Mechanism of early biphasic activation of poly(ADP-ribose) polymerase-1 in response to ultraviolet B radiation

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Summary

The damage to DNA caused by ultraviolet B radiation (280-320 nm) contributes significantly to development of sunlight-induced skin cancers. The susceptibility of mice to ultraviolet B-induced skin carcinogenesis is increased by an inhibitor of the DNA damage-activated nuclear enzyme poly(ADP-ribose) polymerase-1 (PARP), hence PARP activation is likely to be associated with cellular responses that suppress carcinogenesis. To understand the role of activated PARP in these cellular functions, we need to first clearly identify the cause of PARP activation in ultraviolet B-irradiated cells. Ultraviolet B, like ultraviolet C, causes direct DNA damage of cyclobutane pyrimidine dimer and 6, 4-photoproduct types, which are subjected to the nucleotide excision repair. Moreover, ultraviolet B also causes oxidative DNA damage, which is subjected to base excision repair. To identify which of these two types of DNA damage activates PARP, we examined mechanism of early PARP activation in mouse fibroblasts exposed to ultraviolet B and C radiations. The ultraviolet B-irradiated cells rapidly activated PARP in two distinct phases, initially within the first 5 minutes and later between 60-120 minutes, whereas ultraviolet C-irradiated cells showed only the immediate PARP activation. Using antioxidants, local irradiation, chromatin immunoprecipitation and in vitro PARP assays, we identified that ultraviolet radiation-induced direct DNA damage, such as thymine dimers, cause the initial PARP activation, whereas ultraviolet B-induced oxidative damage cause the second PARP activation. Our results suggest that cells can selectively activate PARP for participation in different cellular responses associated with different DNA lesions.

Key words: PARP, ADP-ribose, UV, DNA damage, thymine dimers, oxidative damage

Introduction

Ultraviolet B (UVB, 280-320 nm) is part of the terrestrial solar UV radiation that is implicated in sunlight induced non-melanoma cancers (Black et al., 1997). UVB radiation induces changes in virtually all parts of the cell from nucleus to membranes, but its ability to cause DNA damage by direct and indirect means plays a central role in its carcinogenic action (Black et al., 1997; Cleaver and Crowley, 2002; de Gruijl et al., 2001; Kulms and Schwarz, 2000). Therefore, any cellular process that responds rapidly to UVB-induced DNA damage and that also participates in DNA repair and cell death can play a crucial role in determining the fate of UVB-irradiated cells. Mammalian and higher eukaryotic cells have a nuclear enzyme poly(ADP-ribose) polymerase-1 (PARP), which has many characteristics that make it an ideal candidate for participation in cellular responses to UV (Jacobson et al., 2001). It is activated very rapidly in response to DNA damage by different chemical and physical agents (Burkle et al., 2000), including UVC (230-280 nm) and UVB (Alvarez-Gonzalez and Althaus, 1989; Berger et al., 1980; Chang et al., 2002; Farkas et al., 2002; Jacobson et al., 1983; McCurry and Jacobson, 1981; Yoon et al., 1996). In general, it is known that activated PARP utilizes nicotinamide-adenine dinucleotide (NAD) to form polymers of ADP-ribose (pADPr) on itself and a few selected DNA metabolism-related proteins. This post-translational modification transiently alters normal functions of these proteins until they are restored to their native state by removal of pADPr chains. This metabolism is implicated in various events following DNA damage, such as protection of the damaged DNA from recombination, remodelling of chromatin, transcriptional changes at the damaged site, DNA base excision repair (BER) at lower levels of DNA damage and cell death by apoptosis or necrosis at higher levels of DNA damage (Burkle et al., 2000; Chiarugi and Moskowitz, 2002; Hong et al., 2004; Kraus and Lis, 2003; Le Rhun et al., 1998; Pirrotta, 2003; Zong et al., 2004).

More specifically, in response to UV, PARP activation has been shown to participate in carcinogenesis, DNA repair and cell death. During UVB-induced skin carcinogenesis in mice, the PARP inhibitor 3-aminobenzamide acts as a co-carcinogen by accelerating the onset of tumors and increasing the severity

of cancers (Epstein and Cleaver, 1992). We have also observed that niacin-deficient SKH-1 mice, whose skin is deficient in the PARP-activation response to UVB, become more susceptible to UVB-induced skin cancers (G.M.S., Y. Le Rhun and J. B. Kirkland, unpublished). PARP activation has also been implicated in repair of UVB-induced DNA damage. There are two major types of DNA damage induced by UVB: (1) the intrastrand cross links, such as cyclobutane pyrimidine dimers (CPD) and (6,4)-photoproducts, which are removed by nucleotide excision repair (NER), and (2) oxidant-mediated DNA damage, such as single strand breaks and 8-oxoG, which are removed by BER and single strand break repair (SSBR) (Black et al., 1997; Caldecott, 2003a). PARP is known to be activated during BER process (Lindahl et al., 1995) and it is implicated in SSBR (Caldecott, 2003a; Caldecott, 2003b) and BER (Le Page et al., 2003) of UV-induced oxidative DNA damage through its binding to single strand gaps or breaks (Caldecott, 2003a; Caldecott, 2003b). PARP is not known to be part of the basic steps of NER, however, it is also accepted that the minimal NER system is inefficient when damaged DNA is in its physiological environment, i.e. within chromatin (Balajee and Bohr, 2000). Hence, other factors, such as PARP could participate in efficient execution of NER in organisms with higher order structure of chromatin. Indeed, a recent study has shown that PARP inhibition significantly delays removal of UVB-induced CPD (Flohr et al., 2003). Finally, PARP activation has been linked to UVB-induced apoptosis in cultured cells or in mouse skin (Chang et al., 2002; Farkas et al., 2002; Mi et al., 2003).

Although PARP activation has been implicated in carcinogenesis, DNA repair and cell death after exposure to UV, there is lack of information on exactly when and how PARP is activated in UV-irradiated cells. Many earlier studies focused on examining activation of PARP in UVC-irradiated cells, and suggested that PARP is activated when strand breaks are generated during excision repair (Alvarez-Gonzalez and Althaus, 1989; Berger et al., 1980; McCurry and Jacobson, 1981; Yoon et al., 1996). However, UVC induces very little of oxidative DNA damage that requires BER in which PARP is known to be involved. Most of the UVC-induced DNA damage is direct lesions, such as CPD, which are repaired by NER, and it is not known if strand breaks generated during NER are exposed and available for PARP activation. Recent studies reporting UVB-induced PARP activation in skin (Farkas et al., 2002) and keratinocytes (Chang et al., 2002) suggested that oxidative DNA damage is the cause of PARP activation, although it was not experimentally determined. Therefore, we examined mechanism of early PARP activation in mouse embryonic fibroblasts exposed to UVB and UVC radiation. Here, we show that early PARP activation occurs in two distinct phases around 5 minutes and between 60-120 minutes in the UVB-irradiated cells, whereas only the first phase is observed in response to UVC. Using anti-oxidant treatment, local irradiation, chromatin immunoprecipitation (ChIP) and in vitro PARP binding or activation assays, we show that direct DNA damage by UVB or UVC is the cause for immediate PARP activation; whereas oxidative damage by UVB is the cause of the second PARP activation. The two phases of PARP activation arising from different types of DNA damage suggest that cells can selectively recruit its function for different responses to DNA damage.

