The DNA Repair Endonuclease XPG Binds to Proliferating Cell Nuclear Antigen (PCNA) and Shares Sequence Elements with the PCNA-binding Regions of FEN-1 and Cyclin-dependent Kinase Inhibitor p21*

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Ronald Gary‡, Dale L. Ludwig, Helen L. Cornelius, Mark A. Macllnnes, and Min S. Park§

From the Life Sciences Division, Los Alamos National Laboratory, Los Alamos, New Mexico 87545

Proliferating cell nuclear antigen (PCNA) is a DNA polymerase accessory factor that is required for DNA replication during S phase of the cell cycle and for re-synthesis during nucleotide excision repair of damaged DNA. PCNA binds to flap endonuclease 1 (FEN-1), a structure-specific endonuclease involved in DNA replication. Here we report the direct physical interaction of PCNA with xeroderma pigmentosum (XP) G, a structure-specific repair endonuclease that is homologous to FEN-1. We have identified a 28-amino acid region of human FEN-1 (residues 328–355) and a 29-amino acid region of human XPG (residues 981–1009) that contains the PCNA binding activity. These regions share key hydrophobic residues with the PCNA-binding domain of the cyclin-dependent kinase inhibitor p21Waf1/Cip1, and all three competed with one another for binding to PCNA. A conserved arginine in FEN-1 (Arg339) and XPG (Arg992) was found to be crucial for PCNA binding activity. These results raise the possibility of a mechanistic link between excision and repair synthesis that is mediated by PCNA.

Exposure to UV light causes damage to DNA primarily in the form of cyclobutane pyrimidine dimers and (6-4) photoproducts. These types of DNA lesions, as well as bulky adducts produced by some chemical mutagens, are processed by nucleotide excision repair (NER). The human genetic disorder xeroderma pigmentosum (XP) is the result of defects in this DNA damage repair pathway. Symptoms of XP include extreme sensitivity to sunlight exposure and a greatly elevated risk of skin cancer. In the past few years, much progress has been made in understanding the molecular events associated with NER (1). The DNA-binding protein XPA is involved in damage recognition. In concert with replication protein A, which binds single-stranded DNA, and helicases XPB and XPD, a ¬27–29-base oligonucleotide segment containing the lesion is excised as the result of dual incision by structure-specific endonucleases XPF-ERCC1 and XPG. The XPF-ERCC1 complex cleaves the damaged strand at a 5’ site about 23 nucleotides from the lesion, whereas XPG cleaves the strand approximately 5 nucleotides to the 3’ side of the damage. The resultant gap is filled in by the action of DNA polymerase δ or e, and then DNA ligase seals the nick to complete repair. The re-synthesis step requires proliferating cell nuclear antigen (PCNA; Refs. 2 and 3), a ring-shaped homotrimeric protein that encircles DNA and acts as a “sliding clamp” that links the polymerase to the DNA template (4). PCNA performs the same essential function in replicative DNA synthesis during S phase of the cell cycle. PCNA requires replication factor C, a primer recognition protein that loads the PCNA trimer onto DNA in an ATP-dependent manner (5–7).

XPG is homologous to another structure-specific endonuclease, FEN-1. FEN-1 is involved in Okazaki fragment processing during DNA replication (8), and it is required for avoidance of duplication-type insertion mutations in yeast (9). FEN-1 binds to PCNA (10–12), and this complex can be disrupted by p21Waf1/Cip1 (12), a bifunctional protein that has a C-terminal PCNA-binding domain and an N-terminal domain that inhibits cyclin-dependent protein kinases (13, 14). Here we report domain mapping experiments to pinpoint the PCNA-binding region of FEN-1 and show that the small region responsible for activity is conserved in XPG. This domain in XPG as well as the full-length XPG protein are shown to bind to PCNA. We also provide evidence from in vivo studies indicating that the PCNA-XPG interaction has a role in repair of UV damage. Finally, we identify a convergent evolutionary relationship between the PCNA-binding domains of the DNA damage-inducible inhibitor p21 and the repair endonuclease XPG and show that these domains compete for binding to PCNA.

