Conserved Residues of Human XPG Protein Important for Nuclease Activity and Function in Nucleotide Excision Repair*

(Received for publication, September 21, 1998, and in revised form, December 7, 1998)

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The human XPG endonuclease cuts on the 3’ side of a DNA lesion during nucleotide excision repair. Mutations in XPG can lead to the disorders xeroderma pigmentosum (XP) and Cockayne syndrome. XPG shares sequence similarities in two regions with a family of structure-specific nucleases and exonucleases. To begin defining its catalytic mechanism, we changed highly conserved residues and determined the effects on the endonuclease activity of isolated XPG, its function in open complex formation and dual incision reconstituted with purified proteins, and its ability to restore cellular resistance to UV light. The substitution A792V present in two XP complementation group G (XP-G) individuals reduced but did not abolish endonuclease activity, explaining their mild clinical phenotype. Isolated XPG proteins with Asp-77 or Glu-791 substitutions did not cleave DNA. In the reconstituted repair system, alanine substitutions at these positions permitted open complex formation but were inactive for 3’ cleavage, whereas D77E and E791D proteins retained considerable activity. The function of each mutant protein in the reconstituted system was mirrored by its ability to restore UV resistance to XP-G cell lines. Hydrodynamic measurements indicated that XPG exists as a monomer in high salt conditions, but immunoprecipitation of intact and truncated XPG proteins showed that XPG polypeptides can interact with each other, suggesting dimerization as an element of XPG function. The mutation results define critical residues in the catalytic center of XPG and strongly suggest that key features of the strand cleavage mechanism and active site structure are shared by members of the nuclease family.

The XPG protein is a DNA endonuclease with remarkable structure-specific properties, cleaving near the junctions between duplex and single-stranded DNA with a defined polarity.

* This work was supported by postdoctoral fellowships from the Swiss National Science Foundation (to D. G.) and from l’Association pour la Recherche sur le Cancer (to P. L.), by the Human Frontiers of Science Program (to E. E. and R. D. W.), by Grants 31-36481.92 and 31-52777.97 from the Swiss National Science Foundation (to G. S. C.), and by the Imperial Cancer Research Fund. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: N region, N-terminal region; I region, internal region; NER, nucleotide excision repair; CS, Cockayne syndrome; XP, xeroderma pigmentosum; XP-G, XP complementation group G; RPA, replication protein A; TFIIH, transcription factor IIH; BSA, bovine serum albumin.

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TABLE I
Oligonucleotides used for site-directed mutagenesis

| Mutation   | Oligonucleotide containing altered codon
|------------|---------------------------------------------|
| D77A       | 5'-CTATTTTGGTGTTGCGGGATGCTCCA-3'           |
| D77E       | 5'-CTATTTTGGTGTTGCGGGATGCTCCA-3'           |
| E791A      | 5'-CTATATTGAGAAGCGCGATGGCGGC-3'            |
| E791D      | 5'-CTATATTGAGAAGCGCGATGGCGGC-3'            |
| C794A      | 5'-GCAGAGGCCCAGGCGGATGGCGGC-3'             |
| C794S      | 5'-GCAGAGGCCCAGGCGGATGGCGGC-3'             |

* The altered nucleotides are underlined.

postinfection, the medium was replaced with a transfection mix comprising 3 ml of minimum Eagle’s medium, 5 μg of plasmid DNA (two portions of 2.5 μg for co-expression), and 15 μl of TransfectACE (29). After 3 h at 33 °C, an additional 7 ml of minimum Eagle’s medium supplemented with 5% fetal calf serum was added, and incubation was continued at 33 °C for 12 h. Cells were harvested 24 h later, extracts by phenol-chloroform extraction and resuspended in one pellet volume of 50 ml Tris-HCl (pH 8.0), 300 mM NaCl, 0.1% Nonidet P-40 (Buffer A) complemented with 10% glycerol, 1 mM EDTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, and 1 μg/ml pepstatin. The suspension was incubated on ice for 30 min, and proteins were removed from the cytosolic extract by centrifugation at 14,000 × g for 20 min. For metabolic labeling, HeLa cells were infected-transfected as described above. 20 h later, cells were washed and incubated for 1 h with minimum Eagle’s medium lacking methionine and cysteine before incubation for 4 h with 100 μCi/ml EasyTag Express Protein labeling Mix (NEN Life Science Products) containing [35S]methionine and [35S]cysteine. Cells were lysed in 25 ml HEPES-KOH (pH 6.8), 10% glycerol, 2 mM MgCl2, and 1 mM dithiothreitol (Buffer B) containing 0.15 M KCl, 0.4% Nonidet P-40, 0.1 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, and 1 μg/ml pepstatin. Extracts were cleared as above at 14,000 × g for 20 min.

