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ROLE OF INTERACTION OF XPF WITH RPA IN NUCLEOTIDE EXCISION REPAIR

Laura A. Fisher[1], **Mika Bessho**[1], **Mitsuo Wakasugi**[2], **Tsukasa Matsunaga**[2], and **Tadayoshi Bessho**[1,*]

[1]Eppley Institute for Research in Cancer and Allied Diseases, University of Nebraska Medical Center, Omaha, Nebraska, USA

[2]Laboratory of Human Molecular Genetics, Institute of Medical, Pharmaceutical and Health Sciences, Kanazawa University, Kanazawa 920-1192, Japan

Abstract

Nucleotide excision repair (NER) is a very important defense system against various types of DNA damage and it is necessary for maintaining genomic stability. The molecular mechanism of NER has been studied in considerable detail, and it has been shown that proper protein-protein interactions among NER factors are critical for efficient repair. A structure-specific endonuclease, XPF-ERCC1, which makes the 5' incision in NER, was shown to interact with a single-stranded DNA binding protein, RPA. However, the biological significance of this interaction was not studied in detail. We used the yeast two-hybrid assay to determine that XPF interacts with the p70 subunit of RPA. To further examine the role of this XPF-p70 interaction, a p70-interaction deficient mutant form of XPF that contains a single amino acid substitution in the N-terminus of XPF was isolated by the reverse yeast two-hybrid assay using randomly mutagenized XPF. Biochemical properties of this RPA-interaction deficient mutant XPF-ERCC1 are very similar to wild type XPF-ERCC1 in vitro. Interestingly, expression of this mutated form of XPF in the XPF-deficient Chinese hamster ovary (CHO) cell line, UV41, only partially restores NER activity and UV resistance *in vivo* compared to wild type XPF. We discovered that the RPA-interaction deficient XPF is not localized in nuclei and the mislocalization of XPF-ERCC1 prevents the complex from functioning in NER.

Introduction

UV light penetrates cells, causing damage to DNA including 6-4 photoproducts and cyclobutane pyrimidine dimers (CPDs). These lesions are removed by NER [1; 2; 3] and, if not repaired, they inhibit replication and induce mutations by incorporation of incorrect nucleotides across from the damage [4]. Defects in NER lead to a rare autosomal recessive disease, xeroderma pigmentosum (XP). Patients with this disease are extremely sensitive to sunlight and have a predisposition to skin cancer [5].

Six factors, XPA, RPA, XPC-HR23, TFIIH, XPG and XPF-ERCC1, are essential to reconstitute the excision step of NER *in vitro*[6; 7; 8]. Protein-protein interactions among these

*To whom correspondence should be addressed. Tel: + 1 402 559 7018 tbessho@unmc.edu.

six factors are crucial for the efficient accomplishment of the excision reaction[9; 10; 11; 12; 13; 14]. NER machinery must recognize DNA damage with a high specificity. None of the six NER factors recognizes DNA damage with such a high specificity[15; 16; 17; 18]. It has been recently proposed that cooperative damage recognition by XPA, RPA, XPC-HR23 coupled with the kinetic proofreading by TFIIH provide a requisite damage-specific recognition in NER[16; 19; 20]. XPA, RPA, XPC-HR23 (and TFIIH through the interaction with XPC) forms an unstable complex at the site of DNA damage[16; 17; 18]. This cooperative binding achieves a higher specificity for damage recognition[15; 16]. TFIIH contains the two DNA helicases, XPB and XPD, and the ATP-dependent unwinding by these helicases opens the DNA around the lesion, forming a bubble-like structure[21; 22]. This unwinding by TFIIH further verifies the specificity of damage recognition and also promotes entry of XPG into the complex to further stabilize the damage recognition complex (kinetic proofreading)[17]. Lastly, XPF-ERCC1is recruited through an interaction between ERCC1 and XPA[12; 14; 23] and dual incision by XPG at the 3' of the damage and XPF-ERCC1 at the 5' of the damage removes a short oligonucleotide with the damage[24].

Human RPA is involved in various DNA transactions including DNA replication, repair, and recombination[25]. It was shown that RPA interacts with XPF-ERCC1, suggesting a potential role for this interaction in NER[7; 26]. This interaction alone does not seem to be sufficient for recruiting XPF-ERCC1 to the pre-incision complex [9]; however, it has been suggested that RPA helps position XPF-ERCC1 properly[27]. To define the role of the interaction between XPF-ERCC1 and RPA, we isolated a point mutant form of XPF, which specifically lacks the interaction with RPA, but retains other known biochemical properties. This XPF mutant can only partially restore NER activity and UV resistance in the XPF-deficient UV41 cells. Unexpectedly, cell fractionation studies revealed the RPA-interaction deficient XPF was mislocalized in cytosolic fraction. We conclude that cellular localization of XPF is controlled by the interaction with RPA.

