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UV sensitivity and impaired nucleotide excision repair in DNA-dependent protein kinase mutant cells

Catherine Muller, Patrick Calsou, Philippe Frit, Caroline Cayrol[1], Timothy Carter[2] and Bernard Salles*

Institut de Pharmacologie et de Biologie Structurale (CNRS, UPR 9062), 205 route de Narbonne, 31077 Toulouse Cedex, France, [1]Institut de Recherche Pierre Fabre, Laboratoire de Pharmacologie Cellulaire Cutanée, CHU Rangueil, 31054 Toulouse Cedex, France and [2]Department of Biological Sciences, St John's University, Jamaica, NY 11439, USA

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ABSTRACT

DNA-dependent protein kinase (DNA-PK), a member of the phosphatidyl-inositol (PI)3-kinase family, is involved in the repair of DNA double-strand breaks. Its regulatory subunit, Ku, binds to DNA and recruits the kinase catalytic subunit (DNA-PK$_{cs}$). We show here a new role of DNA-PK in the modulation of the process of nucleotide excision repair (NER) *in vivo* since, as compared with their respective parental cell lines, DNA-PK mutants (*scid* , V-3 and *xrs 6* cells) exhibit sensitivity to UV-C irradiation (2.0- to 2.5-fold) and cisplatin (~3- to 4-fold) associated with a decreased activity (40–55%) of unscheduled DNA synthesis after UV-C irradiation. Moreover, we observed that wortmannin sensitized parental cells *in vivo* when combined with either cisplatin or UV-C light, but had no effect on the DNA-PK$_{cs}$ deficient *scid* cells. Despite a lower repair synthesis activity (~2-fold) measured *in vitro* with nuclear cell extracts from DNA-PK mutants, a direct involvement of DNA-PK in the NER reaction *in vitro* has not been observed. This study establishes a regulatory function of DNA-PK in the NER process *in vivo* but rules out a physical role of the complex in the repair machinery at the site of the DNA lesion.

INTRODUCTION

Correct modulation of chromosome structure and maintenance of DNA integrity are critical to the survival of dividing cells. Although many enzymes are known that either act on DNA directly or use DNA as a template, few enzymes have been described that act potentially as DNA-regulated signal transducers in response to damaged DNA. One such enzyme, recently identified, is the DNA-dependent protein kinase, DNA-PK (1–3). An important feature of DNA-PK is that it binds to DNA directly and needs to be physically associated with DNA to be active. DNA-PK consists of at least two components, a regulatory DNA-binding subunit and the large catalytic subunit, DNA-PK$_{cs}$. The best characterized regulatory subunit is the human autoantigen, Ku, composed of two tightly associated polypeptides of ~70 and 80 kDa (Ku70 and Ku80, respectively), which binds *in vitro* to DNA termini or other discontinuities in the DNA structure (4–6). DNA-PK$_{cs}$ is a 469 kDa serine/threonine kinase which belongs to the phosphatidylinositol (PI)-3 kinase (p110) family. It is recruited to DNA by Ku binding and then acquires the capacity, at least *in vitro*, to phosphorylate many DNA-binding proteins in the vicinity (2,3).

The essential role of DNA-PK in the repair of double-strand breaks (DSBs) and V(D)J recombination has been revealed recently by analysis of mutant cells defective in both pathways. *Scid* (severe combined immune deficient) mice lack mature T and B lymphocytes, due to a dramatic decrease in the coding join formation during the V(D)J recombination process of immunoglobulin and T cell receptor genes (7–10). Subsequent studies demonstrated that non-lymphoid cells in *scid* mice also exhibit hypersensitivity (2- to 4-fold) to ionizing radiations (IR) and other agents that cause DSBs (11–13). The murine *scid* cells belong to complementation group 7 of ionizing radiation-sensitive (IR^s) rodent mutant cells together with the hamster V-3 cells (14). Both are DNA-PK$_{cs}$ mutants: the mutation present in *scid* cells leads to loss of the extreme terminal carboxyl region of DNA-PK$_{cs}$ (15–17) whereas the V-3 cell line contains very reduced DNA-PK$_{cs}$ transcript levels (15). Members of the complementation group 5 of IR^s mutant cell lines were shown to lack a DNA-end binding activity that corresponded to Ku80 (18–22). Ku80-deficient mice have been generated recently and exhibit severe combined immunodeficiency and defective processing of V(D)J recombination intermediates (23,24). Moreover, a direct consequence of the Ku defect is a concomitant deficiency in the kinase activity of DNA-PK (25), indicating that Ku DNA-binding represents a major mechanism for DNA-PK activation *in vivo*.

Taken together, the forgoing results establish that DNA-PK is a crucial component of the mammalian DSB repair/V(D)J recombination apparatus. However, some data suggest that DNA-PK may be involved in another type of DNA repair mechanism. Indeed, some authors have reported that the deficiency in DNA-PK conferred cross-sensitivity to various classes of DNA-damaging agents (26,27). Regarding cross-sensitivity to UV-C light, however, conflicting results have been reported (13,28–30). The main DNA-lesions induced by these classes of genotoxic agents are recognized and processed by the nucleotide excision repair (NER) pathway (31,32). This repair reaction can

*To whom correspondence should be addressed. Tel: +33 5 61 17 59 36; Fax: +33 5 61 17 59 33; Email: salles@ipbs.fr

be summarized as two broad steps: (i) lesion recognition, strand incision and damaged oligonucleotide displacement and (ii) gap filling by DNA polymerization and ligation. This process has been recently reconstituted *in vitro* with partially purified components (33–35) and has been shown in mammalian cells to involve at least 20 polypeptides including proteins that also have a role in DNA replication and transcription.