**Immunoblotting, Immunopurification, and Immunoprecipitation—**

Immunoblotting was performed on cell extracts (10–50 μg of protein), using 4%–12% SDS-PAGE and blots were probed with rabbit serum against XPG (31, 32). After washing, the blots were incubated with horseradish peroxidase-conjugated secondary antibody (New England Biolabs, Beverly, MA) and peroxidase activity was measured on a model bubble substrate. Protein bands were visualized using DAB as a substrate. The immunoblotting was repeated for all samples and the same antibodies. Where indicated, extracts were probed with anti-XPG antibodies prepared with recombinant XPG protein (see below).

**Expression of Mutated XPG Proteins—**

54-mm-diameter Petri dishes containing monolayers of HeLa cells or transiently transfected XPCS1RO fibroblasts were infected with 5 plaque-forming units/cell of vTF7–3, a recombinant vaccinia virus expressing T7 RNA polymerase (28). At 1 h postinfection, the medium was replaced with a transfection mix comprising 3 ml of minimum Eagle’s medium, 5 μg of plasmid DNA (two portions of 2.5 μg for co-expression), and 15 μl of TransfectACE (29). After 3 h at 33 °C, an additional 7 ml of minimum Eagle’s medium supplemented with 5% fetal calf serum was added, and incubation was continued at 33 °C for 12 h. Cells were harvested 24 h later, extracts by phenol-chloroform extraction and resuspended in one pellet volume of 50 ml Tris-HCl (pH 8.0), 300 mM NaCl, 0.1% Nonidet P-40 (Buffer A) complemented with 10% glycerol, 1 mM EDTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, and 1 μg/ml pepstatin. The suspension was incubated on ice for 30 min, and proteins were removed from the cytosolic extract by centrifugation at 14,000 × g for 20 min.
BSA, or purified DNA incision proteins (per reaction, 1.44 pmol of XPA, 0.16 pmol of XPC-hHR23B, 1.84 pmol of RPA, 6.0 μl of TFIIF heparin-Sepharose fraction IV, 0.24 pmol of ERCCI-XPF, 1.4 pmol of XPG, and 0.05 pmol of vaccinia virus-overexpressed wild-type or mutant XPG) in repair buffer containing 20 μM each dATP, dCTP, dGTP, and dTTP, 4 mM NaCl, 1 mM DTT, 0.1 mM EDTA, 15.4% glycerol, and 10 μg of BSA. Repair proteins were preincubated for 10 min at 30 °C. DNA was added, and after a further 15 min of incubation at 30 °C, KMnO₄ (120 mM stock) was added to a final concentration of 6 mM. After 1 min, the oxidation was stopped by adding β-mercaptoethanol to 1×. Purification of the DNA and analysis on a 6% sequencing gel was performed as described (10). A secondary structure prediction (39) of the XPG sequence was manually aligned in this region by mainstreaming using the 8H7 monoclonal antibody. For determination of the catalytic center (38), we were able to test their intrinsic activity on all side chains by considering all possible side chain conformer interactions.

RESULTS

Description and Expression of Mutated XPG Proteins—XP125LO and XP124LO are two sibling XP-G individuals with a very mild XP clinical presentation (41). Their paternal XPG allele contains a premature stop codon at position 960, whereas their maternal allele codes for valine instead of alanine at position 792 (8, 10). Immunoblots of cell extracts from these patients have revealed the presence of full-length XPG carrying the A792V substitution but not the truncated XPG protein, suggesting that the latter or its message is unstable (10). Ala-792 is located in a conserved amino acid sequence core within the I region and is surrounded by glutamate and cysteine residues that have been found in the active site of various DNA nuclease (Ref. 26 and Fig. 1). To test the potential functional significance of these residues, site-directed mutagenesis was used to convert Glu-791 to either Ala or Asp, and Cys-794 to either Ala or Ser. Similarly, the highly conserved Asp-77 in the N region was changed to either Ala or Glu. The mutations were confirmed by sequencing, and appropriate sequenced restriction fragments were subcloned into an otherwise wild-type XPG cDNA background to ensure the absence of any additional undesired mutations. The cDNAs were preceded by the 5′ leader for the human U1 snRNA-associated A protein (27) to improve translational efficiency and were cloned in a plasmid vector behind a promoter for T7 RNA polymerase.

