Synergistic Interaction between Yeast Nucleotide Excision Repair Factors NEF2 and NEF4 in the Binding of Ultraviolet-damaged DNA*

(Received for publication, April 14, 1999, and in revised form, June 2, 1999)

Sami N. Guzder, Patrick Sung‡, Louise Prakash, and Satya Prakash§

From the Sealy Center for Molecular Science, University of Texas Medical Branch, Galveston, Texas 77555-1061 and the Department of Molecular Medicine/Institute of Biotechnology, University of Texas Health Science Center at San Antonio, San Antonio, Texas 78245

Saccharomyces cerevisiae RAD4, RAD7, RAD16, and RAD23 genes function in the nucleotide excision repair (NER) of ultraviolet light (UV)-damaged DNA. Previous biochemical studies have shown that the Rad4 and Rad23 proteins are associated in a stoichiometric complex named NEF2, and the Rad7 and Rad16 proteins form another stoichiometric complex named NEF4. While NEF2 is indispensable for the incision of UV-damaged DNA in the in vitro reconstituted system, NEF4 stimulates the incision reaction. Both NEF2 and NEF4 bind UV-damaged DNA, which raises the intriguing possibility that these two complexes cooperate to achieve the high degree of specificity for DNA damage demarcation required for nucleotide excision repair in vivo. Consistent with this hypothesis, we find that NEF2 and NEF4 bind in a synergistic fashion to UV-damaged DNA in a reaction that is dependent on ATP. We also purify the Rad7 protein and show that it binds DNA but has no preference for UV-damaged DNA. Rad7 physically interacts with NEF2, suggesting a role for Rad7 in linking NEF2 with NEF4.

In eukaryotes, nucleotide excision repair (NER) of ultraviolet (UV)-damaged DNA is a highly intricate process which requires a large number of proteins. A defect in NER in yeast results in an extreme sensitivity to UV light and a hypermutational phenotype, and in humans, defective NER is the underlying cause of the skin cancer-prone syndrome xeroderma pigmentosum, observations that underscore the importance of this repair system in neutralizing the cytotoxicity and genotoxicity of UV light (1).

During NER, the DNA lesion is first bound by damage recognition proteins, followed by the recruitment of additional NER factors and DNA unwinding by the two DNA helicases Rad3 and Rad25, present in TFIIH, to create a DNA "bubble." Dual incision of the damage-containing strand in the unwound DNA, on the 5'-side by the Rad1-Rad10 nuclease and on the 3'-side by Rad2, results in the release of the lesion in the form of an oligonucleotide ~30 nucleotides in length (for a discussion, see Ref. 2). A conserved set of proteins performs the same function in humans (3–5). Biochemical fractionation of Saccharomyces cerevisiae extract has revealed distinct subassemblies of the NER proteins, termed nucleotide excision repair factors or NEFs. Thus, Rad14 associates with the Rad1-Rad10 endonuclease to form NEF1 (6), Rad4 and Rad23 combine to form NEF2 (7), and Rad2 associates with the six subunit RNA polymerase II transcription factor TFIIH to form NEF3 (2). The combination of NEF1, NEF2, NEF3, and the heterotrimeric ssDNA binding factor replication protein A (RPA) is sufficient to mediate dual incision of UV-damaged DNA in an in vitro reconstituted system (7), indicating that the basal NER machinery consists of these factors and also lends credence to the suggestion that NER is mediated by the stepwise incorporation of the aforementioned NEFs and RPA at the damage site (2).

