Atypical protein kinase C stimulates nucleotide excision repair activity ☆

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Received 7 July 2004; revised 8 August 2004; accepted 8 August 2004

Available online 21 August 2004

Edited by Varda Rotter

Abstract Nucleotide excision repair (NER) deals with bulky DNA damages. However, the regulation of this process is still unclear. Here, we show that both cell resistance to genotoxic agents that generate DNA lesions corrected by NER and in vitro NER activity are correlated with atypical protein kinase C (PKC) ζ expression levels. Moreover, repair intermediates are produced and eliminated more rapidly in UV-irradiated PKCζ-overexpressing cells. The expression levels of XPC and hHR23B, two NER proteins, are correlated with PKCζ expression. Altogether, these results strongly suggest that PKCζ could act as a modulator of NER activity by regulating the expression of XPC/hHR23B heterodimer.

Keywords: Protein kinase Cζ; Nucleotide excision repair; XPC; hHR23B

1. Introduction

Cellular DNA is exposed to various genotoxic stresses such as reactive oxygen species, radiations, or carcinogens. Depending on the levels of DNA damages, cells switch either to cell cycle progression arrest in order to allow time for DNA repair and further resume cell cycle, or to apoptosis. However, cells can also tolerate some unrepaired DNA damage potentially leading to mutagenesis and carcinogenesis.

Several mechanisms are available to repair DNA damage (for review, see [1]). Nucleotide excision repair (NER) is mainly implicated in the removal of DNA lesions such as helix-altering or bulky adducts on DNA. NER is a multistep process involving more than 30 proteins [2]. Following lesion recognition and dual incision 3′ and 5′ to damage, 27–29 nucleotides are excised. DNA polymerase fills in the gap before ligation [1,3]. Xeroderma pigmentosum (XP) is a clinical syndrome resulting from NER defects, characterized by hypersensitivity to UV light and very high incidence of skin tumors.

The protein kinase C (PKC) family comprises several proteins that regulate major cell functions such as proliferation, differentiation, motility, and apoptosis. Three classes of PKC have been defined on the basis of their primary structure and biochemical properties: conventional PKC isotypes (α, β and γ), novel PKCs (δ, ε, η, and θ), and atypical PKCs (the closely related ζ and λ/ι isoforms) [4]. Several proteins involved in genome integrity are regulated by PKC, including DNA-dependent protein kinase [5], hMutsα [6,7], O(6)-methylguanine–DNA methyltransferase [8], and topoisomerase II [9]. All these observations suggest a pivotal role for PKC in the coordination of the different DNA repair mechanisms. PKCζ has recently emerged as a critical component of survival pathways activated by receptor and non-receptor tyrosine kinases, and oncogenes [10]. In this study, we investigated whether PKCζ expression could also play a role in cell resistance to genotoxic agents and in maintenance of genetic integrity by modulating DNA repair mechanisms.

2. Materials and methods

2.1. Cell lines and chemicals

U937 and HeLa cell lines were obtained from the ATCC (Rockville, MD, USA). PKCζ-transfected U937 cells were a gift from Dr. K. Ways (Greenville, NC, USA). Briefly, stable co-transfection of U937 cells was done with pMAMneo (Clontech) and either PKCζ plasmid (U937-ζJ and U937-ζB clones) or vector without PKCζ insert (U937-neo) by electroporation [11]. U937 cells were grown in RPMI 1640 with 10% fetal calf serum, 2 mM glutamine, streptomycin and penicillin. PKCζ expression and activity were increased by 4–5- and 3-fold, respectively, in U937-ζJ and U937-ζB cells compared to U937-neo [11–13].

Stable PKCζ gene suppression was done using GeneSuppressor kit [14] according to the manufacturer's recommendations (Imgenex, Clinisciences Montrouge, France). Briefly, human PKCζ oligonucleotide inserts were designed: PKCζ forward 5′TCGAGATCTTCATCACCAGCGTGGAGAGTACTGTCCACGCTGGTGATGAAGATTTTTTT3′; PKCζ reverse: 5′CTGAAAAAATCTTCATCACCAGCGTGGACAGTACTCTCCACGCTGGTGATGAAGATC 3′ [14]. After primer annealing, inserts were cloned in the pSuppressor plasmid containing Neomycin resistance gene. Following stable transfection of HeLa cells by Lipofectamine™ 2000 (Invitrogen, Cergy Pontoise, France) with siRNA PKCζ plasmid, two HeLa cell clones, HeLa Si5 and HeLa Si11, displaying a reduction by 50% and 60%, respectively, in PKCζ gene expression, were used.

