Defining the function of xeroderma pigmentosum group F protein in psoralen interstrand cross-link-mediated DNA repair and mutagenesis

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Many commonly used drugs, such as psoralen and cisplatin, can generate a very unique type of DNA damage, namely ICL (interstrand cross-link). An ICL can severely block DNA replication and transcription and cause programmed cell death. The molecular mechanism of repairing the ICL damage has not been well established. We have studied the role of XPF (xeroderma pigmentosum group F) protein in psoralen-induced ICL-mediated DNA repair and mutagenesis. The results obtained from our mutagenesis studies revealed a very similar mutation frequency in both human normal fibroblast cells and XPF cells. The mutation spectra generated in both cells, however, were very different: most of the mutations generated in the normal fibroblast cells were T¹⁶⁷ → A transversions, whereas most of the mutations generated in the XPF cells were T¹⁶⁷ → G transversions. When a wild-type XPF gene cDNA was stably transfected into the XPF cells, the T¹⁶⁷ → A

mutations were increased and the T¹⁶⁷ → G mutations were decreased. We also determined the DNA repair capability of the XPF cells using both the host-cell reactivation and the *in vitro* DNA repair assays. The results obtained from the host-cell reactivation experiments revealed an effective reactivation of a luciferase reporter gene from the psoralen-damaged plasmid in the XPF cells. The results obtained from the *in vitro* DNA repair experiments demonstrated that the XPF nuclear extract is normal in introducing dual incisions during the nucleotide excision repair process. These results suggest that the XPF protein has important roles in the psoralen ICL-mediated DNA repair and mutagenesis.

Key words: DNA repair, interstrand cross-link (ICL), mutagenesis, mutation spectrum, psoralen, xeroderma pigmentosum group F (XPF).

INTRODUCTION

In living cells, genomic DNA is constantly damaged by both endogenous and exogenous factors. DNA repair plays an important role in maintaining genetic fidelity and stability. Many DNA repair systems have evolved to remove DNA damage from the genome of living organisms. On the basis of the nature of DNA damage, DNA repair can be categorized into base excision repair, nucleotide excision repair (NER), mismatch repair and recombinational DNA repair (double-strand-break DNA repair). The molecular mechanisms for repairing these types of DNA damage have been well studied [1]. Many commonly used drugs such as psoralen and the anticancer drug cisplatin can also generate a very unique type of DNA damage, the DNA ICL (interstrand cross-link) [2,3]. The molecular mechanism for repairing this type of DNA damage, however, is not well understood.

XP (xeroderma pigmentosum) is a genetic disease linked with DNA repair deficiency [4]. XP patients suffer from many distinctive clinical symptoms including high sensitivity to sunlight and predisposition to many types of cancer. Seven clinically distinguishable groups of XP patients (groups A–G) and a variant group (V) have been identified [1]. Each group of the XP patients (A–G) is defective in one of the genes involved in the NER process. The sequential events of the NER process begin with the XPC–HR23B complex recognizing and binding to the damaged sites [5–9]. The XPA and RPA proteins then bind to the damaged sites [6,10,11], resulting in the recruitment of other NER components, including TFIIH, XPG and XPF–ERCC1 complex (where XPF stands for XP group F), to the damaged sites [6–8,12,13]. Next, the helicase activities of the XPB and XPD proteins contained in the TFIIH basal transcription factor unwind the damaged DNA region [14–17], whereby the XPG

protein makes an incision 5 base pairs 3' to the damaged site [18] and the XPF protein makes an incision 22–24 base pairs 5' to the damaged sites [19,20], resulting in the release of a 27–29-base nucleotide from the damaged site [21]. Lastly, the DNA polymerase (Pol ϵ and Pol δ) fills in the gap by using the complementary DNA strand as a template and the DNA ligase seals the gap to complete the repair process. In addition to these proteins, the XPE protein is a DNA damage recognition protein involved in DNA global genome repair, a subfamily of the NER [22–24]; and the XPV protein is an error-prone DNA polymerase that bypasses many DNA translesions [25,26]. Interestingly, in addition to its 5' endonuclease activity, the XPF protein has also been identified to possess some other enzymic activities including the ICL-dependent 3' → 5' exonuclease activity [27,28] and the 3' endonuclease activity [29].