EXPERIMENTAL PROCEDURES

Bacterial Expression Plasmids—The EcoRI-XhoI fragment of human FEN-1 expression plasmid pET-FCH (15) was ligated with EcoRI/XhoI-linearized pGEX-4T-1 vector (Pharmacia Biotech Inc.) to create a plasmid that expresses a fusion protein of glutathione S-transferase (GST) and residues 206–380 of FEN-1. The amino acid fragments 254–363, 254–380, 290–363, and 290–380, 290–328, 328–363, and 328–380 of FEN-1 were amplified by polymerase chain reaction using pET-FCH as template and primer pairs F2 (5’-CCAGAATTCAAGAGCATC-GAGGAGATCGTG-3’), F3 and R2 (5’-GTGCTCGAGTCACAGCC-CTTTGACCCCACTG-3’), F3 and R5 (5’-GTGCTCGAGTATTATTTTCCCCTTT-GTGAATTCCTGAG-3’), R2, and F5 and R3, respectively. The products were digested with EcoRI and XhoI and subcloned into EcoRI/XhoI-linearized pGEX-4T-1 to make GST fusion protein expression plasmids. Expression plasmids for the production of GST fusion proteins containing FEN-1 residues 328–348 and 328–355 were generated by site-

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directed mutagenesis of the GST-FEN328–363 plasmid to convert codon 349 or 356 to a stop codon. The stop codons were introduced using the QuickChange Mutagenesis procedure (Stratagene, La Jolla, CA) and mutagenic primer pairs 5′-CTTCAAGGACGCGCTCTCTTCCACG-3′ and 5′-GGGATCCCTTGGGTTCTGGCTTGCGGCCCTGGGTGCTGCACTAGTGATGGTG-3′ using plasmid kindly provided by Dr. Jerry Harwit, Memorial Sloan-Kettering Cancer Center) to be expressed in Escherichia coli (15). The polyhistidine tag binds tightly to metal chelation affinity resin. The FEN-1 codon arginine 339 in this plasmid was replaced with either an alanine or glutamate codon by QuickChange Mutagenesis to create R339A and R339E single point mutant derivatives using mutagenic primer pairs 5′-CAGAAGGACGCGCTCTCTTCCACG-3′ and 5′-GGGATCCCTTGGGTTCTGGCTTGCGGCCCTGGGTGCTGCACTAGTGATGGTG-3′, respectively. These primer pairs each created a BsaHI or SacI site to facilitate screening.

The second pair of mutagenic primers was also used to generate the R339E derivative of plasmid GST-FEN328–363, whose product was used in PCNA bead binding competition experiments. Truncated FEN-1 proteins comprising amino acids 1–328 or 1–363 with C-terminal polyhistidine tags were created by QuickChange Mutagenesis of pET-FCH-XhoI-linearized pGEX-4T-1. The oligonucleotides corresponded to each strand of the FEN-1 DNA sequence for the specified region, including the stop codon immediately after codon 380 and were flanked by EcoRI and XhoI sites.

Plasmid pET-FCH produces full-length FEN-1 (amino acids 1–380) with six histidine residues appended to the C terminus (15). The polyhistidine tag binds tightly to metal chelation affinity resin. The FEN-1 codon arginine 339 in this plasmid was replaced with either an alanine or glutamate codon by QuickChange Mutagenesis to create R339A and R339E single point mutant derivatives using mutagenic primer pairs 5′-CAGAAGGACGCGCTCTCTTCCACG-3′ and 5′-GGGATCCCTTGGGTTCTGGCTTGCGGCCCTGGGTGCTGCACTAGTGATGGTG-3′, respectively. These primer pairs each created a BsaHI or SacI site to facilitate screening. The second pair of mutagenic primers was also used to generate the R339E derivative of plasmid GST-FEN328–363, whose product was used in PCNA bead binding competition experiments. Truncated FEN-1 proteins comprising amino acids 1–328 or 1–363 with C-terminal polyhistidine tags were created by QuickChange Mutagenesis of pET-FCH-XhoI-linearized pGEX-4T-1. The oligonucleotides corresponded to each strand of the FEN-1 DNA sequence for the specified region, including the stop codon immediately after codon 380 and were flanked by EcoRI and XhoI sites.