☆ Supplementary data associated with this article can be found, in the online version, at doi: 10.1016/j.febslet.2004.08.024.

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Abbreviations: AAS, atomic absorption spectrophotometry; MTT, 3-(4,5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide; NER, nucleotide excision repair; PKC, protein kinase C; XP, xeroderma pigmentosum

0014-5793/$22.00 © 2004 Published by Elsevier B.V. on behalf of the Federation of European Biochemical Societies.
doi:10.1016/j.febslet.2004.08.024

Melphalan (Alkeran) was provided by Wellcome Laboratories (Paris). Other reagents were obtained from Sigma (St Quentin-Fallavier, France).

2.2. Clonogenic and cytotoxicity assays

U937 cells were irradiated with UV-C light (254 nm) in PBS, resuspended in complete medium and seeded on 96-well plates (10 cells/well). After 7 days, the colonies larger than 50 cells were counted and the percentage of cell survival was determined. HeLa cells were plated at 200 cells/35 mm dish and UV-C irradiated next day. One week later, cells were fixed and stained with Crystal violet and colony larger than 50 cells was counted.

For the 3-(4,5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide (MTT) assay, cells (3×10^5 cells/ml) were treated for 1 h with cisplatin or melphalan at 37 °C in complete medium. Cells were washed, then seeded on 96-well plates (30 000 cells/well) for 48 h at 37 °C. MTT solution was added for 1 h at 37 °C. Following centrifugation, the supernatants were removed prior to addition of DMSO (100 μl). Optical density was measured at 590/620 nm and the percentages of surviving treated versus untreated cells were calculated.

2.3. In vitro DNA repair reactions

Whole cell extracts were prepared as described previously [15]. The 2959 bp pBS (pBluescript KS+; Stratagene, Amsterdam, Netherlands) and the related 3738 bp pHM14 plasmid were prepared by alkaline lysis method from *Escherichia coli* JM109. pBS plasmid was irradiated with UV light at 254 nm (300 J/m^2). This dose produces about 10 UV lesions per plasmid (pBS-UV).

The repair synthesis assay was performed as already described [16], Briefly, cell extract proteins (200 μg) were incubated at 30 °C for 3 h with a reaction mixture containing damaged pBS (300 ng), untreated pHM14 closed circular plasmids (300 ng), [α^{32}P] dCTP (800 Ci/mmol) (2 μCi) (ICN, Orsay, France), and glutamic acid potassium salt (60 mM) [15]. Purified plasmid DNA was linearized with EcoRV and electrophoresed on a 1% agarose gel containing 0.5 μg/ml ethidium bromide. Gels were processed on a phosphorimager and the radioactivity was quantified. Specific incorporation of [α^{32}P] dCMP was expressed as radiolabeled incorporation in the damaged versus undamaged plasmid, and was normalized to the same amount of DNA as determined from the fluorograph of the ethidium bromide stained gels.

2.4. Alkaline comet assay

The alkaline comet assay was performed as previously described [17]. Briefly, cells (1×10^5) were mixed with 0.5% low melting point agarose (Invitrogen) (75 μl) and layered onto microscope slide. The slides were sham- or UV-C- irradiated on ice at 15 or 40 J/m^2, and incubated or not in complete medium for 15 or 60 min. The slides were immersed at 4 °C for 1 h in a cold lysis buffer. The slides were placed on a horizontal electrophoresis unit filled with freshly prepared alkaline solution and exposed to alkali for 20 min at RT. Electrophoresis was conducted for 25 min at 25 V, 300 mA. The slides were rinsed with Tris–base (0.4 M, pH 7.5), stained with ethidium bromide, and observed at 40× magnification with a LSM410 invert laser scan microscope Zeiss equipped with excitation laser at 488 nm. Images of randomly selected cells (150 cells for each clone) were analyzed and tail DNA was determined with kinetic analysis comet 4.0.2 software.

2.5. Measurement of platinum–DNA adducts

Exponentially growing cells were treated with 40 μM cisplatin for 1 h at 37 °C, washed and incubated with complete medium. Cells (15×10^6) were harvested after treatment or after additional incubation in drug-free medium for 2, 5 or 23 h and incubated in lysis buffer (0.3 M NaCl, 5 mM EDTA, 0.5% SDS, and 100 μg/ml proteinase K). After extraction using standard procedure, DNA was digested with S1 nuclease. Covalent platinum (Pt)–DNA adducts were measured by flameless atomic absorption spectrophotometry (AAS) as already described [18].

