Nucleotide Excision Repair by Mutant Xeroderma Pigmentosum Group A (XPA) Proteins with Deficiency in Interaction with RPA*

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The xeroderma pigmentosum group A protein (XPA) is a core component of nucleotide excision repair (NER). To coordinate early stage NER, XPA interacts with various proteins, including replication protein A (RPA), ERCC1, DDB2, and TFIH, in addition to UV-damaged or chemical carcinogen-damaged DNA. In this study, we investigated the effects of mutations in the RPA binding regions of XPA on XPA function in NER. XPA binds through an N-terminal region to the middle subunit (RPA32) of the RPA heterotrimer and through a central region that overlaps with its damaged DNA binding region to the RPA70 subunit. In cell-free NER assays, an N-terminal deletion mutant of XPA showed loss of binding to RPA32 and reduced DNA repair activity, but it could still bind to UV-damaged DNA and RPA. In contrast, amino acid substitutions in the central region reduced incisions at the damaged site in the cell-free NER assay, and four of these mutants (K141A, T142A, K167A, and K179A) showed reduced binding to RPA70 but normal binding to damaged DNA. Furthermore, mutants that had one of the four aforementioned substitutions and an N-terminal deletion exhibited lower DNA incision activity and binding to RPA than XPA with only one of these substitutions or the deletion. Taken together, these results indicate that XPA interaction with both RPA32 and RPA70 is indispensable for NER reactions.

Nucleotide excision repair (NER) is a versatile DNA repair system that corrects a broad spectrum of DNA damage, including UV-induced cyclobutane pyrimidine dimers (CPDs) and pyrimidine-pyrimidone (6-4) photoproducts ((6-4)PPs) as well as chemical carcinogen-induced lesions (1). There are two subpathways in NER (2) as follows: transcription-coupled repair, which efficiently removes damage on the transcribed strand of transcriptionally active genes, and global genome repair (GGR), which occurs throughout the genome, including the nontranscribed strand of active genes.

Xeroderma pigmentosum (XP) is an autosomal recessive disease characterized by hypersensitivity to sunlight and a high incidence of skin cancer on sun-exposed skin (1, 3). Cells from individuals with XP are hypersensitive to UV light because of a defect in NER. XP is classified into seven complementation groups (XP-A to XP-G) and a variant form (1). Except for the variant form, the primary defect of XP resides in the early steps of NER as follows: damage recognition and dual incisions of the damaged strand on both sides of the DNA lesion (3). Both transcription-coupled repair and GGR are defective in groups A–G, but not groups C and E, in which only GGR is impaired (4–6). To date, all XP genes have been cloned. In addition, the ERCC1 protein, encoded by a human gene that can correct the repair deficiency in a UV-sensitive rodent mutant cell line, is also involved in NER. A case of human inherited ERCC1 deficiency, diagnosed as cerebro-oculo-facio-skeletal syndrome, has been reported (7). The core reaction of GGR in humans has been reconstructed in vitro with purified XP and other proteins, and the GGR mechanism has been broadly elucidated (8).