At the genomic level, another level of complexity exists in the repair of transcribed versus nontranscribed DNA. The repair of the transcribed strand requires the RAD26 gene, a homolog of the human CSB gene (8), whereas the repair of the nontranscribed strand requires the RAD7 and RAD16 genes (9, 10). Deletion of RAD7 or RAD16 causes an intermediate level of UV sensitivity, and the UV sensitivity of the rad7Δ rad16Δ double mutant is the same as that of the rad7Δ and rad16Δ single mutants. The Rad7 and Rad16 proteins exist together in a stable complex termed NEF4, and the purified Rad7-Rad16 heterodimer binds UV-damaged DNA in an ATP-dependent manner (11). NEF4 also has a DNA-dependent ATPase activity, and UV irradiation of DNA results in a marked inhibition of ATP hydrolysis (12). These observations have suggested a model in which NEF4 utilizes the free energy from ATP hydrolysis to translocate on DNA. Because of the attenuation of the ATPase activity, NEF4 stops translocating at the damage site and becomes stably bound to the damage. In this scenario, NEF4 would be the first protein complex to arrive at the damage site and would serve as the nucleation site for the subsequent assembly of the other repair factors (11, 12). Consistent with this hypothesis, the addition of NEF4 to the reconstituted NER system results in a marked stimulation of the proficiency of the incision reaction (11).

In addition to NEF4, the Rad4-Rad23 complex (NEF2) also recognizes UV damage (13, 14). However, by contrast to the damage binding activity of NEF4, which is strongly dependent upon ATP, NEF2 damage binding shows no such dependence on a nucleotide co-factor (13). To begin delineating the intricacy of the damage recognition step in NER, we examine here whether the combination of NEF2 and NEF4 is more adept at binding UV-damaged DNA than either protein complex alone. Our results indicate a synergistic enhancement of damage binding by NEF2 and NEF4. We also purify the Rad7 protein.
and show that it binds DNA but has no specificity for UV-damaged DNA. Furthermore, we provide evidence for the physical interaction of Rad7 with NEF2, which suggests a specific function for Rad7 as a bridging factor between NEF2 and NEF4.

MATERIALS AND METHODS

Antibodies—The anti-Rad7, anti-Rad4, anti-Rad16, and anti-Rad23 antibodies used in this study were all affinity purified from rabbit antiserum raised against portions of the respective proteins expressed in and purified from Escherichia coli as described (11, 13, 15).

Purification of Rad7 protein—The overexpression of the Rad7 gene in the E. coli strain JM101, using the plasmid pRAD7.7. After induction with 1 mM isopropyl-1-thio-b-D-galactopyranoside, cells were broken and the GST-Rad7 fusion protein, which was localized in the inclusion bodies, was solubilized in 1.5% Sarkosyl buffer containing 0.1 M ethanolamine and 100 mM EDTA (pH 8.0). The crude cell lysate was centrifuged at 100,000 × g for 30 min at 4 °C. The supernatant containing the GST-Rad7 protein was dialyzed extensively against phosphate-buffered saline at 4 °C. The GST-Rad7 protein in this fraction was present at approximately 1.5 mg/ml.

Expression of GST and GST-Rad7 Fusion Protein—The GST-Rad7 fusion protein was overexpressed in the E. coli strain JMB101, using the plasmid pRAD7.4. During the purification procedure, the Rad4 and Rad23 proteins were monitored by Western blotting after staining with ethidium bromide.

RESULTS

Synergistic Action of NEF2 and NEF4 in Damage Binding—Binding of NEF4 to UV-damaged DNA is strongly stimulated by ATP or ATPγS (11, 12), whereas NEF2 shows no dependence on a nucleotide in its binding to UV-irradiated DNA (13). The two NEFs do not bind simultaneously to DNA, but rather one NEF binds first, and then the second NEF binds only if the first NEF is present (13). The affinity of NEF4 for DNA is strongly increased by ATP or ATPγS (13). To determine whether NEF2 and NEF4 interact synergistically in the binding of UV-damaged DNA, we used concentrations of NEF2 and NEF4 at which little binding of the UV-irradiated DNA fragment is seen with either factor alone and then investigated the effect of mixing the two NEFs. Concomitant with the increased binding of the UV-damaged DNA fragment containing the entire Rad7 coding sequence was cloned in plasmid pGEX-3X to yield the GST-Rad7 expression plasmid pRAD7.4.