2.6. Western blot analysis

Whole cell extracts were prepared in Laemmli buffer, and proteins were separated in 10% SDS–PAGE and transferred to nitrocellulose membrane (Amersham Pharmacia Biotech, Saclay, France). Membranes were incubated with polyclonal anti-XPB (Santa Cruz, CA), or anti-XPC, or monoclonal anti-hHR23B (Transduction Laboratories), and anti-β-actin (Neomarkers, Fremont, CA) antibodies overnight at 4 °C, then incubated with horseradish peroxidase-conjugated antibody (Jackson Laboratories, West Grove, PA). Proteins were visualized using the ECL system (Amersham Pharmacia Biotech).

2.7. Statistical analyses

Statistical analyses were performed using the Student's t test, $P < 0.01$ was considered statistically significant.

3. Results

3.1. PKCζ enhances cell resistance to genotoxic stress

We first investigated whether PKCζ could influence the sensitivity of U937 cells to UV-C, cisplatin, and melphalan. Clonogenic assays show that PKCζ-transfected clones exhibit a 3-fold increase in resistance to UV-C compared to control U937-neo cells (Fig. 1(a)). Moreover, PKCζ overexpression results in a 4- and 20-fold increase in resistance to cisplatin or melphalan treatment, respectively, compared to control cells, as measured by MTT assay (Fig. 1(b) and (c)).

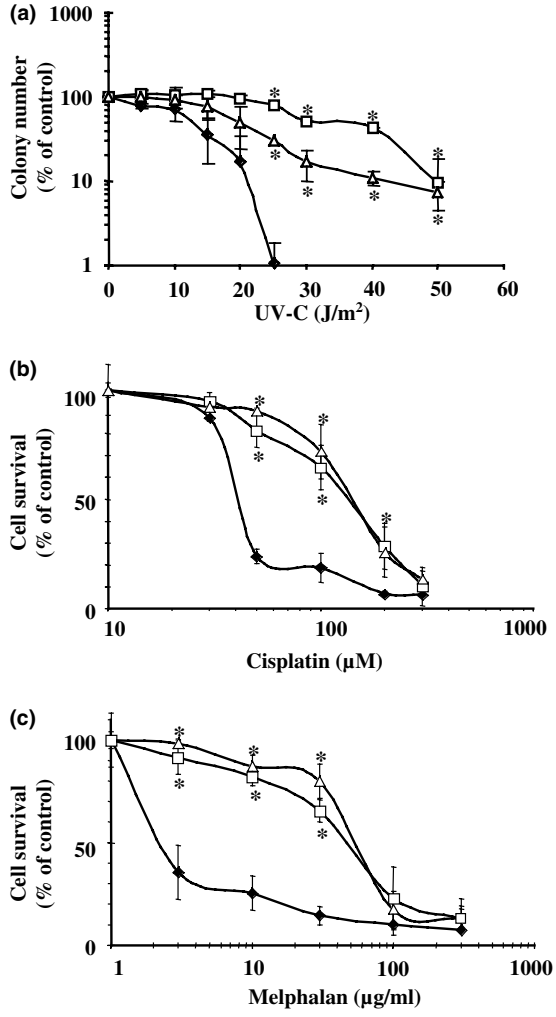

Fig. 1. Effect of PKCζ on cell sensitivity, (a) Clonogenic assay of U937-neo (◆), U937-ζJ (□) and U937-ζB (△) cells following UV-C-irradiation. (b) and (c) Cell viability measured by MTT assays performed following cell treatment with cisplatin (b) or melphalan (c). The results are means ± S.D. of three separate experiments.

3.2. PKCζ increases in vitro NER activity

In vitro NER activity measures the capacity of cell protein extracts to repair UV-C irradiated plasmids [16]. We compared the basal activity in whole cell extracts from control and PKCζ-overexpressing U937 cells. The basal repair activity shows a 7-fold increase in U937-ζJ compared to U937-neo cells (Fig. 2). A 4-fold increase is observed in U937-ζB cell extracts. These results strongly suggest that basal NER activity could be increased in PKCζ-overexpressing U937 cells.

