The Non-catalytic Function of XPG Protein during Dual Incision in Human Nucleotide Excision Repair

Mitsuo Wakasugi, Joyce T. Reardon, and Aziz Sancar
From the Department of Biochemistry and Biophysics, University of North Carolina School of Medicine, Chapel Hill, North Carolina 27599-7260

XPG is a member of the FEN-1 structure-specific endonuclease family. It has 3'-junction cutting activity on bubble substrates and makes the 3'-incision in the human dual incision (excision nuclease) repair system. To investigate the precise role of XPG in nucleotide excision repair, we mutagenized two amino acid residues thought to be involved in DNA binding and catalysis, overproduced the mutant proteins using a baculovirus/insect cell system, and purified and characterized the mutant proteins. The mutation D77A had a modest effect on junction cutting and excision activity and gave rise to uncoupled 5'-incision by mammalian cell-free extracts. The D812A mutation completely abolished the junction cutting and 3'-incision activities of XPG, but the excision nuclease reconstituted with XPG (D812A) carried out normal 5'-incision at the 23rd–24th phosphodiester bonds 5' to a (6–4) photoproduct without producing any 3'-incision. It is concluded that Asp-812 is an active site residue of XPG and that in addition to making the 3'-incision, the physical presence of XPG in the protein-DNA complex is required non-catalytically for subsequent 5'-incision by XPF-ERCC1.

Xeroderma pigmentosum (XP)1 is an autosomal recessive disease caused by defective excision repair (1, 2). XP patients are hypersensitive to sunlight and develop actinic keratoses and skin cancer at a high incidence and frequency. In addition, XP individuals with severe repair defects develop sensorineural and motor neurological symptoms. It has been shown that mutations in seven genes (XP through XPG) cause XP. Recently, the proteins encoded by these genes (with the exception of XPE) have been purified, and it has been established that six repair factors are necessary and sufficient for carrying out dual incisions that remove damage such as cyclobutane pyrimidine dimers and (6–4) photoproducts from DNA. These six repair factors that include these gene products are necessary for subsequent 5'-incision by XPF-ERCC1.

1 The abbreviations used are: XP, xeroderma pigmentosum; CFE, cell-free extract; CHO, Chinese hamster ovary; CS, Cockayne's syndrome; nt, nucleotide(s).

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1 To whom correspondence should be addressed: Dept. of Biochemistry and Biophysics, CB 7260, University of North Carolina, Chapel Hill, NC 27599-7260. Tel.: 919-962-0115; Fax: 919-966-2952.

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substrates (12) were used to test the endonuclease activities associated with XPG. The reaction mixture (7.5 μl) contained 2.5 fmol of DNA in 25 mM Hepes-KOH, pH 7.9, 25 mM KCl, 3 mM MgCl₂, 1 mM dithiothreitol, and 6.5% glycerol. The reaction was at 37 °C for 90 min, and the reaction products were separated on 10% denaturing polyacrylamide gels. Quantitation was done using a PhosphorImager (Molecular Dynamics).

**Repair Assay**—A 136-base pair duplex containing a (6–4) photoproduct (T6–4T) at nt 68–69 of one strand and 32P label at the 5th phosphodiester bond 5′ to the photolesion was used as substrate (16, 17). Complementation assays with cell-free extracts were performed as described previously (18). Reconstitution of the excision nuclease with wild-type or mutant XPG was done using highly purified repair factors and under conditions described elsewhere (3, 4); all of the repair factors described previously (18). Reconstitution of the excision nuclease with wild-type or mutant XPG proteins was incubated with 16 ng of wild-type or mutant XPG protein as indicated. The reaction products were separated on a 10% polyacrylamide sequencing gel. The positions of DNA size markers (51X174 DNA digested with HinFII and radiolabeled by phosphorylation with γ-32P[ATP] are shown to the left of the autoradiogram. Below the autoradiogram the schematic drawing illustrates the bubble-30 substrate, and the XPG incision sites are indicated by arrows.