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**Protein Purification—GST-XPG961–1009, GST-XPG961–1009(R992E), GST-FEN328–363, and GST-FEN328–363(R339E) proteins used in competition experiments were purified by glutathione-agarose chromatography.** Human PCNA was purified from bacterial lysate by Q-Sepharose, S-300 Sephacryl gel filtration, and Phenyl-Superose chromatography (19). Protein concentrations were determined by a Bradford-based assay (Bio-Rad) and verified by Coomassie Blue staining of SDS-PAGE gels.

**PCNA Bead Binding Assay—Purified human PCNA or protease-free bovine serum albumin (BSA; Boehringer Mannheim) at 4 mg/ml in 25 mM NaHCO3, 150 mM NaCl, pH 8.3, was added to cyagenon bromide-activated agarose beads (Sigma) and incubated for 16 h at 4°C to cover the entire surface of the beads. Coating efficiency was 80% or higher for each protein. Unbound protein was removed, and then remaining reactive sites were blocked with 0.1 M glycine, 65 mM Tris-HCl, 150 mM NaCl, pH 8.0. Washed beads were stored on ice. Human XPG was expressed from recombinant baculovirus as described previously (17), except that *Trichoplusia ni* (BTI-TN-5B1-4) insect cells (“High Five” cells) were used. The latter primer pair 5′-ACAGCTCGCGATTGATTCCTTCTTTAGATTAG-3′ and 5′-ACTTCCCTGCTGCCCCACTAGTGATGGTGATGGTGG-3′ generate the R339E derivative of plasmid GST-FEN328–363, whose products were used in competition experiments were purified by glutathione-agarose chromatography. Human PCNA was purified from bacterial lysate by Q-Sepharose, S-300 Sephacryl gel filtration, and Phenyl-Superose chromatography (19). Protein concentrations were determined by a Bradford-based assay (Bio-Rad) and verified by Coomassie Blue staining of SDS-PAGE gels.

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DNase I (Boehringer Mannheim) were added to the washed beads. The beads were incubated for 20 min at 10 °C and were mixed periodically to keep the beads in suspension. After two more washes, proteins were eluted and analyzed. To confirm the activity of DNase I under the conditions used, HindIII-digested Lambda DNA (Life Technologies, Inc.) and DNase I were added at various ratios to washed beads and incubated as described. These confirmatory reactions were terminated by addition of 25 mM EDTA and 0.5% SDS, and the DNA was evaluated by agarose gel electrophoresis and ethidium bromide staining.