3.3. PKCζ accelerates UV-C-induced DNA damage repair

NER involves endonuclease activity that generates repair-induced strand breaks in damaged DNA. We determined the rate of DNA repair after UV irradiation by alkaline comet assay. The tail DNA reflects the appearance of repair-induced strand breaks. In PKCζ-transfected and control cell lines, tail DNA increases with the dose of UV irradiation. However, tail DNA is maximum at 15 and 60 min following UV-irradiation in U937-ζJ and U937-neo cells, respectively (Fig. 3). After 60 min, tail DNA is markedly decreased in U937-ζJ cells but still increased in U937-neo cells. These results suggest that UV lesions are excised more rapidly and that repair process is faster in U937-ζJ than in control cells.

3.4. PKCζ improves covalent Pt–DNA adduct elimination

To further investigate the influence of PKCζ on DNA repair, we measured the amount of covalent Pt–DNA adducts in U937-ζJ, U937-ζB, and U937-neo cells treated with cisplatin

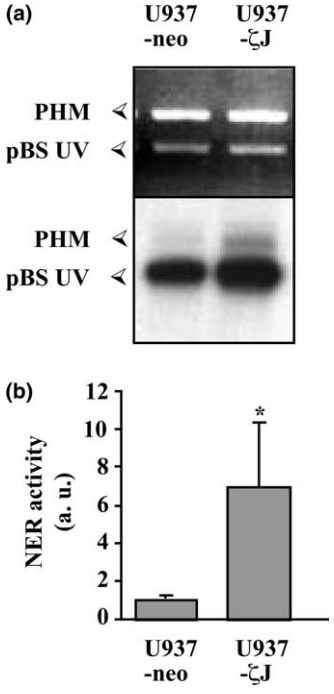

Fig. 2. In vitro NER activity in PKCζ-overexpressing cells, (a) DNA repair synthesis activity in U937-neo and U937-ζJ cell extracts was determined on UV-C damaged plasmids. (a) Ethidium bromide stained gel (upper) and gel autoradiography (lower) are shown, (b) Quantification of NER activity was expressed as the ratio of radiolabeled dCMP incorporation in the damaged versus undamaged plasmid. The NER activity of the U937-neo cell extracts was arbitrary set at 1. Results are means ± S.D. of three separate experiments performed with three independent cell extracts.

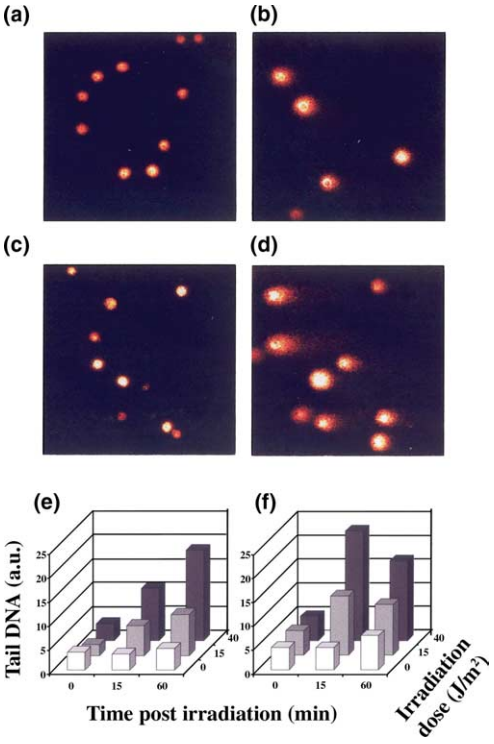

Fig. 3. Effect of PKCζ on removal of DNA photodamage. U937-neo and U937-ζJ cells were irradiated with UV-C light (15 and 40 J/m^2) and DNA strand breaks were measured just after irradiation on ice, or after incubation at 37 °C for 15 or 60 min following irradiation. A representative experiment is shown (a–d). Cells were mock-irradiated (a,c) or irradiated at 40 J/m^2 (b,d) and analyzed 15 min after irradiation. Complete results expressed as means of the tail DNA are shown for U937-neo (e) and U937-ζJ (f) cells.

for 1 h treatment. At the end of treatment, the amount of Pt–DNA adducts is similar (20 ± 3.4 ng Pt/μg DNA) in the three cell lines. This result suggests that PKCζ does not interfere with cisplatin uptake and efflux. Two hours after cisplatin re-

