CPD-photolyase adenovirus-mediated gene transfer in normal and DNA-repair-deficient human cells

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Summary

Cyclobutane pyrimidine dimers (CPDs) are the most frequent and deleterious lesions generated in the mammalian genome after UV-C irradiation. The persistence of these lesions in DNA can be toxic and mutagenic, and also represents a specific signal to apoptosis. To investigate the CPDs repair in situ and consequent UV-induced apoptosis in human cells, we generated a recombinant adenovirus vector containing the gene encoding a CPD-photolyase-EGFP fusion protein (Ad*phr*-EGFP). Ad*phr*-EGFP-infected cells are proficient in photorepair, which prevents apoptotic cell death in comparison with samples kept in the dark, indicating that the fusion protein is functional in CPD recognition and removal. By using local UV irradiation, foci of the photolyase fusion protein were observed in UV-damaged areas of the nuclei in colocalization with NER enzymes. *Phr*-EGFP migration to CPD sites and redistribution after photorepair was followed, and shown to present similar kinetics in normal or DNA-repair-deficient cells. To our knowledge, this is the first report of an investigation of CPDs repair in situ employing a CPD-photolyase-EGFP enzyme. The Ad*phr*-EGFP vector can be an informative tool to investigate the repair and cellular consequences of UV-induced lesions in primary human cells.

Key words: Cyclobutane pyrimidine dimers, Adenovirus, Photorepair, Local UV irradiation, Apoptosis

Introduction

Ultraviolet light irradiation (UV, 254 nm) generates cyclobutane pyrimidine dimers (CPDs) and pyrimidine 6-4 pyrimidone photoproducts (6-4 PPs) as main photolesions in genomic DNA. CPDs are shown to be the most numerous lesions, and if not repaired, are very cytotoxic, being a blockage to transcriptional synthesis, inhibiting RNA polymerase II progression and triggering apoptotic cell death (Ljungman and Zhang, 1996; Nishigaki et al., 1998; Chiganças et al., 2002). Indeed, the removal of these lesions by heterologous photolyase prevents UV-induced apoptosis in human cells (Chiganças et al., 2000), and, because they represent physical hindrance to RNA polymerase II (Donahue et al., 1994), these studies indicate that persistent CPDs in the transcribed strand promote cellular suicide.

CPDs can be repaired by the CPD photolyases or by the nucleotide excision repair system (NER). CPD photolyases are enzymes of the photolyases/blue light receptors family (Todo, 1999; Carell et al., 2001) that mediate either DNA repair or perception/signal transduction of external light signals. These proteins contain chromophores capable of capturing photons of blue light and a mechanism of converting the electronic excitation in CPD removal in a fast and direct repair process (photorepair) (Hearst, 1995; Sancar, 1996). These enzymes occur in bacteria, lower eukaryotes, plants and many animals including marsupials, but photorepair is absent in placental mammals, probably being lost during evolution, with this subclass presenting only NER as a mechanism to eliminate CPDs in the genome (Thompson and Sancar, 2002; Menck, 2002; Schul et al., 2002). The mammalian genes Cry1 and Cry2, homologs of the photolyases, are essential for the maintenance of circadian rhythms, as the knockout mice for these genes present alterations in the biological clock and loss of light entrainment locomotor activity (van der Horst et al., 1999).

In comparison, NER is a versatile and highly conserved DNA repair mechanism, but less efficient for CPD removal in comparison with photorepair. This pathway is able to remove a variety of bulky DNA lesions and needs the coordinated action of about 20 proteins or complexes in human cells (Benhamou and Sarasin, 2000; Costa et al., 2003). This sophisticated process is divided into two sub-pathways: transcription-coupled repair (TCR), a specialized pathway that preferentially eliminates lesions located in the transcribed strand of active genes and requires active RNA polymerase-II-driven transcription; and global genomic repair (GGR), which removes lesions in the entire genome. There are several human syndromes related to the absence or deficiency in NER, which affect how cancer prone an individual will be and even organism development. Xeroderma pigmentosum (XP), for example, is a recessively inherited disorder characterized by the severe photosensitivity, a high incidence of skin cancers and, in some cases, associated neurological abnormalities (Benhamou and Sarasin, 2000; Balajee and Bohr, 2000). The Cockayne syndrome (CS) and Trichothiodystrophy (TTD) are further examples of NER deficiencies, whose clinical features

are also sun sensitivity and mental retardation. Additionally, patients with CS present postnatal growth failure, premature senescence and deficient neurological development, and, in contrast to XP, generally do not develop cancer, presumably because of the elimination of cells with DNA damage by apoptosis (Ljungman and Zhang, 1996; Licht et al., 2003). Mutations in the *CSA* and *CSB* genes are present in most CS patients, and CS cells have been shown to present impaired transcriptional activity per se and deficiency in RNA synthesis recovery after UV; more specifically, on the removal of CPDs by TCR (Venema et al., 1982; Licht et al., 2003). In the case of TTD syndrome, all known patients present a great heterogeneity and a wide spectrum of clinical symptoms; they exhibit dry and easily broken hair, skin changes (ichthyosis), mental retardation, photosensitivity, infertility, skeletal defects and neurological and immunological impairment (Lehmann et al., 1988; Bergmann and Egly, 2001). TTD might be also characterized as a 'transcription syndrome' like CS; however, the absence of increased cancer susceptibility in TTD might involve distinct explanations (Berneburg et al., 2000; Dubaele et al., 2003).

To investigate the role of the presence of CPDs lesions in normal and NER deficient human primary cells, we generated a recombinant adenovirus vector containing the gene of a marsupial CPD photolyase (*phr* gene) fused with the enhanced green fluorescent protein (EGFP; Ad*phr*-EGFP virus). In recent years, there has been considerable interest in developing adenoviruses as defective vectors to carry and express foreign genes for research and therapeutic purposes (Yeh and Perricaudet, 1997; Benihoud et al., 1999; Mountain, 2000). These vectors present several advantages: (1) their genomes are easily manipulated in vitro, (2) give more efficient gene transfer compared with other systems (Mountain, 2000), (3) can transfer and express the gene of interest in many human and non-human primary and transformed cell types (Mizuguchi and Kay, 1998), (4) the carried genes are expressed in large amounts in different target cell lines. There are several recent successful reports of adenovirus-mediated gene delivery to study DNA repair (Francis et al., 1997; Muotri et al., 2002) and apoptosis (Yukawa et al., 2002; Arafat et al., 2003) in human cells.

In this work, normal and NER-deficient diploid human primary fibroblasts and transformed cells were employed to study heterologous photorepair. Results in Ad*phr*-EGFP-infected cells indicate that this fusion protein is functional on CPD recognition and removal; these cells display recovery in clonogenic capacity and prevention of apoptotic cell death when exposed to photoreactivating light (PRL) after UV-irradiation. Analysis of *phr*-EGFP enzyme in locally UV-irradiated sites demonstrates a specific response of migration to CPD-containing sites and redistribution after photorepair. This is the first report of an investigation of photorepair in situ by employing a CPD-photolyase fusion protein.

Materials and Methods

Cell culture

XP12RO-SV (XPA complementation group) cell lines were routinely grown in DMEM (Invitrogen, CA) supplemented with 10% fetal bovine serum (FBS; Cultilab, Campinas, Brazil) and 1% antibiotic-antimycotic (Invitrogen), at 37°C in a humidified 5% CO_2 atmosphere. The human primary fibroblasts and HEK 293 cell lines were routinely grown in MEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS; Cultilab, Campinas, Brazil) and 1% antibiotic-antimycotic (Invitrogen) in the same culture conditions described above. The human primary cells used in this work, 198VI (normal), XP456VI (XPA mutated), XP148VI (XPC mutated) and XP4PA (XPC mutated, see xpmutation.org) were from our laboratory (UPR 2169 Intitut Gustave-Roussy, Villejuif, France) (Queille et al., 2001).

Construction of the recombinant adenovirus vector

The marsupial photolyase gene (*phr*) was amplified by PCR reaction using the following primers: forward 5′ CTCGAGACCCACCATGGACTCCAAAAAGAG 3′ (*Xho*I site); reverse 5′ CCGCGGATCTGCAGGGCTGATCTTTCGTTC 3′ (*Sac*II site). The amplified product was cloned in the pEGFP-N1 plasmid (Clontech, CA, USA) to obtain an inframe copy of the photolyase protein with the EGFP at its C-terminus. The *phr*-EGFP gene was transferred to an adenovirus vector by using the Adeno-X system as previously described (Fig. 1) (Mizuguchi and Kay, 1998).

Production of CsCl-purified adenoviral particles

The production of purified adenoviral particles was performed as described (Graham and Prevec, 1995; Dedieu et al., 1997; Hitt et al., 1998). Briefly, after the cytopathic effect in transfected HEK 293 cells (low passage, $P \leq 50$) (Graham et al., 1977), the cells were lysed and the virus was amplified by three successive infection cycles in HEK 293 cells. The last amplified lysate was used to infect HEK 293 cells. Approximately 40 hours after infection the cells were lysed by 5 cycles of freezing (–80°C) and thawing (37°C), and ultracentrifuged in isopycnic CsCl gradient (Dedieu et al., 1997). The band containing the purified virus was taken and then desalted by dialysis (Dedieu et al., 1997; Hitt et al., 1998). The purified virus preparation was aliquoted and stored at –80°C in Tris-HCl 10 mM pH 7.4, $MgCl_2$ 1 mM and glycerol 10%.

