**Communication**

**Preferred Binding of Yeast Rad4-Rad23 Complex to Damaged DNA**

(Received for publication, July 30, 1998)

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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) 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.

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HTTP hydroxylation (in this case, a linear gradient of KPO4, pH 7.4 in buffer A containing 50 mM NaCl was used; tRad4 peak around 240 mM KPO4), 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 (HR55/5) (350 mM NaCl), which both gave comparable results. The sample was 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 (acylamide:N,N'-methylenebisacrylamide, 37.5:1) gel and run in 25 mM Tris-Glycine, pH 8.5, 1 mA. The gels were run at 4 °C for 6 h. Gels were dried and exposed to Fuji RX film.

RESULTS

DNA Damage Binding by tRad4—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(aged)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 rad4Δ 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 Cu2+ ions.

We have purified tRad4 in two distinct ways: first by sequential chromatography on phosphocellulose, hydroxyapatite, 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. 1A). 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, 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, 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, and in agreement with previous reports (4, 6), this protein was shown to be Rad23 using anti-Rad23 antiserum and immunoblotting (not shown).

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 rad4Δ, rad23Δ, and rad4Δrad23 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 rad4Δ, rad23Δ, and rad4Δrad23 disruption mutants (see also Refs. 5 and 18) is rescued when they are supplemented with tRad4ΔRad23 (Fig. 1B), purified by either of the two approaches described above. This indicates that the complex is functional in NER.

DNA Damage Binding by tRad4—Using the purified functional tRad4-Rad23 complex, we have characterized its DNA damage binding properties. We conducted electrophoretic mobility shift assays using a 32P-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, tRad4ΔRad23-DNA complex formation is markedly increased (Fig. 2). Binding of tRad4ΔRad23...
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. 2A). Complex formation increases with increasing UV dose (Fig. 2B) 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. 3A), showing that damage-induced binding of Rad4-Rad23 is 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. 3B), 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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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.

Rad4-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 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.

Acknowledgments—We are indebted to Dr. Kiran Madura for providing RAD4 clones and anti-Rad23 antiserum and to Dr. J.C. Wang for helpful suggestions, and members of our group for discussion. Drs. Kaoru Sugasawa and Jan Hoeijmakers are acknowledged for communicating results prior to publication.

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