein·DNA complexes. 10 ng of tRad4·Rad23 was incubated with either untreated (−) DNA or DNA treated with 180 μM of NA-AAF (+), in the absence (−) or the presence (+) of 12CA5 antibodies (indicated by *αHA*). Low mobility antibody·protein·DNA complex (labeled as *S*), protein·DNA complexes (labeled as *C*), and free DNA (labeled as *F*) were resolved by polyacrylamide gel electrophoresis and visualized by autoradiography

is not confined to DNA containing UV-induced lesions. To further validate the damaged DNA binding by tRad4·Rad23, we assayed complex formation on a more defined DNA substrate. To this purpose, we constructed a linear 146-bp double-stranded DNA fragment containing a single positioned AAF adduct. Electrophoretic mobility shift analysis using this single AAF-adduct substrate also showed a clear enhancement of complex formation compared with undamaged DNA (Fig. 3*B*), demonstrating that even a single DNA damage induces DNA binding by tRad4·Rad23. Therefore, preferential binding is not confined to DNA fragments containing multiple damaged sites.

In all experiments performed, at least two protein·DNA complexes were observed. Because these complexes are also observed using undamaged DNA or DNA containing a single lesion, the existence of more than one complex cannot be explained solely by the presence of multiple lesions per DNA fragment. To determine whether the protein·DNA complexes

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contained tRad4, we used 12CA5 monoclonal antibodies directed against the HA-epitope. Addition of this antibody to the binding reactions resulted in a supershift of the labeled fragment (Fig. 4), indicating that tRad4 is present in the observed protein·DNA complexes.

DISCUSSION

In this study, we report the preferential binding of the *Saccharomyces cerevisiae* Rad4·Rad23 complex to damaged DNA. We have purified the complex and assayed its binding characteristics to damaged DNA by mobility shift analysis.

Previous reports suggest a role for Rad4 in damage recognition. Rad4-independent repair *in vivo* exists in yeast (11). Likewise, repair in human cells can take place in the absence of XPC (12), the human homolog of *RAD4.* In addition, *in vitro* NER is observed in the absence of XPC for some DNA lesions (13–15). Apparently the lesion structure may circumvent the need for XPC in humans or Rad4 in yeast, suggesting a role for these proteins in recognition of DNA lesions. Our data provide direct evidence for this hypothesis, as we observe preferential binding of tRad4·Rad23 to damaged DNA. Two structurally distinct types of NER substrates (*i.e.* UV-induced photoproducts and AAF adducts) induce binding of tRad4·Rad23 to DNA.

Recently Sugasawa *et al*. (25) have shown by competition assays that human NER is initiated by XPC-hHR23B. Furthermore, they provide direct evidence that purified human XPC-hHR23B preferentially binds to DNA damage by means of an immuno-pull down assay and by DNase I footprinting. Our results of damaged DNA binding by yeast Rad4·Rad23 are consistent with and extend these data, using different methodology and proteins from a different eukaryotic origin. These observations again underscore the homology between human and yeast NER.

Rad23 is in complex with Rad4 but might also have functions independent of Rad4. Repair of rDNA that is independent from Rad4 (11), does depend on functional Rad23 (26). Also, biochemical experiments have shown that purified Rad23 interacts with Rad14 and TFIIH and promotes complex formation between these proteins (8). Furthermore, it has recently been shown that the NER complex can be linked to the 26 S proteasome via Rad23 (6). These observations point to a possible role for Rad23 in NER complex assembly and disassembly. Rad4·Rad23 may therefore act as an intermediate in damage binding and NER complex formation.

We observe a moderate enhancement of DNA binding by tRad4·Rad23 induced by DNA damage. Because a number of factors have now been implicated in damage recognition in yeast, it is possible that a coordinate action of different proteins, such as Rad14 (2), Rad7·Rad16 complex (3), and Rad4·Rad23 (this study) is needed to gain the specificity required at the genomic level. Interactions between Rad4 and Rad7 (5), as well as between Rad23 and Rad14 (8), have been reported, pointing to a link between these damage-recognizing proteins. Along this line, it has recently been suggested that human NER complex formation at the site of the damage only occurs when XPC-hHR23B, XPA, replication protein A (RPA), TFIIH, and XPG are present together, indicating a cooperative mode of binding to the damage (27).

On the basis of the evidence we present in this report, we suggest that Rad4·Rad23 acts early in the formation of the NER complex by directly binding to DNA damage, possibly in concert with other NER factors.

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