Cell infection with recombinant adenovirus

Approximately 1.5×10^5 primary fibroblasts in 60 mm Petri dishes were infected with 20-40 μl of purified adenovirus [multiplicity of infection (m.o.i.) 1.6×10^5] during 1-1.5 hours in a medium without serum and antibiotics. The mock-infected samples received the same volume of the virus stock solution (see above). After this period the medium was replaced by complete medium. Approximately 40 hours after cell infection, the expression of the *phr*-EGFP gene was accessed by analyzing the presence of green fluorescence in infected cells with a fluorescence microscope (Zeiss Axiovert S100, Hamamatsu 3CCD color camera, 60× objective). Cell suspensions in PBS buffer were also analyzed by flow cytometry (FACScalibur, Becton Dickinson, USA) and the EGFP fluorescence was determined (CellQuest Software, Becton Dickinson).

UV irradiation and photoreactivation

For XP12RO-SV cell line, 40 hours after the infection, the cells were washed twice with pre-warmed PBS and irradiated by a low-pressure germicidal lamp (UV light emitting mainly at 254 nm). The cells were then submitted immediately to dark or photoreactivation (PRL) treatments for 2 hours in PBS. For photoreactivation, single cell layers were illuminated 10 cm over fluorescent lights (two daylight lamps, Philips 15W; emission, 400-700 nm), with the temperature kept at 37°C. During photoreactivation, the cell dishes are kept over a glass plate to catch away any trace amounts of UV emission from the fluorescent lamps. The cells were then maintained in a complete medium for the indicated periods of time. Human primary fibroblasts were treated as described above, but maintained in transparent MEM

medium (without phenol red; Invitrogen, CA) during the whole experimental period.

Clonogenic assay

Approximately 40 hours after infection with Ad*phr*-EGFP vector (m.o.i. 4×10^4), 700 cells (XP12RO-SV cell line) were plated in 60 mm Petri dishes 14-16 hours before UV irradiation. This procedure is necessary for cell adhesion and to assure the irradiation of isolated cells. After UV irradiation, control and treated cells were maintained in complete medium for 7-10 days and then fixed with 10% formaldehyde and stained with 1% violet crystal. Colonies with the minimal number of 15 cells were scored. Survival values were obtained as the ratio of the number of colonies from irradiated cells to non-irradiated cells.

Measurement of UV endonuclease sensitive sites (ESS)

Approximately 1.0×10^6 cells, infected with the Ad*phr*-EGFP adenovirus or mock-infected, were plated in 60 mm Petri dishes. After 24 hours, the cells were grown in complete medium containing [^{3}H]-methyl-thymidine (0.50 µCi/ml, Amersham-Pharmacia Biotech, USA) for 24 hours. After this period, the cells were UV irradiated and maintained in dark or photoreactivating light conditions (PRL) in pre-warmed PBS for 2 hours. Nuclei were prepared immediately after this treatment with 0.5% Triton X-100, 0.1 M NaCl and 10 mM EDTA, washed twice with PBS, and incubated in NET buffer, i.e. 100 mM NaCl, 10 mM Tris-HCl and 10 mM EDTA (Spivak and Hanawalt, 1995), with and without T4-endonuclease V, for 30 minutes at 37°C. Molecular weights of untreated and treated DNA were determined by alkaline sucrose gradient sedimentation, as described before (Menck and Meneghini, 1982). From these values, the number of T4-endonuclease sensitive-sites (ESS), which corresponds to the number of CPDs per 10^9 daltons, was calculated.

Flow cytometry analysis

Infected cells were UV irradiated and maintained in dark or photoreactivating light conditions (PRL) in prewarmed PBS for 2 hours. Transformed and human primary cells were harvested 48 and 72 hours after the UV treatment, respectively, and centrifuged at 1500 rpm (700 ***g***) for 10 minutes. Pelleted cells (0.5 to 1.0×10^6 cells) were lysed with 500 µl of a hypotonic fluorochrome solution (50 µg/ml PI in 0.1% sodium citrate plus 0.1% Triton X-100), and incubated at least 30 minutes on ice in the dark (Amarante-Mendes et al., 1998). Then, samples were transferred to microtubes, and PI fluorescence was measured by flow cytometry (FACScalibur, Becton Dickinson, USA). Results were obtained as the percentage of subdiploid nuclei (CellQuest Software, Becton Dickinson, USA), which represent the apoptotic cells.

Morphological analysis of apoptosis

Approximately 3.5×10^5 cells in 35 mm Petri dishes (Nunc) were harvested 48 hours after UV treatment, centrifuged, and resuspended in 20 µl of PBS buffer. Two µl of acridine orange (100 µg/ml) and ethidium bromide (100 µg/ml) solution in PBS buffer (Amarante-Mendes et al., 1998) were added. The cells were analyzed in a fluorescence microscope (Leica DM LB) using a fluorescein filter and a 60× objective.

Local UV irradiation and foci analysis

Approximately 5×10^4 cells were plated on coverslips. As described previously (Moné et al., 2001), 40 hours after infection, the cells were rinsed in PBS. The PBS was then removed, leaving only a thin layer of buffer on top of the coverslips. An isopore polycarbonate membrane filter (Millipore) containing pores of 5 µm (4×10^5 pores/cm^2) was placed on top of the cells. The coverslip with filter was irradiated from above with the UV germicidal lamp (50 J/m^2). The formation of foci was followed by fluorescence microscopy from 2 minutes until 4 hours after the local UV irradiation. To undertake kinetics of the photorepair by counting the number of cells presenting different *phr*-EGFP foci fluorescence intensities (Fig. 6A, pattern A and B), the cells were locally UV irradiated and maintained immediately in dark or PRL conditions up to the period of 2 hours, and then counted. For some samples, the cells were cultivated for 2 additional hours after PRL. *Phr*-EGFP fluorescence in foci was quantified using the Molecular Analyst software (BioRad, CA) for a population of 50 cells (198VI and XP456VI cell lines) and 10 cells (XP148VI cell line) in each treatment. The fluorescence in the area of the foci spots was compared with that of a non-locally irradiated identical area of the same nucleus. For the cells not submitted to local UV irradiation, the fluorescence of two independent areas in the same nucleus was compared.

Immunolabeling of CPDs and NER proteins

The cells were prepared as described (Moné et al., 2001; Volker et al., 2001). Approximately 7×10^4 cells plated on coverslips were washed twice with PBS and then fixed with paraformaldehyde 2% in PBS for 15 minutes. The cells were permeabilized using Triton X-100, 0.5% in PBS for 10 minutes. For CPD immunolabeling, DNA was denatured using 0.1 M HCl in PBS for 15 minutes. Thereafter, the cells were incubated with PBS$^+$ (PBS containing glycine 100 mM; BSA 0.5%) for 10 minutes. Immunolabeling of CPDs was performed using a mouse mAb TDM-2 (Mori et al., 1991); TFIIH labeling was carried out using a mouse mAb against the p62 subunit (kindly provided by J. M. Egly, Illkirsch, France) and XPC labeling was carried out using a mouse mAb anti-XPC (kindly provided by J. H. J. Hoeijmakers, Rotterdam, The Netherlands). Detection was performed by using a Texas-red conjugated anti-mouse IgG (TRITC) for all immunolabelings above mentioned. All incubations were performed at room temperature, except the denaturation of DNA for CPD detection, performed at 37°C. The coverlips were analyzed in a fluorescent microscope (Zeiss Axiovert S100, Hamamatsu 3CCD color camera) using fluorescein and FITC filters, with a 60× objective.

Results

Construction of an adenoviral vector expressing the CPD-photolyase fusion protein (Ad*phr*-EGFP)

The CPD-photolyase gene from *Potorous tridactylus* (gene *phr*) was fused with the EGFP reporter gene and cloned in the Adeno-X vector as illustrated in Fig. 1A. The integrity of the gene *phr*-EGFP in the viral vector was confirmed by PCR (Fig. 1B) and DNA sequencing. The plasmid Ad*phr*-EGFP was used to produce adenoviral particles in HEK 293 cell as described (Graham and Prevec, 1995; Hitt et al., 1998). This plasmid contains the adenoviral type 5 genome with deletions in the genes *E1* and *E3* (Mizuguchi and Kay, 1998), therefore, this plasmid is not able to produce replication-competent particles in normal cells (Graham et al., 1977).