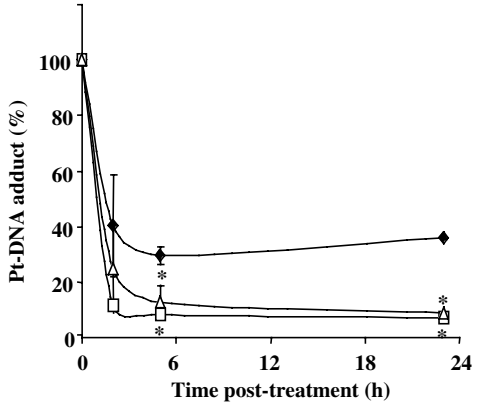

Fig. 4. Kinetics of covalent cisplatin–DNA adduct removal in U937-neo (◆), U937-ζJ (□) and U937-ζB (△) cells. DNA was extracted at different times after treatment with 40 μM cisplatin and platinum (Pt) was quantified by AAS. Results are expressed as percentages of Pt–DNA adduct remaining compared to the amount of Pt–DNA adducts just after treatment. Each data is the mean ± S.D. obtained from three independent experiments performed with three independent preparations of DNA.

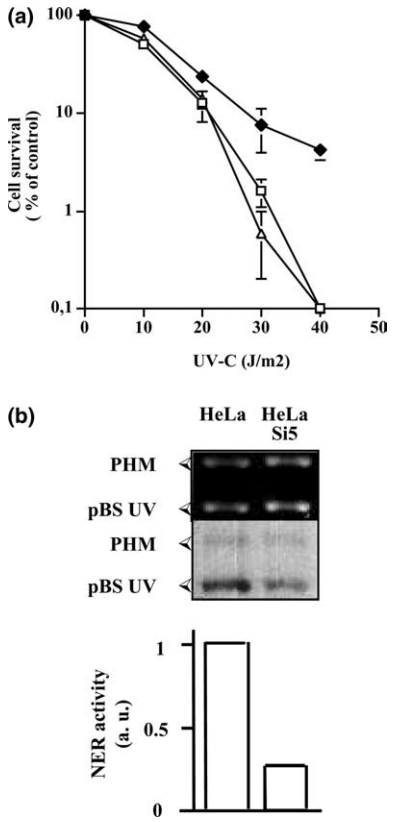

Fig. 5. Effect of PKCζ siRNA on cell response to UV-C. (a) Clonogenic assay of control HeLa (◆), HeLa Si5 (□) and HeLa Si11 (△) cells following cell irradiation. (b) In vitro NER activity in PKCζ siRNA cells. Photograph of ethidium bromide stained gel (upper) and gel autoradiography (lower). DNA repair activity was quantified as in Fig. 2. The DNA repair activity of control HeLa cell extracts was arbitrary set at 1.

moval from medium, 50% of Pt–DNA adducts persist in U937-neo cells while only 10% of Pt–DNA adducts are detected in U937-ζJ or U937-ζB cell DNA (Fig. 4). Twenty-three hours after cisplatin removal, only 10% Pt–DNA adducts still remain in U937-ζJ or U937-ζB cells, contrasting with more than 30% in U937-neo cells. These results suggest that PKCζ enhances Pt–DNA adduct removal.

3.5. PKCζ downregulation sensitizes cells to UV-C irradiation and inhibits NER activity

We investigated the effects of PKCζ depletion on cell survival following UV irradiation and NER activity in HeLa cells. Two stable siRNA-transfected HeLa clones, displaying lower PKCζ expression (Si5 and Si11), were significantly sensitized to UV-C compared to control HeLa cells (Fig. 5(a)). Moreover, in vitro NER activity was significantly decreased in HeLa Si5 cells (Fig. 5(b)). These results support a role for PKCζ in the control of NER activity.

3.6. PKCζ expression levels correlate with some NER protein expression

We hypothesized that PKCζ could interfere with some steps of NER process. We evaluated the expression levels of different NER proteins. Although no changes in PCNA, XPD, XPE (data not shown) and XPB protein expression levels are detected, a 10- and 5-fold increase in XPC and hHR23B protein expression levels, respectively, is observed in PKCζ-overexpressing cells compared to control cells (Fig. 6(a) and (b)). Conversely, in PKCζ siRNA-transfected cells, XPC and hHR23B protein expression levels are decreased (Fig. 6(c) and (d)). Interestingly, XPC and hHR23B mRNA levels are similar in U937-ζJ, U937-ζB and in U937-neo cells, as revealed by semi-quantitative RT-PCR (data not shown). These results suggest that PKCζ could interfere with XPC and hHR23B expression at a post-transcriptional level.