Materials and Methods

Cells

Mouse embryonic fibroblasts derived from *PARP*$^{+/+}$ mice (Wang et al., 1995) were grown at 37°C in a humidified chamber with 5% CO_2 in custom-made dyeless DMEM low glucose medium (Gibco-BRL) supplemented with 10% foetal bovine serum, 100 Units/ml penicillin and 100 μg/ml streptomycin.

Global or local UV irradiations

Cells were irradiated with UVB or UVC while under a layer of dyeless culture medium in Spectrolinker XL-1500 (Spectronics) fitted with six UVB (BLE-1T158, peak 312 nm) or UVC (BLE-1T155, peak 254 nm) fluorescent tubes. UV flux was measured using a UVX digital radiometer (UVP Inc.) equipped with specific probes for UVB or UVC. For all UVB irradiations, even minor cross-contaminating UVC wavelengths were filtered out using 0.0127 cm thick film of Kodacel K6805 cellulose triacetate (Eastman Kodak). For local UVC irradiation (Moné et al., 2001), cells under a thin layer of phosphate buffered saline (PBS) were exposed to UVC through a 5 μm isopore membrane (Millipore), followed by incubation in the culture medium after removal of the filter and PBS. For treatment with anti-oxidants, cells were incubated for 1 hour prior to UVB irradiation with 5000 Units/ml of catalase (Sigma) or 20 mM *N*-acetylcysteine (Sigma) in dyeless serum-free medium. As a positive control for PARP activation, cells were treated with 100 μM H_2O_2 for 10 minutes in dyeless serum-free medium.

Immunofluorescent detection of pADPr and thymine dimers

UV-exposed cells were fixed in 10% TCA, given a series of ethanol washes (Kupper et al., 1990) and probed with anti-pADPr monoclonal 10H (1:50) (Kawamitsu et al., 1984) or anti-thymine dimer (T-T) monoclonal KTM53 (Kamiya, 1:100) antibodies. To study the co-localization of T-T and pADPr, anti-pADPr polyclonal LP96-10 (Aparptosis Inc., 1:250) was used. The primary antibodies were tagged with suitable secondary antibodies tagged with FITC-green (Jackson) or Alexa 594-red (Molecular Probes). Nuclear DNA was counterstained with DAPI and cells were examined at 40× magnification in a Nikon Eclipse E1000 fluorescence microscope. Images were processed by MetaMorph software (Universal Imaging) for merger of two colour fluorescence images and for quantification of fluorescence signals. For quantification of PARP activation, the fluorescent intensity of the pADPr signal was measured in at least 150 nuclei per time point from different fields and normalized for the total nuclear area measured from DAPI-stained fields to calculate relative fluorescence units of pADPr signal.

Analysis of intracellular oxidant

UVB exposed cells were loaded with fluorescent probe 2′,7′-dichlorodihydrofluoresceindiacetate (DCF-DA; Molecular Probes) in the dark at 37°C for 30 minutes and fixed in 2% formaldehyde prior to microscopy (Hempel et al., 1999). Images were processed and quantification of relative fluorescence units was carried out, as described above for pADPr.

Western blot

The cell extracts were prepared and resolved on 8% SDS-PAGE, followed by transfer to nitrocellulose and immunoblotting as described previously (Affar et al., 2002; Shah et al., 1995b). The specific antibodies used were: anti-PARP monoclonal (C-2-10, Aparptosis, 1:10,000) or anti-pADPr monoclonal 10H (Kawamitsu et al., 1984) (1:100) or anti-pADPr polyclonal LP96-10 (Aparptosis, 1:10,000) and anti-β-actin monoclonal (Sigma, 1:20,000).

Electrophoretic mobility shift assay (EMSA)

A 50 bp double stranded oligo with many adjacent pyrimidines was derived from pBlueScript (Stratagene) by *Eco*RI/*Xho*I digestion, and end-labelled with ^{32}P[dCTP] by Klenow. The oligo was irradiated at 3.2 kJ/m^2 UVB and, where specified, treated with 0.7 ng of T4 endonuclease V (T4 EndoV) (Trevigen). For EMSA, 5 ng oligo was reacted at 30°C for 30 minutes with 45 ng (or specified amount) of hydroxyapatite-purified bovine PARP (Aparptosis Inc.; 1341 Units/mg protein) in 10 µl of 25 mM Tris-HCl, pH 8.0 containing 50 mM NaCl, 1 mM $MgCl_2$, 0.5 mM DTT, 50 µg/ml BSA and 5 mM spermidine. The reaction mix was electrophoresed on a 1.5% agarose gel, which was dried for autoradiography. The quantification of PARP-oligo complex bands was carried out by electronic autoradiography using a Canberra Packard Instant Imager. For immune competition, anti-PARP C-2-10 (1:100) was added prior to reaction with the oligo.

PARP activation assays

Covalently closed circular plasmid (pEGFP-N3, Clontech) was exposed to 3.2 kJ/m^2 UVB, and an aliquot of UVB-irradiated DNA was also treated with T4 EndoV (Trevigen). For some assays, activated DNA (Sigma) was used as a positive control. Using 10 µg of the above DNA, 1.2 Units of hydroxyapatite-purified bovine PARP (described above) was activated in vitro in an assay containing 5 µCi, i.e. 1.8 µM [adenylate-^{32}P]NAD (1.8 mCi/ml, NEN) (Affar et al., 2002). Aliquots resolved on 8% SDS-PAGE were probed by autoradiography. To estimate the amount of ^{32}P-labelled pADPr bound to PARP at a given time, an aliquot was precipitated with TCA on Whatman GF/C filter discs, washed with ethanol and ether, and measured by scintillation counting (Shah et al., 1995b). In an alternative PARP activation assay, ^{32}P-labelled NAD was replaced by 200 µM non-isotopic NAD, and samples were western blotted for immunodetection of pADPr-modified PARP, as described above.

Chromatin immunoprecipitation (ChIP) for PARP and T-T

10^7 control or UVB (1.6 or 6.4 kJ/m^2) irradiated cells were cross-linked after 15 seconds with 1% formaldehyde, and the nuclear fraction was sonicated exactly as described earlier for the ChIP protocol (Orlando et al., 1997). ChIP was carried out with 100 µg DNA equivalent of nuclear extracts using a cocktail of polyclonal and polypeptide antibodies to PARP (317, 318, 419 and 422, Aparptosis Inc., each at 1:2,000) or monoclonal antibody to T-T (Kamiya, 1:1,000). IP eluates were not subjected to reversal of cross-linking, but were processed to identify the protein or DNA that was co-immunoprecipitated with the first antibody. From ChIP eluate obtained with anti-PARP antibodies, cross-linked DNA was extracted after deproteinization and examined for the presence of T-T by immunodot-blot. From ChIP eluate obtained with anti-T-T antibody, cross-linked proteins were extracted in SDS-PAGE buffer and immunoblotted for detection of PARP (Affar et al., 2002).