Infection of cells with the Ad*phr*-EGFP vector

Diploid human primary fibroblasts (198VI, XP456VI and XP148VI) were infected with the Ad*phr*-EGFP vector and after 40 hours the expression of the *phr*-EGFP gene was accessed by fluorescence microscopy and flow cytometry. In Fig. 2A is shown the data for 198VI cell line. This DNA repair

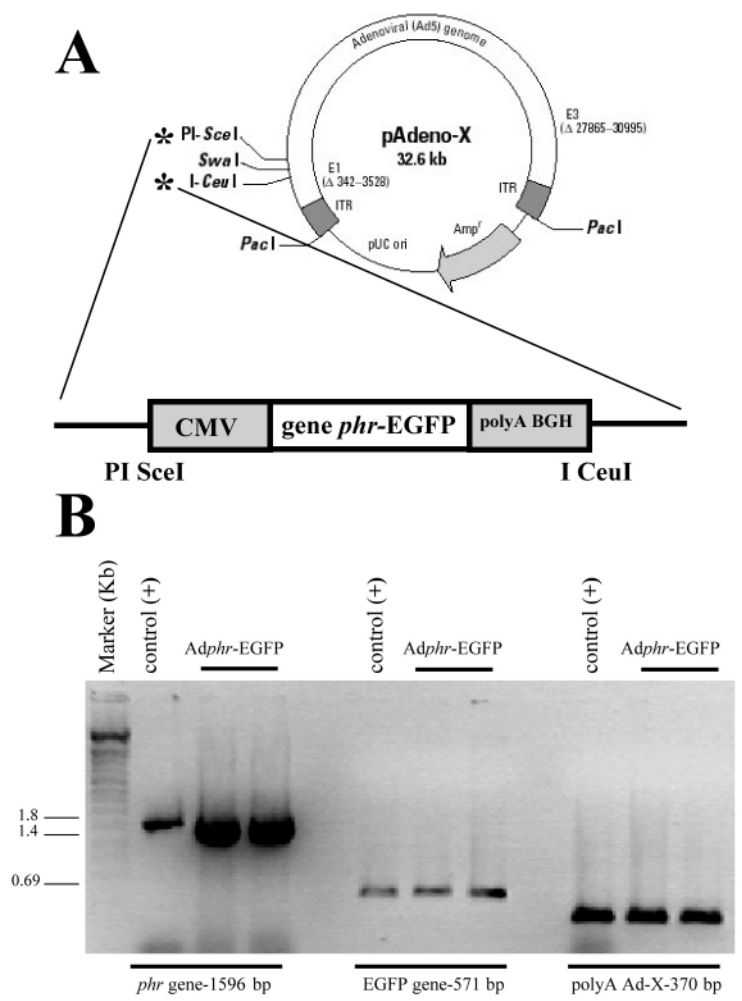


Fig. 1. The Ad*phr*-EGFP adenoviral vector. (A) The marsupial CPD-photolyase gene (gene *phr*) was fused with EGFP in its C-terminal. Thereafter, the *phr*-EGFP expression cassette, under the control of the cytomegalovirus (CMV) promoter and with the bovine growth hormone (BGH) poly(A) signal, was cloned in the pAdeno-X vector by standard recombinant technology at the indicated restriction sites. This plasmid was transfected into low passage HEK 293 cells for the production of recombinant viral particles containing the *phr*-EGFP gene. (B) The integrity of the *phr*-EGFP insert in the recombinant adenovirus vector: the presence of the sequences *phr*, EGFP and poly(A) signal regions from the virus vector were confirmed by a PCR reaction, with the positive controls pCY4B*phr*, pEGFP-N1 and pAdeno-X, respectively.

enzyme presents nuclear localization and a homogeneous pattern of distribution. The percentage of cells presenting the EGFP fluorescence was quantified by flow cytometry, demonstrating that in these infection conditions the whole cell population is expressing the *phr*-EGFP gene.

Recovery of clonogenic capacity in cells infected with Ad*phr*-EGFP

XP12RO-SV (XPA complementation group) cells were infected with the Ad*phr*-EGFP virus and, after approximately 40 hours the expression of the reporter gene was accessed in the same way described in the Fig. 2A. After confirming *phr*-EGFP expression in infected cells, the cells were sub-cultivated in low density to determinate cell survival after UV irradiation (Fig. 2B). Cells infected with the Ad*phr*-EGFP virus and exposed to PRL conditions immediately after UV, displayed improved survival in comparison with parental or mock-infected cells. This survival increase after PRL in infected cells is probably due to CPDs removal by the marsupial CPD-photolyase-EGFP, confirming the action of this heterologous repair enzyme in this cell line.

CPD photorepair in cells infected with Ad*phr*-EGFP

The number of CPDs after UV-irradiation was determined by employing the T4-endonuclease V enzyme. This enzyme recognizes and nicks the DNA specifically at CPD-containing sites, reducing the DNA molecular weight when detected by sedimentation in alkaline sucrose gradients. Thus, the frequency of endonuclease sensitive sites (ESS) corresponds to the relative frequency of CPDs in the cellular DNA (Menck and Meneghini, 1982; Spivak and Hanawalt, 1995). These values were used to calculate the efficiency of CPD removal by photorepair in cells infected with the Ad*phr*-EGFP adenovirus or mock infected. In the data presented in Table 1, there is a drastic reduction in the number of ESS in cells infected with the Ad*phr*-EGFP adenovirus and exposed to PRL conditions immediately after irradiation in comparison with samples kept in dark for the same period. In infected cells treated with 1.0 J/m^2 and exposed to PRL, there is almost a complete removal of CPDs in the cellular genome. The contribution of this heterologous photorepair with a higher UV dose, 2.5 J/m^2, is reduced to 75%. To confirm this specific response of CPD removal performed by the *phr*-EGFP fusion protein, CPD lesions were immunolabeled in human primary fibroblasts (Fig. 3). Normal (198VI) and XPA mutated (XP456VI) primary cells were UV-irradiated with 10 J/m^2 and maintained under dark or PRL during 2 hours immediately or 4 hours after irradiation. The disappearance of the fluorescent signal detected by anti-CPD reveals that *phr*-EGFP photorepair in infected cells can remove all or most of the CPD lesions in the nucleus, even when performed 4 hours after UV. Altogether, the data presented indicate that the *phr*-EGFP fusion enzyme is being functional in CPDs recognition and photorepair.

Prevention of UV-induced apoptosis by the *phr*-EGFP-mediated photoreactivation of CPDs

Previous data demonstrated that CPDs represent an important signal to UV-induced apoptosis in human cells, and heterologous photorepair can prevent the apoptotic response in irradiated cells (Chiganças et al., 2000). The capacity of photorepair performed by *phr*-EGFP to prevent UV-induced apoptosis was investigated in XP12RO-SV infected cells. These DNA-repair-deficient cells were chosen for increased sensitivity to apoptosis measurement in low UV doses, due to its deficiency in NER (Benhamou and Sarasin, 2000). The cells were infected, UV-irradiated at the indicated doses, followed

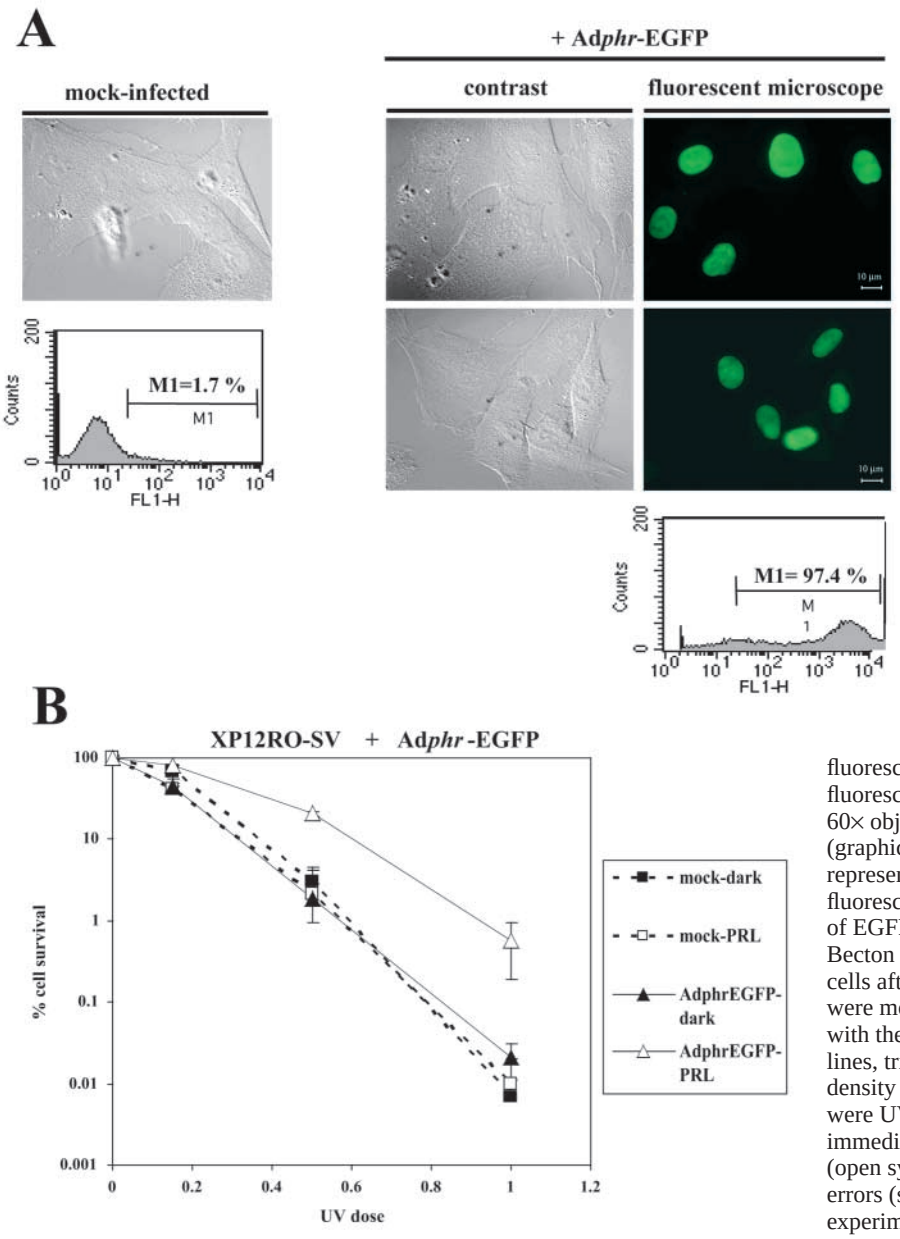


Fig. 2. Photorepair protects Ad*phr*-EGFP-infected cells from UV irradiation. (A) Infection of primary cells with Ad*phr*-EGFP vector. Normal human primary fibroblasts (198VI cell line) were infected with Ad*phr*-EGFP (m.o.i. 1.6×10^5) and the fluorescence of EGFP was analyzed after 40 hours by fluorescence microscope (photos of representative fields; 60× objective) and flow cytometry of cell suspension (graphics). For the graphics of fluorescence, 'counts' represents the number of cells and 'FL1-H' the green fluorescence intensity. M1 corresponds to the percentage of EGFP fluorescence calculated (CellQuest Software, Becton Dickinson, USA). (B) Survival of XP12RO-SV cells after UV irradiation. Approximately 6.0×10^5 cells were mock-infected (dotted lines, squares) or infected with the Ad*phr*-EGFP vector (m.o.i. 4×10^4; unbroken lines, triangles) and, after 40 hours, sub-cultivated in low density for analysis of clonogenic capacity. The cells were UV-irradiated at the indicated doses and immediately exposed to dark (closed symbols) or PRL (open symbols) conditions The bars indicate standard errors (s.d.; each sample in triplicate, two independent experiments).