Luciferase Reporter Plasmid Repair Assay—The repair assay using UV-damaged luciferase reporter plasmid has been described previously (22). Briefly, luciferase expression plasmid pGL-2 (Promega, Madison, WI) was irradiated with 0, 400, or 800 J/m² of 254-nm UV light from a calibrated source. XPG function was provided by pBactin-XPG, a mammalian expression plasmid containing human XPG cDNA under the control of a β-actin promoter. A truncated derivative of this plasmid was created by BamHI digestion to remove amino acids 948–1186 of the XPG coding region and a downstream polyadenylation signal and then calibrated source. XPG function was provided by pBactin-XPG, a mammalian expression plasmid containing human XPG cDNA under the control of a β-actin promoter. A truncated derivative of this plasmid was created by BamHI digestion to remove amino acids 948–1186 of the XPG coding region and a downstream polyadenylation signal and then circularizing the vector fragment. R992A and R992E mutations were generated in pBSK-XPGA using mutagenic primers as described for bacterial expression plasmids. These mutations were introduced into pBactin-XPG by swapping EcoRV-KpnI fragments between plasmids. XPG plasmid concentrations were determined by measurement of absorbance at 260 nm and verified by agarose gel electrophoresis and ethidium bromide staining. The XPG-deficient CHO cell line UV135 (16, 23) was transfected with a mixture of 150 ng of luciferase plasmid, 150 ng of β-galactosidase plasmid (22), and 30 ng of wild-type or mutant XPG plasmid by calcium phosphate precipitation (16). For each XPG transfection set, three to six 60-mm dishes were used. Cells were lysed 48 h after transfection for analysis of luciferase and β-galactosidase activity. For each transfection, the luciferase activity was divided by the corresponding β-galactosidase activity to give relative luciferase activity, a measure of DNA repair that is normalized for transfection efficiency. Statistical analyses of the data were performed using Microsoft Excel version 4.

RESULTS

The PCNA Binding Activity of FEN-1 Resides within a Short Region near the C Terminus—We produced a series of GST fusion proteins that contain various regions of human FEN-1 and assayed their PCNA binding activity using a “pull-down” affinity bead interaction assay (Fig. 1). The binding activity was contained entirely within amino acids 328–355 of FEN-1. All fusion proteins that contained this region bound to PCNA, whereas none of the proteins tested that lacked the complete 28 amino acid sequence displayed binding activity. For example, the fusion protein containing only residues 328–348 of FEN-1 did not bind PCNA.

It has been reported previously that the PCNA-binding domain of human FEN-1 is contained within residues 307–380 and that residues 364–380 are essential for PCNA binding (12). The former conclusion is consistent with the observations reported here, but the latter conclusion is not. Because we observed no requirement for 364–380 in our domain mapping studies, we generated truncated FEN-1 proteins to address the importance of this region in more detail (Fig. 2). Deletion of the PCNA-binding region to give a truncated form of FEN-1 comprising only amino acids 1–328 abolished PCNA binding activity. However, a truncated FEN-1 comprising amino acids 1–363 bound PCNA as effectively as full-length FEN-1 (amino acids 1–380). Thus, we conclude that the C-terminal region from residues 364 to 380 of FEN-1 is not essential for PCNA binding activity and in fact makes little if any contribution to this activity. This contrasts with the previous report that truncated FEN-1 (amino acids 1–363) is unable to bind to PCNA in gel filtration and affinity bead pull-down assays (12). The reason for the difference between those observations and our own is not apparent. We next sought to identify specific residues of FEN-1 that are most important for interaction with PCNA. Arginine 339 of FEN-1, lying within the PCNA-binding region, was found to be crucial for PCNA binding activity. Single point mutagenesis of FEN-1 to convert Arg339 to either alanine or glutamate dramatically decreased the ability of the protein to

Fig. 1. The PCNA binding activity of FEN-1 is contained within amino acids 328–355. A, Coomassie-stained gel of protein complexes bound to glutathione-agarose beads in pull-down binding assay. Molecular mass marker bands of 200, 116, 97, 66, 55, 37, 31, and 21 kDa are shown (lane 1). Beads were mixed with lysate from bacteria expressing GST fusion proteins containing human FEN-1 amino acids 206–380 (52 kDa; lane 2), 254–380 (48 kDa; lane 3), 290–380 (37 kDa; lane 4), 280–328 (31 kDa; lane 5), 328–380 (32 kDa; lane 6), 328–363 (31 kDa; lane 7), 328–355 (30 kDa; lane 8), 328–348 (29 kDa; lane 9), 354–380 (32 kDa; lane 10), 363–380 (29 kDa; lane 11), or GST alone (28 kDa; lane 12). Lysate from bacteria expressing human PCNA (35 kDa) was added to each assay. After washing, protein complexes were eluted and analyzed by SDS-PAGE. Bound PCNA is present in lanes 2–4 and 6–8. The 34-kDa protein seen in lane 11 migrates slightly faster than PCNA. B, schematic diagram summarizing the results of the PCNA binding assays; AA’s, amino acids.