Damage Binding by NEF2 and NEF4

DNA Mobility Shift Assay—A 130-bp double-stranded DNA fragment containing 5′-end with 32P and incubated with the indicated amounts of Rad7, NEF2, and NEF4 in 10 μl of reaction buffer (30 mM potassium HEPES, pH 7.5, 0.3 mM KCl, 5 mM MgCl2, 1 mM dithiothreitol, 100 μg/ml bovine serum albumin) and containing either 50 ng or the indicated amounts of HindIII linearized pHX174 double-stranded DNA as competitor DNA. After 10 min at 30 °C, samples were mixed with 2 μl of gel loading buffer (0.1 × Tris-HCl, pH 7.0, 50% glycerol, and 0.05% Orange G) and electrophoresed in a 4.5% polyacrylamide gel in TAE buffer (40 mM Tris acetate, pH 7.4, 1 mM EDTA) at 4 °C. The gels were dried and subjected to autoradiography using Kodak MR films, and autoradiograms were quantitated by a Bio-Rad GS670 densitometer.

NER Reaction—The NER reaction was carried out as described (7). Briefly, 75 ng of NEF2, 50 ng of RPA, 65 ng of NEF1, 65 ng of NEF3, and 60 ng of NEF4 or Rad7, where indicated, were incubated in reaction buffer (45 mM potassium HEPES, pH 7.9, 8 mM MgCl2, 120 μg/ml bovine serum albumin, 1.5 mM dithiothreitol, 2 mM ATP, an ATP regenerating system consisting of 30 mM creatine phosphate and 200 ng of creatine phosphokinase) with 150 ng of M13mp18 DNA that had been irradiated with UV light emitting at 254 nm to a total dose of 30 J/m². Reaction mixtures were incubated at 30 °C for varying times, deproteinized by treatment with SDS and proteinase K (7), and analyzed in a 0.8% agarose gel in TAE buffer (20 mM Tris acetate, pH 7.4, 0.5 mM EDTA). The DNA species were visualized by staining with ethidium bromide.
ternary complex consisting of the damaged DNA, NEF2, and NEF4. Under the conditions used, undamaged DNA is not bound by the combination of NEF2 and NEF4 (Fig. 1C).

**ATP Is Required for Ternary Complex Formation**—Because NEF4 binds and hydrolyzes ATP, and the presence of either ATP or ATPγS is critical for optimal damage-specific DNA binding by NEF4 (11, 12), it was of considerable interest to examine whether the synergistic cooperation between NEF2 and NEF4 in damage-specific DNA binding is dependent on the presence of ATP. To do this, NEF2 alone, NEF4 alone, and the combination of NEF2 and NEF4 were incubated with undamaged DNA or with UV-irradiated DNA in the absence or the presence of ATP. As shown in Fig. 1, combining NEF2 and NEF4 results in a synergistic increase in the binding of UV-damaged DNA. Significantly, the formation of the NEF2-NEF4-UV-damaged DNA ternary complex was greatly diminished when ATP was omitted from the reaction mixture (Fig. 1C, compare lanes 16 and 17), indicating a requirement for ATP in the formation of the ternary nucleoprotein complex. As reported previously (11, 12) and reiterated here, ATPγS was effective in promoting DNA damage binding by NEF4 (Fig. 1C, compare lanes 14 and 15 with lane 13). ATPγS, however, did not stimulate the formation of the ternary complex of damaged DNA with NEF2 and NEF4 (Fig. 1C, compare lanes 16 and 18). This result stands in contrast to the damage binding by NEF4 alone, and it suggests that either only ATP is effective in inducing a conformation conducive for the interaction of NEF4 with NEF2 or that ATP hydrolysis is in fact indispensable for mediating the interaction of NEF2 with NEF4. No significant binding of the undamaged DNA fragment was seen even with the combination of NEF2 and NEF4, regardless of whether a nucleotide was present or not (Fig. 1C).