by immediate exposure to dark or PRL conditions (Fig. 4A,B). When the pattern of genomic DNA distribution in the cell cycle is accessed by flow cytometry, we observe dose-dependent apoptosis induction, exhibited as the increase in the percentage of cells at the sub-diploid region of the cell cycle (M1; see Fig. 4A). Cells infected with Ad*phr*-EGFP and exposed to PRL after UV show a drastic decrease in the cell population located in the sub-G_1 region of the cell cycle. These results confirm CPDs as being a signal to apoptosis after UV in human cells, and demonstrated that the phenotypical response of *phr*-EGFP enzyme is functional in CPD recognition and repair in light conditions, preventing apoptosis execution in XP12RO-SV cells.

Apoptotic morphological characteristics, such as loss of membrane integrity, nuclear condensation and fragmentation, were also investigated by using acridine orange/ethidium bromide staining in XP12RO-SV cells infected with Ad*phr*-EGFP vector. Apoptotic cells normally appear with orange nuclei, which can be easily distinguished from the green live cells. UV irradiation causes an increase in the percentage of cells with apoptotic morphology (Fig. 4B), confirming this specific type of active cell death in response to UV-induced DNA damage in these cells. For infected cells exposed to PRL conditions after UV, there is a reduction in the frequency of apoptotic cells, once more indicating that the action of *phr*-EGFP in CPD elimination is preventing apoptosis in comparison with dark conditions or mock-infected cells. Infection with Ad*phr*-EGFP does not alter the percentage of non-irradiated cells presenting apoptotic markers, indicating that adenovirus infection or the presence of the heterologous *phr* enzyme are not toxic to these cells. These results add support to the *phr*-EGFP enzyme being functional in

photorepair of CPDs, preventing UV-induced apoptotic cell death in human cells.

The contribution of *phr*-EGFP action in CPD removal and apoptosis was also investigated in human primary cells 72 hours after UV irradiation by flow cytometry (Fig. 4C). Human primary cells, normal (198VI), XPA (XP456VI) and XPC mutated (XP148VI), were irradiated with the indicated doses 40 hours after infection with the Ad*phr*-EGFP vector under the conditions as described above. Cells mutated in the XPA gene, completely defective in NER (Costa et al., 2003), exhibit a similar percentage of apoptosis at a much lower UV dose in comparison with the other cell lines (Queille et al., 2001), and thus confirming that this DNA repair deficiency makes these cells more sensitive to DNA damage-induced apoptosis. In the cells infected with the Ad*phr*-EGFP vector and maintained in PRL conditions, there is a clear reduction in the relative number of sub-G_1 nuclei after UV irradiation, not observed in samples maintained under dark conditions or mock-infected cells. It is important to notice that in mock-infected cells there is no significant difference in the frequency of apoptotic cell death, whether the cells are exposed or not to PRL. These data indicate that this fusion protein is being functional in CPD elimination and confirm these photoproducts as being an important signal to UV-induced apoptosis in human primary cells, as well (Chiganças et al., 2000). Moreover, the expression of the heterologous *phr*-EGFP enzyme does not modify the phenotype of infected cells.

Table 1. Removal of T4-endonuclease sensitive sites (ESS) by Ad*phr*-EGFP-mediated photorepair in XP12RO-SV cells

Treatment	ESS† (per 10^9 Da)	Photorepair (%)‡
UV=0 J/m^2		
UV=0 J/m^2 PRL	0.32±0.05	
UV=0 J/m^2+Ad*phr*-EGFP	0.04±0.23	
UV=1.0 J/m^2 0 hours	3.77±1.01	
UV=1.0 J/m^2 dark 2 hours	3.55±0.65	
UV=1.0 J/m^2 PRL 2 hours	3.85±0.45	
UV=1.0 J/m^2 dark 2 hours+Ad*phr*-EGFP	3.32±0.84	
UV=1.0 J/m^2 PRL 2 hours+Ad*phr*-EGFP	0.13±0.71	96%
UV=2.5 J/m^2 0 hours	6.77±1.69	
UV=2.5 J/m^2 dark 2 hours	6.25±1.98	
UV=2.5 J/m^2 PRL 2 hours	6.90±2.06	
UV=2.5 J/m^2 dark 2 hours+Ad*phr*-EGFP	6.20±1.92	
UV=2.5 J/m^2 PRL 2 hours+Ad*phr*-EGFP	1.55±0.65	75%

†The ESS numbers represent the number of T4-endonuclease-sensitive sites per 10^9 Daltons. Cells were exposed to UV irradiation at the indicated doses, harvested immediately or kept in dark or PRL conditions for 2 hours. The numbers represent the average of at least four independent experiments.
‡Values for photorepair were obtained considering the decrease of ESS in Ad*phr*-EGFP infected cells submitted to PRL in relation to those maintained in the dark. The differences between infected samples exposed to dark or PRL conditions after UV were assessed for statistical significance by Student's *t* test ($P<0.05$).

Analysis of *phr*-EGFP foci in CPD sites after local UV irradiation

Normal primary cells were locally UV irradiated with 50 J/m^2 and the formation of foci of the photolyase fusion protein was investigated in fluorescence microscopy. This technique allows us to UV-irradiate only a confined area of individual cell nuclei, and thus investigate the mobility and lesion access by DNA repair enzymes (Moné et al., 2001; Volker et al., 2001). In Fig.

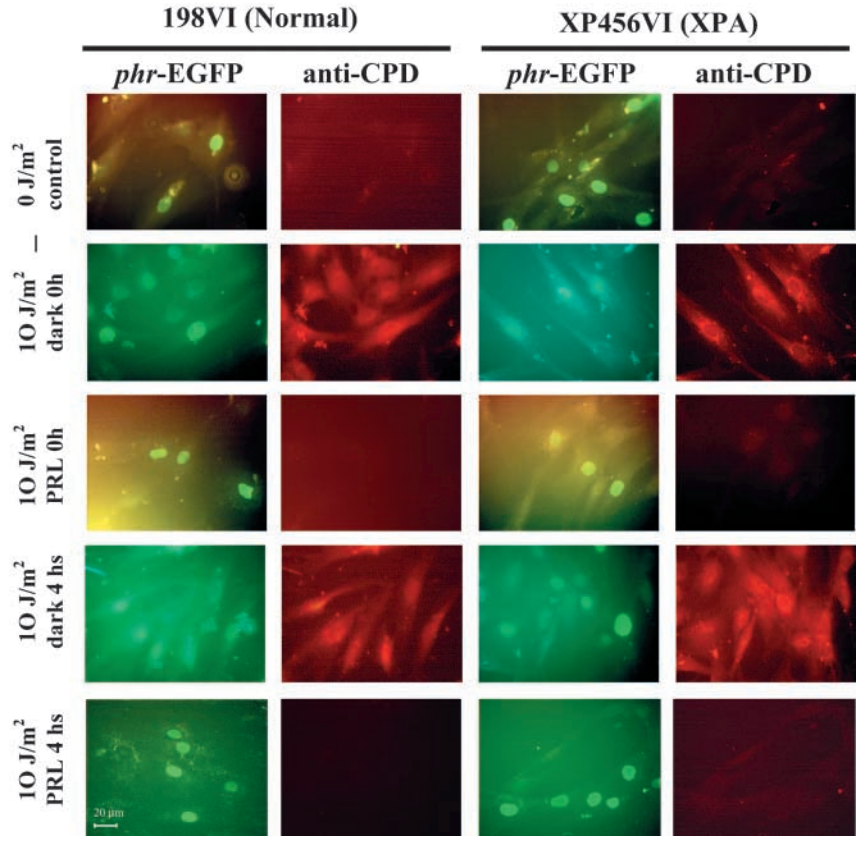


Fig. 3. CPD immunostaining in UV-irradiated 198VI and XP456VI cells. 5.0×10^4 cells were infected with Adphr-EGFP adenovirus and, 40 hours later, the cells were exposed to UV irradiation (10 J/m^2). Different samples were maintained under dark or PRL conditions during 2 hours immediately after a period of 4 hours incubation. Thereafter, the cells were fixed and immunostained for CPD as described in the Materials and Methods. The cells were analyzed in fluorescence microscopy (Zeiss Axiovert S100, Hamamatsu 3CCD color camera). Representative fields of each sample for fluorescein and TRITC filters are shown.