The corresponding XPG proteins were overexpressed in HeLa cells using a vaccinia virus-based expression system (28). Cells were infected with a recombinant vaccinia virus expressing T7 RNA polymerase and then were transfected with the various mutated cDNAs. After overnight incubation, cytosolic extracts were prepared and were examined by immunoblotting to ensure that the wild-type and mutated XPG proteins were equally expressed. All mutated XPG proteins were recognized by the anti-XPG mouse monoclonal antibody 8H7 (14) and migrated on gels at the same position as wild-type XPG (Fig. 2A). All recombinant proteins were strongly overexpressed compared with the endogenous HeLa XPG (Fig. 2A, compare the right lanes with lane CSB).

A Pull-down Endonuclease Assay—Previous studies showed that purified XPG is capable of cleaving a DNA substrate containing a centrally unpaired bubble of 30 nucleotides flanked by two duplex regions of 30 base pairs, specifically at the 3′ end of the unpaired region (6, 14). Relatively pure XPG preparations are needed for this type of assay because other nuclease activities in cruder fractions degrade the bubble substrate. To circumvent this problem, we made use of the 8H7 monoclonal antibody to first immunopurify the overexpressed XPG proteins on protein A-Sepharose beads; the immobilized proteins were then mixed with the bubble substrate that had been 5′-labeled on one strand. This antibody does not inhibit NER reactions in vitro (14) or the structure-specific cleavage of the bubble substrate by purified XPG in solution (data not shown). Similarly, binding of wild-type XPG to the antibody-protein A-Sepharose complex did not affect its endonuclease activity or change its substrate specificity: XPG still cut close to the 3′ junction between single-strand and double-stranded DNA, generating a labeled fragment of ~61 nucleotides (Fig. 2B, lanes 4–6). The activity of the overexpressed wild-type XPG protein was very much higher than any endogenous HeLa XPG background (Fig. 2B, compare lanes 4–6 with lanes 1–3).

The Alanine and Acidic Amino Acid Substitutions Abolish XPG Intrinsic Endonuclease Activity—Because all mutated XPG proteins were immunoprecipitated by the monoclonal antibody with equal efficiency, we were able to test their intrinsic...
endonuclease activity using this pull-down assay. Conversion of the highly conserved Asp-77 and Glu-791 residues to Ala abolished endonuclease activity (Fig. 2B, lanes 7–9 and 13–15). Changing each of these residues to the other acidic amino acid (namely D77E and E791D) also abolished activity in this assay (Fig. 2B, lanes 10–12 and 16–18). In contrast, amino acid changes at Cys-794 only partially reduced endonuclease activity, with C794S being slightly less active than C794A (Fig. 2B, lanes 22–24 and 25–27). The natural A792V substitution present in XP125LO and XP124LO cells also appeared to abolish intrinsic endonuclease activity (Fig. 2B, lanes 19–21). Because wild-type XPG can utilize either Mg\(^{2+}\) or Mn\(^{2+}\) as cofactor, but not Co\(^{2+}\) (30), we assayed the mutated proteins with Mn\(^{2+}\) as the divalent cation. This stimulated endonuclease activity of the overexpressed wild-type XPG protein (Fig. 2C, lanes 1–4). Proteins carrying the Asp-77 or Glu-791 substitutions again showed no detectable activity in this assay (data not shown), but interestingly, weak but measurable activity was found for the A792V protein in the presence of Mn\(^{2+}\) (Fig. 2C, lanes 5 and 6).

The Acidic Amino Acid Substitutions, but Not the Alanine Substitutions, Permit Dual Incision Activity in Reconstituted NER in Vitro—To test the activity of the various mutant XPG proteins in NER, we used a dual incision assay with DNA containing a single cisplatin adduct, purified repair proteins (XPA, XPC-hHR23B, RPA, ERCC1-XPF, and TFIIH), and recombinant XPG produced in the vaccinia virus system. Pilot studies showed that although production of XPG mutant proteins in HeLa cells gave the highest yields, production in XPG-defective XPCS1RO fibroblasts was preferable as it eliminated any residual interference from endogenous XPG activity.

The amount of vaccinia virus-expressed wild-type XPG in cytosolic extracts was determined by comparison to known amounts of purified XPG by immunoblotting. To determine the appropriate amounts of protein to use in repair reactions, the wild-type vaccinia-produced XPG was titrated in the reconstituted repair system (data not shown). Based on this titration, we compared the activity of 1.5 ng of vaccinia-produced wild-type and mutant XPG proteins.

Oligonucleotides excised during repair were detected by endlabelling as described (32). Vaccinia-expressed wild-type XPG (Fig. 3, lane 2) showed the same pattern of excision products as found with baculovirus-expressed XPG (lane 11) or HeLa whole cell extract (lane 12). No incision products were observed when XPG was omitted (lane 1) or when an extract containing overexpressed CSB protein was used (lane 3). The mutant XPG proteins differed in their repair activity. The nonconservative D77A and E791A changes completely inactivated repair function (Fig. 3, lanes 4 and 6), but the acidic substitution mutants D77E and E791D retained considerable activity in the reconstituted system. In this particular experiment, they exhibited 37 and 97% of normal (lanes 5 and 7), but in other experiments, both mutants were close to 100% of normal. The A792V mutant XPG gave a very weak repair signal (~8% of normal), which could be increased by using more mutant protein (data not shown). The C794A and C794S XPG proteins were intermedi-
ate, displaying 63–73% of normal repair activity in this experiment (Fig. 3, lanes 9 and 10).