The XP protein, consisting of 273 amino acid residues, binds to RPA, ERCC1, DDB2, and TFIH, as well as to UV- or chemical carcinogen-damaged DNA (9, 10), and it participates in the formation of an open complex (11). We previously identified two XP-binding proteins, XAB1 and XAB2, by yeast two-hybrid screening (12, 13). Several distinct functional regions in XPA have been identified (Fig. 1). The N-terminal region (residues 4–29) of XPA is responsible for binding to the 32-kDa subunit of RPA (RPA32) (14, 15), and the basic amino acid region (residues 30–42) is important for nuclear localization of the XPA protein (17). The region containing the glutamic acid cluster (E-cluster) (residues 78–84) is important for XPA interaction with ERCC1 (18, 19). The C-terminal region (residues 226–273) binds to TFIH (20, 21). The central region is the minimal polypeptide (residues 98–219) necessary for preferential binding to damaged DNA (22), and within this region residues 98–187 are necessary for binding to the 70-kDa subunit of RPA (RPA70) (14, 15). The region between residues 185 and 225 is also necessary for binding to DDB2 (16). Although XPA has no enzymatic activity, it functions as a core component in NER reactions by interacting with damaged DNA and NER factors. In addition, the binding of XPA to damaged DNA is markedly increased by its interaction with other NER components (18, 21, 23, 24), which could function to coordinate the early stages of NER (16, 25). We have shown that interactions between XPA and...
other NER factors (RPA, ERCC1, and TFIH) are important for NER using anti-XPA monoclonal antibodies (26), but we have been unable to define the interactions between XPA and other factors in the NER reaction that are critical for NER. It has been reported that the interaction between XPA and RPA70 is important for GGR by measuring the RPA binding and NER activities of XPA with deletions of −4 to 8 amino acid residues in its RPA70 binding region (15); however, the damaged DNA binding activities of these deletion mutants were not examined, although it was suggested that the deletions would affect the activity of damaged DNA binding by altering the structure of XPA.

Here, we prepared mutant XPA proteins with amino acid substitutions in the RPA70 binding region as well as XPA with a deletion of the RPA32 binding region, and we analyzed their RPA binding, damaged DNA binding, and GGR activities.

**EXPERIMENTAL PROCEDURES**

**Preparation of Mutant XPA Proteins**—To generate His$_{6}$-tagged XPA expression constructs, wild-type and NΔ29 XPA cDNA (14) were cloned in-frame in pET16b (Novagen). Point mutations in the central region of XPA were introduced into pET16b-XPA and pET16b-XPANΔ29 using a QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer’s instructions. DNA sequencing excluded additional mutations introduced elsewhere in the mutant cDNA.

*Escherichia coli* strain BL21 (DE3) pLysS transformed with each plasmid was grown at 25 °C to an A$_{600}$ of 0.75, and the proteins were induced with 1 mM isopropyl 1-thio-β-D-galactopyranoside in the presence of 10 μM zinc acetate. After 6 h of additional growth at 25 °C, the *E. coli* cells were collected, washed with PBS, and stored at −80 °C. Thawed cells were resuspended in a 1/20 volume of TALON buffer (20 mM Tris-HCl, pH 8.0, 300 mM NaCl, 10% glycerol, 10 mM 2-mercapto-ethanol, 0.02% Triton X-100, and 0.5 mM PMSF) and lysed by sonication on ice. Lysates were centrifuged at 40,000 × g for 20 min, and the cleared lysates were incubated with TALON resin (Clontech) for 1 h at 4 °C. The resin was washed three times with TALON buffer and twice with TALON buffer containing 10 mM imidazole. His$_{6}$-tagged XPA proteins were eluted with TALON buffer containing 100 mM imidazole. The eluted samples were dialyzed against buffer H (30 mM Hepes-KOH, pH 7.5, 10% glycerol, 0.1 mM EDTA, 2 mM DTT, 0.01% Triton X-100, and 0.5 mM PMSF) containing 150 mM NaCl and loaded onto a Mini Q column (GE Healthcare) equilibrated with buffer H containing 150 mM NaCl. The column was washed with 10 bed volumes of the same buffer and eluted with 20 bed volumes of a linear gradient of NaCl from 150 to 350 mM in buffer H. Fractions containing XPA were pooled and dialyzed against Manley’s buffer (25 mM Hepes-KOH, pH 7.9, 100 mM KCl, 12 mM MgCl$_2$, 1 mM EDTA, 17% glycerol, and 2 mM DTT).

**Cell-free Incision Assay**—Covalently closed circular DNA containing a single 1.3-intrastrand d(GpTpG)-cisplatin adduct (Pt-GTG) was prepared as described previously (27). Whole cell extracts (100 μg of protein) from each transfected or XP12ROSV (XP-A) cells were preincubated with 200–250 μg of the purified XPA protein for 5 min at 30 °C, and an incision assay was carried out as described previously (11, 26) using 150 ng of Pt-GTG in a total volume of 25 μl.