The present study was designed to test the putative involvement of DNA-PK in the nucleotide excision repair activity. The sensitivity of cells deficient in either the Ku or DNA-PK_{cs} component of DNA-PK to DNA lesions repaired by NER was assessed precisely and three different strains of DNA-PK deficient cells were found to be hypersensitive to cisplatin and UV-C irradiation. This sensitivity appeared to result from reduced NER activity in mutant cells but DNA-PK did not appear to be involved physically in the NER reaction analysed in an *in vitro* repair assay.

MATERIALS AND METHODS

Cell culture

The *scid* mouse fibroblastic cell line, SC-3T3/W (29), was obtained from Dr M. Mezzina (Villejuif, France) and was designated *scid* cell line in our work. The wild-type fibroblastic cell line, Balb-3T3, was from the American Type Culture Collection (Rockville, MD).

The parental CHO-K1 and the Ku80-deficient *xrs 6* cell line were obtained from the European Collection of Animal Cell Culture (Salisbury, UK). The *xrs 6* cell line stably transfected with the human Ku80 gene, *xrs 6*/Ku80, was generously donated by Dr P. A. Jeggo (Brighton, UK) (19). Dr G. Whitmore (Toronto, Ontario) generously provided us with the AA8 and the DNA-PK_{cs} mutant V-3 cell lines. CHO repair proficient (AA8) and two different UV-C-sensitive DNA repair-deficient mutant cell lines UV4 and UV135 were kindly provided by Dr M. Defais (Toulouse, France). The HeLa S3 cell line was obtained from the stock of European Molecular Biology Laboratories (Heidelberg, Germany). Cell lines were grown in RPMI 1640 medium (Gibco-BRL) for HeLa, or αMEM medium (Gibco-BRL) for the other lines, supplemented with 10% fetal calf serum (FCS; except for Balb-3T3 and *scid* cell lines where 15% FCS was used), 2 mM glutamine, 125 U/ml penicillin and 125 µg/ml streptomycin. The growth medium for *xrs 6*/Ku80 cell line was supplemented with 5 mg/ml mycophenolic acid and 50 mg/ml xanthine. Cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2. All cell lines were sub-cultured bi-weekly by trypsinization to maintain exponential cultures.

The doubling times of mutants and their corresponding wild-type cell lines were as follows: CHO xrs6 (28.1 h)/CHO K1 (17.3 h); scid (32.2 h)/Balb-3T3 (29.4 h); V-3 (24.2 h)/AA8 (19.1 h). Cell cycle phase distributions were performed as described previously (36) and results expressed in percentage of G_1/S/G_2 M were as follows: CHO xrs6 (28.7/52.1/19.2)/CHO K1 (24/48.7/27.3); scid (40.6/43.2/16.2)/Balb-3T3 (40.1/44.8/15.1).

Chemicals and enzymes

Bleomycin and methyl methanesulphonate (MMS) were purchased from Sigma Chemicals Co. Cisplatin (cis-diamminedichloro-platinum-II) was a gift from Roger Bellon Co. Bleomycin (3 mg/ml stock solution) and cisplatin (0.5 mg/ml stock solution) were dissolved in water and 150 mM NaCl, respectively. Solutions were stored at –20°C in small aliquots and used only once. Wortmannin was obtained from Sigma Chemical Co. and dissolved in DMSO (100 mM stock solution) immediately before use. Restriction enzymes were from Gibco-BRL and purified human DNA-PK was from Promega.

Cell survival assays with DNA-damaging agents

Cells growing in exponential phase in tissue culture flasks were trypsinized and 300–500 cells were plated into 60 mm tissue culture dishes and left to attach for 24 h. After removal of culture medium, cells were washed with phosphate-buffered saline (PBS) pH 7.2 and then subjected to UV-C-irradiation (254 nm) with a germicidal lamp (Bioblock, France) at a fluence of 0.5 $J/m^2/s$. Immediately after irradiation, fresh medium was added and dishes were returned to the incubator. Exposures to bleomycin, cisplatin or MMS were for 1 h, after which medium containing drug was removed and fresh medium added. Following all treatments, the dishes were incubated at 37°C and 5% CO_2 for 6 days. Dishes were washed with 150 mM NaCl and colonies stained with crystal violet (2 mg/ml). For each survival curve, duplicate dishes were seeded per point. Colonies containing >50 cells were scored under an inverted microscope. Three independent experiments for the mutant and wild-type cell lines were carried out in parallel.

In the experiments using wortmannin, cells were pretreated with 20 µM wortmannin or DMSO (solvent) for 2 h, then incubated for 1 h with drugs at the indicated doses. After 1 h, medium containing drug was removed and cells were incubated further with 20 µM wortmannin for 15 h. Cells were then washed in PBS and fresh medium added. Dishes were then incubated at 37°C and 5% CO_2 for 6 days and colonies scored as described (see above).