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bind PCNA, showing the importance of this region of FEN-1 and of this residue in particular. Although PCNA binding activity of the R339A and R339E mutants of FEN-1 was severely impaired, each mutant retained endonuclease activity as determined by rapid kinetic flow cytometry (15, 24) using a fluoresceinated 5'-flap DNA substrate (data not shown).

Identification of a PCNA-binding Domain in XPG—The amino acid sequence of the PCNA-binding region of FEN-1 is significantly conserved in XPG (Fig. 3). We sought to determine whether the function of this region is conserved in XPG as well. We made a GST fusion protein (GST-XPG) containing the 29-amino acid sequence of human XPG (residues 981–1009) that is homologous to the PCNA-binding region of FEN-1. GST-XPG bound human PCNA very efficiently when bacterial lysates containing these proteins were mixed (Fig. 4). GST alone lacked XPG sequence had no observable affinity for PCNA. The resulting from a single amino acid substitution in GST-XPG binding activity in FEN-1 (Fig. 2). Replacing this arginine in amino acid sequence of human XPG (residues 981–1009) that is homologous to the PCNA-binding region of FEN-1. GST-XPG and PCNA were mixed (Fig. 4). GST alone lacked PCNA binding activity. The dramatic decrease in bound PCNA resulting from a single amino acid substitution in GST-XPG attests to the specificity of this assay.

The PCNA-binding domain of FEN-1 displayed robust binding activity under all conditions tested; however, the binding activity of the corresponding region of XPG exhibited profound salt dependence (Fig. 5). Binding of GST-XPG and PCNA was almost undetectable in a buffer containing 60 mM NaCl. Adding 100 mM potassium phosphate to this buffer produced maximal binding, and adding 25 mM potassium phosphate, 100 mM KCl, or 300 mM KCl increased binding to about 25, 25, and 100% of maximum, respectively. Thus, divalent anion was especially effective in aiding binding. In contrast to the behavior of GST-XPG, GST-FEN (residues 328–355) displayed nearly maximal binding in any of these buffers. Varying pH from 6.0 to 8.0 had little effect on the association of GST-XPG and PCNA, and this complex was stable to repeated washing with either 1% Nonidet P-40 detergent or 1.0 M NaCl (data not shown).

Full-length XPG Binds to PCNA—An affinity bead assay was used to evaluate the interaction of PCNA and full-length XPG. Purified human PCNA was covalently attached to beads. Human XPG was expressed in insect cells that had been infected with a recombinant baculovirus strain that contains XPG cDNA (17). XPG present in baculovirus-infected cell lysates bound to PCNA beads but not to control beads made with BSA (Fig. 6A). The specificity of the PCNA-XPG interaction was further demonstrated in competition experiments. The binding of XPG to PCNA beads was blocked by the addition of free PCNA to the lysate but was unaffected by addition of BSA (Fig. 6B). Binding was also inhibited by the addition of GST-XPG981–1009, the fusion protein containing the PCNA-binding fragment of XPG. However, the R992E derivative of this fusion protein, which lacks PCNA binding activity (Fig. 4), was unable to compete with XPG for binding to the beads. Similarly, GST-FEN928–363, but not the R339E mutant of GST-FEN328–363, competed with XPG for binding to the PCNA beads. It appears that the FEN-1 domain binds to PCNA with higher affinity than the XPG domain, because competition by GST-FEN328–363 was complete, whereas that by GST-XPG981–1009 at similar concentration was partial. Because PCNA and XPG are both DNA-binding proteins, we questioned whether DNA might mediate their association indirectly. DNase I treatment of the PCNA-XPG complex failed to remove XPG (Fig. 6C), proving that the two proteins were not simply linked by DNA. Prior to treatment, the complex was washed to remove free DNA and actin, components of crude insect cell lysate that could decrease the effectiveness of DNase I. Under the reaction conditions employed, the DNase I used in the treatment was sufficient to digest 25 μg of HindIII-cut Lambda DNA to completion and to convert 250 μg of DNA to a low molecular weight smear (data not shown). The lack of effect of DNase I treatment, together with the competition experiments, prove conclusively that the interaction of XPG and PCNA is direct.