**RPA Binding Assay**—Recombinant RPA and RPA32 were purified as described previously (14, 28). One hundred nanograms of purified XPA or XPANΔ29 was incubated in 100 μl of PBS containing 1 mM DTT (PBS/DTT) for 1 h at 37 °C for adsorption to 96-well plates. Unbound proteins were washed out with PBS/DTT. Subsequent steps were performed at room temperature. The wells were incubated with PBST (PBS/DTT, 0.1% Tween 20) containing 5% skim milk for 1 h and washed twice with PBST. The XPA-immobilized wells were incubated with RPA (100 ng) or RPA32 (30 ng) in PBST containing 1% skim milk for 2 h. Unbound proteins were removed by washing three times with PBST. The wells were then incubated with anti-RPA32 monoclonal antibody (29) in PBST containing 1% skim milk for 1 h and washed five times with PBST. Next, the wells were incubated with alkaline phosphatase-conjugated anti-mouse IgG in PBST containing 1% skim milk for 1 h. After washing the wells five times with PBST and twice with AP buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, and 5 mM MgCl$_2$), the wells were incubated with 100 μl of AP buffer containing 1 mg/ml p-nitrophenyl phosphate. The reaction was stopped with 100 μl of 1 N NaOH, and absorbance was measured at 405 nm.

Mutant XPA (600 ng) was incubated with anti-XPA polyclonal antibody (1 μg; Santa Cruz Biotechnology, FL-273) in NETN buffer (50 mM Tris-HCl, pH 7.8, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, and 0.5 mM PMSF) for 4 h at 4 °C and immobilized to protein G-Sepharose. The XPA beads were incubated with in vitro translated [35S]methionine-labeled RPA70 (14) or purified RPA in NETN containing 1% skim milk for 2 h at 4 °C. The beads were washed five times with NETN, and the bound proteins were separated by SDS-PAGE and analyzed by an image analyzer or by Western blotting using anti-RPA70 antibody.

**Damaged DNA Binding Assay**—The nitrocellulose filter binding assay was performed as described previously (22) with some modifications. The labeled DNA was incubated with XPA protein for 1 h at 4 °C. DNA bound to XPA was collected by filtration on a nitrocellulose filter.

**Isolation and UV Survival of Stable Transfectants**—For expression in mammalian cells, the XPA cDNAs were cloned into pcDNA3. XP12ROSV cells were transfected with the XPA expression constructs using Effectene transfection reagent (Qiagen) according to the manufacturer’s recommendations. Stable transfectants were selected in the presence of G418 (500 μg/ml). By immunoblot analysis using anti-XPA antibody, a similar level of expression of XPA was observed in the transfectants.

UV survival of the transfectants was assessed by measuring colony-forming ability after UV irradiation. Exponentially growing cells were plated at 1 × 10$^4$ cells per 100-mm dish and exposed to UV-C light at various dosages ~14 h after plating. The cells were then cultured for 7–10 days, fixed with 3.7% formaldehyde, and stained with 0.1% crystal violet. Colonies were counted using a binocular microscope.
Interaction of XPA with RPA in NER

Measurement of UV-induced Photoproducts in Genomic DNA by Slot-blot Analysis—Cells were irradiated with 12 J/m² of UV-C light and incubated for various times. Genomic DNA was isolated using DNeasy Blood and Tissue kit (Qiagen). For the control, DNA was prepared from unirradiated cells. DNA was denatured by heating at 95 °C for 10 min followed by rapid chilling in an ice bath. To quantify the DNA lesions, denatured DNA (100 ng for CPD and 200 ng for (6-4)PP) was spotted on to Hybond-N⁺ (GE Healthcare) with 5 × SSC using a slot-blot apparatus (Schleicher & Schuell). The filter was baked at 80 °C for 2 h. Quantification of CPDs and (6-4)PPs was carried out using anti-CPD antibody (Kamiya Biomedical) and anti-(6-4)PP antibody (Cosmo Bio) (30). Antibodies bound to CPDs and (6-4)PPs were detected using the ECL plus Western blotting detection reagents (GE Healthcare) and analyzed by ImageQuant LAS-4000 (GE Healthcare).