Unscheduled DNA synthesis

Cells were grown on cover-slips (in 30 mm wells) for 16–18 h in αMEM with 15% FCS. Cells were then washed twice with PBS and further incubated for 24 h in serum-free medium. Cells were washed once with PBS, and UV-C (254 nm) irradiated. Cells were labeled in complete medium during 4 h with [^{3}H]thymidine (100 µCi/ml) in the presence of 10^{-6} M fluorodeoxyuridine (Sigma) and 4 mM hydroxyurea (Sigma). Cells were then washed at 37°C for 30 min in medium containing an excess of unlabeled thymidine (10^{-5} M). After two washes with PBS, cells were fixed with methanol/acetic acid (3/1 vol:vol) for 15 min at room temperature. Autodiograms were performed and for each preparation the average number of grains over 30 labeled nuclei was counted.

In vitro repair reactions

Preparation of plasmids and treatment with damaging agents. The 2959 bp plasmid pBS (pBluescript KS$^+$; Stratagene) and the related 3738 bp pHM14 plasmid (gift from Dr R. D. Wood, ICRF, UK) were prepared by the alkaline lysis method from *Escherichia coli* JM109 (relevant genotype: *recA*1, *endA*1, *gyrA96*, *hsdR*17). Both plasmids were carefully purified by one caesium chloride and two neutral sucrose gradient centrifugations as described (24) to obtain plasmids in a closed-circular form. pBS plasmids were irradiated with UV-C light (peak wavelength = 254 nm) at 450 J/m^2 to produce ~15 photoproducts per circle, or treated with cisplatin as described (3,37) in order to obtain ~20 cisplatin adducts per circle.

Cell extracts. HeLa whole cell extracts were prepared as described previously (37). Nuclear extracts were prepared from fibroblastic rodent and murine cells as follows: ~5×10^7 exponentially growing cells were scraped from the dishes (170 cm^2) in PBS using a rubber policeman and centrifuged at 4°C for 5 min at 1500 r.p.m. Cell pellets were immediately used to prepare nuclear extracts as described previously (38) except that the final dialysis was performed for 3 h in 50 mM Tris–HCl, pH 7.5, 10% glycerol, 100 mM potassium glutamate, 1 mM EDTA, 1 mM dithiothreitol. After preparation, all extracts were immediately frozen and stored at –80°C. The extracts from the mutant and wild-type cell lines were prepared in parallel.

Repair synthesis assay. Standard 50 µl reaction mixtures contained 200 ng each of damaged and untreated closed-circular plasmids, 74 kBq of [α-^{32}P]dCTP (110 TBq/mmol, Amersham), cell extract as indicated and 60 mM potassium glutamate in reaction buffer containing 45 mM HEPES–KOH, pH 7.8, 7.4 mM $MgCl_2$, 0.9 mM dithiothreitol, 0.4 mM EDTA, 2 mM ATP, 20 µM each of dGTP, dATP and dTTP, 4 µM dCTP, 40 mM phosphocreatine, 2.5 µg of creatine phosphokinase (Type I, Sigma), 3.4% glycerol and 18 µg of bovine serum albumin as described (37). Reactions were carried out at 30°C for 3 h. Plasmid DNA was purified from the reaction, linearized with *Eco*RV and electrophoresed overnight on a 1% agarose gel containing 0.5 µg/ml ethidium bromide. The dry gel was then exposed for autoradiography. For data presentation, gels and autoradiographs were scanned and processed with Adobe Photoshop 3.0 software.

Quantitation of repair. Data were quantified by scintillation counting of excised DNA bands and densitometry of the photographic negative of the gel to normalize for plasmid DNA recovery in each reaction sample (Scanning Laser Densitometer, Biocom, France).

DNA-PK assay

Kinase assays were performed as described elsewhere with some modifications (25). Briefly, 50 µg of HeLa extracts or 100 µg of extracts from rodent cells were incubated at 4°C for 1 h under agitation with 20 µl of pre-swollen double-stranded DNA–cellulose (Sigma) in a total volume of 40 µl of Z buffer (25 mM HEPES–KOH, pH 7.9, 50 mM KCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, 0.1% Nonidet P-40 and 20% glycerol). The dsDNA–cellulose was then washed twice with 500 µl of Z buffer, resuspended in 40 µl of the same buffer and divided into two aliquots. 185 kBq of [γ-^{32}P]ATP (Du Pont-NEN), ATP (to a 200 µM final concentration) and 10 µg (corresponding to ~0.25 mM final concentration) of either DNA-PK specific peptide substrate (SQE peptide: EPPLSQEAFADLWKK) or negative control peptide (SEQ peptide: EPPLSEQAFADLWKK) were added prior to a 30 min incubation at 30°C. SQE and SEQ peptides derived from the sequence of p53 and contain either a 'wild-type' or a mutated DNA-PK phosphorylation site. Reactions were spotted onto DE81 paper (Whatmann), washed as described (25) and analyzed by scintillation counting. DNA-PK activity was expressed in fmol AMP as the value obtained with the SQE peptide from which was subtracted the value corresponding to the reaction with the SEQ peptide.

DNA-PK depletion

Anti DNA-PK mouse monoclonal antibodies mAb 18-2, 42-27 and 25-4 were reported previously (12). Each mAb in hybridoma supernatant was coupled to magnetic anti-mouse IgG beads (Dynabeads M-450, Dynal), according to the manufacturer's recommendations. Under 20 µl final volume, 250 µg of HeLa whole cell extracts were incubated at 4°C for 60 min with 20 µl of wet anti-DNAPK beads (equivalent to ~2 µg anti DNA-PK IgG) or control anti IgG beads in extract dialysis buffer under gentle agitation. The supernatant was removed over a magnet (Dynal MPC, Dynal). When necessary, a second depletion was performed immediately under the same conditions. An aliquot (equivalent to 50 µg of protein extract) was assayed for DNA-PK activity and the remaining protein fraction was assayed for repair activity under standard conditions as described above. The beads were washed twice with 500 µl of Z buffer , then incubated at 4°C for 60 min under agitation in 20 µl of Z buffer in the presence of 1 µg of activated calf thymus DNA (Sigma), washed twice with 500 µl Z of buffer and then split for DNA-PK activity assays with SQE or SEQ peptides under conditions as described above except that the adsortion step on DNA–cellulose was omitted.