Extensive studies have been performed in bacteria and some in mammalian cells to study ICL-mediated DNA repair [27,28,30–36]. Results of the studies on bacteria indicate that two important DNA repair pathways, the NER pathway and the recombinational DNA repair pathway, are involved in the ICL DNA repair process [30]. The ICL DNA repair process begins with the enzymes in the NER pathway making dual incisions on one of the ICL-damaged DNA strands, resulting in a small single-stranded gap around the ICL-damage site. Following this, the second DNA strand (the complementary DNA strand) is repaired by the recombinational DNA repair pathway and the damaged strand is replaced with an undamaged copy of the same DNA sequence. Once this occurs, a DNA polymerase uses the newly repaired DNA strand as a template to synthesize DNA in the gap region of the first strand. The DNA ligase completes the DNA repair process by sealing the gap. A similar but more complicated DNA repair system seems to be involved in the ICL repair in mammalian

Abbreviations used: ddATP, dideoxy ATP; HCR, host-cell reactivation; ICL, interstrand cross-link; MA, monoadduct; NER, nucleotide excision repair; NF, normal fibroblast; TdT, terminal transferase; TFO, triplex-forming oligonucleotide; XP, xeroderma pigmentosum; XPF, xeroderma pigmentosum group F.

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systems although a detailed DNA repair process has not been established [27,28,31–36]. In addition, some published results also suggest the requirement of DNA polymerase ζ (Pol ζ), for the ICL-translesion bypass [37,38], as well as the existence of a recombination-independent DNA repair pathway for the ICL DNA damage in mammalian cells [36].

In the present study, we investigated the role of the XPF protein in DNA repair and mutagenesis mediated by the DNA cross-link reagent psoralen. The psoralen ICL DNA damage was generated at a specific site of the pSupFG1 plasmid via a TFO (triplex-forming oligonucleotide)-mediated gene targeting strategy [39]. The psoralen ICL-mediated DNA repair and mutagenesis was then determined in both human NF (normal fibroblast) cells, which carry a normal XPF protein and are proficient in DNA repair, and XPF cells, which carry a defective XPF protein and are defective in the NER pathway. The results obtained from our targeted mutagenesis experiments indicated that the mutation frequency caused by psoralen ICL in both the NF and XPF cells was similar (8.7 % in NF cells versus 9.7 % in XPF cells). The mutation spectra generated by the psoralen ICL in both cells, however, were very different: most of the mutations generated in the NF cells were T¹⁶⁷ → A transversions (65 %), whereas most of the mutations generated in the XPF cells were T¹⁶⁷ → G transversions (76 %). When a normal XPF protein was expressed in the XPF cells, however, the T¹⁶⁷ → A mutations were increased from 11 to 37 % and the T¹⁶⁷ → G mutations were decreased from 76 to 26 %. We further determined the DNA repair capability of the XPF cells using both HCR (host-cell reactivation) and *in vitro* DNA repair assays. The results obtained from our HCR experiments indicated that the XPF cells were normal in the reactivation of the luciferase reporter gene from a psoralen-damaged plasmid. The results obtained from our *in vitro* DNA repair experiments demonstrated that the XPF nuclear extract was normal in making dual incisions that caused the release of an approx. 30 nt DNA fragment from the psoralen-damaged plasmid vector. All these results suggest that the XPF protein has important roles in the NER process and the cisplatin-mediated mutagenesis and that these roles are separate from each other. This knowledge provides an important understanding for the molecular mechanism of psoralen ICL-mediated DNA repair and mutagenesis. It also has important clinical implications for the treatment of many diseases including cancer.

MATERIALS AND METHODS

Cell lines, oligonucleotides and plasmids

All the human cells used in the present study were obtained from NIGMS Human Genetic Cell Repository (Coriell Institute, Camden, NJ, U.S.A.). The human NF (GM00637) and XPF (GM08437) cells were maintained in MEM medium supplemented with 10 % (v/v) foetal bovine serum and 2× essential amino acids, non-essential amino acids and vitamins. The XP group C (XPC) cells (GM00671) were maintained in MEM medium supplemented with 20 % foetal bovine serum and 2× essential amino acids, non-essential amino acids and vitamins. The XP group A (XPA) cells (GM02250) were maintained in RPMI 1640 medium supplemented with 15 % heat-inactivated foetal bovine serum. Both the GM00637 and the GM08437 cells were SV40-transformed cells. The mutation(s) contained in the XPF gene of the GM08437 cell line has not been determined.

A psoralen-conjugated TFO, psoAG10, described previously [40], was used in this study to introduce site-specific psoralen ICL damage into plasmid constructs. The psoAG10 oligo was synthesized by Oligos Etc. (Wilsonville, OR, U.S.A.).