5A, foci of the *phr*-EGFP protein can be observed as early as 2 minutes after the local UV irradiation. Colocalization of this fusion protein with the CPD lesion (Fig. 5B) confirms the specificity of heterologous CPD-photolyase behavior; rapidly migrating to CPD and forming a stable complex in a light-independent manner (Sancar, 1994). Labeling in un-irradiated parts of locally damaged nuclei was indistinguishable from background levels of un-irradiated control cells in a separate experiment, indicating that the filter material efficiently absorbs 254 nm UV light. The same response was observed for the NER deficient primary cell lines (data not shown).

Colocalization of *phr*-EGFP with NER proteins in CPD-containing sites

We know that the pattern of distribution of NER proteins in nuclei changes after UV irradiation, and that the XPC protein and the transcription factor IIH (TFIIH) colocalize with the CPD lesions in human cells (Volker et al., 2001). Normal human primary cells infected with Ad*phr*EGFP were incubated with anti-XPC and anti-p62 1 hour after local UV irradiation to verify the colocalization of photolyase with these NER enzymes (Fig. 5B). The CPD-photolyase fusion protein colocalizes with NER sites, containing the XPC protein and the TFIIH factor subunit p62 (Seroz et al., 1995). These data confirm the specificity of the migration of the *phr*-EGFP enzyme to CPD-containing sites that are recognized and repaired by the regular NER pathway.

Redistribution of the *phr*-EGFP protein in the nuclei after photorepair

Experiments of local UV irradiation were performed for normal and NER-deficient human primary cell lines, and the cells being subsequently exposed or not to PRL after different periods. In dark conditions, strongly-fluorescent foci are formed at the damaged sites at least up to 1 hour later, indicating that the photorepair enzyme recognizes and binds to CPD sites (Fig. 6A, foci pattern A indicated by blue arrows). In samples submitted to PRL conditions for 1 hour, *phr*-EGFP foci in UV-irradiated sites appear with less intense fluorescence (Fig. 6A, foci pattern B indicated by yellow arrows), suggesting a redistribution of the enzyme after CPD photorepair in light conditions. This response is more drastic in samples exposed to PRL and analyzed 4 hours after UV.

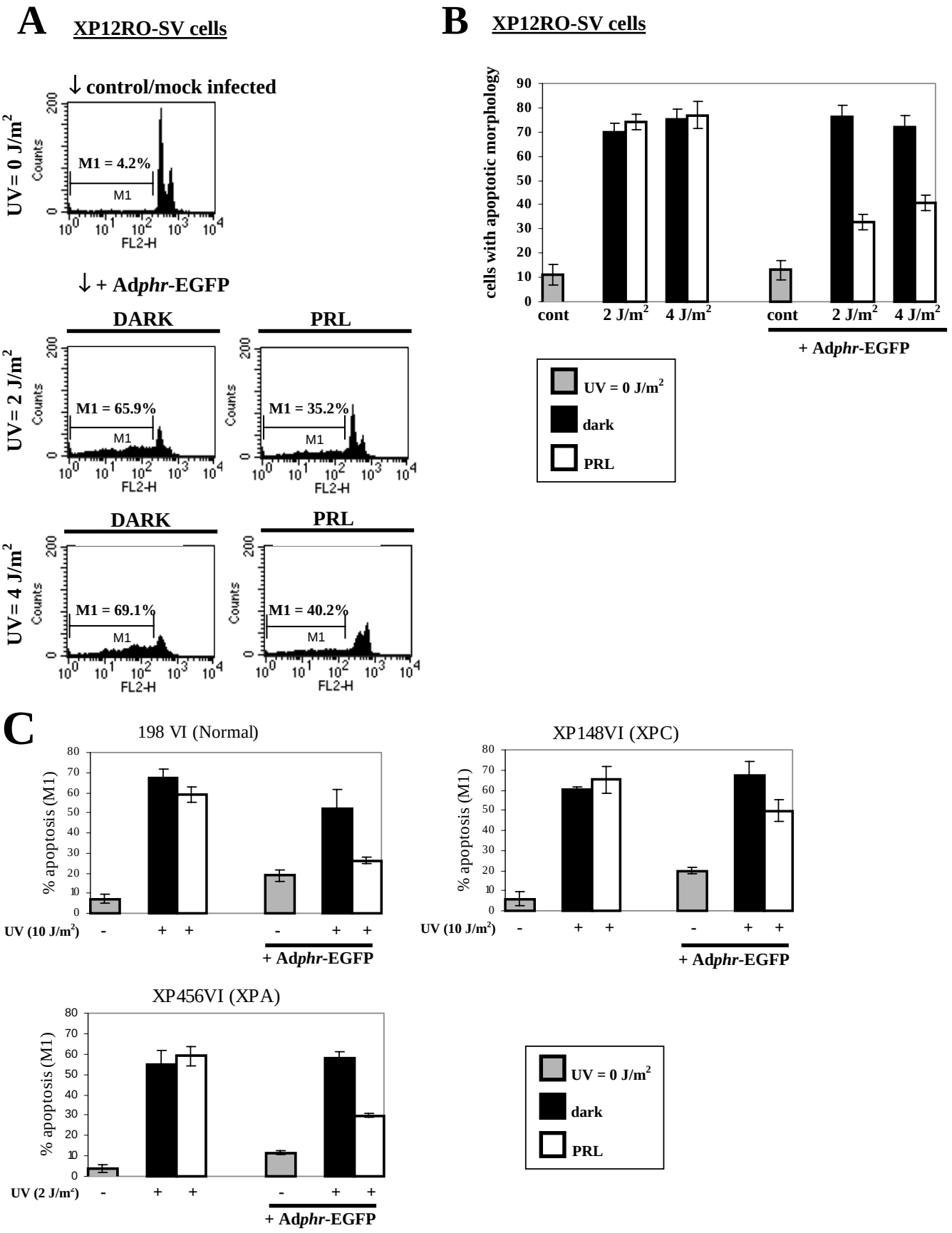
To further confirm whether this response corresponds to a redistribution of the *phr*-EGFP enzyme after CPD elimination, cells maintained in dark or PRL conditions for different periods of time after UV irradiation were tested for the presence of lesions with anti-CPD. The reduction of fluorescent signal in the foci for CPDs lesions (red) is similar to the pattern of *phr*-EGFP fluorescence (green; Fig. 6B). These results indicate a specific response of redistribution of the heterologous photolyase fusion protein after photorepair in situ. In contrast, cells that were not exposed to PRL present a strong-fluorescent signal for the CPD antibody at least up to 4 hours after UV exposure (data not shown).

The number of cells presenting different *phr*-EGFP fluorescent signals in UV-damaged spots was quantified. The two different patterns of foci exemplified in Fig. 6A, pattern A (blue arrows) and B (yellow arrows) of fluorescence, were considered as categories for this analysis. In Fig. 7, we show the results for normal, XPA and XPC mutated primary cell lines in different periods after local UV irradiation. There is a time-dependent response of change in fluorescence intensity of *phr*-EGFP in CPD-containing sites, even in cells kept in the dark. Curiously, this redistribution of the *phr*-EGFP enzyme in dark conditions also occurs in XPA and XPC mutated cell lines. However, for the three cell lines analyzed, the decrease of fluorescence of the *phr*-EGFP enzyme in UV-irradiated spots is much more pronounced if the cells are exposed to PRL: 4 hours after local UV irradiation, 75-80% of the cells present pattern B of *phr*-EGFP fluorescent signal. Most probably, for cells exposed to PRL, this enzyme redistribution in situ is due to CPD-removal. The XPA and XPC mutated cell lines do not present a different behavior of the photolyase enzyme, indicating that this enzyme can access and repair CPDs even in the presence of these mutated NER proteins.

The ratio of *phr*-EGFP fluorescence intensity in foci sites in relation to non-exposed areas of the same nucleus was also determined (Fig. 8). After local UV irradiation and maintenance in dark during 1 hour, the *phr*-EGFP signal in UV-irradiated sites is approximately 3.5-4.2 stronger than non-exposed areas, in contrast to approximately 2.0 in samples exposed to PRL for the same period of time. In samples analyzed 4 hours after local UV irradiation, the fluorescent signal *phr*-EGFP/nucleus in photoreactivated cells is 1.6-1.2, which are values close to those observed in cells not exposed to local UV irradiation. These results confirm that CPD-photolyase enzyme leaves the site of damage after photorepair to redistribute in the whole nucleus, and this response presents similar kinetics independent of NER status. In agreement with the results in Fig. 7, there is a change in *phr*-EGFP signal even in dark conditions in all cell lines employed.