RESULTS

Analysis of Mutant XPA Proteins with N-terminal Deletions—XPA interacts with RPA32 through its N-terminal region (Fig. 1). To investigate the role of the XPA interaction with RPA32 in NER, we prepared and examined the repair activities of mutant XPA proteins with N-terminal deletions. We have reported that the XP-A transfectant expressing a mutant XPA protein with a deletion of the N-terminal 36 amino acid residues (NA36) was more sensitive to UV irradiation than the transfectant expressing wild-type XPA and that the repair of the CPD is decreased in NA36-expressing cells (31).

In this study, we used cell-free NER assays to evaluate the repair activity of the mutant XPA protein with the N-terminal deletion. Whole cell extracts were prepared from XP-A cells expressing either wild-type or NA36 XPA and incubated with closed circular DNA containing a single 1,3-intrastrand (dGdTdG)-cisplatin cross-link. Platinated 23- to 30-mer oligonucleotides released by dual incision were detected after incubation of the DNA with whole cell extracts from HeLa cells and XP-A (XP12ROSV) cells stably expressing wild-type (WT) or NA36 XPA (lanes 1–3) or from XP12ROSV cells (lanes 4 and 7) plus 200 ng of WT or NA29 XPA protein (lanes 5 and 6) were incubated with closed circular DNA containing a single 1,3-intrastrand (dGdTdG)-cisplatin cross-link in the reaction mixture. After incubation for 30 min at 30 °C, reaction products were purified, digested with HindIII and XhoI, separated in a denaturing polyacrylamide gel, transferred to a charged nylon membrane, and hybridized with a 32P-labeled oligonucleotide probe complementary to the DNA sequence surrounding the cisplatin-DNA adduct. 6, 1 ng of 32P-labeled and UV-irradiated (8 kJ/m²) or unirradiated DNA was incubated with WT or NA29 XPA for 1 h at 4 °C. DNA bound to the protein was collected by filtration on nitrocellulose filters, and the radioactivity retained on the filters was measured using a scintillation counter. Closed circle, UV-irradiated DNA; open circle, unirradiated DNA. C, RPA and RPA32 were incubated in WT- or NA29-immobilized wells. Bound protein was detected using anti-RPA32 monoclonal antibody.

To examine the DNA binding activity of XPA, a nitrocellulose filter binding assay was employed. Wild-type XPA preferentially bound to UV-irradiated DNA, and the binding activity of the NA29 mutant for UV-irradiated DNA was comparable with that of wild-type XPA (Fig. 2B).

Next, the RPA binding activity of XPA was examined. Wild-type or NA29 XPA was immobilized to the wells of a 96-well plate, and then the wells were incubated with RPA32 or RPA. Bound RPA32 and RPA were detected with an anti-RPA32 antibody. As expected there was little binding of the NA29 deletion mutant to RPA32 (Fig. 2C), but the deletion mutant bound to RPA because NA29 XPA retains the RPA70
binding region. These results indicate that the binding of the N-terminal region of XPA to RPA32 is important for NER reactions.

**Analysis of Mutant XPA Proteins with Amino Acid Substitutions in the DNA/RPA70 Binding Region**—The region of XPA necessary for binding to RPA70 is located in the central region of XPA, which overlaps with the region essential for the binding to damaged DNA (Fig. 1). To investigate the functional significance of the binding of XPA to RPA70, we introduced amino acid substitutions into the central region. We have reported previously that the interaction between XPA and RPA is sensitive to high salt concentrations (33) and that signal losses in NMR spectroscopy occur at some amino acid residues in this region when the XPA central domain is incubated with the RPA70 single-stranded DNA binding domain (34). Therefore, we changed the conserved and charged amino acid residues in this region to alanines. Twenty-three mutant XPA proteins (Table 1) were prepared, and their NER, RPA70 binding, and damaged DNA binding activities were examined.