DNA immunodot-blot for T-T

For immunodot-blot, either 100 ng of ChIP input DNA or an equal volume of DNA extracted from different ChIP eluates were loaded on Hybond N+ nitrocellulose (Amersham), denatured in 1.5 M NaCl, 0.5 M NaOH for 10 minutes, neutralized for 5 minutes in 1.5 M NaCl, 0.5 M Tris-HCl, pH 7.2, 1 mM EDTA and baked for 2 hours at 80°C. The membrane was immunoblotted with anti-T-T (Kamiya, 1:10,000).

Results

Biphasic activation of PARP in response to UVB

To understand the mechanism of PARP activation in response to UVB, we first determined the early time-course of PARP activation in PARP-replete mouse embryonic fibroblasts exposed to 1.6 kJ/m^2 UVB (Fig. 1). Since PARP is constitutively present in the nucleus, its catalytic activation was monitored by detection of its product pADPr by indirect immunofluorescence (Fig. 1A). A strong nuclear punctate fluorescence of pADPr was seen as early as 15 seconds and it peaked at 5 minutes, followed by decay in signal between 15 and 30 minutes. There was a second strong activation of PARP between 60-120 minutes, which was quenched by 240 minutes. The quantification of fluorescence signal for 150 nuclei from different fields for each of the many

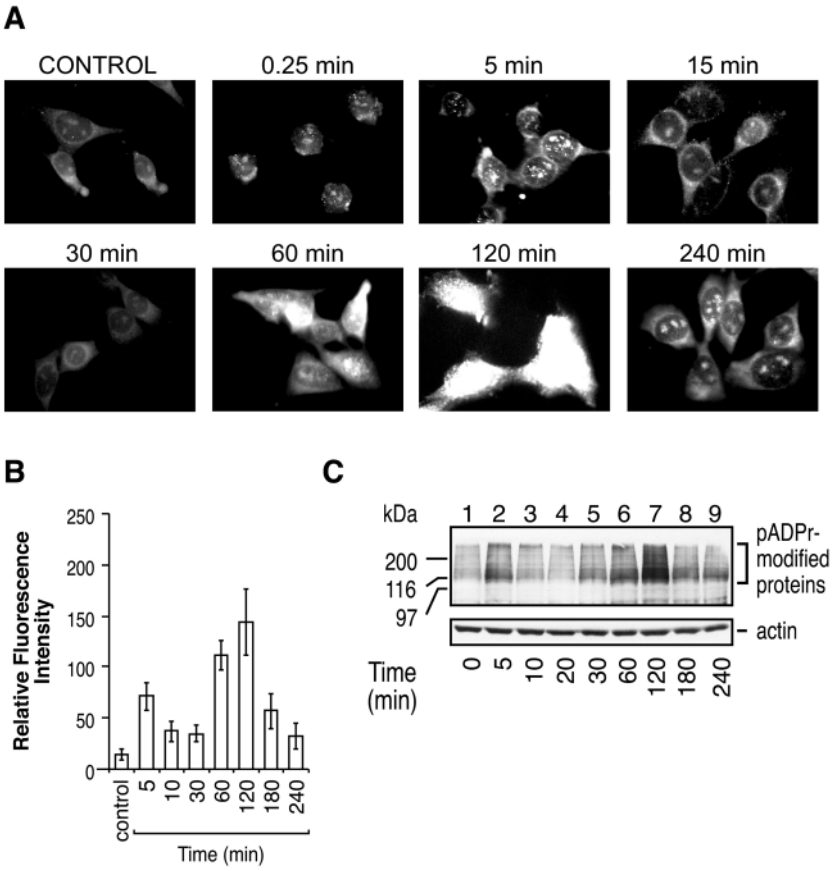

Fig. 1. Biphasic activation of PARP by UVB. (A) Time course of PARP activation in UVB-irradiated cells. Cells were exposed to 1.6 kJ/m^2 UVB and pADPr was visualized by indirect immunofluorescence using monoclonal 10H antibody. Data represent one of five experiments with identical results. (B) Quantification of pADPr signal in UVB-exposed nuclei. Cells were exposed to UVB, as described for A and relative fluorescent intensity was measured in at least 150 nuclei per time point from different fields. Data are expressed as mean±s.d. of relative fluorescence units. (C) Polymer immunoblotting of cells exposed to UVB. Extracts of cells treated with 1.6 kJ/m^2 UVB were western blotted for pADPr with polyclonal LP96-10 or for actin as a loading control. One of six replicates is shown.

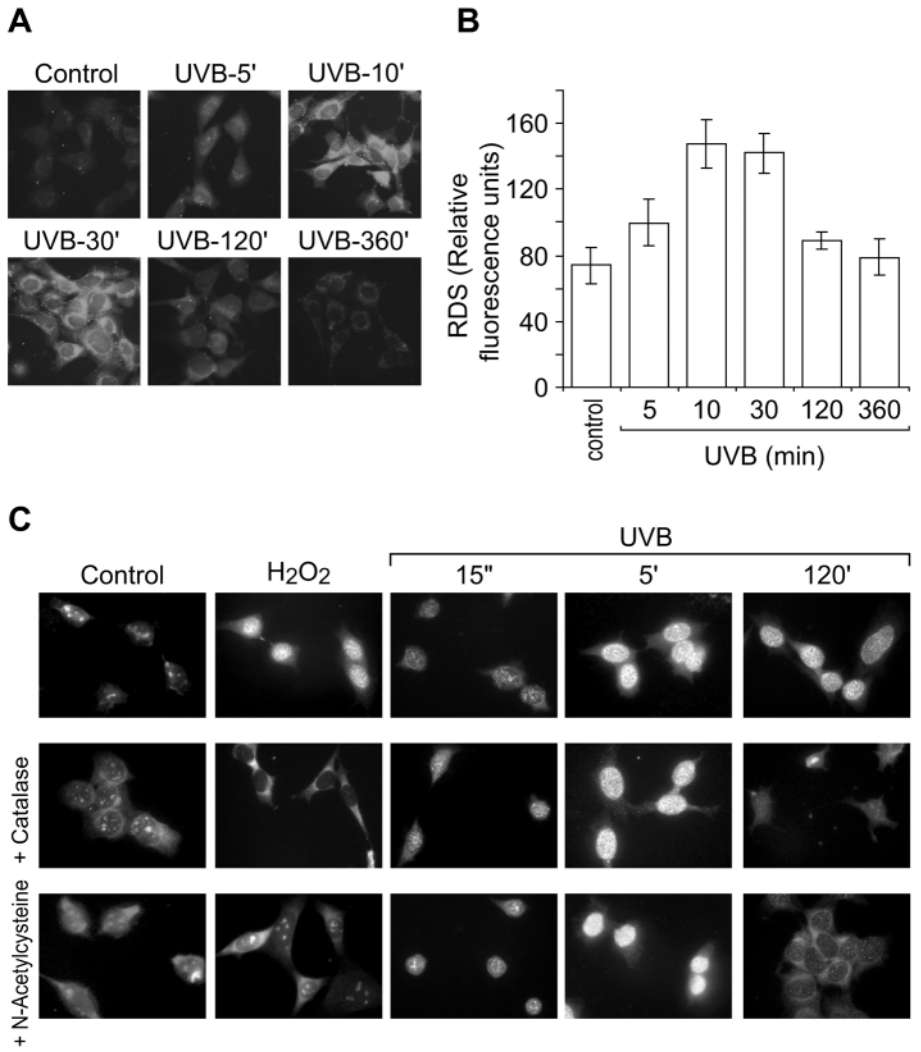

Fig. 2. The UVB-induced second peak of PARP activation. (A) Time course of oxidant formation in UVB-irradiated cells. Cells exposed to 1.6 kJ/m^2 UVB were loaded with DCF-DA prior to harvesting to detect the oxidant load in the cytoplasm by immunofluorescence. (B) The quantification of oxidant signal in UVB-exposed cells. The cytoplasmic fluorescence of UVB-exposed cells described above was quantified in 150 cells per time point. Data are expressed as average fluorescence/cell (mean±s.d.). (C) Suppression of only the second peak of PARP activation by antioxidants. Cells were incubated for 1 hour with 5000 Units/ml catalase or 20 mM *N*-acetylcysteine prior to exposure to 1.6 kJ/m^2 UVB. Cells were then harvested at 15 seconds, 5 minutes or 2 hours. As a control, cells were treated with 100 μM H_2O_2 for 10 minutes. PARP activation was monitored by immunofluorescent detection of pADPr as described for Fig. 1. Data are representative of three experiments with identical results.