Discussion

The experimental tool of heterologous photorepair in mammalian cells to investigate the role of CPDs in apoptosis has been very informative in different systems (Asashina et al., 1999; Kulms et al., 1999; Chiganças et al., 2000; Stege et al., 2000; Chiganças et al., 2002). CPD-photolyases are enzymes that can recognize and form a stable complex with the CPD in a light-independent manner (Sancar, 1994; Todo, 1999), removing the photoproduct in the presence of visible light, thereby restoring the native state of the bases in the DNA molecule. By using the heterologous expression of photolyases, we showed that CPDs represent an important signal for UV-induced apoptotic suicide in human cells (Chiganças et al., 2000), mainly those present in the transcribed strand of active genes, by representing a complete block to RNA polymerase II progression (Donahue et al., 1994; Ljungman and Zhang, 1996; Chiganças et al., 2002). In this work, we generated a recombinant adenovirus carrying a CPD-photolyase-EGFP fusion gene, employed to study the behavior of this DNA repair enzyme in human cells. This was shown to be an efficient (almost 100% of cells are transduced) and reproducible gene-transfer system, being harmless and not interfering in repair and apoptosis analysis in these cells. Using local UV irradiation, foci of *phr*-EGFP enzyme were detected in CPD-containing areas in the nucleus, and photolyase redistribution after photorepair in situ was followed. These foci


A
XP12RO-SV cells
↓ control/mock infected
UV= 0 J/m2
M1 = 4.2%
↓ + Adphr-EGFP
DARK
PRL
UV= 2 J/m2
M1 = 65.9%
M1 = 35.2%
UV= 4 J/m2
M1 = 69.1%
M1 = 40.2%
Counts
FL2-H
B
XP12RO-SV cells
cells with apoptotic morphology
cont
2 J/m2
4 J/m2
+ Adphr-EGFP
UV = 0 J/m2
dark
PRL
C
198 VI (Normal)
XP148VI (XPC)
XP456VI (XPA)
% apoptosis (M1)
UV (10 J/m2)
UV (2 J/m2)
+ Adphr-EGFP

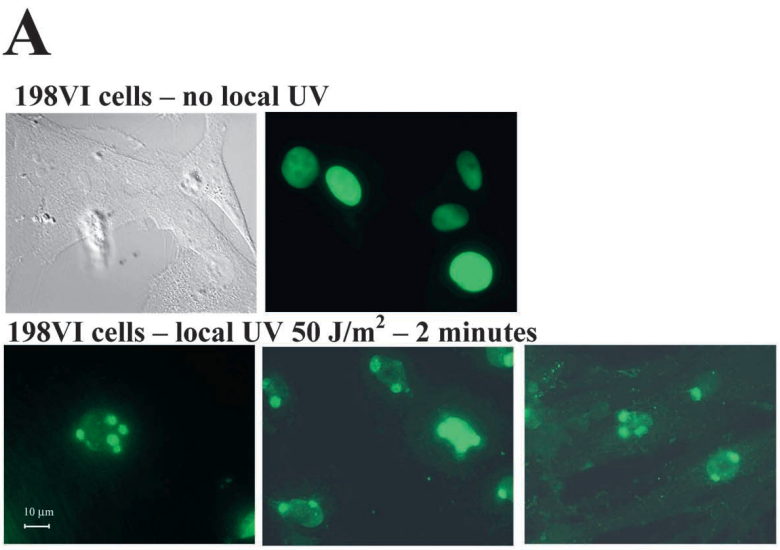


Fig. 4. Photorepair performed by the *phr*-EGFP recombinant enzyme prevents UV-induced apoptosis in XP12RO-SV and diploid human fibroblasts. (A) Apoptosis analysis by flow cytometry in UV-irradiated XP12RO-SV (XPA mutated) cells. Ad*phr*-EGFP and mock-infected cells were UV-irradiated at the indicated doses and exposed immediately to PRL or dark conditions. The percentage of apoptosis was accessed 48 hours after UV irradiation by flow cytometry analysis. In the graphics presented, 'counts' corresponds to the number of cells and 'FL2-H' to the amount of DNA measured by P.I. fluorescence. M1 represents the percentage of cells in sub-G1 region of the cell cycle, that is apoptotic cells. (B) Photorepair performed by *phr*-EGFP prevents UV-induced morphological apoptotic markers in XP12RO cells. Mock and Ad*phr*-EGFP-infected samples were UV-irradiated and treated as described in (A). The morphology of cells was analyzed 48 hours after UV-irradiation by fluorescence microscopy using acridine orange/ethidium bromide staining. Shaded bars represent control samples (UV=0 J/m^2), black bars irradiated samples kept in the dark, and white bars, irradiated samples exposed to PRL. (C) Apoptosis analysis by flow cytometry in UV-irradiated human primary cells. 198VI (Normal), XP456VI (XPA mutated) and XP148VI (XPC mutated) were treated as described in (A), the samples being analyzed 72 hours after UV irradiation by flow cytometry. The frequency of subdiploid nuclei was calculated and plotted for the three cell lines employed. Shaded bars represent control samples (UV=0 J/m^2), closed bars irradiated samples kept in dark, and open bars irradiated samples exposed to PRL. The data for each cell line represent the average of two independent experiments.

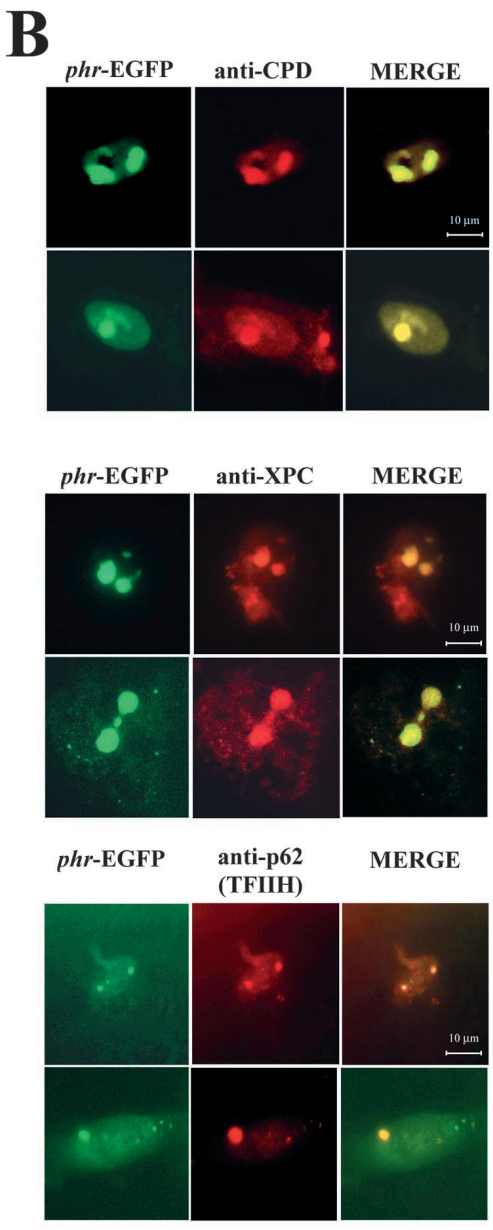


Fig. 5. Investigation of foci of *phr*-EGFP in CPD-containing areas in primary cells. (A) Normal human primary fibroblasts (198VI cell line) were infected with Ad*phr*-EGFP and, after 40 hours, locally UV irradiated with 50 J/m^2. The formation of foci of *phr*-EGFP was analyzed after 2 minutes in culture by fluorescence microscopy. (B) Normal human primary fibroblasts (198VI cell line) infected with Ad*phr*EGFP were incubated with anti-CPD, anti-XPC and anti-p62 1 hour after 50 J/m^2 of local UV irradiation. Two rows of representative fields in fluorescence microscopy (60× objective) are presented for each colocalization experiment. Each photo contains a single cell/nucleus presenting independent foci of *phr*-EGFP in UV-irradiated areas.

should represent accumulation of photolyase in CPD-containing sites in locally irradiated areas; NER enzymes, previously described as colocalizing with UV-induced photolesions, are also detected in the same sites.

This is the first report of a CPD-photolyase fusion protein being used to investigate CPD access and repair in situ, as well the consequences of these lesions in the apoptotic response in human cells. In light conditions, the specific removal of CPD photoproducts in infected cells was demonstrated by sedimentation in alkaline sucrose gradients and CPD immunostaining, indicating the *phr*-EGFP fusion protein is functional in photorepair. The