The plasmid pSupFG1 was previously described [39] and used in this study as a reporter for DNA repair and mutagenesis. The *supF* reporter gene carried in the pSupFG1 plasmid contains a psoAG10 binding-site sequence, enabling a single psoralen ICL to be introduced into the *supF* reporter gene of the plasmid by the psoAG10 oligo. The plasmid pUSAG15 was described previously [41] and used in this study for determining the DNA repair capacity of the XPF cells. The pUSAG15 plasmid carries a cytomegalovirus promoter-driven luciferase reporter gene and a psoAG10-binding site. It enables a single psoralen ICL to be introduced into the plasmid with the psoAG10 oligo.

TFO psoralen-mediated mutagenesis

The pSupFG1 plasmid DNA (20 μ g) was incubated with the psoAG10 oligonucleotide (10 μ M) in 1× triplex binding buffer (20 mM MgCl₂/10 mM Tris, pH 8.0/1 mM spermidine) at 37 °C for 2 h. The samples were irradiated with long-wavelength UV light (UVA, 365 nm, 1.8 J/cm²; Photochemical reactor RPR-200, Southern New England Ultraviolet Company, Branford, CT, U.S.A.) to introduce the psoralen ICLs into the plasmid. The free psoAG10 oligo was removed from the plasmid by filtration using a centricon YM30 apparatus (Millipore, Bedford, MA, U.S.A.). The ICL-containing pSupFG1 plasmid DNA (10 μ g/100 mm dish) was transfected into both NF and XPF cells by a Superfect Transfection Reagent (Qiagen, Chatsworth, CA, U.S.A.). The cells were incubated in a tissue culture incubator at 37 °C for 2 days for DNA repair and plasmid replication to occur. The plasmid DNA was then isolated from the cells using a method described previously [39].

Detection of mutations occurring in the *supF* reporter gene and determination of the mutation spectrum

The pSupFG1 plasmid DNA isolated from the transfected cells was first digested with the restriction enzyme *DpnI* to eliminate any unreplicated plasmid molecules. The plasmid DNA was then transformed into an *Escherichia coli* SY204 *lacZ* (amber) strain and plated on to Luria–Bertani agar plates that contained both ampicillin (100 μ g/ml) and X-gal (20 μ g/ml). The plates were incubated at 37 °C overnight and the mutations occurring at the *supF* reporter gene were detected on the plates. Since the *E. coli* SY204 strain carries an amber mutation in the *lacZ* gene, a functional *supF* gene would correct the mutation of the *lacZ* gene and produce blue colonies whereas the mutant *supF* reporter gene would result in colourless colonies. The mutation frequency was determined by the ratio of the mutant colonies to the total number of colonies appearing on the plates. Plasmid DNA was isolated from the mutant colonies using a QIAprep Spin Miniprep Kit (Qiagen) and analysed by agarose gel electrophoresis to determine the size of the mutant plasmid. The plasmid DNA was further sequence-analysed using a dsDNA Cycle Sequencing System (Life Technologies, Gaithersburg, MD, U.S.A.) [39]. The mutation spectrum was determined in both XPF and NF cells.

Establishment of XPF cells stably transfected with XPF cDNA

The XPF gene cDNA clone, cER4-34 [42] plasmid (10 μ g) was mixed with 50 μ l of Superfect Transfection Reagent (Qiagen) and 250 μ l of MEM medium (serum-free) at room temperature (20 °C) for 40 min. The mixture was diluted into 3 ml of cell growth medium and added to a 100 mm tissue culture dish that contained the XPF cells grown to 50–60 % confluence. The cells were incubated with the transfection reagent in a tissue culture incubator (37 °C) for 8 h. The liposome-containing medium was

then replaced with fresh cell-growth medium and the cells were cultured in a tissue culture incubator overnight. The cells were harvested from the dish and diluted into 6-well plates. The cells were maintained in G418-containing medium (300 µg/ml) for 2 weeks until individual colonies appeared on the plates. Individual colonies were subcultured and the level of the XPF protein was determined using a Western-blot hybridization assay.