different time points from 0-240 minutes confirmed biphasic activation of PARP (Fig. 1B).

Polymer immunoblotting using antibody to pADPr is another method to assess average PARP activation response of an entire cell population (Fig. 1C). In polymer-immunoblots of cells exposed to different DNA damaging agents, proteins including PARP, which are modified by pADPr chains of heterogeneous sizes, appear as a smear between 100-200 kDa (Ha and Snyder, 1999; Halappanavar et al., 1999; Yu et al., 2002). In UVB-irradiated fibroblasts, the signal for pADPr-modified proteins was seen initially at 5 minutes and later in a much stronger peak at 60-120 minutes (Fig. 1C). The early biphasic activation of PARP in response to UVB was confirmed in another mouse fibroblast and in two different human skin fibroblasts. The extent of PARP activation for both phases was dose-dependent from 0.8-12.8 kJ/m^2 UVB with minor variations in exact timing of the peak (data not shown). Absence of pADPr signal in UVB-irradiated *PARP-1*$^{-/-}$ cells or *PARP-1*$^{+/+}$ cells treated with PARP inhibitor (data not shown), indicated a major catalytic role played by PARP-1, but not by other members of PARP family in response to UVB.

UVB-induced oxidant is the cause of the second peak of PARP activation

It has been suggested earlier that oxidative DNA damage induced by UVB is the most probable cause of PARP activation in UVB-irradiated cells (Caldecott, 2003b; Chang et al., 2002; Farkas et al., 2002). Therefore, we examined whether both the phases of PARP activation were due to a UVB-induced oxidant (Fig. 2). Since oxidants are generated in the cytoplasm and PARP is activated in the nucleus, we first determined the time course of formation of oxidant using DCF-DA, a redox fluorescent intracellular probe that is highly responsive to intracellular H_2O_2 (Chan and Yu, 2000; Gniadecki et al., 2002). A faint cytoplasmic fluorescence was observed in UVB-irradiated cells at 5 minutes, which increased from 10-30 minutes and then declined at 2-3 hours (Fig. 2A). The quantification of fluorescent signals from at least 150 cells per time point confirmed that oxidant signal peaked between 10-30 minutes (Fig. 2B). Thus, the majority of intracellular oxidants were generated well after the first wave of PARP activation but preceding the second peak.

The cause and effect relationship between oxidant and two phases of PARP activation was more directly examined by pre-treatment of cells with anti-oxidants prior to irradiation (Fig. 2C). Incubation of cells with catalase is often used for confirming the role of H_2O_2 in cellular responses to UV (Huang et al., 2001; Mahadev et al., 2001). As a control, we observed that catalase pre-treatment quenched PARP activation, i.e. pADPr formation in the cells treated with 100 μM H_2O_2 for 10 minutes (Fig. 2C, second column). In the cells treated with UVB, catalase could quench PARP activation only at 120 minutes, but not at 15 seconds or 5 minutes (Fig. 2C, second row), implying that a UVB-induced oxidant, specifically H_2O_2, was the major cause of the second but not the first peak of PARP activation. We observed that pre-

treatment with a general anti-oxidant *N*-acetylcysteine also inhibited the second but not the first peak of PARP activation (Fig. 2C, third row). Similar results were observed with other general anti-oxidants, such as DMSO or Na-azide (data not shown). Thus, the UVB-induced oxidant accounted for only the second phase of PARP activation at 1-2 hours, but not the immediate activation of PARP from 15 seconds to 5 minutes.

Monophasic activation of PARP in response to UVC

UVC radiation, unlike UVB, principally causes direct DNA damage and much less of the oxidative DNA damage (de Gruijl et al., 2001), hence we examined early PARP activation response in UVC-irradiated fibroblasts. The cells were exposed to 0.01 kJ/m^2 UVC and PARP activation was monitored by polymer immunoblotting using polyclonal antibody to pADPr (Fig. 3A, top panel). As a positive control, cells exposed to 100 μM H_2O_2 for 10 minutes showed a strong signal for pADPr-modified proteins. In UVC-irradiated cells, a strong signal for pADPr-modified proteins was seen at 5 minutes, which was suppressed by 15 minutes and a much weaker signal for pADPr emerged once again between 60-120 minutes. In order to exclude the possibility that polyclonal antibody to pADPr may have failed to detect different size pADPr chains formed at 1-2 hours, we reprobed the blot with 10H, a monoclonal antibody that detects larger pADPr chains (Kawamitsu et al., 1984), however, the signal at 1-2 hours remained weak with this antibody (Fig. 3A, middle panel), confirming that UVC irradiation induced a practically monophasic activation of PARP with a strong first peak at 5 minutes and a feeble second peak at 1-2 hours.

A recently developed technique of local irradiation through a polycarbonate filter containing irregularly distributed holes of about 5 μm size allows visualization of events occurring directly at the site of UVC exposure and distinguishes them from events occurring as a result of diffusible factors in non-irradiated parts of the cell (Moné et al., 2001). We used this technique to identify whether PARP is activated throughout the nucleus or only in the UVC-irradiated spots. To obtain a response similar to irradiation without a filter, we had to use 10-times higher dose of UVC (0.1 kJ/m^2) with the filter. This was required because without the filter all the radiation that is coming at different angles to the surface can reach the cells, whereas with the filter, only the radiation that is strictly perpendicular to small holes in the filter can reach the cells and the rest is absorbed by the filter. The UVC-irradiated spots in the nuclei were identified by immunofluorescent staining for T-T photolesions (green) and PARP activation was