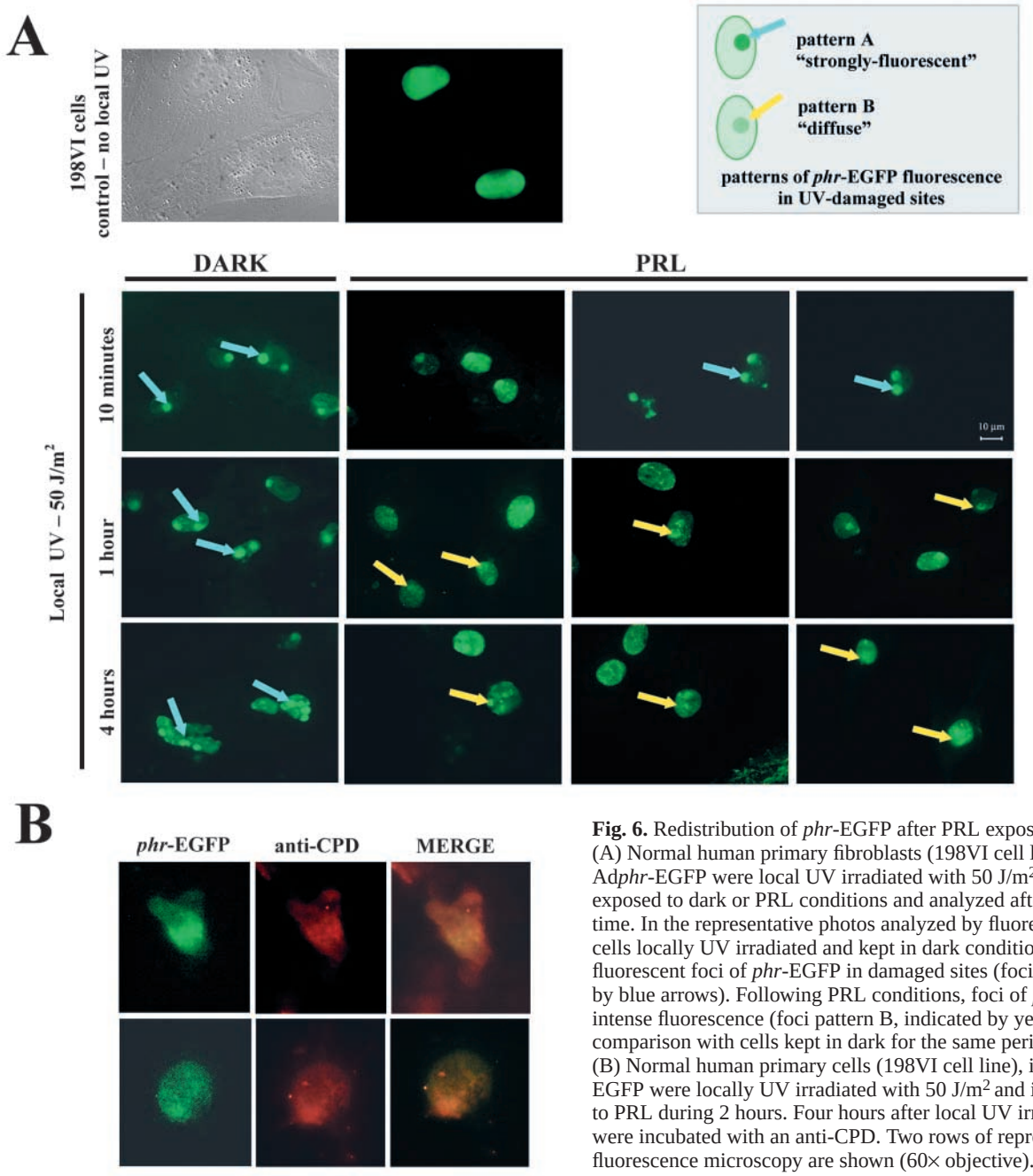


Fig. 6. Redistribution of *phr*-EGFP after PRL exposure in primary cells. (A) Normal human primary fibroblasts (198VI cell line) infected with Ad*phr*-EGFP were local UV irradiated with 50 J/m^2, immediately exposed to dark or PRL conditions and analyzed after different periods of time. In the representative photos analyzed by fluorescence microscopy, cells locally UV irradiated and kept in dark conditions display strongly-fluorescent foci of *phr*-EGFP in damaged sites (foci pattern A, indicated by blue arrows). Following PRL conditions, foci of *phr*-EGFP with less intense fluorescence (foci pattern B, indicated by yellow arrows) in comparison with cells kept in dark for the same period of time. (B) Normal human primary cells (198VI cell line), infected with Ad*phr*-EGFP were locally UV irradiated with 50 J/m^2 and immediately exposed to PRL during 2 hours. Four hours after local UV irradiation the cells were incubated with an anti-CPD. Two rows of representative fields in fluorescence microscopy are shown (60× objective).

heterologous removal of CPDs after PRL exposure clearly increases the clonogenic capacity and prevents induction of apoptosis in UV-irradiated cells, confirming that these photoproducts are important signals to cell death after UV, as previously described (Miyaji and Menck, 1996; Chiganças et al., 2000; Chiganças et al., 2002), and demonstrating that *phr*-EGFP fusion protein is effective in CPD recognition and photorepair whatever the NER status of the cell. CPDs are a physical hindrance to the transcriptional complex progression (Donahue et al., 1994); the RNA polymerase II complex stalled by persistent photoproducts in the transcribed strand is an active signal to cellular suicide after UV irradiation (Ljungman and Zhang, 1996; Chiganças et al., 2002). The removal of these CPD lesions, by the photolyase fusion enzyme in human cells infected with Ad*phr*-EGFP and exposed to PRL, prevents apoptosis induction probably by eliminating the signal to death in active genes, allowing for the RNA synthesis to restart, and thus promoting progression in the cell cycle and survival.

Human primary cells infected with the Ad*phr*-EGFP vector display nuclear localization and a homogeneous pattern of distribution of this DNA repair enzyme (see Fig. 2A, Fig. 5A). These observations demonstrate that, under normal growth conditions, this enzyme is not confined to specific regions of chromatin within the nucleus of human cells, being only recruited after the generation of UV-induced lesions. The formation of *phr*-EGFP foci in damaged areas was detected as

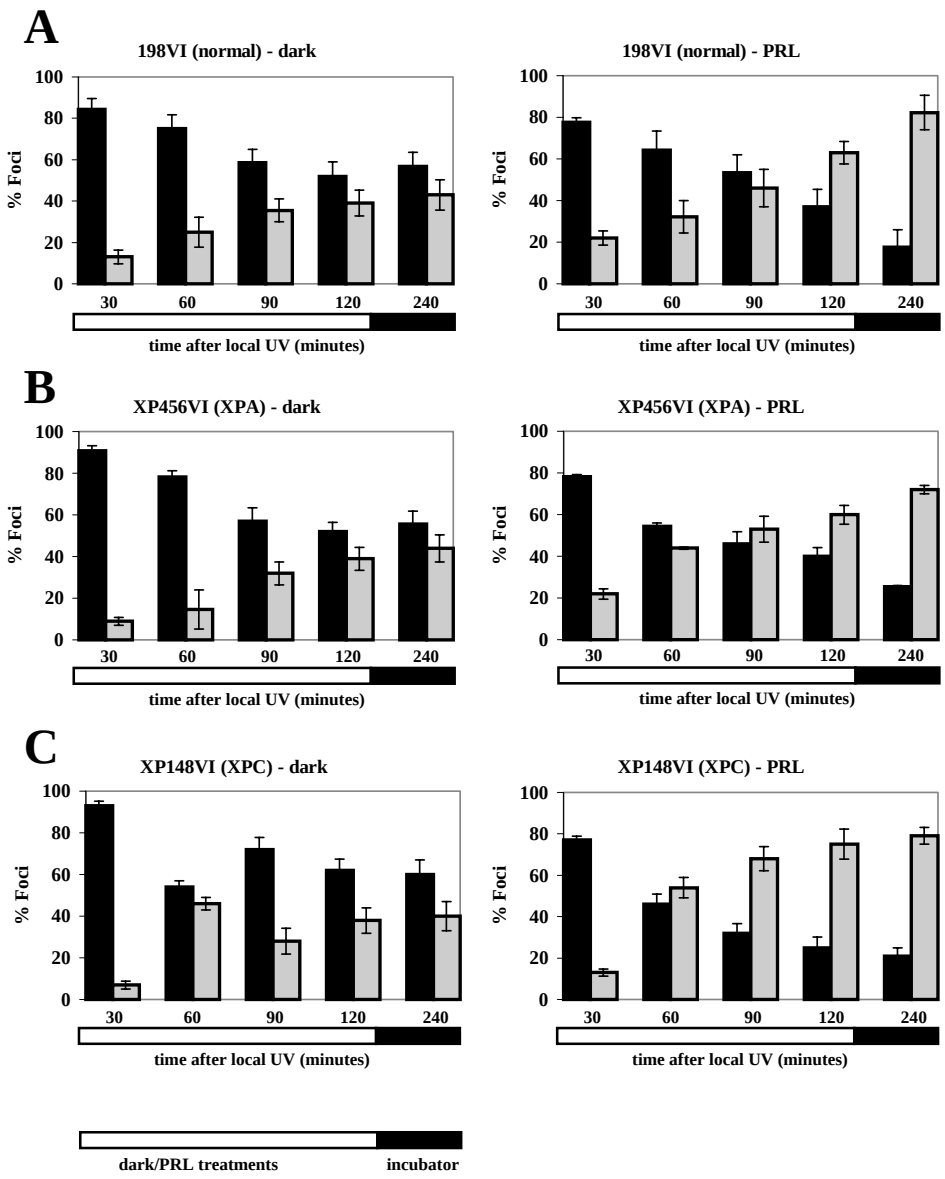


Fig. 7. Quantification of foci pattern A or B (as shown in Fig. 6A) in human primary cells locally UV irradiated. Normal (A; 198VI), XPA (B; XP456VI) and XPC mutated (C; XP148VI) human primary fibroblasts infected with Ad*phr*-EGFP were locally UV irradiated with 50 J/m^2. The cells were exposed to dark or PRL treatments immediately after local UV irradiation. These treatments were performed during different periods of time, but up to a maximum period of 2 hours, the samples then being kept in the incubator until analysis by fluorescence microscopy (see bar in legend). The number of cells with foci strongly-fluorescent (pattern A, black bars) or with a less intense fluorescence (pattern B, gray bars) was counted by fluorescence microscopy in different periods of time after local UV irradiation. We counted 100 for each sample, and the data thereby represent the average of three independent experiments.

early as 2 minutes after local UV irradiation (Fig. 5A), colocalizing with the CPD lesion in situ. The XPC protein and the TFIIH-subunit p62 were also detected in CPD sites with the CPD-photolyase, both participating in NER and already reported to access photoproducts in UV-exposed areas (Volker et al., 2001). These observations add support to heterologous *phr*-EGFP enzyme being functional in CPD access in sites that are subject to repair by the regular NER pathway.