Activity of Mutant XPG Proteins in Open Intermediate Formation—The mutant XPG proteins were tested for their ability to sustain formation of a key preincision open intermediate in NER. Open intermediates with single-stranded character were detected by their sensitivity to a 1-min pulse of KMnO$_4$ following a 15-min incubation of damaged DNA with repair proteins, as described (14).

Because of the distortion caused by the cisplatin adduct, there is an intrinsic permanganate sensitivity of the T residues at positions $-5$, $-4$, $-2$, and 0, and at G(+1) as revealed by the BSA-only sample (Fig. 4A, lane 14). NER-related open intermediates produced during the 1-min period of KMnO$_4$ modification are detectable by intensification of sensitivity at these positions, and the appearance of new diagnostic bands at positions $-7$, $-9$, and $-10$ can take place in the absence of XPG protein (Fig. 4, lanes 3 and 6). This XPG-independent opening is probably equivalent to the preincision complex that has been designated PIC2 (42).

When XPG protein with nuclease activity was included in reaction mixtures, 3' incisions were formed and accumulated throughout the 15-min incubation period (Fig. 4A, lane 4). To ensure the assignment of these bands, reactions were done without permanganate treatment so that incision products could be visualized separately. The 3' incision bands were absent in a reaction mixture containing HeLa cell extract instead of purified incision proteins (Fig. 4B, lane 1), because the extract can carry out DNA synthesis and ligation after incision.
The results show that vaccinia-expressed wild-type XPG (Fig. 4B, lanes 5 and 6) and the mutants D77E (lane 9), E791D (lane 11), A792V (lanes 12 and 13), C794A (lane 14), and C794S (lane 15) are all capable of carrying out 3' incision during this 15-min period, in the context of the rest of the repair complex. Two mutants, D77A and E791A, gave no detectable 3' incision (Fig. 4B, lanes 8 and 10). However, bands indicating opening were even more intense, indicating accumulation of open intermediates (Fig. 4A, lanes 7 and 9). The band at T(+5) was particularly intense, and further bands at T(+7) and T(+8) were
detected. These are the most 3’T residues before the sites of the major 3’ incisions (Fig. 4C). These bands were not detectable in the absence of KMnO₄ treatment (Fig. 4B), showing that they represent oxidation-sensitive T residues with single-stranded character.

Because DNA is labeled at the 3’ end in these experiments, it is also possible to detect “uncoupled” 5’ incisions that occur in the absence of 3’ incision. These are normally rare events (31). With most XPG mutant proteins, uncoupled 5’ incisions occurred at similarly low levels. However, 5’ incisions readily accumulated in reaction mixtures with the D77A and E791A XPG proteins (Fig. 4B, lanes 8 and 10). Indeed, the intensity of these uncoupled 5’ incisions was similar to the intensity of the 3’ incisions seen with wild-type XPG protein. This indicates that the NER reaction proceeds normally with the alanine substituted XPG proteins, except that the 3’ incision does not occur. The D77A and E791A proteins therefore are well folded and assemble normally into the repair complex. Uncoupled 5’ incisions were barely if at all detectable with the A792V protein (Fig. 4B, lane 12), even with a 4-fold excess (lane 13), suggesting that this mutant XPG protein does not remain stably bound in the repair complex.

The Acidic Amino Acid Substitutions, but Not the Alanine Substitutions, Permit Complementation of XP-G Cell Lines in Vivo—To test the in vivo relevance of these results, we cloned the various mutated XPG cDNAs into plasmid EBO-pLPP, an Epstein-Barr virus-based episomal vector (33), and transfected them into two lymphoblastoid cell lines derived from XP-G patients XP3BR and XP125LO (23, 24). Following hygromycin selection, stable transfectants were irradiated with 254-nm light, and their survival was assayed 48 h later with Alamar blue, a redox indicator that both fluoresces and changes color in response to chemical reduction of the medium resulting from cell growth (35). As revealed by immunoblotting, all mutated forms of XPG were expressed in the transfectants, and in levels comparable to that of XPG in a wild-type lymphoblastoid line (data not shown).