RESULTS

Cells deficient in Ku or DNA-PK_{cs} are sensitive to UV-C irradiation and cisplatin

We investigated the sensitivity to various DNA-damaging agents of three DNA-PK deficient cell lines, as compared with their respective parental cell line. The cell lines used in our study included: (i) two different mutant cell lines defective in the catalytic subunit of DNA-PK, the hamster V-3 and the murine *scid* cell lines; (ii) a hamster mutant cell line with a defect in the Ku80 DNA-ends binding activity and (iii) an *xrs 6*/Ku80 cell line tranfected with the human Ku80 cDNA which has regained Ku DNA-end binding activity (20) and DNA strand break rejoining activity (19). The results of the clonogenic experiments are summarized in Table 1.

First, cross sensitivity to bleomycin was investigated since the drug acts on chromosomes as a radiomimetic agent (39). As expected (13,28,29,40), the mutant cell lines were 4- to 9-fold more sensitive to bleomycin than their respective parental cell line (determined by the ratio of IC_{50} values of mutant versus wild-type cells, see Table 1) whereas survival of *xrs 6*/Ku80 cells after exposure to bleomycin was similar to its parental cell line CHO-K1. We next addressed the question of the survival of these DNA-PK mutant cell lines after exposure to DNA damaging agents that do not induce DSBs, including UV-C light, cisplatin and MMS. In contrast with the effects of bleomycin on DNA, UV-C light and cisplatin induce lesions that are repaired by the NER process (31,41). As shown in Figure 1, all the DNA-PK deficient cells exhibited significantly increased sensitivity to killing by UV-C irradiation (2.0- to 2.5-fold, see Table 1). As in the case of bleomycin sensitivity, the *xrs 6*/Ku80 cell line also regained a sensitivity to UV-C similar to the parental cell line CHO-K1. DNA-PK mutants were also sensitive to cisplatin (3- to 4-fold), when compared with their respective parental cell line. In contrast, parental and mutant cells exhibited the same sensitivity to MMS, a drug that leads to the formation of base methylation mainly recognized by specific enzymes like DNA glycosylase and DNA methyl-transferase (Table 1).

Table 1. Sensitivity of DNA-PK-deficient cells to DNA-damaging agents

Cell line	Defect	Bleomycin (µg/ml)	UV (J/m^2)	Cisplatin (µM)	MMS (µM)
CHO-K1	–	27.0 ± 2.4[a]	13.5 ± 1.3	8.4 ± 1.1	0.75 ± 0.03
xrs 6	Ku80	2.8 ± 0.32 **(9.7)**[b]	6.1 ± 0.4 **(2.2)**[b]	3.01 ± 0.92 **(2.8)**[b]	0.68 ± 0.05 **(1.1)**
xrs 6/Ku80	corrected	32.0 ± 1.5 **(0.84)**[b]	14.8 ± 1.6 **(0.91)**	NT	NT
AA8	–	46.2 ± 3.3	12.5 ± 1.6	11.6 ± 0.8	NT
V-3	DNA PK_{cs}	11.0 ± 2.5 **(4.2)**[b]	5.0 ± 0.4 **(2.5)**[b]	2.9 ± 0.8 **(4.0)**[b]	NT
Balb-3T3	–	29.0 ± 4.2	5.8 ± 0.5	10.7 ± 1.6	1.2 ± 0.15
scid	DNA PK_{cs}	6.2 ± 1.3 **(4.7)**[b]	2.9 ± 0.6 **(2.0)**[b]	2.8 ± 0.7 **(3.8)**[b]	1.3 ± 0.11 **(0.92)**

[a]Concentration of drug that inhibited cell survival by 50% (IC_{50}).
[b]$P < 0.01$.
Numbers in brackets represents the fold increase in sensitivity compared with the parental cell line.
All values are the mean of at least three experiments (±SD).
MMS, methylmethane sulfonate; NT, not tested.

DNA-PK mutant cells are deficient in the repair of UV-C damage *in vivo*

Since the cells deficient in DNA-PK activity are sensitive to agents that induced DNA lesions recognized by the NER process, the extent of unscheduled DNA synthesis (UDS) after UV-C irradiation was determined. In these experiments, under conditions that inhibit DNA replication, radiolabelled deoxynucleotides are specifically incorporated during the repair synthesis step, allowing measurement of the extent of DNA repair. After cell fixation, the average number of grains per nuclei was counted from autoradiography, allowing determination of the extent of NER DNA synthesis. First, we checked that a significant 80% decrease in UDS activity was detected under our experimental conditions in the NER-deficient mutant cell line, UV4 (complementation group 1 of UV-sensitive mammalian cell lines), as compared with its parental cell line, AA8 (Fig. 2B). After 20 J/m^2 irradiation, UDS activity (expressed as the average number of grains per nucleus) was lower in the UV-C treated DNA-PK mutants, from 40 to 55% of the values obtained in their respective parental cells (Fig. 2). Decreased UDS activity was also observed in mutant cells after 10 and 30 J/m^2 irradiation (data not shown). Furthermore, the UDS activity was restored in the *xrs 6*/Ku80 cells to the parental level (Fig. 2C).