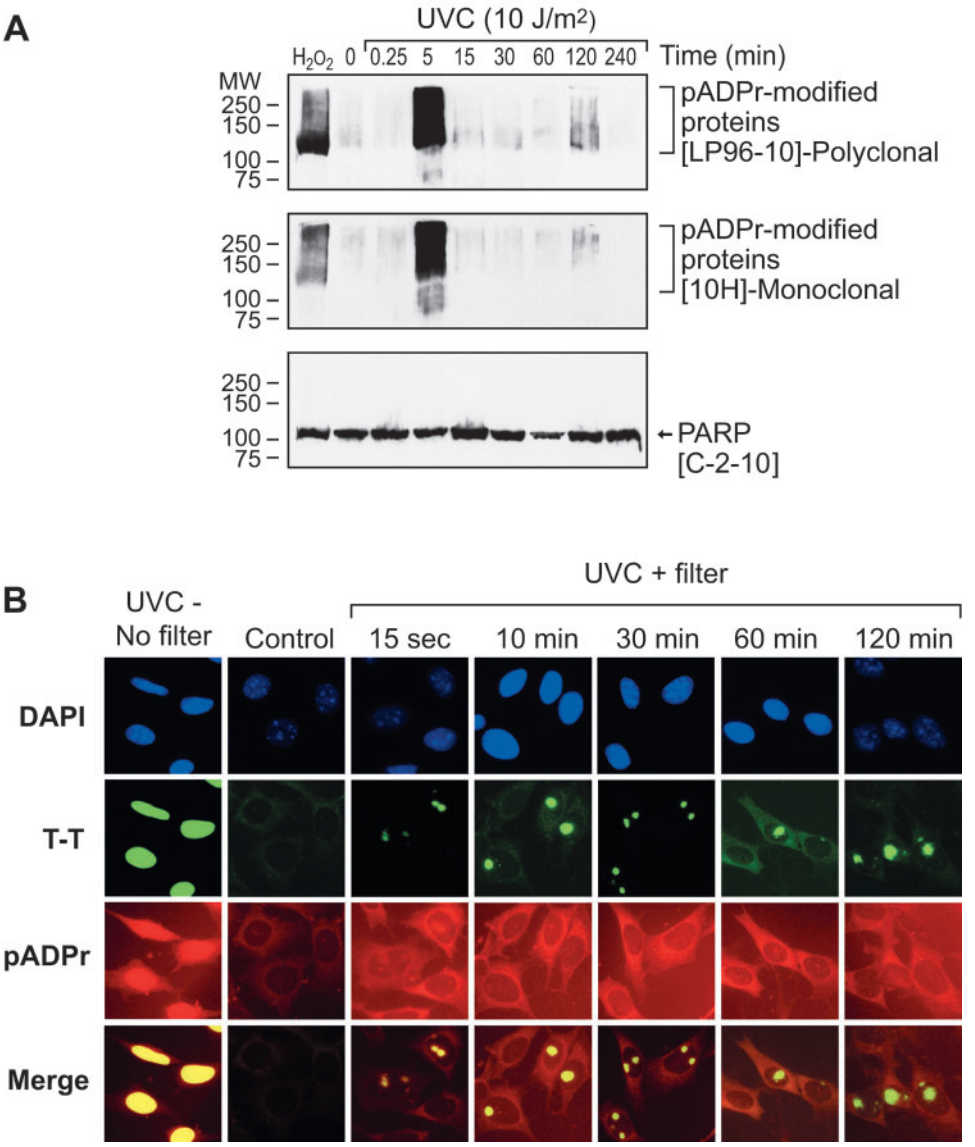

Fig. 3. Monophasic activation of PARP by UVC. (A) Time course of PARP activation in UVC-irradiated cells. Cells were exposed to 0.01 kJ/m^2 UVC or 100 μM H_2O_2 for 10 minutes. The pADPr-modified proteins were detected by immunoblotting with polyclonal LP96-10 or monoclonal 10H antibodies. Blots were reprobed with PARP antibody C-2-10. (B) Immediate activation of PARP at the site of local UVC irradiation. Cells were exposed directly or through a polycarbonate filter to 0.1 kJ/m^2 UVC. Samples were fixed using the TCA protocol at times ranging from 15 seconds to 2 hours after irradiation. The signals for T-T (FITC green) or pADPr (Texas red) were detected by indirect immunofluorescence; where the signals co-localized, a yellow colour was present in the merged images. Nuclear DNA was stained with DAPI. Images shown are representative of four experiments with identical results.

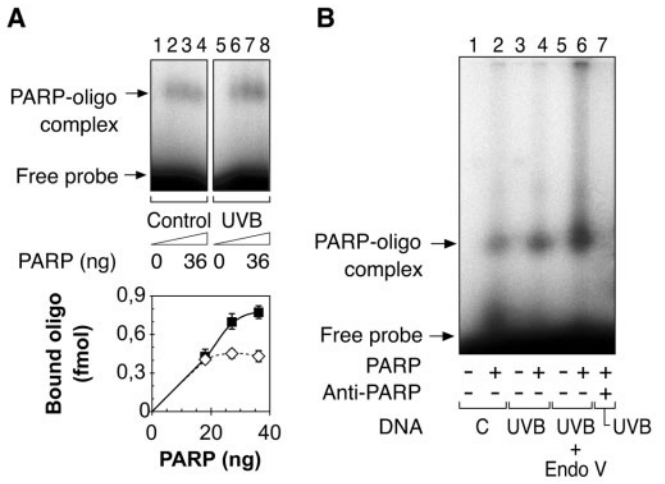

Fig. 4. EMSA of UVB-irradiated oligo by PARP. (A) Dose dependent binding of PARP to UVB-irradiated oligo. The unirradiated and UVB-irradiated 50 bp oligo were reacted with 0-36 ng of PARP and resolved on neutral 1.5% agarose-TBE gel. Gels were dried and analysed using an instant imager (top panel). The cpm in each band of oligo-PARP complex from four independent experiments were pooled and shown here as mean±s.d. (bottom panel). (B) Specificity of interaction of PARP with UVB-irradiated oligo. Purified PARP (45 ng) was reacted with 5 ng of one of the three oligos: unirradiated control (lanes 1 and 2), 3.2 kJ/m^2 UVB-irradiated (lanes 3 and 4) or UVB + T4 EndoV-treated (lanes 5 and 6). Samples were resolved on agarose gel and analysed, as described above. For immune competition study, anti-PARP C-2-10 (1:100) was added prior to reaction with UVB-irradiated oligo (lane 7). Data are from one of three experiments with identical results.

identified by staining for pADPr with polyclonal LP96-10 antibody (red). When the cells were exposed to UVC without the filter, a uniform signal was observed after 15 seconds throughout the nucleus for T-T photolesions and pADPr, which upon merger of the images gave yellow nuclei indicating their co-localization (Fig. 3B, first column). When the cells were exposed to UVC through the filter, T-T signal was confined to positions on the nuclei that were directly below the 5 μm holes, and it was detectable from 15 seconds to 120 minutes (Fig. 3B, second row). Interestingly, at 15 seconds after UVC irradiation, PARP was also activated exactly in the same 5 μm areas where the signal for T-T was detected, and this was evident from yellow spots in the merged panel (Fig. 3B, third column). We reconfirmed this co-localization of T-T and pADPr signals at 15 seconds in hundreds of nuclei from several independent experiments (data not shown). PARP activation at the site of UVC-irradiation was much reduced by 10 minutes, because merged spots were fainter yellow (Fig. 3B, column 4) and no PARP activation was observed from 30-120 minutes, as merged spots were only green from the T-T dimers (Fig. 3B, columns 5-7). The local irradiation technique established that the cause for immediate PARP activation was present only in the directly irradiated area of the nucleus and that it was not a diffusible factor that could have arrived from unexposed areas of the nucleus or from UVC-exposed 5 μm spots in the cytoplasm. Finally, lack of activation of PARP at the site of UVC-irradiation after 10 minutes, despite the presence of unrepaired T-T, indicated that PARP may be participating only in the early events at the site of direct DNA damage by UVC.