The PCNA Binding Activity of XPG Is Needed for Maximum Repair Efficiency—PCNA and XPG both participate in excision repair, raising the expectation that the interaction of these proteins occurs in that context. Therefore, we sought to investigate the role of this interaction in NER in vivo. CHO-UV135 cells lack a functional XPG homolog. They are hypersensitive to UV-induced cell mortality and are severely defective in the repair of UV-damaged DNA. Both phenotypic defects can be corrected by transfection with an XPG expression plasmid (16, 22, 26). This system provides a model of NER function in vivo that is dependent upon exogenously supplied XPG. Because arginine 992 is critical for full PCNA binding activity of XPG, we transfected UV135 cells with R992A or R992E single point mutant derivatives of XPG to produce cells in which the XPG-PCNA interaction was impaired relative to wild-type XPG transfectants. XPG-dependent repair activity of wild-type and mutant transfectants was compared to assess the importance of the XPG-PCNA interaction in NER (Fig. 7). In this assay, in vivo...
vivo repair of a UV-damaged luciferase reporter plasmid results in expression of luciferase commensurate with the extent of repair. Fully reconstituted repair activity is exemplified by cells transfected with wild-type XPG. Absence of repair activity is shown by cells transfected with truncated XPG lacking residues 948–1186. The deletion of 3'-untranslated sequence in this mutant probably destabilizes the mRNA, and the coding region truncation removes the nuclear localization signal to encode a mutant protein unable to enter the nucleus (20, 21). Lying between these benchmark indicators of maximum and minimum repair activity were those cells dependent upon R992A and R992E XPG mutants for NER function. A small but reproducible decrease in repair efficacy was observed for each of these mutants. This suggests that impairment of the PCNA-XPG interaction adversely affects NER but that the magnitude of the defect is small compared with complete absence of function.

The PCNA-binding Domains of FEN-1 and XPG Compete with That of p21—The PCNA-binding region of the cyclin-dependent kinase inhibitor p21 has been localized to amino acids 141–160 (27). A 39-mer synthetic peptide corresponding to amino acids 126–164 of p21 competes with full-length FEN-1 for binding to PCNA (12). The high resolution domain mapping of FEN-1 and XPG reported here allowed for a useful sequence alignment between the PCNA-binding regions of these nucleases and p21 (Fig. 8). Comparison of the amino acid sequences of the PCNA-binding domains of FEN-1 and p21 reveals a structural similarity that suggests direct competition for a single binding site on PCNA. Furthermore, the mapping data predict competition between p21 and XPG for binding to PCNA. Competition between the isolated PCNA-binding domains of these proteins was confirmed using the pull-down affinity bead interaction assay (Fig. 9). Interestingly, the p21 peptide was more potent than the XPG peptide in competing with the GST-XPG fusion protein for binding to PCNA, suggesting that the p21-PCNA interaction (2.5 nM $K_D$; Ref. 31) is higher affinity than the XPG-PCNA interaction. As expected, the p21 peptide competed the PCNA-binding GST-FEN fusion protein (data not shown), confirming the previously reported competition between p21 peptide and full-length FEN-1 (12).
PCNA plays a major role in DNA replication and DNA damage repair. In addition to its core function of tethering DNA polymerases $\delta$ and $\epsilon$ to the DNA template, PCNA also interacts with the cell cycle regulator p21, the replication endonuclease FEN-1, and, as shown here, the repair endonuclease XPG. Similarity in the primary structures of the PCNA-binding domains of p21, FEN-1, and XPG (Fig. 8) strongly suggests that these proteins all use an analogous arrangement of key hydrophobic residues to bind to a hydrophobic pocket formed primarily by the interdomain connector loop of PCNA, as is known to be the case for p21 (28). This similarity predicts that competitive binding between these proteins would be the result of direct competition for a single binding site on PCNA, rather than steric hindrance arising from adjacent but distinct sites. Experiments using the isolated PCNA-binding domains from these proteins are consistent with this prediction. In contrast, the previously observed competition between FEN-1 and p21 was interpreted as being due to ligand-induced conformational change in PCNA, rather than occupation of the same site on PCNA (12). Because p21 is otherwise unrelated to the nucleases, the common structural elements in the PCNA-binding regions of p21 and FEN-1/XPG appear to have arisen by convergent evolution.