**RESULTS**

**XPG Active Site Mutants**—Analyses of eucaryotic nucleases belonging to the FEN-1/XPG family and the exonuclease domains of procaryotic DNA polymerases led to the identification of eight amino acid residues that are conserved in the entire set (14). Based on this sequence conservation as well as on analysis of the exonuclease domains of the polymerases, it was predicted that these eight residues may be essential for substrate binding or catalysis, and an elegant study with human FEN-1 endonuclease revealed that the Asp-34, Asp-86, and Asp-181 residues of this 380-amino acid-long polypeptide contribute to the active site of the enzyme (14). We wished to know if the corresponding residues in XPG are involved in forming the active site of the protein. Toward this goal we made alanine substitutions at positions Asp-77 and Asp-812 of the XPG protein, corresponding to Asp-86 and Asp-181 of FEN-1, two of the three residues most critical for FEN-1 catalytic activity. Fig. 1 shows the wild-type and mutant proteins analyzed by SDS-polyacrylamide gel electrophoresis. All three proteins are overproduced to comparable levels and behave similarly on chromatographic resins. Hence, it is reasonable to assume that the mutant proteins folded properly and that any change in activity would reflect the function of those specifically altered amino acids.

**Nuclease Activities of Mutant XPG Proteins**—Since XPG is a 3′-junction cutting nuclease (11, 12), the D77A and D812A proteins were tested for junction cutting activity using a substrate with a 30-nt bubble. Neither the wild-type nor the mutant XPG proteins have endonuclease activity when double stranded DNA is the substrate (Fig. 2, lanes 1–4). D77A retains about 75% of the wild-type activity while D812A is totally defective in junction cutting with the 30-nt bubble substrate (Fig. 2, lanes 5–8). These results suggest that Asp-77 is not essential for catalysis but are uninformative with regard to the essentiality of Asp-812 since lack of activity could be due to misfolding of the protein, failure to bind to DNA, or absence of catalytic activity. Although these issues can be addressed by a variety of means, we reasoned that the most direct test would be to assay the mutant proteins for DNA repair activity.

**Excision Nuclease Complementing Activity with Mutant XPG Proteins**—The effect of mutations at the presumptive active site resides on excision nuclease activity was tested by a complementation assay. Mutant proteins were added to XP-G cell-free extracts prepared from the Chinese hamster ovary (CHO) cell line UV135 or the human lymphoblastoid line AG08802. Fig. 3 shows the results obtained with XPG-D77A. As is apparent, this mutant protein complements the excision activity of the XP-G cell-free extract to wild-type level as indicated by the presence of excision products 24–28 nt in length. Thus, it appears that the D77A mutation has near-normal functions with regard both to interactions with other general repair factors and 3′ incision activity as evidenced by complementation of excision nuclease activity in XP-G but not XP-B extracts (Fig. 3B). In addition, we observe 91–92-nt-long fragments corresponding to incisions at the 23rd and 24th phosphodiester bonds 5′ to the (6–4) photoproduct (17). In a small fraction of the excision complex mutant XPG assembles with the other repair factors and apparently fails to make the 3′ incision but enables XPF-ERCC1 to make the 5′ incision, thus generating the DNA fragments that migrate at 91–92 nt (4, 12).

**Fig. 1.** XPG proteins used in this study. The purified proteins were separated by electrophoresis through an 8% SDS-polyacrylamide gel and were visualized by silver staining. The positions of molecular mass size markers are indicated on the left side.

**Fig. 2.** Junction cutting activity of wild-type and mutant XPG proteins. 5′-End labeled double-stranded (DS) or bubble-30 (B30) substrates were incubated with 16 ng of wild-type or mutant XPG protein as indicated. The reaction products were separated on a 10% polyacrylamide sequencing gel. The positions of DNA size markers (51X174 DNA digested with HinFII and radiolabeled by phosphorylation with γ-32P[ATP] are shown to the left of the autoradiogram. Below the autoradiogram the schematic drawing illustrates the bubble-30 substrate, and the XPG incision sites are indicated by arrows.
FIG. 3. Complementation of XP-G mutant CFE with XPG-D77A mutant protein. A, excision with CFE (50 μg) from HeLa and CHO-UV135 cells supplemented with 30 ng of wild-type or mutant XPG as indicated. B, excision with CFE (50 μg) from CHO-UV135 (XP-G) or CHO-UV24 (XP-B) supplemented with XPG-D77A as indicated or cross-complementation with UV135 + UV24 CFEs. The reaction products were separated on 8% denaturing polyacrylamide gels. On the left, the positions of DNA size markers are shown. In the middle are schematic drawings indicating the incision patterns giving rise to the bands. Open arrows indicate a band generated by a nonspecific 3′→5′ exonuclease (particularly potent in rodent CFE) that digests DNA up to the vicinity of the lesion, (6–4) photoproduct in this assay. The excision products (24–28 nt in length) are indicated by smaller arrows.