Binding and activation of PARP by UVB damaged DNA in vitro

The local irradiation technique with a polycarbonate filter can not be used for UVB, because unlike UVC, UVB passes through this filter. Hence, to determine whether direct DNA damage by UVB was also the cause of the first peak of PARP activation, two different in vitro assays were used in which capacity of PARP to directly bind to and be activated by UVB-damaged DNA was evaluated (Figs 4 and 5). These assays use UVB-damaged DNA along with purified PARP, and do not contain cellular extracts or NER factors, therefore, initial UVB-induced damage to DNA is not processed further to generate DNA strand breaks. The capacity of PARP to bind to UVB-damaged DNA was examined by electrophoretic mobility shift assay (EMSA) using a ^{32}P-labelled 50 bp oligo containing numerous sites with adjacent pyrimidines (Fig. 4A). When increasing amounts of PARP was reacted with 5 ng of ^{32}P-labelled UVB-irradiated or control oligo, there was a dose-dependent increase in binding of only the UVB-irradiated oligo to PARP (Fig. 4A, top panel). In contrast, a basal level of PARP binding to control oligo, probably due to weak interaction with free DNA termini (Sastry et al., 1989) or inner regions of DNA (de Murcia et al., 1983), did not increase with increasing amounts of PARP. Earlier EMSA studies also reported a weak interaction between blunt ended duplex oligo and PARP-like zinc fingers (Petrucco, 2003) or PARP (Mendoza-Alvarez and Alvarez-Gonzalez, 2001); the latter study also showed that increasing the amount of PARP from 7 to 70 ng did not increase the extent of this non-specific retention of oligo by PARP. Hence, concentration-dependent increase in binding between PARP and UVB-irradiated oligo indicated specific interaction between PARP and UVB-induced changes in the oligo. The quantification of ^{32}P-labelled oligo retained in the PARP-oligo complex, from four assays, showed that nearly twice the amount of UVB-irradiated oligo was bound to 36 ng of PARP, as compared with control oligo (Fig. 4A, bottom panel).

PARP was recognizing the oligo with intact DNA damage in the form of CPD, because when UVB-irradiated oligo was treated with T4 EndoV to create nicks near CPD sites, the resultant nicked DNA reacted much more strongly with PARP (Fig. 4B, lane 6), which was expected because PARP has very high affinity for single strand breaks (Burkle et al., 2000). Pre-incubation of reaction mix with anti-PARP antibody completely blocked the interaction between PARP and UVB-irradiated oligo (Fig. 4B, lanes 4 and 7), confirming that the oligo was indeed retained by PARP.

We next examined whether PARP is activated as a consequence of binding to UVB damaged DNA (Fig. 5). In this assay, we used closed circular plasmid DNA to reduce the extent of PARP binding and activation by free DNA termini. The plasmid DNA was exposed to 3.2 kJ/m^2 UVB, and it was confirmed in agarose gel electrophoresis that no linear or nicked molecules were generated in the plasmid DNA irradiated with this dose or even a four times higher dose of UVB. The capacity of unirradiated and UVB-irradiated plasmid to activate PARP was examined in an in vitro assay using ^{32}P-labelled NAD (Affar et al., 2002), followed by

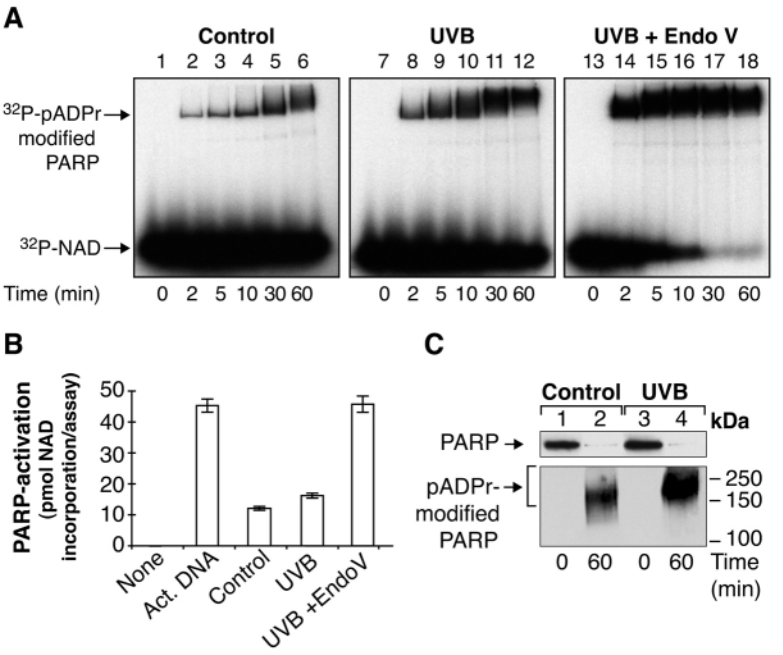

Fig. 5. PARP activation by UVB-irradiated closed circular plasmid DNA. (A) PARP activation in vitro with UVB-irradiated plasmid. Purified PARP was activated with control, UVB (3.2 kJ/m^2)- or UVB + T4 EndoV-treated circular plasmid DNA in the presence of 1.2 μM ^{32}P-NAD. Aliquots were resolved on SDS-PAGE prior to autoradiography. Results of one of four experiments with identical results is shown. (B) Quantification of PARP activation in the in vitro assays. PARP was reacted with three plasmids, as described above, along with activated, i.e. extensively nicked DNA. Reaction was terminated at 30 minutes and ^{32}P-pADPr-modified PARP was TCA precipitated and counted. This experiment was carried out four times with each assay conducted in triplicate and data are presented as mean±s.e.m. of 12 observations. (C) In vitro PARP activation assay with non-isotopic NAD. In vitro PARP activation assay with control and UVB-irradiated plasmid DNA was carried out with 200 μM non-isotopic NAD for 60 minutes. The unmodified PARP and pADPr-modified PARP were detected by immunoblotting with antibodies specific for PARP and pADPr. Data represent four experiments with identical results.

autoradiographic detection of pADPr-modified PARP (Fig. 5A). At every stage of this reaction starting from 2 minutes until 60 minutes, a significantly higher amount of ^{32}P-pADPr-modified PARP was formed in response to UVB-irradiated plasmid as compared to control DNA. Moreover, progressive depletion in the substrate NAD was evident only in the assay with UVB-irradiated plasmid and not with control plasmid. In this assay, we also observed that UVB-irradiated DNA that was nicked adjacent to CPD sites, by treatment with T4 EndoV, stimulated PARP activity very strongly, resulting in complete exhaustion of the NAD substrate between 10 and 30 minutes (Fig. 5A, lanes 16-18). Thus, stronger activation of PARP was observed in response to UVB-irradiated plasmid bearing T-T lesions as compared to unirradiated control plasmid.