XPG and PCNA are involved in distinct and experimentally separable steps of NER, namely excision and repair resynthesis, respectively. The identification of PCNA binding activity in XPG suggests that the excision and resynthesis machinery might communicate with one another in some manner. The structural and functional conservation of PCNA binding in FEN-1 and XPG reflects a selective pressure to preserve this structural and functional conservation of PCNA binding in sis, respectively. The identification of PCNA binding activity in separable steps of NER, namely excision and repair resynthesis, suggests that these proteins may act to subvert the repair process.

**FIG. 6. Specific interaction of full-length XPG and PCNA.** A–C, Western blots showing XPG bound to affinity beads. Purified BSA or human PCNA was covalently coupled to CNBr-activated Sepharose to make affinity beads. Beads were mixed with lysate from insect cells infected with a recombinant baculovirus that directs expression of human XPG. After washing, noncovalently bound proteins were eluted, resolved by SDS-PAGE, and analyzed by anti-XPG Western blot. A, XPG bound to BSA-CNBr or PCNA-CNBr affinity beads. B, XPG bound to PCNA-CNBr beads after competition by exogenous purified proteins. 0.4 mg of BSA, PCNA, GST-XPGG91-1009, the R992E mutant of GST-XPGG91-1009, or the R339E mutant of GST-FEN-328-363 was added to XPG lysate prior to incubation with beads. C, PCNA-CNBr beads were incubated with XPG lysate and then washed to remove free DNA and actin (a DNase I inhibitor). 10 mM MgCl$_2$ was added to each of the washed bead suspensions, and one received 250 units of DNase I to degrade any tightly associated DNA that might be present. After 20 min of incubation, the ± DNase I-treated beads were washed again, and bound proteins were eluted for Western analysis.

**FIG. 7. Mutant XPG defective in PCNA binding is unable to fully complement nucleotide excision repair activity in a repair-deficient cell line.** NER-defective UV135 cells lacking functional XPG were transiently co-transfected with XPG, luciferase, and $\beta$-galactosidase expression plasmids. Wild-type XPG (●), point mutant R992A (■), point mutant R992E (▲), or truncated XPG (×) were assayed for ability to complement the UV135 repair defect. The luciferase reporter plasmid was unirradiated (0 J/m$^2$) or damaged by UV light (400 or 800 J/m$^2$) prior to transfection. $\beta$-Galactosidase was used as an internal standard to normalize co-transfection efficiency. XPG-dependent repair is plotted as the mean relative luciferase activity ($\gamma$-galactosidase activity, arbitrary units) ± S.E. Analysis of variance was performed for each dose of UV damage, testing the null hypothesis that the mean relative luciferase activity is equal between the transfected groups. Considering all four groups (wild type, R992A, R992E, and truncated), $p = 0.21$ at 0 J/m$^2$, $p = 0.000014$ at 400 J/m$^2$, and $p = 0.00991$ at 800 J/m$^2$. Considering only wild type, R992A, and R992E, $p = 0.34$ at 0 J/m$^2$, $p = 0.016$ at 400 J/m$^2$, and $p = 0.086$ at 800 J/m$^2$. The analyses indicate no significant differences between groups when undamaged reporter plasmid was used but significant differences between XPG transfection groups in the repair of UV-damaged plasmid. One-tailed $t$ tests showed a significant decrease in XPG-dependent repair for each point mutant compared with wild-type XPG at the same UV damage dose: at 400 J/m$^2$, $p = 0.011$ for R992A and $p = 0.032$ for R992E; at 800 J/m$^2$, $p = 0.052$ for R992A and $p = 0.051$ for R992E. There was no significant difference between R992A and R992E groups at any dose. The results shown represent one out of a total of six independent experiments that were conducted; in all six experiments, R992A and R992E each exhibited a repair deficiency similar in magnitude to that shown.