The incision activity of the mutant XPA proteins with the XP-A cell extract was measured using single cisplatin cross-link-containing DNA. As shown in Fig. 3, the dual incision activity was moderately affected by K145A (lane 21) and K167A (lane 14), and it was considerably affected by D114A (lanes 4 and 7), K141A (lane 29), T142A (lane 30), K167A/K168A (lanes 8 and 15), and K179A (lane 17). When the K141A/T142A double mutant was used, few incisions were detected (Fig. 3, lane 12). The results are summarized in Table 1.

Next, the RPA70 binding activities of the mutant XPA proteins were examined. Mutant XPA proteins immobilized on protein G-Sepharose via anti-XPA polyclonal antibody were incubated with *in vitro* translated RPA70, and the amount of bound RPA70 was measured. Seven mutants (K141A, T142A, K141A/T142A, K145A, K167A, K167A/K168A, and K179A) showed substantially reduced RPA70 binding activity as compared with wild-type XPA (Fig. 4 and Table 1). Three mutants (K151A, K168A, and K204A) showed slightly lower RPA70 binding activity than wild-type XPA.

The damaged DNA binding activities of the mutants were then examined as described above. Three mutants (K141A/K142A, K145A, and K167A/K168A) exhibited modest loss of damaged DNA binding activity (50–60% of wild-type; Fig. 5 and Table 1) and RPA70 binding activity, with fewer DNA incisions in the cell-free DNA incision assay. Four other mutants (K141A, T142A, K167A, and

### TABLE 1

**Summary of mutant XPA with amino acid substitutions in the DNA/RPA70 binding region**

| No. | Mutation   | Incision | RPA70 binding | Damaged DNA binding |
|-----|------------|----------|---------------|---------------------|
| 1   | Wild type  | ++       | ++            | ++                  |
| 2   | D101A      | ++       | ++            | ++                  |
| 3   | E106A      | ++       | ++            | ++                  |
| 4   | E111A      | ++       | ++            | ++                  |
| 5   | D114A      | ++       | ++            | ++                  |
| 6   | D122A      | ++       | ++            | ++                  |
| 7   | D127A      | ++       | ++            | ++                  |
| 8   | K141A      | +        | ++            | ++                  |
| 9   | T142A      | +        | ++            | ++                  |
| 10  | K141A/T142A| +        | +             | +                   |
| 11  | K145A      | +        | +             | +                   |
| 12  | K151A      | ++       | ++            | ++                  |
| 13  | D154A      | ++       | ++            | ++                  |
| 14  | R158A      | ++       | ++            | ++                  |
| 15  | E159A      | ++       | ++            | ++                  |
| 16  | K167A      | +        | ++            | ++                  |
| 17  | K168A      | ++       | ++            | ++                  |
| 18  | K167A/K168A| +        | +             | +                   |
| 19  | K179A      | +        | +             | +                   |
| 20  | K183A      | ++       | ++            | ++                  |
| 21  | E198A      | ++       | ++            | ++                  |
| 22  | E201A/E202A| ++       | ++            | ++                  |
| 23  | K204A      | ++       | ++            | ++                  |
| 24  | E205A      | ++       | ++            | ++                  |

*Note:* +++, ++, and + indicate >67, 3–67, and <33% of the wild-type level, respectively. Minus means that no incision products were detected.

**FIGURE 3.** *In vitro* dual incision assay using mutant XPA with amino acid substitutions in the DNA/RPA70 binding region. The assay was performed as described in Fig. 2A using whole cell extracts (100 μg of protein) from XP12ROSV cells and 250 ng of mutant XPA protein.
K179A) showed reduced RPA70 binding and fewer DNA incisions but had normal damaged DNA binding activity. These results indicate that the binding of XPA to RPA70 is also important for NER and that amino acid substitutions in some XPA mutants result in both reduced damaged DNA binding and RPA70 binding activities.