In both XP-G cell lines, transfectants containing the EBO-pLPP vector alone were highly sensitive to UV, whereas the presence of wild-type XPG restored UV resistance to normal levels (Ref. 26 and Fig. 5A). UV resistance was not regained by transfectants expressing either the D77A or E791A forms of XPG but was partially restored by the C794A and C794S substitutions (Fig. 5, A and B). These results were corroborated by an alternative assay for cell viability, the uptake of [3H]thymidine during a short pulse 2 days after UV irradiation (36). This assay measures the proportion of cells that enter S-phase and is a more direct and sensitive assay of the effect of DNA damage on viable cell number than the redox indicator. XP125LO cells expressing the EBO-pLPP vector alone or XPG with the D77A or E791A substitutions were comparably UV-sensitive by this assay. UV resistance was again partially restored in the C794A and C794S transfectants, with the C794A protein being slightly more active than XPG carrying the C794S substitution (Fig. 5, C and D). In contrast to their inactivity on the bubble substrate (Fig. 2), the D77E and E791D XPG proteins were able to fully restore UV resistance to both XP-G cell lines in both assays (Fig. 5, A–D). Identical results were obtained (data not shown) when the mutant XPG cDNAs were transfected into lymphoblastoid line AG08802 from XP-G/CS patient XP20BE (43). These in vivo results thus corroborate the conclusion that the acidic substitutions permit XPG endonuclease activity when in the presence of the other NER proteins.

The Alanine Substitutions Render Transfected Raji Cells UV-sensitive—The accumulation of uncoupled 5’ incisions found with the D77A and E791A proteins (Fig. 4) raised the possibility that these alanine substitutions may be able to sequester the NER machinery into inactive complexes to generate a dominant negative phenotype in wild-type cells. To test this possibility, cDNAs for the various XPG proteins were cloned behind the strong SRo promoter in the EBS-PL episomal vector (34) and then were transfected into Raji cells derived from a Burkitt lymphoma. These cells contain wild-type XP genes but carry mutations in both alleles of the p53 gene (44) that are expected to make them more resistant to UV cytotoxicity and to exhibit less UV-induced apoptosis than cells expressing wild-type p53 (45, 46). Untransfected Raji cells were indeed very UV-resistant (Fig. 5E). This resistance was not impaired by transfection of the EBS-PL vector alone or by overexpression of XPG proteins having partial or complete complementation activity in the XP-G cell lines (Fig. 5, E and F, see A792V, wt, D77E, and E791D). However, overexpression of the D77A and E791A XPG proteins made the Raji cells significantly more UV-sensitive (Fig. 5F). These results are thus consistent with the in vitro studies and suggest that these alanine substituted proteins are capable of binding both to the DNA substrate and to other components of the NER preincision complex.

The A792V Substitution Permits Weak Complementation in Vivo—The A792V protein is the only stably expressed form of XPG in XP124LO and XP125LO lymphoblasts (10). This mutant protein is of particular interest because it is unable to complement these cells by one fluorescence assay (8), yet the Alamar blue assay suggests that it is able to restore UV resistance to lymphoblasts from patient XP3BR (47). To investigate this further, we examined transfectants of both cell lines with the [3H]thymidine uptake assay. This more sensitive assay revealed that the XP3BR cells are significantly more UV-sensitive than the XP125LO cells (Fig. 5G). As expected, and consistent with the earlier fluorescence assay (8), expression of the A792V protein in XP125LO cells did not alter their UV sensitivity. However, expression of this mutant protein in XP3BR cells increased their UV resistance up to the XP125LO level (Fig. 5G). We conclude that the A792V XPG protein has very low but detectable nuclease activity in cells that permits removal of some UV damage.

The Monomeric versus Multimeric State of Human XPG Protein—The 5’ cut in human NER is made by a structure-specific nuclease formed by a heterodimer of the XPF and ERCC1 proteins (16, 17). Although XPG is able to make a structure-specific 3’ cut without additional proteins (6, 14), and Fig. 2), it is conceivable that it does so as a multimer. It is also noteworthy that some related nucleases, such as FEN-1/DNase IV, load onto a single-stranded DNA end (48), whereas XPG requires an open structure within a contiguous stretch of DNA (14). For these reasons, we used two experimental approaches to investigate whether XPG could multimerize.