Results

XPF interacts with p70 through its N-terminus region

To determine which subunit of RPA, p70, p34, or p14, interacts with XPF, we performed the two-hybrid assay. XPF, fused to the DNA binding domain of GAL4, and each RPA subunit, fused to the activating domain of GAL4, were co-expressed in the tester strain of yeast, CG1945. Interaction was detected as growth on a restrictive medium between XPF and the p70 subunit of RPA, to a lesser degree between XPF and the p34 subunit, and no interaction was detected with the p14 subunit (Figure 1A). As a control, XPF was co-expressed in yeast with ERCC1 fused to the activating domain of GAL4. The binding between XPF and ERCC1 is very strong and did result in a positive signal in this assay (Figure 1A).

To define the RPA interaction domain of XPF, a reverse two-hybrid assay was employed based on a screening strategy described by Krejci et. al.[28]. Random mutations were introduced into the XPF gene by propagating the XPF- plasmid in a mutator strain of *E. coli*, XL-1 Red. The randomly mutagenized XPF plasmids and wild type p70 plasmids were co-transformed into a tester yeast strain. Colonies that failed to grow on the selective plates, indicating a lack of interaction between XPF and p70, were selected from the growth medium plates and the plasmid DNA was isolated (Figure 1B). As a control, CG1945 yeast cells were also co-transformed with randomly mutated XPF and wild type ERCC1 to screen for a disruption in XPF-ERCC1. Twelve candidates for a lack of RPA interaction and twelve candidates for lack of interaction with ERCC1 were found.

To verify that the lack of interaction was due to a mutation in the XPF gene rather than elsewhere on plasmid, the XPF gene was subcloned from the isolated mutated plasmids into

a new pGBT9 plasmid All the isolated XPF mutants were due to possible mutations in the XPF gene (Figure 1C and data not shown). Six of twelve candidate XPF mutants were sequenced and one mutant with a single amino acid substitution, Proline to Serine at amino acid 85 (P85S) was identified. We also isolated a mutant XPF that lacks the interaction with ERCC1 by the same procedure. Five of twelve candidate genes for this mutation were sequenced. Two contained the same point mutation, a Lysine to Proline substitution at position 801 (L801P), which is within the known ERCC1 interaction domain[29] (Figure 2A).

RPA interaction deficient XPF mutants retain ERCC1 binding and endonuclease activities

To properly assess the role of the XPF and RPA interaction in NER, we need to verify that an isolated mutant XPF only lacks the RPA-interaction but retains other known biochemical activities. To this end we examined the impact of the XPF(P85S) mutation on other known biochemical properties of XPF-ERCC1, the XPF-ERCC1 interaction and the structure specific endonuclease activity of XPF.

To investigate the interaction of XPF(P85S) with ERCC1, the XPF(P85S) gene in pGBT9 plasmid was co-expressed with ERCC1-pGAD424 in the yeast two-hybrid system. XPF(P85S) interacted with ERCC1 as efficiently as wild type XPF in this assay but XPF(L801P), which was isolated as an ERCC1-binding deficient mutant in the reverse two-hybrid assay, failed to interact with ERCC1 (Fig. 2A). Additionally, the interaction between XPF(P85S) and ERCC1 was confirmed by efficient purification of XPF(P85S) as a complex with ERCC1. A FLAG tag was added to the XPF(P85S) and wild type XPF genes. FLAG tagged XPF (^{FLAG}XPF) and His tagged ERCC1 (ERCC1^{His}) were co expressed in SF-9 insect cells and purified as a complex using an anti-FLAG antibody column, followed by a Ni-agarose column. ^{FLAG}XPF can be purified only as a complex with ERCC1^{His} after the Ni-agarose under this purification scheme. We were able to purify XPF(P85S)-ERCC1 as efficiently as wild type XPF-ERCC1 (Fig. 2B). These results also indicate that XPF(P85S) retains the ERCC1 binding activity.

Next, we examined the structure-specific endonuclease activity of XPF(P85S)-ERCC1. XPF-ERCC1 participates in NER as a 5’-incision nuclease, and an endonuclease deficient XPF cannot support NER. Thus, any mutation that abolishes the endonuclease activity of XPF-ERCC1 is expected to result in defective NER. To verify that the P85S mutation does not compromise the nuclease activity of XPF-ERCC1, the endonuclease assay was performed using a radio-labeled splayed DNA substrate [30]. Purified wild type XPF-ERCC1 or the RPA-interaction deficient XPF(P85S)-ERCC1 was incubated with the labeled substrate. XPF-ERCC1 makes an incision on double-stranded (ds) DNA 3’ to a junction between dsDNA and single-stranded DNA and generates a 33 nt fragment with this substrate (Supplemental Figure 1A). The endonuclease activity of XPF(P85S) -ERCC1 (Fig. 2C, lane 4) was equivalent to the wild type XPF-ERCC1 complex (Fig. 2C, lane 3) at each concentration we tested (Figure 2C in the graph under the gel). The results confirm that the P85S mutation does not affect the endonuclease activity of XPF-ERCC1. Thus, we isolated an XPF mutant, which is specifically defective in the interaction with p70 subunit of RPA, but retains the ERCC1 binding and endonuclease activities.