**Analysis of Mutant XPA Proteins Defective in RPA Binding**—Next, we examined the mutant XPA proteins that were defective in both RPA32 and RPA70 binding but normal in damaged DNA binding activity. On the basis of the above results, four mutant XPA proteins were prepared. The mutants (NΔ29/K141A, NΔ29/T142A, NΔ29/K167A, and NΔ29/ K179A) have a deletion of 29 N-terminal amino acid residues and single amino acid substitutions to alanine at Lys-141, Thr-142, Lys-167, and Lys-179, respectively, that affect RPA70 binding. The RPA binding activities of the mutant XPA proteins were examined in co-immunoprecipitation experiments using purified proteins (Fig. 6A). Mutants that had only a single amino acid substitution in the central region (K141A, T142A, K167A, and K179A) showed RPA binding activity comparable with that of wild-type XPA. These results suggest that the loss of RPA70 binding activity has little effect on RPA binding activity. In contrast, the RPA binding activity of NΔ29 was slightly decreased. This is consistent with the result shown in Fig. 2C. The double mutants with a 29-amino acid deletion and a single amino acid substitution in the central region showed less RPA binding activity than corresponding mutants that had only a single amino acid substitution or the NΔ29 deletion. Only a small amount of RPA bound to NΔ29/K141A and NΔ29/K179A. It was confirmed that the damaged DNA binding activity of the XPA mutants was comparable with that of wild-type XPA (Fig. 6B).

The NER activities of the mutant XPA proteins were then examined in the cell-free incision assay (Fig. 6C). The double mutants showed much fewer dual incisions than the mutant proteins with either a corresponding single amino acid substitution or the NΔ29 deletion. In addition, the amount of dual
incision products correlated with the RPA binding activity of the mutants.

**In Vivo NER Activity of the XPA Mutants Defective in RPA Binding**—To elucidate the consequence in vivo of the deficiency of XPA in RPA binding, we examined cell viability after UV irradiation in XP-A cells expressing NΔ29/K141A or NΔ29/K179A. We isolated stable transfectants that expressed the wild-type, NΔ29/K141A, or NΔ29/K179A XPA at nearly normal levels. Consistent with the in vitro results, UV survival experiments demonstrated that the cells expressing NΔ29/K141A or NΔ29/K179A were more sensitive to UV irradiation compared to the wild-type cells.

**FIGURE 6.** *In vitro* RPA binding, damaged DNA binding, and dual incision activities of mutant XPA with an N-terminal deletion and single amino acid substitutions in its central region. **A,** mutant XPA protein (125 ng) was immobilized on protein G-Sepharose via anti-XPA polyclonal antibody. The XPA beads were then incubated with 75 ng of purified RPA. After the beads were washed, the bound proteins were separated by SDS-PAGE and analyzed by Western blotting using anti-RPA70 monoclonal antibody. **B,** nitrocellulose filter binding assay was performed as described in Fig. 2B. Closed circle, UV-irradiated DNA; open circle, unirradiated DNA. **C,** in vitro incision assay was performed as described in Fig. 2A using whole cell extract (100 μg of protein) from XP12ROSV cells and 200 ng of mutant XPA protein. **D,** stick representation of the structure of the DNA/RPA70 binding domain of XPA. The side chains of Lys-141, Thr-142, Lys-167, and Lys-179 are indicated in green. Red indicates acidic amino acid residues; blue indicates basic amino acid residues.
K141A and NΔ29/K179A were more sensitive to UV light than the cells expressing wild-type XPA (Fig. 7A). We further investigated DNA repair efficiency by determining the levels of UV-induced (6-4)PPs and CPDs in the transfectants. Up to 2 h after UV irradiation, the removal of (6-4)PPs in cells expressing NΔ29/K141A or NΔ29/K179A was slightly lower than that in cells expressing wild-type XPA, but it was equal to that in cells expressing wild-type XPA at 4–8 h after UV irradiation (Fig. 7B). At 24 h after UV irradiation, however, the removal of CPDs in the mutant XPA transfectants was still low (Fig. 7C). These results suggest that the UV hypersensitivity of the mutant XPA-expressing cells is caused by a delay in CPD removal.