The PI3-kinase inhibitor wortmannin sensitizes wild-type but not DNA-PK_{cs} mutant cells to bleomycin, cisplatin and UV-C

In order to confirm that the kinase activity associated with DNA-PK could somehow be implicated in the NER process *in vivo*, we have performed survival experiments in the presence of wortmannin. In combination with ionizing radiation, wortmannin has been characterized as an effective radiosensitizer in various cell lines (42). This radiosensitization relied on a defect of DSBs rejoining compatible with an inhibition of the DNA-PK kinase activity (11). Therefore, we tested wortmannin as a possible drug sensitizer towards non-double-strand breaking agents in both wild-type and DNA-PK mutant cells. We first investigated whether wortmannin alone exhibited any cytotoxicity at doses necessary to observe a radiosensitization effect. Since murine fibroblasts were less sensitive to the toxic effect of wortmannin than hamster fibroblasts (data not shown), we further used the murine cells only (Balb-3T3 and *scid* cells). At the non-toxic dose of 20 µM wortmannin, the survival of control cells exposed to increasing concentration of bleomycin decreased (4-fold) whereas no effect was observed in *scid* cells (Table 2). A similar dose of wortmannin was necessary to observe a radiosensitization effect in murine fibroblasts (42). In combination with wortmannin, cisplatin and UV-C irradiation toxicities were also

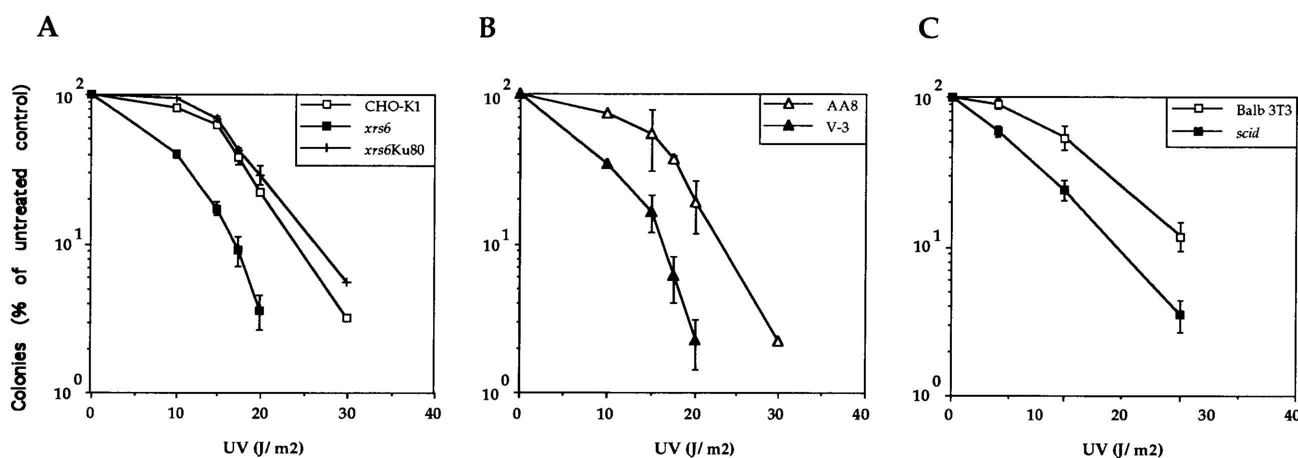

Figure 1. Survival of DNA-PK mutants and parental cell lines to UV-C light.

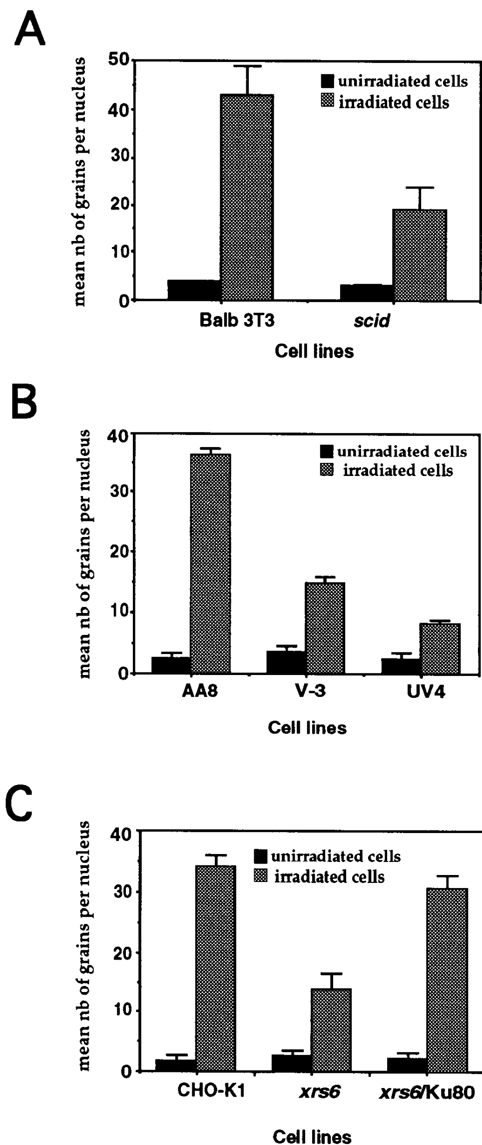

Figure 2. Unscheduled DNA synthesis activity in DNA-PK mutant cells. UDS activity expressed as the average number of grains per nuclei (over 30 labeled cells) in 20 J/m^2 UV-C irradiated cells. (**A**) Balb-3T3 and *scid* cells; (**B**) AA8, V-3 and UV4 cells and (**C**) CHO-K1, *xrs 6* and *xrs* 6/Ku80cells—mean of three independent experiments with standard deviations indicated as error bars. Note that for UV4 cells, the dose of UV-C irradiation used was 5 J/m^2.