To examine the contribution of the XPF-RPA interaction in NER, we performed in vitro excision assay[31]. A defined substrate DNA with a single (6-4)photoproduct was prepared to analyze NER activity in vitro. ^{32}P was introduced near the (6-4)photoproduct in the defied substrate. Dual incision by NER releases a labeled 24–32 nt oligonucleotide with the (6-4)photoproduct and these short oligonucleotides can be detected by a sequencing gel (Supplemental Figure 1B). The extract prepared from UV41 cells was defective in NER in vitro as previously shown[7] (Supplemental Figure 2). We added purified XPF-ERCC1 to the UV41 extract to investigate if the complex restores NER activity. An RPA-binding defective

XPF(P85S)-ERCC1 complemented the *in vitro* NER defect in UV41extract cells as effectively as wild type XPF-ERCC1 at two different concentrations (Figure 2D and the graph next to the gel). These results show that XPF(P85S)-ERCC1 retains intact NER activity in vitro and the XPF-RPA interaction is dispensable for or has very little impact on in vitro NER activity.

Disruption of the interaction between XPF and RPA reduces NER efficiency in Chinese hamster ovary cells

The Chinese hamster ovary (CHO) cell line, UV41, is deficient in XPF and thus sensitive to UV irradiation. To study the in vivo role of the interaction between XPF and RPA in NER, wild type human XPF or the RPA-interaction deficient mutant XPF(P85S) was expressed stably in UV41. The cellular survival of clones expressing either wild type human XPF or the XPF(P85S) mutant was investigated following exposure to UVC irradiation. These cells expressed the XPF and XPF(P85S) at similar levels (Supplemental Figure 3). It was reported that expression of wild type human XPF in UV41 cells restores their resistance to UV light to a level similar to their NER proficient parent cell line, AA8, while an endonuclease deficient XPF mutant failed to restore the UV resistance in UV41[30]. Importantly, the UV41 cells expressing XPF(P85S) showed only a partial restoration of the UV resistance, while UV41 expressing wild type XPF displayed a similar level of the UV resistance to NER proficient AA8 (Fig 3A). These data show that the RPA-interaction defective XPF mutant cannot support NER fully, suggesting a specific role for the interaction of XPF with RPA in NER.

To further examine the role of the interaction between XPF and RPA in NER, we measured the *in vivo* repair of 6-4 photoproducts (Figure 3B). The same UV41 cells expressing human wild type or the XPF(P85S) mutant used for the cellular sensitivity study were exposed to UVC light at 10 J/m^2. Untransfected UV41 cells were used as control. The cells were collected at various time points after the UVC exposure (repair incubation) and the number of 6-4 photoproducts remaining in the genomic DNA was determined by ELISA. Interestingly, only 25% of the 6-4 photoproducts were removed in UV41 cells expressing XPF(P85S) mutant, while nearly 80% of the lesions were removed after 24 hours in UV41 cells expressing wild type XPF (Fig 3B). Very little repair of 6-4 photoproducts was detected in control UV41 cells. We also measured repair of pyrimidine dimers from the genome. However, due to very little repair of pyrimidine dimers from the genome in rodent cells, we did not detect any difference between UV41 cells expressing wild type XPF-ERCC1 and XPF(P85S)-ERCC1 mutant (data not shown). These *in vivo* repair experiments clearly demonstrate that the interaction between XPF and RPA contributes to efficient NER. These *in vivo* experiments are the first demonstration of the biological significance of the interaction between XPF and RPA.

RPA-interaction deficient mutant XPF(P85S) is mislocalized in UV41 cells

Our *in vivo* results clearly demonstrate the significance of the XPF-RPA interaction in efficient NER, while an RPA-interaction defective XPF(P85S)-ERCC1 retains intact NER activity in vitro. One possible role of this interaction is to regulate the cellular localization of XPF. It was reported that a mutation in the XPF-binding domain in the ERCC1 gene resulted in a reduced protein level of the XPF-ERCC1 complex and mislocalization of ERCC1 in cytoplasm[32].

The level of wild type XPF and XPF(P85S) in UV41 cells was determined in the nuclear and cytosolic fraction (Figure 4). XRCC1, which is required for the DNA repair synthesis/ligation step in NER, is known to localize in the nuclear fraction [33; 34; 35]. XRCC1 was only detected in the nuclear fraction (Figure 4, lanes 4–6). Approximately 90% of p70 subunit of

RPA was also detected in the nuclear fraction as expected (Figure 4, lanes 4–6). Two known cytoplasmic proteins, Heat Shock Protein 90 (HSP90) and α-tubulin, were detected mostly in cytosolic fraction (Supplemental Figure 4, lanes 1–3) and ~70% of p32 subunit of RPA was detected in nuclear fraction (Supplemental Figure 4, lanes 4–6). Thus, there is little or no leak of the nuclear proteins in the cytosolic fraction under the conditions used. Unexpectedly, 80–90% of XPF(P85S) was localized in the cytosolic fraction, while ~70% of wild type XPF was in the nuclear fraction (Figure 4). The significant reduction of XPF(P85S) in nuclear fraction could explain the reduced removal of UV photoproducts and partial restoration of UV resistance in UV41 by XPF(P85S). These results demonstrate that the RPA-interaction deficient XPF is mislocalized in cytoplasm, therefore, it only partially restores NER activity in UV41 cells. We propose that RPA regulates cellular localization of XPF and as a consequence, it controls NER.