In the first approach, we measured the Stokes radius and the sedimentation coefficient of purified XPG recombinant protein produced in the baculovirus system. Gel filtration chromatography on a fast protein liquid chromatography Superose 6 column revealed that in 1 M KCl, XPG protein and structure-specific endonuclease activity eluted as a single peak (Fig. 6A) with an estimated Stokes radius of 70.3 Å. For S value determination, pure XPG was loaded onto a 10–50% glycerol gradient in either 0.4 or 1 M KCl, and after centrifugation, XPG-containing fractions were revealed by immunoblotting. The protein sedimented at 5.1 S in both salt concentrations (Fig. 6, B and C). Overexpressed XPG in a crude vaccinia-infected HeLa extract migrated in a gradient with 0.4 M KCl to exactly the same fractions as the pure protein (Fig. 6C). Combining the two measurements made in identical buffer conditions (1 M KCl), the native molecular mass of the protein was
calculated to be 146 kDa by the equation of Siegel and Monty (49), assuming a partial specific volume of 0.725 cm$^3$/g. This figure is close to the predicted monomeric molecular mass of 133 kDa. Thus, under these conditions, the bulk of XPG appears to exist most stably as a monomer.

In the second approach, we asked whether interactions between XPG polypeptides could be detected by immunoprecipitation. For this approach, we engineered a truncated XPG protein lacking the N-terminal 136 amino acids. This deletion eliminates the conserved N region, including the first 109 amino acids that were used previously as the immunogen to raise an anti-XPG rabbit polyclonal antibody (30). Recombinant proteins were again produced in the vaccinia expression system, but in this case, the infected-transfected HeLa cells were incubated overnight and then labeled with [35S]methionine and [35S]cysteine for 4 h before cell lysis. As revealed by autoradiography after gel electrophoresis, the wild-type and ΔN136 XPG proteins were stable, easily distinguishable major

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**Fig. 5. Viability of transfected lymphoblastoid cells after UV irradiation.** A and B, LBL463 cells from patient XP3BR were stably transfected with the indicated XPG clones in the EBO-pLPP vector and were assayed by Alamar blue fluorescence 48 h after irradiation. Results are expressed as percentage of the fluorescence of nonirradiated cells. Error bars show the standard deviations of groups of four measurements. C and D, XPG83 cells from patient XP125LO were stably transfected with the indicated XPG clones in the vector EBO-pLPP and were pulse-labeled for 2 h with [3H]thymidine 48 h after irradiation. Results are expressed as percentage of thymidine incorporation of nonirradiated cells. Error bars show the standard deviations of groups of four measurements. C and D, Raji lymphoblasts were stably transfected with the indicated XPG clones in the vector EBS-PL and were assayed by Alamar blue fluorescence 48 h after irradiation. Results are expressed as percentage of the fluorescence of nonirradiated cells. Error bars show the standard deviations of groups of four measurements. E, and F, cells from patients XP125LO and XP3BR were stably transfected with the indicated XPG clones in the vector EBO-pLPP vector and were pulse-labeled for 2 h with [3H]thymidine 48 h after irradiation. Results are expressed as percentage of thymidine incorporation of nonirradiated cells. Error bars show the standard deviations of groups of four measurements.
When separately expressed, wild-type XPG protein, but not the ΔN136 version without the epitope, was immunoprecipitated by the polyclonal antibody in a buffer containing 0.15 M KCl and 0.4% Nonidet P-40 (Fig. 7B, compare lane 1 with lanes 7 and 9). However, the truncated protein was found in the immunoprecipitate after the two proteins were co-expressed in the same cells (Fig. 7B, lanes 2–6 and 8). The same result was obtained when the buffer contained 50 μg/ml ethidium bromide to abolish nonspecific interactions mediated via DNA (50), when MgCl₂ was omitted from the buffer, or when the salt concentration was raised to 1 M KCl (data not shown). However, ΔN136 XPG was not found in the immunoprecipitate when the two proteins were expressed separately and then mixed (Fig. 7B, lane 10). To confirm the identities of the protein bands, unlabeled extracts were immunoprecipitated with the anti-N region polyclonal antibody and then immunoblotted with the anti-XPG 8H7 monoclonal antibody. The ΔN136 XPG protein was again found in the immunoprecipitate with co-expressed wild-type XPG (Fig. 7C, lanes 5 and 6). We conclude that when co-expressed, the full-length and truncated XPG proteins are able to interact with one another and that this interaction does not require the conserved N region.

To estimate the stoichiometry of the interaction, infected HeLa cells were co-transfected with various ratios of the two cDNAs to try to obtain equimolar expression of the full-length and truncated proteins. When this was achieved (Fig. 7A, lane 4), the subsequent immunoprecipitation was found to contain the wild-type and ΔN136 XPG proteins in a 3:1 molar ratio (Fig. 7B, lane 4). Because this method detects wild-type/wild-type and wild-type/ΔN136 interactions, but not ΔN136/ΔN136 interactions, this result implies an interaction between an even number of molecules, most simply that XPG can dimerize.