Western-blot hybridization assay

The mouse monoclonal antibody against the XPF protein (4431-MC-100) was purchased from Trevigen (Gaithersburg, MD, U.S.A.). The cells were grown to 70–80 % confluence and then harvested from the cell-culture flasks. The cells were lysed in the RIPA cell-lysis buffer (1× PBS/1 % Nonidet P 40/0.5 % deoxycholic acid/0.1 % SDS). The cell lysates (20 µg of total protein) were mixed in 20 µl of 1× SDS gel-loading buffer [60 mM Tris, pH 6.8/1 % SDS/1 % 2-mercaptoethanol/10 % (v/v) glycerol/0.1 % Bromophenol Blue] and boiled for 10 min. The boiled cell lysates were analysed by SDS/PAGE (10 % gel). The proteins were transferred to a PVDF membrane. The membrane was blocked in the TBST (25 mM Tris/135 mM NaCl/2.7 mM KCl/0.05 % Tween 20, pH 7.4)-5 % (w/v) dry milk solution for 1 h at room temperature and then hybridized with the XPF antibody (1:500) in the TBST/5 % dry milk solution overnight at 4 °C. The membrane was washed three times in the TBST solution (15 min each) and then hybridized with sheep anti-mouse secondary antibody (1:2000; Amersham Biosciences, Arlington Heights, IL, U.S.A.) for 1 h at room temperature. The membrane was washed three times in the TBST solution and then reacted with the ECL[®] reagent (Amersham Biosciences) for 1 min to detect the XPF protein. The same membrane was then stripped in a stripping solution (62.5 mM Tris, pH 6.8/2 % (w/v) SDS/0.7 % 2-mercaptoethanol) and hybridized with β-actin antibody (Oncogene, San Diego, CA, U.S.A.) to determine the level of β-actin in each sample. The level of the XPF protein was calculated relative to the level of β-actin in each sample to minimize the experimental variations.

Detection of the excised DNA fragments from the *in vitro* DNA repair assay

The pSupFG1 plasmid DNA was incubated with psoAG10 oligo in 1× triplex binding buffer for 2 h to form the triplex structure. The reactant was then irradiated with either visible light (417 nm) or UVA to introduce the psoralen MA (monoadduct) and ICLs respectively into the plasmid. The free psoAG10 oligo was removed from the plasmid by filtration using a centricon YM30 apparatus (Millipore). The bound psoAG10 oligo was labelled with α-³²P-labelled ddATP (dideoxy ATP) using TdT (terminal transferase). The DNA repair experiment was performed in 20 µl of a solution, containing 1× HeLa buffer (20 mM Hepes, pH 7.9/0.1 M KCl/0.2 mM EDTA), 1 µg of psoAG10-damaged pSupFG1, 10 µl of nuclear extract, 200 µM ddNTPs and 200 µM rNTPs at 30 °C for 2 h. The reaction was stopped by the addition of 10 µl of stop solution [95 % (v/v) formamide, 10 mM EDTA]. The reactants were denatured at 94 °C for 10 min and then analysed by using a DNA sequencing gel with 7 M urea and 8 % polyacrylamide. The excised DNA fragment was detected in the gel by autoradiography with X-ray films. Quantification of the excised DNA fragment was determined by phosphorimaging analysis using a Bio-Rad GS250 Phosphorimager.

HCR assay

The psoralen-damaged pUSAG15 plasmid DNA (10 µg) was transfected into the XPF cells for DNA repair and luciferase

reporter gene expression. The psoralen-damaged pUSAG15 plasmid DNA was also transfected into NF cells, which are proficient in DNA repair and XPC cells, which are deficient in DNA repair, for luciferase reporter gene expression. As a control, the undamaged pUSAG15 plasmid DNA was also transfected into the XPF, NF and XPC cells for luciferase expression. The pRL-CMV plasmid DNA (1 µg), which carries a renilla luciferase reporter gene (Promega, Madison, WI, U.S.A.), was co-transfected with the pUSAG15 plasmid in each experiment. The cells were incubated for 2 days to allow for DNA repair and gene expression to occur and then harvested from the dishes. The cells were lysed in cell lysis buffer and both the firefly and renilla luciferase activities were determined from the cell lysates using a Dual Luciferase Activity Detection System (Promega, Madison, WI, U.S.A.). The level of luciferase expression from the psoralen-damaged pUSAG15 plasmid was calculated as a percentage of the level of luciferase expression from the undamaged pUSAG15 plasmid.

RESULTS

Determining the mutation frequency of the *supF* reporter gene generated by psoralen in the XPF cells

To study the role of XPF protein in the psoralen ICL-mediated DNA repair and mutagenesis, it is important to determine the mutations generated by the psoralen ICL in the XPF cells. In our earlier studies, single psoralen ICL was introduced into the pSupFG1 plasmid with high efficiency via a TFO-mediated gene targeting strategy [39,40,43,44]. This strategy was also used in the present study for introducing single psoralen ICLs into the *supF* reporter gene of the pSupFG1 plasmid. The psoAG10 oligonucleotide was used in this study to introduce the psoralen ICL into the *supF* reporter gene of the pSupFG1. The results obtained from our earlier studies revealed that over 90 % of the plasmid template contained single psoralen ICL damage when treated with the psoAG10 and irradiated with UVA [39,44]. In addition, the results obtained from our earlier studies indicated that the triplex structure formed by the psoAG10 does not have any effect on the psoralen damage-mediated DNA repair and mutagenesis [40].