**Purification of Rad7 Protein**—The Rad7 protein does not contain any known conserved sequence motifs that might suggest a biochemical function for the protein. To gain insight into the biochemical role of Rad7, we overexpressed the protein in yeast cells (Fig. 2A) and purified it to near homogeneity (Fig. 2B). For the overexpression of Rad7 protein, the RAD7 gene tagged with a 6-histidine sequence at the amino terminus was placed under the control of the GAL-PGK promoter to yield plasmid pR7.8. For purifying Rad7 protein, extract from 300 g of the protease-deficient yeast strain LY2 harboring plasmid pR7.8 was subjected to ammonium sulfate precipitation, followed by chromatographic fractionation in columns of Q-Sepharose, SP-Sepharose, hydroxyapatite, nickel NTA-agarose, and finally in Mono-S, as described under “Materials and Methods.” The elution of Rad7 from the various columns was monitored by immunoblotting until the hydroxyapatite step, where Rad7 could be identified by Coomassie Blue staining of polyacrylamide gels in which the column fractions had been run. When 1 μg of Rad7 protein eluting from the last step of purification in Mono-S column (Fraction VI Rad7 protein) was analyzed by denaturing polyacrylamide gel electrophoresis and Coomassie Blue staining, only the Rad7 protein was seen, indicating a high degree of purity of the preparation (Fig. 2B). The purified Rad7 preparation was devoid of Rad16 protein, as no Rad16 was detected when 1 μg of the Rad7 preparation was subjected to immunoblot analysis with anti-Rad16 antibodies.
Rad7 Is a DNA Binding Protein—Because NEF4 has a UV damage-specific DNA binding activity, we examined whether Rad7 protein alone can bind UV-damaged DNA. The results in Fig. 3A show that Rad7 has a DNA binding activity as indicated by the formation of a nucleoprotein complex with the 32P-labeled DNA fragment. However, unlike the Rad7-Rad16 complex, Rad7 protein by itself does not discriminate between the UV-damaged and undamaged DNA, as essentially the same level of nucleoprotein complex was formed with both DNA species (Fig. 3A). The same results were obtained when ATP was omitted from the reaction buffer (data not shown). Furthermore, the binding of Rad7 to either the UV-irradiated or unirradiated DNA substrate can be effectively competed away by adding low amounts of a nonlabeled DNA to the preformed nucleoprotein complex (Fig. 3A), suggesting that the Rad7-DNA nucleoprotein complex is not very stable. In sharp contrast, NEF4 binds specifically and stably to UV-irradiated DNA even in the presence of a 100-fold excess of competitor DNA (Fig. 3B, lane 7). These results indicate that Rad7 is a DNA binding protein but it is devoid of the ability to recognize UV lesions.

Rad7 Interacts with NEF2—In further characterizing the function of Rad7 protein, we considered the possibility that Rad7 may act as a physical link between NEF2 and NEF4. Because even with overexpression, only a small amount of Rad7 protein could be purified from yeast cells, to facilitate our function of Rad7 protein, we considered the possibility that Rad7 may act as a physical link between NEF2 and NEF4.

Rad7 Does Not Enhance the Incision Reaction—Rad7 by itself shows no damage-specific binding (Fig. 3A), and it did not enhance the binding of UV-damaged DNA by NEF2 (data not shown). In a previous study (11), we showed that the addition of NEF4 to an in vitro NER reaction reconstituted with highly purified NEF1, NEF2, NEF3, and RPA markedly increased the efficiency of the damage-specific incision reaction. Because Rad7 interacts with NEF2, we examined whether the repair efficiency could be enhanced by the inclusion of the Rad7 protein in the reconstituted NER reaction. The addition of Rad7 to the basic incision reaction, however, did not result in significant stimulation or inhibition of the UV damage-specific incision (Fig. 5A, compare lanes 6 and 7, respectively, and Fig. 5B), whereas, as reported previously, the inclusion of NEF4 enhances the incision reaction by 4–5-fold (Fig. 5A, compare lanes 8 and 9 with lanes 6 and 7, respectively,
and Fig. 5B). These biochemical results and the results from previous genetic studies are consistent with the suggestion that both Rad7 and Rad16 proteins are required for the functional integrity of NEF4 in UV damage recognition and in NER enhancement.