Cells expressing *phr*-EGFP and exposed to PRL present a decrease in *phr*-EGFP fluorescence in CPD sites (Fig. 6A, foci pattern B indicated by yellow arrows). Under the same experimental conditions, the anti-CPD fluorescent signal becomes less intense, probably due to the disappearance of this lesion under PRL conditions, thus indicating a redistribution of this marsupial CPD-photolyase after photorepair in the nuclei of human cells (see Fig. 6B). This response is reduced in samples kept under dark conditions after UV irradiation and analyzed immediately or up to 4 hours later (Fig. 6A and Fig. 7), thus demonstrating the persistence of most of CPD lesions in the genome under these conditions. Recently, an investigation of recruitment of NER proteins in damaged sites containing UV-induced photoproducts demonstrated a time-dependent, and gradual, decrease in the recruitment of the XPC protein in these sites in increasing times after local UV irradiation in human cells (Wang et al., 2003). The results of CPD-photolyase re-distribution after CPD elimination add support to this model of dispersion of repair proteins after excision of damaged DNA segments and/or lesion removal.

We quantified this response in different periods after local UV exposure in normal and NER-deficient cell lines. A time-dependent decrease in the intensity of foci fluorescence in damaged spots was observed even in cells maintained under dark conditions (Figs 7 and 8). This response occurs in normal and NER-deficient cells, indicating that it is neither influenced by NER activity nor by the persistence of 6-4 photoproducts, lesions that are almost completely eliminated 3-4 hours after UV in NER-proficient cells (Riou et al., 1999). The time-dependent decrease of *phr*-EGFP fluorescent signal in UV-

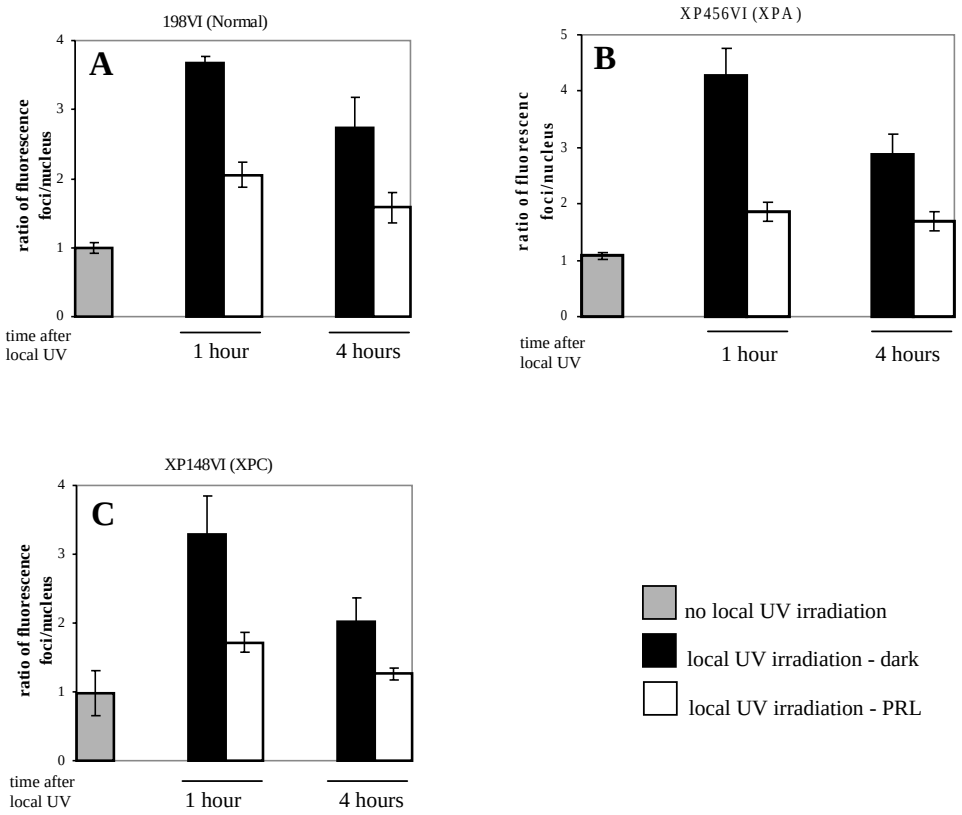


Fig. 8. Quantification of *phr*-EGFP fluorescence in foci sites in relation to non-irradiated areas. Normal (A; 198VI), XPA (B; XP456VI) and XPC mutated (C; XP148VI) human primary fibroblasts infected with Ad*phr*-EGFP were locally UV irradiated with 50 J/m^2. The cells were exposed to dark (black bars) or PRL (white bars) treatments immediately after local UV irradiation and then analyzed after the indicated periods (1 hour and 4 hours). The intensity of green fluorescent signal in UV-irradiated areas (foci) was quantified using the Molecular Analyst software (BioRad, CA) for a population of 50 cells (198VI and XP456VI cell lines) and 10 cells (XP148VI cell line) and compared to non-irradiated areas in the same nucleus.

irradiated areas, even under dark conditions, might indicate a putative general response of chromatin in the presence of DNA damage, thus rendering the CPD less accessible to *phr*-EGFP enzyme. The tumor suppressor gene p53 acts as a chromatin accessibility factor, allowing the NER proteins to reach the lesion site, and revealing a pathway of chromatin reorganization after DNA damage detection in cells (Rubbi and Milner, 2003; Wang et al., 2003). In fact, nucleosome organization in yeast modulates photolyase access to damaged DNA (Gaillard et al., 2003). The time-dependent response in the fluorescence of *phr*-EGFP in foci under dark conditions might be due to chromatin dynamics after the introduction of DNA photoproducts. Another possible explanation is that the CPD-photolyase might dissociate from the CPD in the DNA molecule in response to the interference of other cellular DNA repair proteins that are also supposed to monitor photoproducts in the genome. UV-damaged DNA binding (UV-DDB), XPC-hHR23B and TFIIH complexes present a normal function of lesion recognition in GGR, and RNA polymerase II elongation complexes also sense the presence of CPDs in TCR; these factors might compete and influence *phr*-EGFP permanence in CPD-containing sites after longer periods of time in the dark. These complexes might also access the lesions, and compete with *phr*-EGFP, even in the NER-deficient cells employed in this work.

The NER factors, such as XPC-hHR23B and TFIIH complexes, dissociate from locally irradiated areas around 4 hours after UV exposure (Volker et al., 2001; Uchida et al., 2002; Wang et al., 2003), even with the persistence of the majority of CPD lesions during this period of time (Riou et al., 1999; Wang et al., 2003). These data suggest that this early response in the assembly of repair enzymes in locally damaged sites is following the kinetics of 6-4 photoproducts elimination (3-4 hours) (Riou et al., 1999; Wang et al., 2003), a lesion that generates a larger distortion in the DNA double helix in comparison with CPD, supposedly being preferentially recognized by DNA repair recognition proteins, as the XPC-hHR23B complex (Sugasawa et al., 2001). Thus, the mechanism responsible for NER pre-incision complexes dissociation from damaged sites still containing CPD lesions remains unclear. A similar signal might be dictating the behavior of the heterologous *phr*-EGFP enzyme inside the nuclei of human primary cells, as approximately 40% of the cell population kept in dark after UV present a decrease in *phr*-EGFP fluorescent signal in foci 4 hours after UV exposure, indicating that the molecules might be dissociating from CPD-containing sites. However, cells exposed to PRL after UV display an increase in the number of cells with foci pattern B (75-80% of cell population, see Fig. 7) in comparison with dark conditions, suggesting that the redistribution of photolyase is improved after lesion removal by photorepair (Sancar, 1994).

The XPB protein, involved in TCR, cannot re-distribute after DNA damage in nuclei of NER deficient cells, it remains in CPD sites for long periods after UV irradiation (Volker et al., 2001). In contrast, analysis of the recruitment of NER proteins in UV-irradiated sites reveals that in p53-null cell lines there is an impaired mobility of XPC protein and TFIIH complex to damaged sites (Wang et al., 2003). These data indicate that, as expected, the chromatin and NER status influences DNA damage detection and the mobility of repair enzymes. Previous results from our laboratory, also demonstrate a decreased response in photolyase contribution to the recovery of RNA synthesis in XPB mutated cells (Chiganças et al., 2002). These observations suggest that NER enzymes, when mutated, might

affect the heterologous photorepair kinetics, since these repair enzymes and photolyases access the same damaged sites in the nucleus. In fact, the transduction of a CPD-photolyase fusion protein by the versatile recombinant adenovirus might be used to investigate competition among normal and/or mutated DNA repair enzymes to access CPDs in DNA in situ. In this work, when employing primary human cells with distinct NER phenotypes, the mutated XPA and XPC proteins in the cell lines employed do not seem to impair photolyase activity, as judged by efficient photorepair. XPC-hHR23B is one important recognition complex, having access to lesion sites in the GGR process, this being a putative candidate to encounter the *phr*-EGFP molecules in locally UV irradiated regions in the nucleus. However, data reveal that the heterologous photolyase is able to recognize and photorepair CPDs immediately after irradiation even in the presence of XPC and XPA mutated proteins.

In conclusion, this work represents the first report of a CPD-photolyase fusion protein used to investigate CPDs recognition and repair in situ, as well as cellular responses to the presence of these photoproducts in the genome. The Ad*phr*-EGFP vector was shown to be an efficient and reproducible vehicle to deliver a heterologous photolyase in human primary cells. The action of *phr*-EGFP in photorepair prevents the induction of an active cellular suicide, apoptosis, after UV irradiation. When investigating *phr*-EGFP action and redistribution after photorepair in normal and NER-deficient human primary cells, we confirmed that the *phr*-EGFP enzyme under PRL conditions protects human cells from cell death by apoptosis, acting specifically in CPD sites also prone to NER activity.

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