**DISCUSSION**

NER is an enzymatic pathway involving more than 30 polypeptides. At least in mammalian cells, NER factors are considered to assemble sequentially at DNA damage sites (35–38). XPA is a critical factor in NER because a deficiency of XPA results in a high sensitivity to killing by UV light. Although the XPA protein has no enzymatic activity, it functions as a core component in NER reactions by interacting with damaged DNA, RPA, ERCC1, DDB2, and TFIIH. XP-A cells expressing XPA with a deletion of the binding region for RPA32, but the incision reaction required XPA binding to RPA32.

The minimal DNA binding domain has been assigned to the 122-amino acid region between Met-98 and Phe-219 that contains a C4-type zinc finger motif (22) and overlaps with the RPA70 binding region (14). The structure of the DNA/RPA70 binding domain was determined by NMR spectroscopy (34, 40). The domain consists of a zinc-containing subdomain and a C-terminal subdomain (Fig. 6D). From the NMR spectra of the complex of XPA(98−219) and RPA70(181–422) that contains the single-stranded DNA binding domain of RPA70, we previously deduced that the zinc-containing subdomain serves as an RPA70-binding surface. The individual substitution of several acidic amino acid residues in the zinc-containing subdomain with alanine did not affect the binding of XPA to RPA70. In this study, we used full-length XPA and RPA70, which may be the reason why the substitutions had no effect on RPA70 binding.

The D114A mutation affected the incision activity but not the RPA70 binding and damaged DNA binding activities, which were comparable with those of wild-type XPA. The reason why the D114A substitution caused a reduction in incision activity remains to be clarified.

Among the 23 mutant XPA proteins examined, 8 (D114A, K141A, T142A, K141A/T142A, K145A, K167A, K167A/K168A, and K179A) displayed reduced incision activity, and 7 of these 8 had a mutation in the C-terminal subdomain. The seven mutant proteins had either reduced RPA70 binding or both damaged DNA binding and RPA70 binding activities. These results are not consistent with our previous study using RPA70(181−422) (34). However, the earlier finding, obtained by chemical shift mapping, which showed that RPA70(1−326) made contact with both the zinc-containing and C-terminal subdomains (41), is consistent with the results of this study. RPA70 and DNA were shown to share a binding surface in the C-terminal subdomain (41). Using direct cross-linking experiments, RPA70 and XPA were found to be positioned in close proximity to the damage site (42). This may be why some mutants showed reduced binding activity to both damaged DNA and RPA70.

K141A, T142A, K167A, and K179A, which are located near each other (Fig. 6D), showed a reduction in incision activity with no change in damaged DNA binding activity. It was indicated that the interaction between these amino acid residues and RPA70 is critical for NER reactions. A previous report...
showed that Lys-141 and Lys-179 are involved in the interaction of XPA with damaged DNA (43). Electrophoretic mobility shift and photochemical cross-linking assays were conducted for damaged DNA binding using glycine and glutamic acid substitution mutants. The contradictory results might be due to the particular substitution and/or the methods used.

XPA binds to RPA32 and RPA70 through discrete regions, and several binding ratios are possible (e.g. 1:1, 1:2, 2:1, etc.). XPA forms a homodimer that then makes a complex with RPA (XPA2-RPA) (44). Additionally, the loading of RPA on DNA lesions is not dependent on XPA (45). Consistent with this, RPA and the XPA mutants without RPA binding activity (NΔ29/K141A and NΔ29/K179A) accumulated at the damaged sites after local UV irradiation (data not shown). Taken together, these results suggest that the interaction between XPA and RPA at damaged sites, but not in solution, is important for the early stages of NER. Detailed analyses should be performed to determine the spatial positioning of XPA and RPA at damaged sites during NER reactions.