**DISCUSSION**

**Conserved Catalytic Residues in the XPG Family**—The results presented here provide insight into human XPG function. Structurally, the active site geometry of this protein is expected to be very similar to that of other nucleotide cleaving enzymes in the XPG family. Fig. 8A shows a superposition of three relevant proteins for which structures have been solved by x-ray crystallography: T. aquaticus DNA polymerase (37), T5 D15 exonuclease (3), and T4 RNase H (1). The central β-sheets superimpose extremely well and more closely than the surrounding α-helices. The metal coordinating side chains in the active sites are all clustered at the top of the β-sheets and are highly conserved both in sequence (Fig. 1) and in position.

Fig. 8B shows a model of XPG around the conserved active site region, which consists of both the N and I regions. These come together with remarkable exactness despite the very long sequence (>600 amino acids) between these two regions. All residues mutated in this study lie within this highly conserved active site region. It is possible that XPG co-ordinates two metal ions as found for T4 RNase H (Fig. 8B). Although not necessarily expected to have high precision at the atomic level, the model provides a useful visual aid for interpreting the consequences of each mutation examined in this study, and it helps to predict additional mutations that potentially could be useful in the future to probe the XPG incision mechanism in finer detail.

**Consequences of Mutating the Conserved Acidic Residues**—In most nucleolytic enzymes for which detailed information is available, the active site region contains several acidic residues that are highly conserved both in sequence (Fig. 1) and in position. The nature of these residues is likely to be relevant both for catalysis and to help to predict additional mutations that potentially could be useful in the future to probe the XPG incision mechanism in finer detail.
available, a carboxylate is required either to directly capture a 
proton from a water molecule (in this case the divalent ion 
stabilizes the reaction intermediate) or to bind a divalent ion 
that promotes the formation of a hydroxyl ion. In either case, 
the resulting OH\(^-\) attacks the scissile phosphodiester bond 
(51–55). For the XPG nuclease family, there is an extensive 
network of charged side chain to metal interactions that link 
both the two metal cations (Fig. 8). The two acidic residues 
mutated in this study, Asp-77 and Glu-791, are both involved 
in this network. In this regard, the report of nuclease activity 
in a D77A XPG mutant (56) is very surprising because substitu-
tion of this neutral side chain would be expected to disrupt this 
electrostatic network. We found instead that changing either 
Asp-77 or Glu-791 to Ala completely inactivated the nuclease 
function of XPG (Figs. 2–4). These results strongly suggest that 
the D77A and E791A substitutions, like the homologous mutations in FEN-1 and T4 
RNase H, still permit substrate binding, and they provide 
further evidence that XPG needs to be in the preincision 
complex to permit ERCC1-XPF to make the 5' cut (56).

XPG proteins carrying the D77E and E791D substitutions 
were unable to cut the bubble substrate but were highly active 
in the dual incision assay and in vivo (Figs. 2, 4, and 5). This is 
in accord with a strict requirement for carboxylates at positions 
77 and 791. These results further suggest that other NER 
components are needed to reveal the endonuclease activity of 
these mutated proteins. Minor modifications in the active 
surface of the protein are likely to affect the strict relative orien-
tations of the single-stranded DNA, the metal ion, and the 
attacking water molecule needed for efficient catalysis. Ap-
parently, the repair partners of the D77E and E791D XPG proteins 
can modulate the impact of these mutations on nucleolysis.

One way this might occur is to subtly adjust the confor-
mation of the mutated XPG proteins to better fit the substrate. An-
other possibility is that even if the kinetic parameters (\(K_m\) and  
\(V_{max}\)) for the acidic substitution mutants are low for the 
isolated enzymes, their effects are negligible compared with the 
thermodynamic gain of having specific contacts with other 
repair proteins and with an optimally positioned DNA sub-
strate within the open complex.

Implications for the XPG Incision Mechanism—In the crystal 
structure of T4 RNase H, the short peptide between the N and 
I regions, located above the cleft with the two Mg\(^{2+}\) ions, shows 
nine disordered residues that have been proposed to be in-
volved in substrate binding (1). The corresponding sequence in 
the Taq DNA polymerase also shows disordered residues (37).
In the T5 D15 exonuclease, the corresponding region is part of a helical arch that could perfectly accommodate DNA (3). It has been proposed that this arch may allow T5 D15 nuclease to thread-through single-stranded DNA until it reaches the branch point, where it cleaves. Evidence for a thread-through mechanism has been found for other members of the nuclease family. The flap endonuclease activity of FEN-1 is inhibited by protein binding or by annealing of a complementary strand to the single-stranded arm (61). Proliferating cell nuclear antigen physically associates with FEN-1 and stimulates its nuclease activity at branch substrates (48). It has been proposed that FEN-1 diffuses down the single-stranded flap from the 5′ terminus to the single-stranded double-stranded junction, where it is stabilized by proliferating cell nuclear antigen (48). T4 gene 32 single-stranded DNA-binding protein inhibits the flap endonuclease of T4 RNase H (62). Consistent with a thread-through model, the 5′ nucleases of Taq and E. coli polymerase I (58, 63), FEN-1 (5), and T5 D15 exonuclease (3) absolutely require a free single-stranded terminus to display nucleolytic activity.