**DISCUSSION**

the repair patch. Because the PCNA binding and catalytic domains of XPG are quite close to one another (Fig. 3), the PCNA-XPG interaction may take place near the 3' incision site. However, the PCNA-polymerase complex must initiate repair synthesis at the 5' end of the repair patch and move along the template in a 5' to 3' direction. Therefore, PCNA might be most likely to encounter XPG at the 3' end of the repair patch upon completion of resynthesis.

In excision assays, the DNA undergoing repair remains tightly associated with exinuclease proteins even after comple-
PCNA Binds XPG

FIG. 8. Alignment of PCNA-binding regions of FEN-1, XPG, and p21 reveals key hydrophobic residues in common and suggests a similar mode of interaction with PCNA. Hydrophobic residues of p21 shown in bold type occupy a hydrophobic pocket on the surface of PCNA when the two are complexed, as determined by structural analysis of the co-crystal (29). These residues of p21 have also been shown to be particularly important for interaction with PCNA by mutational analysis (27, 29, 30). Several residues in the PCNA-binding region of human p21 are identical or conservatively substituted in human FEN-1 and XPG, including the key hydrophobic residues Met314, Phe330, and Tyr211.

Fig. 9. The amino acid 139–160 peptide of p21 competes with the PCNA-binding domain of XPG for interaction with PCNA. Coomassie-stained gel of protein complexes bound to glutathione-agarose beads in pull-down binding assay is shown. Beads were mixed with bacterial lysates containing PCNA and the GST-XPG polypeptide (lanes 1–7). Synthetic peptide corresponding to amino acids 139–160 of human p21 was added at 10 μM (lane 2) or 40 μM (lane 3) final concentration, and peptide corresponding to amino acids 981–1009 of human XPG was added at 10 μM (lane 5), 40 μM (lane 6), or 160 μM (lane 7).

PCNA-dependent kinase inhibitor, p21 promotes cell cycle arrest that is thought to ameliorate DNA damage by allowing increased time for repair to take place. The inhibition of PCNA activity by p21 is a more paradoxical aspect of the p21 response, however. Although the association of p21 and PCNA might serve to disrupt replicative synthesis and thereby contribute to cell cycle arrest, PCNA in an active state is nonetheless essential for excision repair. Thus, p21 might be expected to inhibit DNA repair by sequestering PCNA. It has been found by some investigators (40), although not all (41, 42), that p21 does indeed inhibit NER, and this inhibition can be alleviated by the addition of PCNA. On the other hand, p21-deficient cells exhibit a modest UV repair defect, a phenotype that can be reversed by providing wild-type p21 but not truncated p21 lacking PCNA binding activity (43). This suggests that the interaction of p21 and PCNA somehow makes a beneficial contribution to the damage response. The situation in vivo appears complicated, perhaps involving subtle regulation of the repair process or differences in the temporal expression or subcellular localization of PCNA, p21, and XPG that allow these proteins to work together appropriately.

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