We confirmed that UVB-irradiated plasmid was a better activator of PARP than unirradiated plasmid by acid-precipitation of an aliquot of each of these assays at 30 minutes and counting the ^{32}P-pADPr modified PARP collected on a filter according to the conventional PARP activation assay (Shah et al., 1995b) (Fig. 5B). Of course, DNA that was significantly nicked, such as commercially available activated DNA (Sigma) or UVB-irradiated DNA that was treated with T4 EndoV was a much stronger activator of PARP (Fig. 5B). It is known that because of the requirement of high concentrations of radiolabelled NAD, the above mentioned assay uses suboptimal concentration of NAD (1.2 μM), which is almost a hundred times less than the K_m of PARP for NAD (Shah et al., 1995a). Therefore, we carried out the same PARP activation assays using non-isotopic NAD at a concentration of 200 μM and detected pADPr-modified PARP by immunoblotting (Fig. 5C). At this optimum concentration of NAD, the PARP immunoblot revealed that PARP present at 0 minutes in both the reactions disappeared at the end of the reaction, at 60 minutes, owing to pADPr-modification (Fig. 5C, top panel). However, polymer-immunoblot revealed that UVB-irradiated plasmid was a much stronger activator of PARP, as observed in the stronger signal for pADPr-modified PARP at a higher apparent molecular mass, indicating more extensive poly(ADP-ribosyl)ation (Fig. 5C, bottom panel, lanes 2 and 4). Thus, PARP could bind to and be activated by UVB-irradiated DNA.

Co-immunoprecipitation of T-T and PARP from UVB-irradiated cells

Having observed that PARP can be rapidly activated directly at the site of UVC-induced DNA damage, and that it can be activated in vitro by UVB-irradiated DNA with intact photolesions, we next examined by chromatin immunoprecipitation technique (ChIP) whether in the cells irradiated with UVB, PARP can be found in close proximity to direct photolesions, such as T-T, as early as 15 seconds after UVB irradiation (Fig. 6). Normally, this technique is used with anti-protein antibody to identify DNA sequences that are co-immunoprecipitated with specific protein after cross-linking in vivo (Orlando et al., 1997), but we extended the technique by using it with both anti-protein (PARP) and anti-DNA (T-T) antibodies to examine if both are cross-linked in vivo immediately after UVB irradiation. The control or UVB-exposed (1.6 and 6.4 kJ/m^2) cells were cross-linked at 15 seconds and subjected to ChIP with PARP antibodies (Fig. 6A). We started with identical amounts of input DNA (100 μg) from control and UVB-irradiated cell extracts, as seen from analysis of an identical aliquot of input DNA by ethidium bromide staining (Fig. 6A, first panel). In the ChIP input DNA from UVB-irradiated cells, there was a dose-dependent increase in the signal for T-T photolesions (Fig. 6A, second panel). At the end of the ChIP procedure using antibody to PARP, the DNA co-immunoprecipitated with PARP was examined first for amount and then for T-T content. First, there was a dose-dependent increase in the amount of DNA immunoprecipitated from UVB-irradiated cells (Fig. 6A, third panel). This confirmed that PARP was not pulling down any DNA from cell extract in a non-specific manner, because having started with identical amounts of input DNA, we would have recovered the

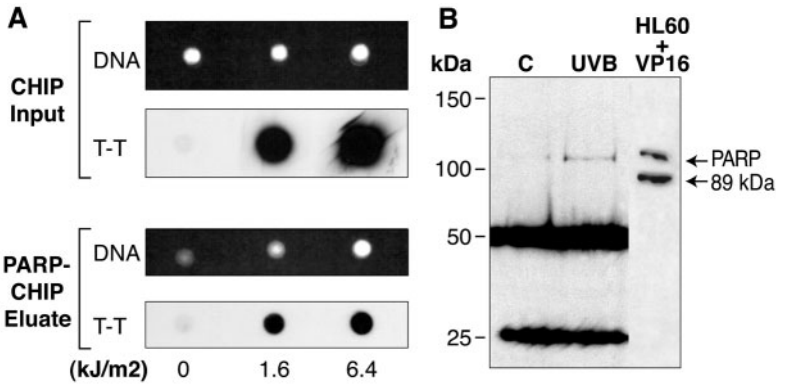

Fig. 6. ChIP assay for in vivo interaction between PARP and T-T. (A) Co-immunoprecipitation of DNA containing T-T photolesions with PARP. ChIP input DNA (top two panels): Chromatin extracts of control or UVB (1.6 and 6.4 kJ/m^2 for 15 seconds)-irradiated cells were adjusted prior to ChIP to 100 μg input DNA and an equal aliquot from each extract was assayed for DNA content by ethidium bromide staining and for T-T content by immunodot-blot. ChIP eluate using antibodies to PARP (bottom two panels). DNA was extracted from PARP-ChIP eluates of control and UVB-treated cells, and an equal portion of each eluate was assayed for DNA content by ethidium bromide and for T-T content by immunodot-blot. (B) Recovery of PARP cross-linked to T-T. ChIP was carried out for extracts from control and UVB (6.4 kJ/m^2)-irradiated cells with anti-T-T, and proteins eluted in SDS-PAGE sample buffer were immunoblotted for PARP. The 25 and 50 kDa bands are subunits of antibodies used in immunoprecipitation. Apoptotic HL-60 cells were loaded for identification of PARP. Data are from one of five experiments with identical results.

same amount of DNA in the ChIP eluate from control or UVB-treated cells. Instead, PARP specifically bound to and immunoprecipitated, in a dose-dependent manner, more of the DNA from UVB-exposed cells than control cells. Immunodot-blots of the ChIP eluate DNA from UVB-irradiated cells also revealed a dose-dependent increase in the signal for T-T photolesions (Fig. 6A, fourth panel). As a corollary, we examined whether ChIP carried out with antibodies to T-T would co-immunoprecipitate PARP (Fig. 6B). Since DNA of control cells do not have any T-Ts, no DNA was pulled down and of course, no PARP was found, which confirmed that we were not pulling down non-specific proteins or DNA in the ChIP assay. However, PARP was detected in the ChIP eluate of UVB-irradiated cells using antibodies to T-T lesions, supporting the argument that immediately after UVB irradiation, PARP is attached to the chromatin DNA that carries the direct photolesions.

Discussion

PARP is known to be activated rapidly in response to different DNA damaging chemical and physical agents, but precise information about where and how PARP is activated within the nucleus of damaged cells was not known until recently. Earlier studies that monitored only NAD levels or pADPr levels could not give precise information about intra-nuclear location or possible multiple phases of PARP activation. Immunohistological visualization of PARP activation often revealed a punctate pattern of signal for pADPr, despite the general presence of PARP in the nucleus (Amé et al., 2000), suggesting that PARP activation is not a brute response of activating all available PARP molecules, but a more controlled process. We have now shown that cells can selectively and repeatedly activate PARP at the site of different types of DNA damages induced by UV. We have shown that UVB-irradiated mouse fibroblasts rapidly activate PARP in two distinct phases, first from 15 seconds to 5 minutes and later between 1 and 2 hours. In contrast, UVC-irradiated fibroblasts show a practically monophasic activation of PARP within the first 5 minutes. We also identified the mechanism for each phase of PARP activation. Using antioxidants, local irradiation, chromatin immunoprecipitation and in vitro PARP assays, we show that UV-induced direct DNA damage, such as thymine dimers causes initial PARP activation, whereas oxidative damage by UVB causes a second wave of PARP activation. Since the types of DNA damages caused by UVB are identical in different cells, we should expect similar PARP-activation responses in other types of cells, although it will be pertinent to specifically verify it in skin keratinocytes, which are one of the targets for UVB-induced skin carcinogenesis.