In contrast, XPG must load onto DNA sites where there is no free 5′ end and therefore may use a different mechanism to accomplish the 3′ cut. During NER, a heterodimer composed of XPF and ERCC1 proteins is responsible for the 5′ incision (16, 17). Given that XPG is able to make a structure-specific 3′ cut without additional proteins (Refs. 6 and 15 and Fig. 2), the ability of XPG to multimerize (Fig. 7) may be relevant to its incision mechanism. Also relevant may be a helix-loop-helix motif found within the I region of XPG (64). Although no evidence was found for XPG multimers in the latter study, a recombinant peptide containing this helix-loop-helix motif was able to dimerize (64). In the present work, co-expressed full-length XPG protein and XPG lacking the conserved N region were found together by immunoprecipitation. Such multimers, most likely dimers, were resistant to high concentrations of detergent and salt and to ethidium bromide, thereby indicating that the interactions are specific and are not mediated by DNA. However, these results are not in accord with hydrodynamic measurements indicating that XPG exists as a monomer in high ionic strength (Fig. 6). This may simply mean that the observed self-interaction is reversible. Indeed, condition-dependent variability of native structure has been observed for many proteins, including the E. coli RuvC endonuclease (65) and members of the Bcl-2 family (4, 66). Further studies with XPG protein and its substrates will be required to test the potential relevance of multimerization to the XPG incision mechanism.

Implications for XP-G Clinical Phenotypes—A striking feature of individuals belonging to XP group G is that they rarely develop skin cancers, even though their cells are among the most UV-sensitive of the eight XP complementation groups. Several XP-G individuals suffer from a very severe early onset form of Cockayne syndrome, which is correlated with an inability to produce full-length XP-G protein (10, 67) and an inability to carry out transcription-coupled repair of oxidative base damage (9). Due to their hospitalization and early demise, these XP-G/CS patients have had limited exposure to UV. In contrast, the XP124LO and XP125LO siblings have been exposed to UV for over two decades, yet despite only occasional use of sun protection, they have no skin cancers.

The results presented here suggest an explanation for this paradox. At a cellular level, transcription-coupled repair of UV damage cannot be detected in XP125LO fibroblasts (10), and unscheduled DNA synthesis is at background levels after UV treatment of XP125LO lymphoblasts (26). The thymidine uptake assay showed, however, that the XP125LO lymphoblasts, although very UV-sensitive, are significantly more UV-resistant than lymphoblasts from XP-G patient XP3BR (Fig. 5G). Moreover, when the A792V protein, the only stably expressed form of XPG detectable in the XP124LO and XP125LO siblings (10), was expressed in XP3BR lymphoblasts, it increased their UV resistance up to the XP125LO level (Fig. 5G). These results strongly suggest that the XP124LO and XP125LO siblings exhibit a very mild XP phenotype because their A792V XPG protein retains some residual repair capacity.

The latter suggestion implies that the A792V protein has some 3′ endonuclease activity, and this is borne out by the in vitro studies. Although A792V XPG was unable to cleave the bubble substrate in the presence of Mg2+, it was able to do so to...
some extent with Mn$^{2+}$ as metal ion cofactor (Fig. 2). Similar Mn$^{2+}$-driven partial activity has been reported for active site mutants of BamHI (68), EcoRV (69), T4 RNase H (59), and Murl I (70) nucleases. Residue Ala-792 is at the beginning of an α-helix and just after the presumed metal coordinating side chain Glu-791 (Fig. 6B). Valine side chains are β-branched and are less favored within α-helices than non-β-branched side chains (71). Hence, this particular α-helix may unwind slightly in the A792V protein, and this in turn may interfere with the ability of Glu-791 to accurately coordinate a metal cation. The 3' endonuclease activity in the presence of Mn$^{2+}$ may reflect partial correction of such a distortion in the metal binding site. This missense mutation appears to have additional effects, however, because the A792V protein exhibited the lowest level of uncleaved 5' incisions (Fig. 4). This indicates that it does not stay firmly bound in the repair complex, because an altered property of this naturally occurring mutation indicate that it would be very valuable to identify the XPG defects in other mildly affected XP-G patients.

Acknowledgments—We thank C. Arlett and J. Cole for helping to establish the LBL463 cell line from patient XP3BR and G. Mottet and J.-B. Marq for help with the vaccinia expression system.

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