enhanced by factors of 3- and 2-fold respectively, this effect again being restricted to the parental cells (Table 2). On the contrary, no effect of wortmannin was observed on MMS toxicity either in wild-type or in mutant cells. In all cases where wortmannin acted as a drug-sensitizer, the post-incubation period in the presence of the kinase inhibitor was necessary to observe this effect (data not shown).

The NER activity defect present in DNA-PK deficient cells is reproduced *in vitro*

Since UDS activity is assumed to reflect *in vivo* NER activity, our results show that DNA-PK mutant cells are defective in this process. NER activity can be directly measured in a cell-free system by incubating cell extracts in the presence of UV-C or drug-damaged plasmid DNA (37,43). Thus, we used this assay to test a direct function of DNA-PK in the NER mechanism.

The extent of DNA repair synthesis obtained *in vitro* with rodent whole cell extracts was relatively low compared with similar extracts from human cells (44 and our unpublished results). In order to improve the repair efficiency of rodent extracts, various protein extraction procedures have been evaluated. In our hands, the most specific repair activity (incorporation in the treated versus untreated plasmid DNA) was obtained with nuclear extracts. We established that the incorporation of radiolabeled nucleotide in UV-C-treated plasmid DNA was due to NER activity by the following control experiments: (i) nuclear extracts from the UV-C-sensitive DNA-repair deficient rodent mutant cell lines, UV4 and UV135 (complementation groups 1 and 5 respectively), yielded only 15–25% of repair synthesis activity compared with extracts from the parental cell line AA8 (data not shown) and (ii) by mixing two NER defective extracts from the two different complementation groups (1 and 5), repair synthesis incorporation was increased up to the value obtained with the parental cell extract (data not shown). Thus, the damage-dependent radiolabel incorporation exhibited by these rodent nuclear cell extracts corresponds to DNA repair synthesis activity by NER.

As illustrated in Figure 3, in the presence of UV-C-damaged plasmid DNA, nuclear extracts from the three different DNA-PK-deficient cells included in our study showed a decrease in damage-specific repair signal as compared with extracts from their respective parental cells. As shown in Table 3, we found consistently that the overall NER activity of extracts from DNA-PK deficient cells was significantly decreased to ~50% of the activity obtained with extracts from their respective parental cell lines. In addition, as shown in a preliminay report, the *in vitro* NER activity is restored in a *xrs 6* revertant cell line which has regained Ku/DNA end binding activity and DNA strand breaks rejoining activity (45).

Modulation of DNA-PK activity *in vitro* does not affect NER activity in cell extracts

In order to test if DNA-PK was physically implicated in the process of NER, we next performed a set of experiments.

First, a preparation of purified DNA-PK was added to DNA-PK deficient as well as control cell extracts, then DNA-PK and NER activities were measured in parallel (Fig. 4). Whereas purified DNA-PK released the profound defect in DNA-PK activity in the extracts from the V-3 cells (Fig. 4A), no significant change in the repair activity was observed (Fig. 4B).

Second, a complementation experiment was performed by mixing extracts from *xrs 6* cells deficient in the Ku DNA end-binding activity with extracts from V-3 cells deficient in the large catalytic subunit, DNA-PK$_{cs}$ (Fig. 5). Previous studies have established that *xrs 6* cells retained normal expression of the DNA-PK$_{cs}$ subunit (16) and that, conversely, V-3 cells exhibited a normal DNA-end binding activity (22 and data not shown). As expected, DNA-PK activity was recovered upon mixing V-3 and *xrs 6* extracts (Fig. 5A). In contrast, no restoration of the NER activity was observed in comparison with the activity in extracts from mutant and parental cells (Fig. 5B).

Table 2. Effect of wortmannin on drug toxicity in Balb-3T3 and *scid* cells

Drug	Cell line			
	Balb-3T3		*scid*	
	Control	Wortmannin (20 μM)[a]	Control	Wortmannin (20μM)[a]
Bleomycin (μg/ml)	31.0[b]	7.8 (4.0)	5.9	6.5 (0.9)
Cisplatin (μM)	10.8	3.6 (3.0)	2.5	2.3 (1.1)
UV (J/m^2)	6.2	3.1 (2.0)	3.2	3.1 (1.0)
MMS (μM)	1.3	1.1 (1.2)	1.5	1.6 (0.9)

[a]Optimal wortmannin concentration which induces a marginal effect on cell growth (<20%). Numbers in brackets represent the IC_{50} for drug alone (control) divided by IC_{50} for drug in combination with wortmannin.
[b]Concentration of drug that inhibited cell survival by 50% (IC_{50}). Mean of two experiments.

Table 3. Nucleotide excision repair activity in DNA-PK mutant cells

Cell line	% of residual activity[a]
scid (Balb-3T3)	50.7 ± 17.5
V-3 (AA8)	51.6 ± 12.6
xrs 6 (CHO-K1)	60.5 ± 11.8

[a]The residual NER activity (mean ± SD) in mutant cells is expressed as the percentage of the activity of their respective parental cell line (name of the parental strain indicated in brackets).
At least two independant extracts were used. In each case, extracts were prepared in parallel for mutant and wild-type cells. Plasmids damaged with either UV-C photoproducts or cisplatin.