Discussion

The molecular mechanism of NER has been studied in detail using both *in vitro* and *in vivo* assays. Protein-protein interactions among NER factors play crucial roles for efficient and proper assembly of the factors[36]. RPA interacts with XPF-ERCC1, however, the biological significance of the interaction and its role in NER were poorly defined[7; 26]. In this study, we isolated an RPA-interaction deficient XPF mutant, XPF(P85S), by using a reverse yeast-two-hybrid assay to investigate the role of this interaction in NER. This mutant specifically lacks the RPA-interaction, but retains the ERCC1 binding and endonuclease activities *in vitro*. This mutant also can restore the NER defect in UV41 in vitro as efficient as wild type XPF-ERCC1. Interestingly, the expression of XPF(P85S) in the XPF-deficient UV41 cells only partially restored the repair of 6-4 photoproducts in the genome *in vivo*. As a consequence, the UV41 cells with XPF(P85S) showed mild UV-sensitivity. Unexpectedly, cell fractionation experiments revealed that this partial *in vivo* NER defect in the XPF(P85S)-expressing UV41 cells is due to mislocalization of XPF(P85S). We conclude that RPA regulates cellular localization of XPF through the direct interaction.

XPF forms a heterodimer with ERCC1 and XPF-ERCC1 is a structure-specific endonuclease[7; 26; 27]. All the known functional domains of XPF are located in the C-terminus of the gene[5]. Both a catalytic domain (GDX_nERKX_3D) and a DNA binding domain (two consecutive helix-hairpin-helix motifs) in the C-terminus of the gene, determine the structure specific binding/endonuclease activity of XPF-ERCC1. The ERCC1-interaction domain is also located in this region[37]. In contrast, a function of the N-terminus of the XPF is unclear, and this region is often described as an “SF2 helicase-like” domain with no helicase activity due to amino acid substitutions at essential catalytic residues for ATPase activity. Interestingly, we identified the RPA interaction domain in this evolutionarily conserved region at the N-terminus of the XPF gene. To investigate a contribution of this region to NER, we generated two additional point mutations in this region. Conserved amino acids in this region, R86 and T89, were substituted with Alanine and the impact of these point mutations was examined. UV41 cells expressing these XPF mutants (R86A or T89A) showed a mild UV sensitivity similar to cells expressing XPF(P85S) (Supplemental Figure 5), while these mutant XPF-ERCC1 possess similar biochemical properties to wild type XPF-ERCC1. These results suggest an important contribution of these conserved amino acids to efficient NER.

Sub-cellular localization of a protein could be influenced by nuclear localization signal (NLS) and/or nuclear export signal (NES). Using a program developed by Kosugi et al.[38], we identified two potential NLSs in XPF. One is located in N156-F164 and the other is in R483-T495 of XPF. It is very interesting to investigate that these two regions control nuclear localization of XPF and, if they do, how the P85S mutation influences these NLSs.

Interestingly, the XPF(R153P) that was found in XP51RO is also localized in cytoplasm and the cell line derived from the patient showed a significant defect in NER[39]. The R153P mutation could negatively influence NLS in N156-F164 in XPF due to the close proximity of the point mutation. During the preparation of this report, mislocalization of XPF-ERCC1 was shown to be a common cause of the reduced NER in XP-F cells[40]. The authors proposed that mis-foldings of XPF induced by disease-causing mutations cause mislocalization of these mutant XPFs. Because a mis-folding of a protein might have an impact on protein-protein interactions, some of these disease-causing mutant XPFs might have a defect in the binding to RPA. It is also possible that a mis-folding induced by the P85S mutation is the cause of the mislocalization of the XPF(P85S). Although a disease-causing mutation in the RPA-binding domain in XPF we identified has not been reported, a proper regulation of cellular localization of XPF by RPA is a critical mechanism to maintain active NER.

Materials and Methods

Random Mutagenesis

XL1 Red cells were transformed with the pGBT9-XPF and incubated at 37°C with shaking for 24 to 48 hours. The cells were harvested and the randomly mutagenized plasmid DNA was isolated using a Qiagen kit.

Yeast two-hybrid Assay

We utilized the pGBT9 vector that contains the DNA binding domain, and pGAD424 that contains the activating domain of Gal 4 from the Matchmaker Gal4 Two-Hybrid system from Clontech. Because XPF fused to the activating domain, and ERCC1 to the DNA binding domain resulted in a high background, the XPF gene was inserted into the pGBT9 vector and ERCC1, p70, p34, and p14 genes were each cloned into the pGAD424 vector and co-transformed into the CG1945 tester strain. Screening was preformed following the manufacturer's protocol. Briefly, co-transformants were plated on growth plates and patches from the plates were replica-plated on growth and selective plates. For detection of interaction deficient mutants, colonies that showed little to no growth on the selective plates were picked from the growth plates and then replica-plated again onto growth and selective plates. Colonies that still failed to grow on the selective plates were isolated from the growth plates as candidates. The mutated pGBT9-XPF plasmid was isolated from each candidate clone and the candidate XPF genes were re-cloned into pGBT9 and co-transformed into CG1945 yeast cells with the p70 subunit containing pGAD plasmid. The genes that maintained the defect in XPF to RPA interaction were selected and the DNA sequenced.