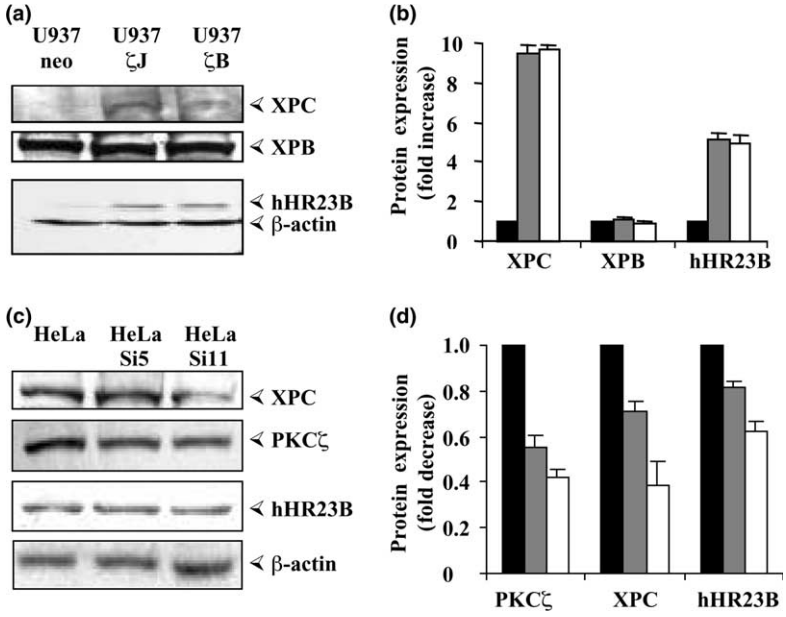

Fig. 6. Expression of some NER components. Expression of XPB, XPC and hHR23B proteins in (a–b) U937-neo (black), U937-ζJ (grey) and U937-ζB (white) cells, and (c–d) HeLa (black), HeLa Si5 (grey) and HeLa Si11 (white) cells. The Western blots are representatives of three independent experiments and the quantification is the mean ± S.D. of these experiments.

4. Discussion

In this study, we provide evidence that PKCζ expression can affect cell response to genotoxic agents. Overexpression of PKCζ markedly increases resistance to these agents. This resistance could be related to increased basal NER activity, as suggested by both in vitro NER activity measurements and comet assays. Quantification of Pt–DNA adducts and kinetic studies of Pt–DNA adduct removal indicate that PKCζ overexpression does not protect cells by decreasing the number of initial DNA lesions, but rather improves Pt–DNA adduct removal. Therefore, our data strongly suggest that PKCζ could play an important role in the regulation of NER activity.

We found that PKCζ overexpression correlates with increased XPC/hHR23B protein expression, suggesting that PKCζ could improve the cell capacity to detect DNA damage. The improvement of this step has been shown to increase DNA repair efficiency [19]. Therefore, our study suggests that the regulatory function of PKCζ on NER activity could be mediated by XPC/hHR23B overexpression. As transcriptional regulation of XPC/hHR23B expression can be ruled out, PKCζ probably interferes at a post-transcriptional level. We then investigated whether PKCζ could interact with XPC and/or hHR23B. Co-immunoprecipitation experiments failed to show any interaction between these proteins (data not shown). No modification of the phosphorylation status of XPC and/or hHR23B was observed (data not shown). No differences in the stability of XPC and hHR23B were detected between control and PKCζ-overexpressing cells (data not shown).

Therefore, the mechanism by which PKCζ modulates NER expression is likely to result from XPC/hHR23B overexpression, but the mechanism by which PKCζ modulates expression of XPC/hHR23B proteins remains unclear.

Recently, the activation of ERK1/2 MAPK in response to lead acetate was recently reported to increase NER activity as well as cell resistance and anti-mutagenicity [20]. Whether the effects we observed in our cell lines are mediated by ERKl/2 MAPK, one of the targets of PKCζ, remains to be demonstrated.

It was generally believed that the protective function of PKCζ against genotoxic agents was mediated by its anti-apoptotic function [21–24]. Our study suggests that PKCζ could also affect cell response to some genotoxic agents by enhancing DNA repair. As PKCζ could upregulate NER activity and prevent genotoxicity, PKCζ could act as an important contributor to genome integrity.

Acknowledgements: We thank Dr. D.K. Ways (Greenville, N.C.) for the gift of U937-neo, U937-ζJ and U937-ζB cells, and Dr. R.D. Wood for the gift of anti-XPC antibody RW028. T.L. is recipient of a grant from the Ministere de l'Education Nationale, de la Recherche et la Technologic (MENRT) and the Association pour la Recherche centre le Cancer.

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