The pSupFG1 plasmid DNA was incubated with psoAG10 and then irradiated with UVA to introduce the psoralen ICL into the Tpa site of the *supF* reporter gene, which is located at positions 166–167 of the gene. The psoralen-damaged pSupFG1 plasmid DNA was then transfected into the XPF cells to allow for DNA repair and mutagenesis to occur. As a positive control, the damaged pSupFG1 plasmid DNA was transfected into the NF cells for DNA repair and mutagenesis processing. The undamaged pSupFG1 plasmid DNA was also transfected into both the NF and XPF cells to determine the background mutation frequency in both cell lines. The plasmid DNA was isolated from the cells 2 days after the transfection and transformed into an *E. coli* SY204 strain for the detection of mutations occurring in the *supF* reporter gene (Table 1). A total of 11 015 colonies derived from the NF cells were screened and 959 colonies carried mutations in the *supF* reporter gene (8.7 %). A total of 30 084 colonies derived from the XPF cells were tested and 2916 mutant colonies were detected (9.7 %).

Determination of the mutation spectrum generated by psoralen ICL in both NF and XPF cells

We further analysed the mutations generated in both NF and XPF cells by DNA sequencing (Figure 1). A total of 31 mutations derived from the NF cells were sequenced. Among these

Table 1 TFO psoralen-directed mutations of the *supF* reporter gene in both NF and XPF cells

Cell lines	No. of mutant colonies	Total no. of colonies	Mutation frequency (%)
Untreated pSupFG1 plasmid			
NF	3	13 000	0.02
XPF	3	10 800	0.03
pSupFG1 plasmid treated with psoAG10 but without UVA			
NF	4	12 055	0.03
XPF	3	10 700	0.03
XPF (corrected)	5	13 220	0.04
pSupFG1 plasmid treated with psoAG10 and UVA			
NF	959	11 015	8.7
XPF	2916	30 084	9.7
XPF (corrected)	1403	10 636	8.2

mutations, 20 were T¹⁶⁷ → A transversions (65%), two were T¹⁶⁷ → G transversions (6%), three were T¹⁶⁷ → C transitions (10%), three were C¹⁶⁸ → T transitions (10%), one was an A¹⁶⁵ → G transition (3%), one was an A¹⁶⁶ → T transversion (3%) and one contained a T¹⁶⁷ deletion (3%) (Table 2A). A total of 46

mutations derived from the XPF cells were sequenced. Among these mutations, 35 were T¹⁶⁷ → G transversions (76%), five were T¹⁶⁷ → A transversions (11%), one was a T¹⁶⁷ → C transition (2%), two were A¹⁶⁶ → T transversions (4%), one was an A¹⁶⁶ → C transversion (2%), one was a C¹⁶⁸ → T transition (2%) and one was a C¹⁶⁸ → G transversion (2%) (Table 2B). Comparison of the results revealed very different mutation spectra generated by the psoralen ICL in NF and XPF cells: most of the mutations generated by the psoralen ICL in the NF cells were T¹⁶⁷ → A transversions (65%), and most of the mutations generated by the psoralen ICL in the XPF cells were T¹⁶⁷ → G transversions (76%).

Defining the function of XPF protein in the psoralen ICL-mediated targeted mutagenesis

The very distinctive mutation spectrum generated by psoralen in the XPF cells suggests that the XPF protein might play an important role in determining the nature of mutation caused by the psoralen ICL. However, other factors might also contribute to the altered mutation spectrum in the XPF cells. To determine if the altered mutation spectrum is indeed caused by the defect in the

A. Mutations of the *supF* reporter gene generated by psoralen in NF cells.

B. The *supF* reporter gene mutations generated by psoralen in XPF cells.

C. The *supF* reporter gene mutations generated by psoralen in XPF (corrected) cells.

Figure 1 TFO psoralen-generated *supF* reporter gene mutations in both the NF and XPF cells

Mutations of the *supF* reporter gene generated by psoralen in (A) NF cells (total of 31 mutations), (B) XPF cells (total of 46 mutations) and (C) XPF gene cDNA stably transfected XPF cells (total of 35 mutations). The point mutations are shown above the *supF* gene sequence and the deletions are shown under the *supF* gene sequence with broken line indicating the lost region. The number in parentheses indicates the total number of mutants with the same mutation (underlined). Positions of the *supF* gene sequence are labelled under the *supF* gene (starting at position 91 and ending at position 175).