Cell culture

UV41 cells were transfected with pcDNA3.1 vector containing the XPF gene using Lipofectamine 2000. Cells that were stably transfected with the plasmid were selected with 0.5 mg/ml G418 in MEM α medium supplemented with 10% FBS. Individual clones of the stably transfected UV41 cells were isolated and grown in MEM α with 10% fetal bovine serum at 37°C with 5% CO_2.

Western blotting

The expression level of XPF in the UV41 clones was determined by Western blotting. Cell lysates were prepared using lysis buffer (1% NP40, 1% Triton X-100, 50 mM Tris pH 7.5, and 150 mM NaCl). Proteins were separated on a 10% SDS gel, transferred to nitrocellulose membrane, and probed with anti-XPF antibody (Ab-1, NeoMarkers) that recognizes human XPF, but not rodent XPF.

Colony-forming assay

Stably transfected clones of UV41 cells were seeded at 500 cells/10 cm dish and allowed to attach overnight. NER proficient AA8 cells were used as a positive control. The next day the medium was removed and the cells were washed with PBS prior to exposure to UV light (254 nm) at 2 or 4 J/m^2. Fresh medium was added and the cells were incubated at 37°C in 5% CO_2 for one week. Cells that were able to repair the damage grew into colonies, which were stained with Giemsa and counted. The surviving fraction is expressed as the percent of cells that formed colonies relative to the number of colonies on control plates.

Protein expression and purification

A FLAG tag was attached to the N-terminus of each form of XPF by ligating an oligonucleotide containing the FLAG tag sequence at the N-terminus of the XPF gene in the pFastBac1 vector. The proteins were then expressed in Sf-9 or Sf-21 cells for purification. The cells from 24 10 cm dishes were harvested and lysed in five times the cell pellet volume insect cell lysis buffer (50mM Tris-HCl pH 7.5, 150 mM NaCl, and 1 % NP-40) for 30 minutes with rocking at 4°C. The cell debris was removed by centrifugation and the cell lysate was applied to a 250 μl anti-FLAG agarose (M2, Sigma). The anti-FLAG agarose was washed five times with 5 ml 1M KCl Tris buffer (20 mM Tris-HCl pH 7.5, 0.1 mM EDTA, 1 M KCl, 10% glycerol), then once with starting buffer (20 mM Tris HCl pH 7.5, 0.1 mM EDTA, 0.8 M KCl, 10% glycerol, 10 mM imidazole). The protein was eluted with 2 mg/ml FLAG peptide in starting buffer by rotating at 4°C for 1–2 hrs. This elution was repeated once and then the resin was washed with the starting buffer. Peak elution fractions were pooled and incubated with 100 μl Ni-agarose overnight with end-over-end rotation at 4°C. The resin was washed with P-1000 buffer (40mM HEPES pH 7.5, 1M NaCl, 10% v/v glycerol) containing 10 mM imidazole and then with PI-10 buffer (40mM HEPES pH 7.5, 100 mM NaCl, 10% v/v glycerol, 10 mM imidazole). The protein was eluted twice with 100 μl PI-100 (40mM HEPES pH 7.5, 100 mM NaCl, 10% v/v glycerol, 100 mM imidazole) by rotating 4°C for 30 min then by 100 μl PI-300 (40mM HEPES pH 7.5, 100 mM NaCl, 10% v/v glycerol, 300 mM imidazole) twice. Purity was assessed by silver staining and Western blots.

Recombinant human RPA was expressed and purified as described in Henricksen et al.[41].

DNA Substrates—The splayed DNA substrate was prepared by labeling the Ytop oligonucleotide at the 5’ end with [γ^{32}-P]-ATP and annealing it to the partially complementary Y3 oligonucleotide, listed below. Double stranded DNA was isolated from a 5% native gel.

Ytop 5’-
ACGTTGTAAAACGACGGCCAGTGAATTCGAGCTCGGTACCCGGAGATCCTC
TAGAGTCGACCTGCAGTGGCTT-3’

Y3: 5’-
CCTAACAGTACTTGATCAGAGCTCTTCGAGAATTTTACCGAGCTCGAATTCA
CTGGCCGTCGTTTTACAACGT-3’ (the complementary region is underlined)

The 6-4 photoproduct (6-4 PP) containing 136 mer was constructed as described [31] with some modifications. A 10 mer containing a 6-4 PP (5'-CGTAT<>TATGC-3', <> denotes the 6-4PP, purchased from Synthetic Organic Chemistry Core Laboratory at the University of Texas Medical Branch at Galveston) was labeled with [γ^{32}-P]-ATP. The six overlapping oligonucleotides listed below; S1, S2, S3, S4, C and the 6-4PP containing 10 mer, were annealed and then ligated. The full-length substrate was purified from a 5% native gel.