DISCUSSION

Genetic studies have indicated that the RAD4 gene is indispensable for the NER of UV-damaged DNA in vivo, whereas the RAD23 gene affects the efficiency of the repair process (15). Previous studies from our laboratory have shown that the Rad4 and Rad23 proteins are tightly associated in a stoichiometric complex called NEF2. Consistent with the genetic results, NEF2 was shown to be indispensable for the dual incision of UV-damaged DNA in the in vitro NER reconstitution studies (7). Because the Rad4 and Rad23 proteins contain no identifiable sequence motifs that would predict an enzymatic activity, it had remained unclear as to whether NEF2 was important for NER by providing a distinct biochemical function or whether it primarily served as a scaffold in the assembly of the NER machinery. In fact, our previous studies have demonstrated that Rad23 interacts with Rad14 protein and with TFIIH (16).

More recently, we showed that NEF2 possesses a DNA binding ability that is highly specific for UV-damaged DNA, but Rad23 by itself shows no DNA binding ability (13), indicating that either Rad4 is the damage recognition subunit of NEF2, or Rad4 and Rad23 proteins are both required for the DNA damage binding activity of NEF2. Similar to the DNA damage recognition ability of NEF2, the equivalent human NER complex consisting of the XPC and HHR23B proteins, also binds preferentially to UV-damaged DNA (18, 19). Taken together, the biochemical studies have provided tangible evidence that during NER, NEF2 serves as a damage recognition factor, and in addition, plays an important role in the assembly of the incision machinery at the damage site via specific protein-protein interactions (16).

The RAD7 and RAD16 genes affect the efficiency of NER in vivo with a particular requirement of these genes in the repair of nontranscribed DNA (9, 10). The addition of Rad7-Rad16 complex, NEF4, to the reconstituted NER reaction results in a marked stimulation of the incision of UV-damaged DNA (11). Consistent with the presence in Rad16 of Walker-type sequence motifs suggestive of the ability to bind and hydrolyze a nucleoside triphosphate, our biochemical studies have indicated that NEF4 contains an intrinsic ATPase activity. The NEF4 ATPase activity requires DNA, and double-stranded DNA is more effective than single-stranded DNA for its activation (12). Like NEF2, NEF4 also binds specifically to UV-damaged DNA. However, unlike NEF2, which shows no dependence on ATP for damage binding, NEF4 requires ATP for maximal binding to UV-damaged DNA. ATPγS is also effective, but to a lesser degree than ATP, in promoting DNA damage binding by NEF4 (12), which has led to the deduction that ATP binding alone is sufficient to induce a conformational change in NEF4 conducive for damage binding. The ATPase activity of NEF4 is markedly inhibited by the presence of UV damage in the DNA (12), suggesting that the free energy derived from ATP hydrolysis may fuel the translocation of NEF4 on DNA and that the movement of NEF4 is arrested upon encountering a DNA lesion. The lesion-bound NEF4 may then serve as the nucleation site for the loading of the remaining NER factors, including NEF1, NEF2, NEF3, and RPA. In addition to NEF2, Rad14, which is a component of NEF1, and RPA also bind UV-damaged DNA specifically (17, 20, 21); however, the sequence by which these damage recognition factors assemble at the site of lesion-bound NEF4 remains to be determined.

To begin addressing the hypothesis that multiple damage
recognition factors including NEF2 and NEF4 function together to achieve a high degree of specificity in damage demarcation during NER, we have now examined NEF2 and NEF4 for their possible cooperation in DNA damage recognition. Our results indicate that combining NEF2 and NEF4 results in synergistic binding of UV-irradiated DNA. We also find that the synergistic action of NEF2 and NEF4 in damage recognition is an ATP-dependent process, suggesting a role of ATP binding/hydrolysis in this reaction.

To explore the role of Rad7 in NER, we overexpressed and purified this protein to near homogeneity. Our results show that Rad7 binds DNA but has no specificity for UV-damaged DNA template. Consistent with this result, addition of NEF4, that Rad7 binds DNA but has no specificity for UV-damaged DNA, purified this protein to near homogeneity. Our results show that the synergistic action of NEF2 and NEF4 in damage recognition is an ATP-dependent process, suggesting a role of ATP binding/hydrolysis in this reaction.