Multiple phases of PARP activation require that pADPr is degraded rapidly after each activation phase, and this is seen in our results and it is supported by reported $T_{\frac{1}{2}}$ of 3-6 minutes for pADPr in UVC-exposed human fibroblasts (Jacobson et al., 1983) or rat hepatocytes (Alvarez-Gonzalez and Althaus, 1989). Our findings are compatible with subsequent waves of PARP activation from other causes, such as apoptotic DNA fragmentation, as shown in keratinocytes, 4-24 hours after exposure to UVB (Chang et al., 2002). Of course, in skin carcinogenesis protocols involving repeated exposures to UVB (Epstein and Cleaver, 1992), these cycles of PARP activation could occur after each UVB exposure. Our data imply that each phase of PARP activation may make a unique contribution to cellular responses to UVB. This notion is supported by observations that inhibiting PARP activation delays DNA repair of CPD and oxidative damage (Flohr et al., 2003), inhibits apoptosis (Mi et al., 2003) and aggravates UVB-induced carcinogenesis (Epstein and Cleaver, 1992).

The cause and effect of immediate PARP activation in response to UVB and UVC

We show that the cause of immediate PARP activation within the first 15 seconds to 5 minutes after UVB and UVC irradiation is direct DNA damage. Using local irradiation, an elegant technique that allows visualization of events occurring in UVC-irradiated zones of the nuclei (Moné et al., 2001), we show that PARP activation occurs at the site of UVC exposure as rapidly as reported accumulation of p48/UV DNA damage binding protein 2 (UV-DDB2) (Fitch et al., 2003; Tang and Chu, 2002). Since DDB protein complex is thought to be responsible for initial recognition of CPD damage (Hanawalt, 2002; Tang and Chu, 2002), our results indicate that PARP

activation occurs concomitant with DDB2 binding to the direct DNA lesions and occurs prior to the start of NER process at the damaged site.

In our study, we have used a T-T antibody as a tool to identify either nuclear areas (in the local irradiation assay) or chromatin DNA fragments (in the ChIP assay) that contain direct photolesions, hence we can not exclude the possibility that direct DNA lesions other than T-T, such as other CPDs (e.g. T-C or C-C) or 6,4-photoproducts can serve to attract and activate PARP. All of these lesions distort DNA structure and it is likely that PARP can recognize and bind to this unusual DNA structure. We have already shown in the in vitro assays that PARP can bind to and be activated by UVB-irradiated DNA in the absence of damage processing repair enzymes. Moreover, PARP is known to be activated by unusual DNA structures without DNA strand breaks, such as cruciform, curved and bent structures (Rolli et al., 2000) or linear and stem-loop structures (Kun et al., 2002). Furthermore, PARP activation and chromatin loosening in the absence of DNA strand breaks has also been observed during transcriptional activation of genes in *Drosophila* salivary gland chromosome (Tulin and Spradling, 2003).

Recently, PARP activation in UVB-irradiated cells was shown to occur at the site of single strand breaks created near the T-T by transfected UV damage endonuclease (Okano et al., 2003). In this study, direct activation of PARP due to CPD alone, equivalent to our first PARP activation, was not reported in UV-irradiated cells that were not transfected with endonuclease. It is possible that direct activation by CPD was deemed to be very weak, perhaps close to background, compared to the extremely strong PARP activation seen in cells with endonuclease-induced DNA strand breaks; this was also seen in our PARP activation assay with T4 EndoV-treated DNA (Fig. 5). Moreover, the TCA fixation protocol used in our study is known to prevent any degradation of pADPr during sample preparation (Kupper et al., 1990).

There are many possible functions for the immediate activation of PARP at the site of direct DNA damage by UVB or UVC. The UVB dose-dependent nature of PARP activation indicates that the amount of pADPr formed in the damaged nuclei and their localization in the vicinity of DNA damage may signal the global levels of intact photolesions and their precise sub-nuclear location to cellular checkpoint and transcriptional machineries to initiate DNA repair. This role of PARP activation as a signalling mechanism to alert the cell to the location and the extent of DNA damage is also suggested in another study using localized proton microbeam ionizing radiation (Tartier et al., 2003). In that study, PARP activation occurs in the same time frame as recruitment of DNA repair proteins ATM and Rad51, but PARP acts only as a sensor of DNA damage rather than a participant in the double strand break repair process.

The rapidity of initial PARP activation may make it an unlikely participant in the much slower NER process, although most of the NER enzymes have also been shown to leave the damaged site around 4 hours after UV exposure, even with persistence of unrepaired photoproducts (Costa et al., 2003; Wang et al., 2003). Thus, early PARP activation at the damaged site may initiate a process that helps in subsequent repair of these lesions. This is supported by the observation that PARP inhibition decreases removal of CPD (Flohr et al., 2003). PARP could achieve this through its control of p53 function (Wieler et al., 2003), which in turn controls DDB2 induction in response to UV (Hwang et al., 1999). Another possible role of PARP activation at this site could be in the remodelling of chromatin (Amé et al., 2004), which is known to facilitate NER in chromatin DNA (Hara et al., 2000; Ura et al., 2001). It is known that PARP activation and histone H1 and H2B modification contributes to relaxation of the 30 nm chromatin fibre in vivo (Poirier et al., 1982). Moreover, there is non-covalent interaction between pADPr chains and histones, which may also contribute to destabilization of nucleosomal structure (Pleschke et al., 2000). More studies are required to establish whether immediate PARP activation participates in NER of UV-induced DNA lesions.

The cause and effect of second phase of PARP activation in response to UVB

Our study clearly shows that the second phase of PARP activation in response to UVB is due to UV-induced oxidative damage. Although oxidants generated by UVB can cause damage in different parts of the cell, from nucleus to membranes, its damage to DNA is likely to be the prime cause of PARP activation. We have observed that H_2O_2 is the major cause for PARP activation in the UVB-irradiated fibroblasts, and H_2O_2 is known to cause different types of DNA damages ranging from single strand breaks to 8-oxoG. It has been suggested that PARP is activated initially by binding to single strand breaks being subjected to SSBR (Caldecott, 2003a), and later when stand breaks are created during the BER of oxidative DNA damages (Caldecott, 2003a; Le Page et al., 2003). It will be interesting to examine whether the second peak of PARP activation, caused by oxidative DNA damage by UVB, contributes positively towards repair of these oxidative lesions by BER.

It is also possible that both phases of early PARP activation are associated with cell death, because early and rapid PARP activation in cells exposed to high levels of DNA damage by alkylating agents has been associated with subsequent apoptotic or necrotic deaths (Hong et al., 2004; Zong et al., 2004). However, we have observed biphasic PARP activation in response to low sub-lethal and high lethal doses of UVB (0.4-12.8 kJ/m^2). Therefore, at least in response to lower levels of UVB, early peaks of PARP activation are not associated with cell death. Moreover, apoptosis-associated PARP activation has been reported to occur between 4 and 24 hours in UVB-irradiated keratinocytes (Chang et al., 2002). Our results show that PARP activation is a dynamic and flexible metabolism that can be targeted to the site of specific DNA damage at a desired time to participate in a specific response of higher eukaryotic cells to DNA damage.

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