Table 2 Comparison of the mutation spectra generated by psoralen in both NF and XPF cells

Cell line and type of mutation	No. of mutation	Percentage
(A) Mutations generated in NF cells		
A ¹⁶⁵ → G	1	3
A ¹⁶⁶ → T	1	3
T ¹⁶⁷ → A	20	65
T ¹⁶⁷ → G	2	6
T ¹⁶⁷ → C	3	10
C ¹⁶⁸ → T	3	10
A167 deletion	1	3
Total	31	100
(B) Mutations generated in XPF cells		
A ¹⁶⁶ → C	1	2
A ¹⁶⁶ → T	2	4
T ¹⁶⁷ → G	35	76
T ¹⁶⁷ → A	5	11
T ¹⁶⁷ → C	1	2
C ¹⁶⁸ → T	1	2
C ¹⁶⁸ → G	1	2
Total	46	100
(C) Mutations generated by TFO psoralen in XPF (corrected) cells		
G ¹⁶⁴ → C	1	3
A ¹⁶⁶ → T	2	6
A ¹⁶⁶ → G	1	3
T ¹⁶⁷ → A	13	37
T ¹⁶⁷ → G	9	26
T ¹⁶⁷ → C	1	3
C ¹⁶⁸ → T	6	17
C ¹⁶⁸ → A	1	3
C ¹⁶⁹ → T	1	3
Total	35	10

XPF protein, a wild-type XPF gene cDNA expression plasmid, cER4-34 [42], was stably transfected into the XPF cells and the psoralen ICL-mediated mutagenesis was then studied in these cells (Table 1 and Figure 1). The mutation frequency of the *supF* reporter gene caused by the psoralen ICL was 8.2% in the XPF gene cDNA stably transfected XPF cells, which is similar to the mutation frequencies caused by the psoralen in both the NF and XPF cells (Table 1). The mutations generated in the stably transfected XPF cells were also sequenced (Figure 1C). Among the 35 mutations that were analysed, 13 were T¹⁶⁷ → A transversions (37%), nine were T¹⁶⁷ → G transversions (26%), six were C¹⁶⁸ → T transitions (17%), one was a T¹⁶⁷ → C transition (3%), one was a C¹⁶⁸ → A transversion (3%), one was an A¹⁶⁶ → G transversion (3%), one was a G¹⁶⁴ → C transversion (3%) and one was a C¹⁶⁸ → T transition (3%) (Table 2C).

To determine the level of the XPF protein in the stably transfected XPF cells, a Western-blot hybridization assay was performed using the cell lysates prepared from both untransfected and the XPF gene cDNA-stably transfected XPF cells (Figure 2). As a positive control, the cell lysates prepared from the NF cells were also used in the Western-blot hybridization assay. When a mouse monoclonal antibody raised against the human XPF protein C-terminal region (amino acids 854–916) was used in the assay, a strong XPF protein band was detected in the NF cells and no XPF protein was detected in the XPF cells (Figure 2, upper panel: lane 1 versus lane 2). In comparison, the XPF protein was detected in the stably transfected XPF cells; however, the level of the XPF protein in the stably transfected XPF cells was lower than that of NF cells (Figure 2, upper panel: lane 3 versus lane 1). As an internal control, the level of β -actin remained unchanged in all the cell lysates (Figure 2, lower panel). These results suggested that the increases in the T¹⁶⁷ → A mutations and the decreases in the T¹⁶⁷ → G mutations were caused by the

Figure 2 Determination of the XPF protein level by Western-blot hybridization assay

The cell lysates of the NF, XPF and XPF gene cDNA-stably transfected XPF cells (corrected) (10 μ g of total protein of each sample) were separated by SDS/PAGE and transferred on to a PVDF membrane. The membrane was first hybridized with the XPF antibody to determine the XPF protein level and then stripped and re-hybridized with β -actin antibody to determine the level of β -actin in each sample.

Table 3 Detection of luciferase activity from psoralen-damaged pUSAG15 plasmid in NF, XPF and XPC cells by HCR

Cell line	pUSAG15 plasmid	Luciferase activity (%)
NF cells (GM00637)	Undamaged	100
	Psoralen-damaged	87
XPF cells (GM08437)	Undamaged	100
	Psoralen-damaged	73
XPC cells (GM00671)	Undamaged	100
	Psoralen-damaged	20

presence of a functional XPF protein in the stably transfected XPF cells. The lower frequency of the T → A mutations in the stably transfected XPF cells compared with that of the NF cells (37% versus 65%) is probably caused by the presence of a lower level of the functional XPF protein in the cells.