The oligonucleotides used for the 6-4PP containing 136 mer are;

S1 5'-
TTACGCCAGATATCAAATTAATACGACTCACTATAGGGAGAAGCTTGCATG
CCACGCGTCCACT-3'

S2 5'-
CGAGAGGTCGACTCTAGAGGATCTCGAATTCACTGGCCGTCGTCCGTCGCA
ACGTCGA-3'

S3 5'-
GACGCGTGGCATGCAATATCTGGCGTATTTGAGTGAGTCGTATTAAGCTTCT
CCCTATAA-3'

S4 5'-
TCGACGTTGCGACGGACGACGGCCAGTGAATTCGAGATCCTCTAGAGTCGA
CCT-3'

C 5'-GTGAGCATAATACGGCTC-3'

The 6-4PP containing 10 mer 5'-CGTAT<>TATGC-3'.

In vitro excision assay

In vitro excision assays was performed as in Reardon and Sancar[31]. A 136 bp internally ^{32}P-labeled DNA substrate containing a 6-4PP were incubated with UV41 cell extract and purified proteins in reaction buffer (35 mM HEPES pH 7.5, 10 mM Tris pH 7.5, 60 mM KCl, 40 mM, NaCl, 6 mM $MgCl_2$, 0.8 mM DTT, 0.4 mM EDTA, 12 μg BSA, and 3% glycerol) at 30°C for the indicated time. 20 μg Proteinase K and SDS to a final concentration of 0.37% was added and incubated at 37°C for 15 minutes. Proteins were removed by phenol/chloroform extraction and the DNA was precipitated with ethanol. The products were analyzed on an 8% denaturing polyacrylamide gel.

Endonuclease assay

The endonuclease assays were preformed as in Fisher et al. [30]. Briefly, 5'-^{32}P-labeled DNA substrate was incubated with purified proteins in the reaction buffer (10 mM HEPES, 25 mM KCl, 0.05 mM EDTA, 0.5 mM DTT, 10% glycerol, and 5 mM $MgCl_2$) at 30°C for 45 min. Proteins were removed by phenol/chloroform extraction and DNA precipitated with ethanol, the reaction products were analyzed on a 10% denaturing polyacrylamide gel. The gel was dried and exposed to a PhosphorImager screen. After scanning with a Typhoon 9700, the image was quantitated using ImageQuant software. The percent incised was determined by dividing the value of the 33nt band by the total value of DNA loaded in the lane.

DNA Damage Removal

UV41 cells, as well as UV41 cells expressing wild type human XPF or XPF(P85S) were exposed to 10 J/m^2 UVC light and then grown for the indicated period of time. Genomic DNAs were purified with the DNeasy kit (Qiagen), and the amounts of CPDs and 6-4PPs were determined by an enzyme-linked immunosorbent assay (ELISA) using specific monoclonal antibodies, TDM-2 or 64M-2, respectively, as described previously [42; 43].

Cell fractionation

CHO cells were resuspended into hypotonic buffer (20 mM HEPES-KOH (pH 7.8), 5 mM KCl, 1.5 mM $MgCl_2$, 1 mM DTT, and 15 % glycerol) equal to cell pellet volume (CPV) and incubate on ice for 15 min. Cells were passed through a 26-gauge syringe needle ten times and incubated on ice for 30 min. Cells were centrifuged at 2000g for 10 min and supernatant containing cytoplasmic proteins was transferred to a tube. Isolated crud nuclei were

resuspended into buffer C (20 mM HEPES-KOH (pH 7.8), 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, and 25 % glycerol) equal to CPV. Nuclei were extracted by passing through a 26-gauge syringe needle ten times followed by incubating for 30 min with rocking. Soluble nuclear fraction was isolated by centrifuging at 25,000g for 30 min and transferred to a new tube.

50 μg of nuclear and cytosolic fractions were separated by 10% SDS-PAGE and level of XPF protein in each fraction was determined by western blot with anti-XPF antibody (NeoMarkers, Ab-1). XRCC1 protein and p70 subunit of RPA was used as nuclear protein and also as loading control. XRCC1 and RPA were detected with anti-XRCC1 antibody (Abcam, 3-3-25) and anti-RPA(p70) antibody (BETHYL Laboratories), respectively.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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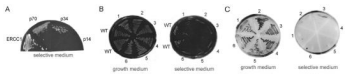

Figure 1. Isolation of a mutant XPF that is defective in interaction with RPA

(A) *XPF interacts with the p70 subunit of RPA.* Wild type XPF was co-expressed in CG1945 yeast with each of the RPA subunits or ERCC1 as a control and grown on synthetic growth medium (SD-trp-leu). Colonies were streaked on selective medium (SD-trp-leu-his) to detect an interaction. (B) *Screening of RPA interaction deficient XPF mutants.* Yeast colonies co-expressing randomly mutated XPF and p70 were streaked onto growth and selective medium. Colonies that did not grow on the selective plates were taken from the growth medium and the plasmid DNA was isolated. Two colonies co-expressing wild type XPF and p70 were streaked as positive controls along with six colonies arising from co-expression of p70 and randomly mutated XPF, indicated as 1–6 in panels B and C. (C) *Identification of a mutation in the RPA interaction deficient mutant XPF*. A repeat of the yeast two hybrid showing six of twelve candidate XPF genes that were re-cloned into fresh plasmids and co-transformed with the p70 gene.