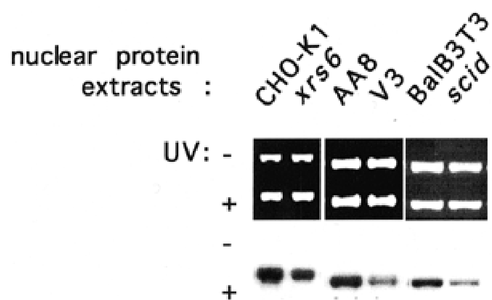

Figure 3. *In vitro* repair activity of nuclear extracts from DNA-PK-deficient cell lines. The repair synthesis assay was performed under standard conditions with 200 μg of nuclear protein of the indicated cell line in the presence of plasmid DNA. UV-damaged and undamaged plamids were denoted UV+ and UV–, respectively. Results were analyzed by agarose gel electrophoresis (upper panel) followed by autoradiography (lower panel).

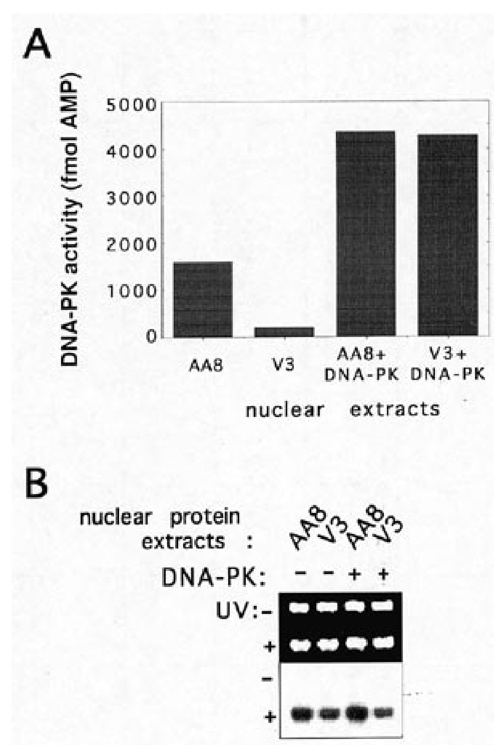

Figure 4. Effect of addition of purified DNA-PK to extracts from rodent cells on DNA-PK and NER activities. 300 μg of nuclear extracts from AA8 and V-3 cells were mixed with 3 μl of purified DNA-PK (Promega) or 3 μl of the enzyme storage buffer. (**A**) 100 μg of the mixture were then assayed for DNA-PK activity on DNA–cellulose as described in Materials and Methods. (**B**) 200 μg of the mixture were used in parallel in a standard repair assay in the presence of UV-damaged and undamaged plamids (denoted UV+ and UV–, respectively). Upper panel, agarose gel electrophoresis; lower panel, autoradiography of the dry gel.

Third, we used monoclonal antibodies to immunodeplete HeLa cell extracts of DNA-PK. Two successive immunoprecipitations with mAb 25-4 removed >90% of the DNA-PK activity and the activity was efficiently recovered on the magnetic anti DNA-PK beads (data not shown). However, no significant difference was observed in the NER efficiency of the extracts treated with anti DNA-PK or control beads, although the overall NER activity decreased in both extracts probably due to the non-specific binding of repair factors to the beads (data not shown).

Finally, since DNA-PK is inhibited, at least *in vitro*, by wortmannin (46), we performed a standard repair experiment with HeLa extracts in the presence of increasing concentrations of inhibitor. Although some non-specific inhibition of background DNA synthesis occured on both damaged and undamaged plasmid DNA, no significant variation in the extent of repair activity was observed with up to 50 μM wortmannin (data not shown).

DISCUSSION

It has been reported previously that a defect in DNA-PK causes sensitivity to ionizing radiation in relation to a defect in DNA DSB repair (13,29,40). Conflicting results about UV-C sensitivity of DNA-PK defective cells have been reported so far (13,28–30). Our results agree with the more controlled experiments of

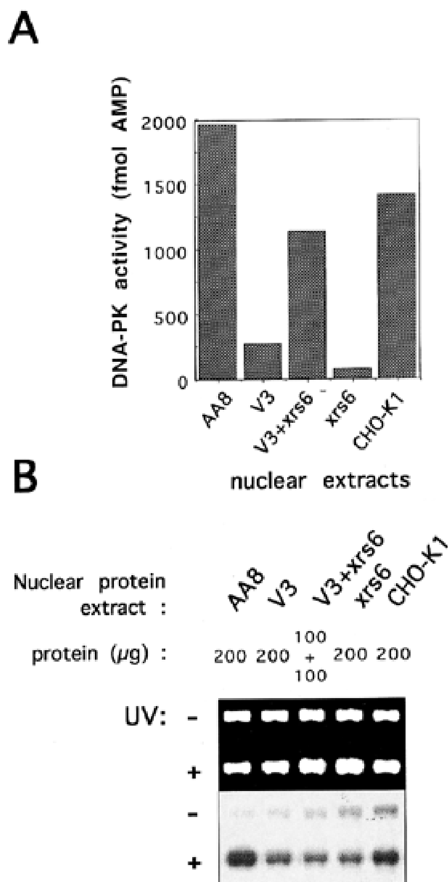

Figure 5. Effect of mixing extracts missing Ku or DNA-PK$_{cs}$ subunits on DNA-PK and NER activities. 300 µg of extracts from control (AA8, CHO-K1) or DNA-PK mutant cells (V-3, *xrs 6*) or mixtures (150 + 150 µg) of V-3 and *xrs 6* extracts were preincubated under a final volume of 30 µl of dialysis buffer for 30 min at 30°C. (**A**) 100 µg of extracts were then assayed for DNA-PK activity on DNA–cellulose as described in Materials and Methods. (**B**) 200 µg of extracts were used in parallel in a standard repair assay in the presence of UV-damaged and undamaged plamids (denoted UV+ and UV–, respectively). Upper panel, agarose gel electrophoresis; lower panel, autoradiography of the dry gel.