Determination of the DNA repair capacity of the XPF cells in repairing psoralen-induced ICL damage

We performed an HCR assay to determine the DNA repair capacity of the XPF cells in repairing the psoralen-generated ICL DNA damage. The plasmid pUSAG15, which carries a CMV promoter-driven luciferase reporter gene, was treated with the psoAG10 oligo and irradiated with UVA to introduce the psoralen ICLs into the plasmid. The psoralen-damaged plasmid DNA was then transfected into both the NF and XPF cells to allow for DNA repair and luciferase gene expression to occur. As a control, the undamaged pUSAG15 plasmid DNA was also transfected into both the NF and XPF cells for luciferase expression. Effective repair of the psoralen-caused ICLs in the plasmid led to the expression of the luciferase reporter gene. In contrast, if the psoralen ICLs were not repaired in the plasmid, expression of the luciferase reporter would be blocked. The results of the HCR experiments are shown in Table 3. When the psoralen-damaged pUSAG15 plasmid DNA was transfected into the NF cells, approx. 87% of the luciferase activity was detected. When the psoralen-damaged pUSAG15 plasmid DNA was transfected into XPF cells, approx. 73% of the luciferase activity was detected. As a negative control, when the psoralen-damaged pUSAG15 plasmid DNA was transfected into the XPC

cells, which are defective in NER repair, only 20 % of luciferase activity was detected (Table 3). These results suggest that the XPF cells effectively repair the psoralen-caused ICLs, leading to the reactivation of the luciferase reporter gene in the cells.

Determination of the NER capacity of the XPF cells

We determined the NER capacity of the XPF cells using an *in vitro* DNA repair assay with the XPF nuclear extracts. The NER capacity of the XPF cells was determined by detection of the excised DNA fragment generated during the NER process. The pSupFG1 plasmid DNA was incubated with psoAG10 oligo and irradiated with either UVA (365 nm) or visible light (417 nm) to generate psoralen ICL or MA DNA damage into the template. The bound psoAG10 oligo was end-labelled with [α - 32 P]ddATP using TdT. The psoralen-damaged pSupFG1 plasmid DNA was then incubated in the XPF nuclear extracts that were supplemented with rNTPs and ddNTPs for the generation of dual incisions during the NER process. Dual incisions of the psoralen-caused MA damage should lead to the release of an approx. 28 nt DNA fragment attached to the α - 32 P-labelled psoAG10 through the psoralen covalent linkage [40]. The excised DNA fragment was detected by a DNA sequencing gel and quantified by DNA phosphorimaging analysis (Figure 3). As controls, the psoralen-damaged pSupFG1 plasmid DNA was also incubated with the HeLa nuclear extract, which was proficient in DNA repair, and an XPA cell nuclear extract, which was defective in the NER. Repair of the psoAG10-caused DNA damage in the HeLa nuclear extract led to the release of an approx. 30 nt DNA fragment attached to the psoAG10 (Figure 3A, lanes 2 and 6, as indicated by the arrow). In comparison, repair of the psoAG10-caused DNA damage in the XPF nuclear extract led to the release of an increased amount of the approx. 30 nt DNA fragment (a 1.5-fold increase; Figures 3A and 3B: lanes 3 and 7 versus lanes 2 and 6). However, when the damaged plasmid DNA was incubated with the XPA nuclear extract, 50 and 60 % decreases in the levels of the released DNA fragment from the ICL and MA-damaged pSupFG1 plasmid respectively were detected (Figures 3A and 3B: lane 4 versus lane 2 and lane 8 versus lane 6).

DISCUSSION

In this study, we have investigated the function of the XPF protein in the psoralen ICL-mediated DNA repair and mutagenesis. Using a TFO-mediated gene targeting strategy, we first determined the role of XPF protein in the psoralen ICL-mediated mutagenesis. When the psoralen-damaged pSupFG1 plasmid DNA was transfected into the XPF cells, the mutation frequency of the *supF* reporter gene was 9.7 %. As a control, the mutation frequency of the *supF* reporter gene caused by the TFO psoralen in the NF cells was 8.7 %. This result suggests that a similar DNA repair capability exists in both the NF and XPF cells for psoralen-mediated ICL DNA damage. We also determined the DNA repair capacity of the XPF cells using an HCR assay. Our HCR results revealed an effective reactivation of the luciferase reporter gene from the psoralen-damaged plasmid in both the NF and XPF cells (87 % reactivation in the NF cells versus 73 % reactivation in the XPF cells). These results suggest that the XPF cells effectively repair psoralen-caused ICL DNA damage.