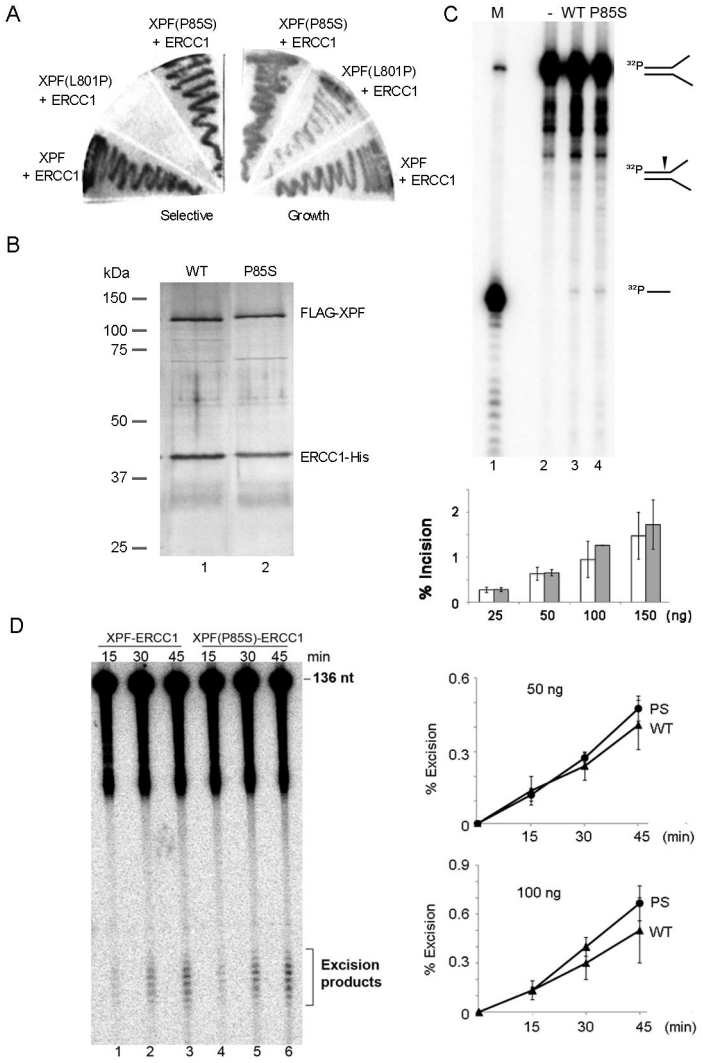

Figure 2. XPF(P85S) retains intact biochemical activities

(A) *XPF(P85S) retains the interaction with ERCC1*. Pair wise yeast two hybrid assay of ERCC1 co-expressed with the RPA interaction deficient XPF(P85S), the ERCC1 interaction deficient XPF(L801P), and wild type XPF are shown. (B) *Purification of XPF-ERCC1 from insect cells.* ^{FLAG}XPF or ^{FLAG}XPF(P85S) was co-expressed with ERCC1^{HIS} in Sf-9 cells and purified with an anti-FLAG agarose followed by a Ni-agarose column. 100 ng of each purified complex was analyzed on a 10% SDS gel and silver stained. (C) *XPF(P85S)-ERCC1 retains the structure specific endonuclease activity in vitro.* ^{FLAG}XPF- and ^{FLAG}XPF(P85S)-ERCC1 ^{HIS} complex was incubated with a 73 nt long splayed DNA substrate with a 38 nt complementary region at the 5'-end and then resolved on an 8% sequencing gel. Both XPF- and XPF(P85S)-ERCC1 make an incision on the dsDNA five nt 5' to the junction, producing a 33 nt product (marked with an arrow). Lane 1, SacI digested substrate shows the 33nt product; lane 2, no enzyme; lane 3, 100 ng XPF-ERCC1; lane 4, 100 ng XPF(P85S)-ERCC1. The average of percent incision with wild type XPF-ERCC1 (open bars) or XPF(P85S)-ERCC1 (gray bars) at the indicated amounts was plotted. Error bars indicate standard deviation from three independent experiments. (D) *XPF(P85S)-ERCC1 restores in vitro NER activity in UV41 cell extract as efficiently as wild type XPF-ERCC1*. (Gel) An internally labeled 136 bp DNA substrate containing a 6-4 PP was

incubated with UV41 cell free extract complimented with 100 ng of either wild type XPF-ERCC1 (lanes 1–3) or XPF(P85S)-ERCC1 (lanes 4–6) at 30°C. Aliquots were withdrawn at 15 min (lanes 1 and 4), 30 min (lanes 2 and 5), and 45 min (lanes 3 and 6). Following Phenol/chloroform extraction and ethanol precipitation, the reaction products were analyzed on an 8% sequencing gel. The gel was dried, exposed to a PhoshorImager screen, and quantitated using Image Quant software. The percent of damaged DNA removed (marked as "Excision products") was calculated by dividing the value of the DNA excised with the total value of DNA in the lane. The percent of DNA excised was: lane 1, 0.1%; lane 2, 0.4%; lane 3, 0.7%; lane 4, 0.2%; lane 5, 0.5%; lane 6, 0.8%. (Graphs) The average of percent excision with wild type XPF-ERCC1 (triangles) or XPF(P85S)-ERCC1 (circles) at each amount of the complex was plotted. Error bars indicate standard deviation from three independent experiments.