FIG. 4. Complementing activity of XPG-D812A mutant. A, effect on CFE from CHO-UV135 (XP-G) mutant. B, complementation of CFE from CHO (UV135) and human (AG08802) XP-G cell lines. C, effect on CFEs from CHO-XPG and -XPB (UV24) mutants. Excision assays were conducted with the indicated cell-free extracts in the absence (–) or presence of wild-type or mutant XPG proteins (30 ng) where indicated. The solid arrow at 91–92 nt indicates uncoupled 5′ incision, and the arrows at 24–28 nt indicate excision products. In the last lane of panel C, CFEs from XP-G and XP-B complementation groups were mixed. The open arrows indicate the band generated by a nonspecific exonuclease that is particularly potent in rodent cell lines and is blocked by the lesion.

compare lanes 2 and 4). These results suggest that while the Asp-77 residue may be involved in 3′ incision, the D77A mutation does not have a significant effect on this catalytic activity of XPG.

In contrast, the D812A mutation completely abolished the excision-complementing activity of the XPG protein (Fig. 4A, lane 5; Fig. 4B, lanes 3 and 6). Addition of mutant protein to the CHO-UV135 (XP-G) extract gave rise to 5′-uncoupled incision products (91–92 mers) equal in intensity to the level of excision (coupled incisions, 24–28 mers) achieved with addition of the wild-type protein (Fig. 4A, lane 5 versus lane 4; Fig. 4B, lane 3 versus lane 2). Qualitatively similar results were obtained with the extract prepared from the human XP-G cell line AG08802 (Fig. 4B, lane 6 versus 5), however, both the excision signal obtained with wild-type XPG (lane 5) and the band arising from the 5′-uncoupled incision (lane 6) were weak due to poor quality of CFE from this human lymphoblastoid cell line. The high level of uncoupled 5′-incision events is unique to complementation experiments with XP-G mutant CFE and D812A mutant protein as no such uncoupled incision is observed when XPG-D812A is added to a CFE from an XP-B mutant (compare Fig. 4C, lanes 2 and 4). These data show that D812A is in the nuclease active site of XPG. Of greatest importance, our data demonstrate that XPG (in addition to making the 3′ incision) is necessary for assembly of a preincision complex to enable XPF-ERCC1 to make the 5′ incision. Furthermore, these data show that it is the physical presence of XPG and not the 3′ incision that is required for the XPF-ERCC1 complex to make the 5′ incision.

Uncoupled 5′ Incision in Reconstituted System—The data presented above are consistent with XPG being involved in the formation of a preincision complex that recruits XPF-ERCC1 to the 5′ incision site. However, experiments with CFE do not exclude the requirement for other proteins for priming the preincision complex for XPF-ERCC1 action. Hence, we decided to carry out reconstitution experiments with the active site mutant XPG protein. Fig. 5 shows that the excision nuclease reconstituted with wild-type XPG makes dual nicks almost exclusively (lane 1, oligonucleotides in the 24–28 nt range); in contrast, dual incisions do not occur when the enzyme system is reconstituted with XPG-D812A (lane 2), but a 5′ incision (dependent on XPF-ERCC1; compare lane 3 with lane 2) equal in intensity to the excision seen with wild-type protein is observed. The data are in agreement with that obtained with CFE and support the conclusion that XPG plays at least two roles in excision repair: making the 3′ incision and forming the proper protein-protein interactions to enable XPF-ERCC1 to make the 5′ incision.