We further determined the mutation spectra generated by psoralen in both the NF and XPF cells. Most of the mutations generated by psoralen in the NF cells were T¹⁶⁷ → A transversions (65 %), which is consistent with the results obtained from our earlier studies and the results published by others [3,40]. However,

Figure 3 Detection of DNA repair-mediated DNA fragment release from the pSupFG1 plasmid

The pSupFG1 plasmid DNA was incubated with psoAG10 and then irradiated with either UVA (365 nm) or visible light (417 nm) to introduce psoralen ICL or MA DNA damage into the plasmid. The attached psoAG10 oligo was labelled with [α - 32 P]ddATP by TdT. Then the damaged pSupFG1 plasmid DNA was incubated in the nuclear extract supplemented with ddNTPs at 30 °C for 2 h for generation of dual incisions and prevention of DNA repair synthesis-mediated digestion of the released DNA fragments. (A) Autoradiogram of the DNA sequencing gel indicated a release of the excised DNA fragment. (B) Quantification of the excised DNA fragment using phosphorimager. Results are the mean values of four independent experiments.

most of the mutations generated by psoralen in the XPF cells were T¹⁶⁷ → G transversions (76 %). To determine if the altered mutation spectrum was caused by the defect of the XPF protein, a wild-type XPF gene cDNA was stably transfected into the XPF cells; psoralen ICL-mediated targeted mutagenesis was then studied in the stably transfected XPF cells. Indeed, expression of the wild-type XPF gene cDNA in the XPF cells led to an increase in the T¹⁶⁷ → A mutation (39 % versus 11 %) and a decrease in the T¹⁶⁷ → G mutation (27 % versus 76 %) for the psoralen ICL-mediated mutagenesis. The Western-blot hybridization results further revealed that a low level of XPF protein was expressed in the stably transfected XPF cells. These results suggest that a functional XPF protein is responsible for the T → A mutations generated by the psoralen ICLs, suggesting an important role of the XPF protein in the psoralen ICL-mediated mutagenesis.

We have also tried to determine the defect of the XPF protein carried in the XPF cells. The results obtained from our *in vitro* DNA repair experiments demonstrated the release of an approx. 30 nt DNA fragment from the XPF nuclear extract, indicating the functional 5' endonuclease activity of the XPF protein contained in the XPF cells. Therefore, the altered mutation spectrum of the

psoralen-mediated mutagenesis in the XPF cells must be caused by other defects of the XPF protein. One possibility is that the functional XPF protein interacts with some specific error-prone DNA polymerases to bypass the psoralen ICL translesion and results in the T → A mutations, whereas the mutant XPF protein interacts with different error-prone DNA polymerases to bypass the psoralen ICL translesion and results in the T → G mutations. Another possibility is that the XPF protein contained in the XPF cells may carry other defects. The results published by Mu et al. [27] demonstrated that the XPF protein possesses an ICL-stimulated 3' → 5' exonuclease activity that cleaves the damaged DNA strand around the psoralen lesion and generates a single-stranded DNA gap, which is essential for the recombinational DNA repair step. The results obtained from the study of *Mus81*, a yeast homologue of the human XPF gene, indicated that the endonuclease activity of the *Mus81* is essential for the resolution of the Holliday junction during the recombinational DNA repair process [45,46]. It is also possible that the XPF protein contained in the XPF cells carries either a defective ICL-dependent 3' → 5' exonuclease activity or a defective endonuclease activity required for the recombinational repair. The incomplete repairing of the psoralen-caused ICL forces different error-prone DNA polymerases to bypass the ICL translesion, resulting in an altered mutation spectrum. However, the results obtained in this study favour the first hypothesis since the psoralen ICL was effectively repaired in the XPF cells. In fact, the results published by others also support this possibility. For example, it has been demonstrated that both the DNA polymerase κ (Pol κ) and the DNA polymerase ι (Pol ι) predominantly cause T → G transversions [47–50]. It is possible that the defective XPF protein may interact with one of these error-prone DNA polymerases to bypass the psoralen-caused ICL translesion in the XPF cells and lead to predominantly T → G transversions. Further studies are needed to define the defects of the XPF protein that lead to the altered mutation spectrum for the psoralen ICL-mediated mutagenesis.

Many commonly used anticancer drugs such as cisplatin can generate both intra- and interstrand cross-links. Many cancer cells can also quickly develop resistance to chemotherapy and cause treatment failure. The relationship between DNA repair and the tumour-cell resistance to chemotherapy needs to be determined. The results obtained from this study may provide some valuable insight into the molecular mechanism of tumour-cell drug resistance and eventually lead to an improved cancer treatment.

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