previous reports indicating that both DNA-PK$_{cs}$ and Ku mutant cell lines are significantly sensitive to UV light (13,28). The UV-C sensitivity of the *xrs 6* strain is related to the DNA-PK defect because enhanced resistance to UV-C was regained in the *xrs 6*/Ku80 cell line stably transfected with the human Ku80 gene (Fig. 1A and Table 1). Regarding sensitivity to cisplatin, our results also extend other reports (26,27). Taken together, these results indicate that DNA-PK activity modulates the cell sensitivity to other DNA-damaging agents in addition to agents that cause DNA DSBs. In addition, the fact that wortmannin, as an inhibitor of the protein kinase activity of the DNA-PK complex, sensitized wild-type but not *scid* cells towards UV-C light and cisplatin indicates that this sensitivity depends at least on the kinase activity of DNA-PK.

The UV-C sensitivity of DNA-PK mutant cells relies, at least in part, on a reduction in NER activity, as measured *in vivo* by the extent of UDS in UV-irradiated cells and *in vitro* in three different DNA-PK mutants. UDS activity was decreased ~2-fold in mutant cells but restored to the parental level in the irradiated Ku80 mutant transfected with the Ku80 cDNA. The repair defect was reproduced *in vitro* with independent nuclear extracts from both Ku and DNA-PK$_{cs}$ mutants in the presence of damaged plasmid DNA, when compared with the repair activity of extracts from their respective parental cells. This observed *in vitro* NER defect is not related to differences in the cell cycle distribution between mutants and wild-type cell lines (see Materials and Methods). Furthermore, this reduced NER activity cannot be complemented either by addition of DNA-PK or by mixing extracts from various deficient cell lines suggesting a non-direct role of DNA-PK in the NER repair steps. This result is in agrement with the lack of effect of the addition of DNA-PK in the NER reaction reconstituted *in vitro* with purified proteins (R. D. Wood, personnal communication). In addition, the repair defect observed in DNA-PK deficient cells appears less profound than the defect observed in cells deficient in any known essential component of the NER process (47 and our results).

How might DNA-PK modulate NER activity *in vivo*? Concerning DSB repair, although DNA-PK binding to DNA ends has been well documented *in vitro* (4,5), a physical involvement of the complex in any step of the repair mechanism has not been demonstrated yet. In addition, the attractive hypothesis of DNA ends protection by the Ku subunit has not been confirmed by analyzing DNA ends during the V(D)J recombination process in Ku80 knock-out mice (23). An alternative function has been suggested in which DNA-PK could act as a DNA damage-sensor that might regulate DNA repair via transcription modulation. A recent report substantiated this hypothesis by demonstrating a functional interaction between DNA-PK and the c-abl protein tyrosine kinase (48) which is activated in response to various DNA-damaging agents (49) and can, in turn, modulate phosphorylation of RNA polymerase II (50). In addition, RNA polymerase II and various transcription factors are known DNA-PK substrates (1) and DNA-PK has been shown potentially to regulate the transcription of target genes via sequence-specific recognition by Ku (51). Although the suggestions of a DNA-PK involvement in p53 accumulation and cell cycle control after DNA damage have been ruled out by recent reports (52–55), the possibility that DNA-PK regulates DNA repair in a global cellular response to the presence of DNA lesions still remains. Hence, a DNA-PK-dependent regulatory pathway could modulate both DSBs repair and NER. In that view, it is interesting to note that at the protein level some overlap exists between recombination and excision pathways as reviewed recently (56). In addition to being activated when cells are submitted to DNA-damaging treatments, the DNA-PK signalling pathway might also be triggered by DNA breaks that arise during normal cellular metabolism. This could explain the slight increase in spontaneous mutant frequency reported in the DNA-PK mutant cells analyzed (57,58) and both the growth defect observed in Ku80 knock-out mice and the early decrease in proliferation exhibited by the fibroblasts derived from these mice which might accumulate unrepaired spontaneous DNA damage (24). Such a basal level of DNA-PK activity in normal cellular metabolism could explain the repair defect that we observed in extracts from unirradiated DNA-PK mutant cells. Finally, since it has been reported that Ku can bind to specific regulatory DNA sequence (51) , we checked by western blotting experiments that the level of expression of XPA, XPD, XPG and p62-TFIIH was not affected in *xrs 6* mutant cells compared with the parental CHO-K1 cells (data not shown).

This study establishes a regulatory function of DNA-PK in the NER process *in vivo* and rules out a physical role of the complex in the repair mechanism at the site of the DNA lesion. A similar dissection of the function of DNA-PK in the repair of DSBs still awaits the development of an *in vitro* strand break repair assay which would allow the biochemical understanding of DNA-PK activity.

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