Dominant-negative Effect of D812A Mutation—When normal XPG protein was added to HeLa cell-free extract and incubated with substrate DNA, there was no effect on excision activity.2 However, when XPG-D812A was added to the HeLa CFE/substrate mixture, we observed a decrease in the level of excision (dual incision) activity. Concomitant with this decrease in excision was the appearance of and XPG concentration-dependent increase in 5′-uncoupled incision (Fig. 6). Apparently, the mutant protein effectively competes with the wild-type protein in HeLa cell-free extract and replaces the normal XPG protein in a fraction of the preincision complexes with the net effect of reduced dual incisions (excision) and increased uncoupled 5′ incisions by XPF-ERCC1.

**DISCUSSION**

Nucleotide excision repair in humans is rather complex with 13 polypeptides in six repair factors required for the dual

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2 T. Matsunaga and A. Sancar, unpublished observation.
incision event preceding the resynthesis step (3, 4, 15, 19). Significant progress has been made toward defining the roles of the individual polypeptides in damage recognition and formation of the preincision complex (20, 21). Work with model substrates (9–12) and with anti-XPG antibodies (13) implicated XPG in $3' \rightarrow 5'$ incision. Addition of anti-XPG antibodies to HeLa CFE and the reconstituted excision repair system inhibited excision and perhaps gave rise to uncoupled $5' \rightarrow 3'$ incision (13). Furthermore, in the reconstituted excision nuclease system it was found that omission of XPF-ERCC1 from the reaction resulted in uncoupled $3' \rightarrow 5'$ incision, that omission of XPG generated no incisions, and that in the complete reaction, $3'$ incision preceded the $5'$ incision (4). In addition, it was shown that XPG is an essential constituent of the pre-$3'$-incision nucleoprotein complex (4). In these reconstitution experiments it was not possible to unambiguously distinguish between two scenarios: 1) $3'$ incision by XPG is a prerequisite for $5'$ incision by XPF-ERCC1 or 2) that the presence of XPG in the preincision complex enables XPF-ERCC1 to make the $5'$ incision.

In the E. coli (A)BC excinuclease system, not only the presence of UvrB but also the $3'$ incision by this subunit (perhaps aided by UvrC (22, 23)) is absolutely required for the $5'$ incision by the UvrC subunit (24). Here we demonstrate that the human excinuclease has a different mechanism. The presence of XPG in the preincision complex (but not the $3'$ nick by this subunit) is a prerequisite for $5'$ incision. In fact, because of the absolute requirement for XPG in the dual incision complex for the $5'$ incision to occur it is conceivable that XPG is directly involved in both incisions. Although the facts that the active site mutant D812A can promote $5'$ incision and that the XPF-ERCC1 complex has $5'$ junction cutting activity (12) strongly suggest that XPG plays an indirect role in $5'$ incision, a direct role in catalysis of $5'$ incision by XPG cannot be formally eliminated.

Since xeroderma pigmentosum is well characterized as an autosomal recessive disease (1, 2), it was of special interest to find that under our conditions the XPG-D812A mutation acted as a dominant-negative mutation with regard to damage removal. A similar dominant-negative effect was observed in this in vitro system with a special XPA mutant protein as well (25). Furthermore, it has been found that overexpression of an ATPase active site mutant of XPD in a wild-type cell also results in dominant-negative phenotype as evidenced by increased sensitivity to UV irradiation. $^3$ These data raise the possibility that certain mutations in some of the XP genes would have a co-dominant effect in heterozygotes, assuming that the wild-type and mutant protein are expressed to the same levels. It is possible that careful screening of the XP kindreds would reveal partial repair defects in heterozygotes in a subset of XP patients.

Finally, the isolation of an XPG active site mutant described in this study makes it now possible to isolate preincision complexes containing only those polypeptides necessary for making dual incisions upon addition of XPF-ERCC1. Such a complex should be useful in identifying the polypeptides present during the dual incision events and the structure of the DNA prior to the dual incision reaction.

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$^3$ S. Kadkhodayan and L. H. Thompson, personal communication.
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