Thus, it appears that NEF4 plays a multifunctional role in NER, viz. in the very initial step of damage recognition in cooperation with NEF2 as shown in our studies here and also in the post-incision phase (23), perhaps in the turnover of the incision protein machinery, and in the recruitment of factors to perform the DNA repair synthesis reaction.

REFERENCES

1. Friedberg, E. C., Walker, G. C., and Siede, W. (1995) DNA Repair and Mutagenesis, ASM Press, Washington, D. C.
2. Habraken, Y., Sung, P., Prakash, S., and Prakash, L. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 10718–10722
3. Mu, D., Park, C.-H., Matsunaga, T., Hsu, D. S., Reardon, J. T., and Sancar, A. (1995) J. Biol. Chem. 270, 2415–2418
4. Mu, D., Hsu, D. S., and Sancar, A. (1996) J. Biol. Chem. 271, 8285–8294
5. Mu, D., Wakahashi, M., Hsu, D. S., and Sancar, A. (1997) J. Biol. Chem. 272, 28971–28979
6. Guzder, S. N., Sung, P., Prakash, L., and Prakash, S. (1996) J. Biol. Chem. 271, 8893–8910
7. Guzder, S. N., Habraken, Y., Sung, P., Prakash, L., and Prakash, S. (1995) J. Biol. Chem. 270, 12973–12976
8. van Gool, A. J., Verhage, R., Swagemakers, S. M. A., van de Putte, P., Brouwer, J., Treestra, C., Bootsma, D., and Hoeijmakers, J. H. J. (1994) EMBO J. 13, 5361–5366
9. Verhage, R., Zeeman, A.-M., de Groot, N., Gleig, F., Gang, D. D., van de Putte, P., and Brouwer, J. (1994) Mol. Cell. Biol. 14, 6135–6142
10. Mueller, J. P., and Smerdon, M. J. (1995) Nucleic Acids Res. 23, 3457–3464
11. Guzder, S. N., Sung, P., Prakash, L., and Prakash, S. (1997) J. Biol. Chem. 272, 21665–21668
12. Guzder, S. N., Sung, P., Prakash, L., and Prakash, S. (1998) J. Biol. Chem. 273, 6292–6296
13. Guzder, S. N., Sung, P., Prakash, L., and Prakash, S. (1998) J. Biol. Chem. 273, 31541–31546
14. Jansen, L. E. T., Verhage, R. A., and Brouwer, J. (1998) J. Biol. Chem. 273, 33111–33114
15. Watkins, J. F., Sung, P., Prakash, L., and Prakash, S. (1993) Mol. Cell. Biol. 13, 7757–7765
16. Guzder, S. N., Bailly, V., Sung, P., Prakash, L., and Prakash, S. (1995) J. Biol. Chem. 270, 8383–8388
17. Guzder, S. N., Sung, P., Prakash, L., and Prakash, S. (1995) Proc. Natl. Acad. Sci. U. S. A. 90, 5433–5437
18. Reardon, J. T., Mu, D., and Sancar, A. (1996) J. Biol. Chem. 271, 19451–19456
19. Sugasawa, K., Ng, J. M. Y., Masutani, C., Iwai, S., van der Spek, P. J., Eker, A. P. M., Hanaoka, F., Bootsma, D., and Hoeijmakers, J. H. J. (1998) Mol. Cell 2, 223–232
20. Clugston, C. K., McLaughlin, K., Kenny, M. K., and Brown, R. (1992) Cancer Res. 52, 6375–6379
21. Burns, J. L., Guzder, S. N., Sung, P., Prakash, S., and Prakash, L. (1996) J. Biol. Chem. 271, 11607–11610
22. Prakash, S., Sung, P., and Prakash, L. (1999) Annu. Rev. Genet. 27, 33–70
23. Reed, S. H., You, Z., and Friedberg, E. C. (1998) J. Biol. Chem. 273, 29481–29486