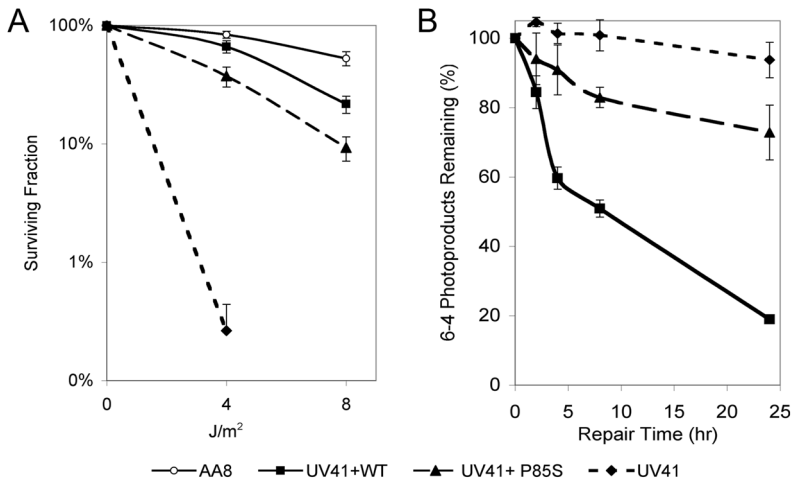

Figure 3. XPF(P85S)-ERCC1 only partially restores a defect in nucleotide excision repair in UV41

A deficiency in the XPF-RPA interaction reduces the ability of cells to repair 6-4 photoproducts and to survive exposure to UVC radiation. (A) *XPF(P85S)-ERCC1 fails to fully restore UV resistance in UV41.* AA8 and UV41 cells, alone or expressing XPF or XPF(P85S) were exposed to 4 or 8 J/m^2 UVC. Surviving cells were allowed to grow into colonies and counted after one week. The surviving fraction is expressed as the percent of colonies that survived the treatment compared to an untreated control. (B) *XPF(P85S)-ERCC1 fails to fully restore removal of 6-4 photoproducts in UV41.* UV41 cells alone or cells expressing XPF or XPF(P85S) were exposed to 10 J/m^2 UVC. Genomic DNA was isolated at the indicated time points and the number of 6-4 photoproducts was measured by ELISA and expressed as a percent of lesions in an untreated control. Error bars signify the standard deviation from three independent experiments. The stable UV41 clones, which express a similar level of XPF or XPF(P85S) were used in the experiments (Supplemental Figure 4).

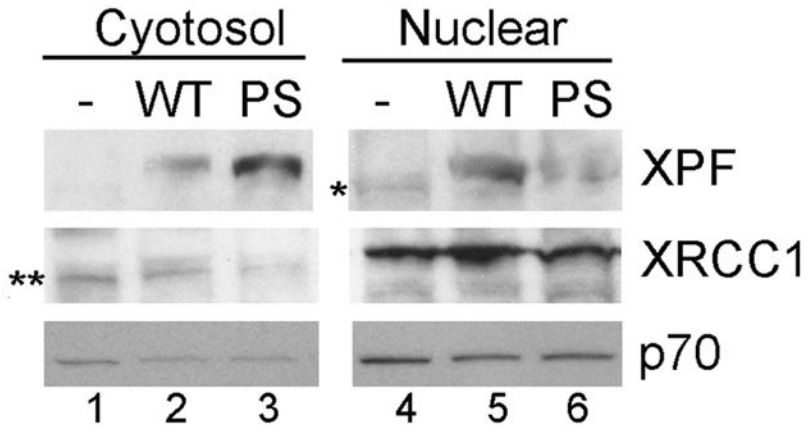

Figure 4. Mislocalization of XPF(P85S) in UV41 cells

A majority of XPF(P35S) was found in cytosolic fraction, while wild type XPF was in nuclear fraction. Cells were fractionated into cytosolic and nuclear fractions. 50 μg of the cytosolic and nuclear fractions from UV41 cells, UV cells expressing wild type XPF, and UV41 cells expressing XPF(P85S) were analyzed on a 10% SDS-PAGE for Western blot. The top, middles and bottom panels were probed with anti-XPF (Ab-1, NeoMarkers), anti-XRCC1 (33-2-5, Abcam) and anti-RPA p70 (BETHYL Laboratories), respectively. Lanes 1–3, cytosolic fraction; lanes 4–6, nuclear fraction. Lanes 1 and 4, fractions from UV41; lanes 2 and 4, fractions from UV41 expressing wild type XPF; and lanes 3 and 6, fractions from UV41 expressing XPF(P35S). Nearly all XRCC1 and RPA (p70) were in nuclear fraction. A single asterisk and double asterisks are non-specific proteins reacted with anti-XPF and anti-XRCC1 antibody, respectively.