REFERENCES

1. Friedberg, E. C., Walker, G. C., Siede, W., Wood, R. D., Schultz, R. A., and Ellenberger, T. (2006) DNA Repair and Mutagenesis, 2nd Ed., American Society for Microbiology, Washington, D. C.
2. Hanawalt, P. C., and Spivak, G. (1999) in Advances in DNA Damage and Repair (Dizdaroglu, M., and Karayaka, A. E., eds) pp. 169 – 179, Kluwer Academic/Plenum Publishers, New York
3. Boetsma, D., Kreamer, K. H., Cleaver, J. E., and Hoeijmakers, J. H. (1998) in The Genetic Basis of Human Cancer (Vogelstein, B., and Kinzler, K. W., eds) pp. 245 – 274, McGraw Hill Inc., New York
4. Evans, M. K., Robbins, J. H., Ganges, M. B., Tarone, R. E., Nairn, R. S., and Bohr, V. A. (1993) J. Biol. Chem. 268, 4839 – 4847
5. Venema, J., van Hoffen, A., Natarajan, A. T., van Zeeland, A. A., and Mullenders, L. H. (1990) Nucleic Acids Res. 18, 443 – 448
6. Venema, J., van Hoffen, A., Karcagi, V., Natarajan, A. T., van Zeeland, A. A., and Mullenders, L. H. (1991) Mol. Cell. Biol. 11, 4128 – 4134
7. Jaspers, N. G., Raams, A., Silengo, M. C., Wiigers, N., Niedernhofer, L. J., Robinson, A. R., Giglia-Mari, G., Hoogstraten, D., Kleijer, W. J., Hoeijmakers, J. H., and Vermeulen, W. (2007) Am. J. Hum. Genet. 80, 457 – 466
8. de Laat, W. L., Jaspers, N. G., and Hoeijmakers, J. H. (1999) Genes Dev. 13, 768 – 785
9. Jones, C. J., and Wood, R. D. (1993) Biochemistry 32, 12096 – 12104
10. Asahina, H., Kuraoka, I., Shirakawa, M., Morita, E. H., Miura, N., Miyamoto, I., Ohtsuka, E., Okada, Y., and Tanaka, K. (1994) J. Biol. Chem. 269, 5052 – 5056
11. Evans, E., Moggs, J. G., Hwang, J. R., Egly, J. M., and Wood, R. D. (1997) J. Biol. Chem. 272, 22991 – 22994
12. Kuraoka, I., Morita, E. H., Saji0, M., Matsuda, T., Morikawa, K., Shirakawa, M., and Tanaka, K. (1996) Mutat. Res. 362, 87 – 95
13. Nakatsu, Y., Asahina, H., Citterio, E., Rademakers, S., Vermeulen, W., Houtsmuller, A. B., and Vermeulen, W. (2000) Mol. Cell. Biol. 20, 5396 – 5402
14. Wakisugi, M., Kasahima, H., Fukase, Y., Imura, M., Imai, R., Yamada, S., Cleaver, J. E., and Matsunaga, T. (2009) Nucleic Acids Res. 37, 516 – 525
15. Miyamoto, I., Miura, N., Niwa, H., Miyazaki, J., and Tanaka, K. (1992) J. Biol. Chem. 267, 12182 – 12187
16. Naga0, A., Saji0, M., Kuraoka, I., Matsuda, T., Kodo, N., Nakatsu, Y., Miki0, T., Min0, M., Biggert0, M., Wood, R. D., Sijbers, A., Hoeijmakers, J. H., and Tanaka, K. (1995) Biochem. Biophys. Res. Commun. 211, 960 – 966
17. Li, L., Eldredge, S. J., Peterson, C. A., Bales, E. S., and Lesters, R. J. (1994) Proc. Natl. Acad. Sci. U.S.A. 91, 5012 – 5016
18. Park, C. H., Mu, D., Reardon, J. T., and Sancar, A. (1995) J. Biol. Chem. 270, 4896 – 4902
19. Nocentini, S., Coin, F., Saji0, M., Tanaka, K., and Egly, J. M. (1997) J. Biol. Chem. 272, 768 – 785
20. Nakatsu, Y., Asahina, H., Citterio, E., Rademakers, S., Vermeulen, W., Houtsmuller, A. B., and Vermeulen, W. (2003) Mol. Cell. Biol. 23, 5755 – 5767