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DNA repair defect in poly(ADP-ribose) polymerase-deficient cell lines

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ABSTRACT

To investigate the physiological function of poly(ADP-ribose) polymerase (PARP), we used a gene targeting strategy to generate mice lacking a functional *PARP* gene. These *PARP*$^{-/-}$ mice were exquisitely sensitive to the monofunctional-alkylating agent *N*-methyl-*N*-nitrosourea (MNU) and γ-irradiation. In this report, we have analysed the cause of this increased lethality using primary and/or spontaneously immortalized mouse embryonic fibroblasts (MEFs) derived from *PARP*$^{-/-}$ mice. We found that the lack of PARP renders cells significantly more sensitive to methylmethanesulfonate (MMS), causing cell growth retardation, G_2/M accumulation and chromosome instability. An important delay in DNA strand-break resealing was observed following treatment with MMS. This severe DNA repair defect appears to be the primary cause for the observed cytoxicity of monofunctional-alkylating agents, leading to cell death occurring after G_2/M arrest. Cell viability following MMS treatment could be fully restored after transient expression of the *PARP* gene. Altogether, these results unequivocally demonstrate that PARP is required for efficient base excision repair *in vivo* and strengthens the role of PARP as a survival factor following genotoxic stress.

INTRODUCTION

Poly(ADP-ribose) polymerase (PARP) is a nuclear zinc finger DNA-binding protein that detects DNA strand breaks. At a site of breakage, PARP catalyses the transfer of the ADP-ribose moiety from its substrate, NAD^+, to a limited number of protein acceptors (heteromodification) involved in chromatin architecture (histones H1, H2B, Lamin B) or in DNA metabolism (Topoisomerases, DNA replication factors), including PARP itself (automodification) (reviewed in 1–3). These modified DNA-binding proteins, carrying chains of negatively charged ADP-ribose polymers, generally lose their affinity for DNA and are rapidly inactivated (4). Degradation of ADP-ribose polymers is rapidly catalysed by poly(ADP-ribose) glycohydrolase (PARG), which cleaves the ribose–ribose bond (5,6). Poly(ADP-ribosylation) is therefore an immediate and transient post-translational modification of nuclear proteins induced by DNA lesions (DNA nicks and base damage generating nicks) mainly repaired by the base excision repair (BER) pathway. Detection and translation of signals emanating from DNA interruptions, as well as their amplification by poly(ADP-ribose) formation, are the main characteristics of this enzymatic activity, catalyzed by a highly conserved protein (7).

PARP interacts with X-ray repair cross complementing factor-1 (XRCC1) (8,9), an adaptor protein which has also two interfaces with two important base excision repair enzymes, DNA ligase III (10) and DNA polymerase β (11). As a consequence of these interactions, XRCC1 stabilizes DNA ligase III (12), but negatively regulates PARP activation following oxidative stress, presumably in a transient manner (8). Therefore, PARP is probably associated with a multifunctional complex including, at least, XRCC1, DNA polymerase β and DNA ligase III. Both enzymes are involved in the BER, the most frequently solicited DNA repair pathway in mammalian cells (13).

Previous studies have shown that a decrease in PARP activity has deleterious effects on cells exposed to genotoxins that trigger the BER pathway (14–18). To assess the biological consequences of PARP deficiency, more recently, *PARP*$^{-/-}$ mice have been generated by homologous recombination (19,20–22). Wang *et al*. showed that a null mutation in the *PARP* gene has no influence on excision repair of DNA damaged with *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) and that, unexpectedly, PARP null mice are obese and display skin hyperplasia in a mixed genetic background (20). In contrast, we have reported that *PARP*$^{-/-}$ mice are hypersensitive to genotoxic agents, like γ-rays and monofunctional-alkylating agents compared with their wild-type litter mates. Mutant mice displayed genomic instability as shown by an increased rate of sister chromatid exchanges (SCEs) and an increased occurrence of chromosome breaks (19,22). Using cells derived from *PARP*$^{-/-}$ mice, we and others have established that apoptosis occurs in the absence of PARP (23) and that PARP cleavage by the caspases proteases (24) is not absolutely required for the execution phase of apoptosis. Moreover, *PARP*$^{-/-}$ splenocytes exposed to *N*-methyl-*N*-nitrosourea (MNU) underwent much more rapid apoptosis than wild-type cells (19).

To understand the causes of the hypersensitivity of *PARP*$^{-/-}$ mice towards monofunctional-alkylating agents, in this work we have monitored several physiological parameters, including cell

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viability, cell-cycle distribution and chromosome stability in *PARP*$^{-/-}$ primary mouse embryonic fibroblasts (MEFs) exposed to methylmethanesulfonate (MMS). We demonstrate, for the first time, that PARP-deficient cell lines performed very limited DNA repair during the first 6 h after DNA damage by alkylating agents. This dramatic decrease in DNA strand-break rejoining is most likely at the origin of the acute hypersensitivity and the high genomic instability of PARP null mice to alkylating agents (19). These studies extend the *in vivo* analysis of the *PARP*$^{-/-}$ defect and provide important insights into the role that PARP plays in repair of DNA single-strand breaks.

MATERIALS AND METHODS

Isolation and immortalization of MEFs

Primary MEFs were harvested from 13.5-day-old embryos according to Abbondanzo *et al*. (25). Cells were cultured at 37°C (5% CO_2) in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum, 4.5 mg/ml glucose and 0.5% gentamicin (Sigma) until immortalization. MEFs of each genotype were used at early passages (passages 2–3). The mean doubling time of primary MEFs was found to be 49 and 45 h for (*PARP*$^{+/+}$) and (*PARP*$^{-/-}$) MEFs, respectively. Immortalized cells were first passed twice in the presence of 5 µg/ml mycoplasma removal agent (ICN Pharmaceuticals), and subsequently subcultured at 7 (*PARP*$^{+/+}$) or 10 (PARP$^{-/-}$) days interval at a density of 8×10^3 cells/cm^2. The mean doubling times were found to be 24 and 36 h for (*PARP*$^{+/+}$) and (*PARP*$^{-/-}$) MEFs, respectively.

Cell growth rates and thymidine incorporation

Exponentially growing primary MEFs at passage two or three (10^5 cells per 30 mm dish) were treated in triplicate with 0–0.1 mM MMS for 30 min at 37°C. Cells were counted daily for three days to determine the cell growth rate. [Methyl-^{3}H]thymidine (5 µCi/ml) incorporation was measured after 3 days of cultivation following damage.

Restoration of cell viability using a *lac-Z* reporter gene

Immortalized MEFs were co-transfected by electroporation, using 15 µg DNA of either pECV (empty vector) (26) or pECV PARP (27) and 3 µg of a plasmid containing the bacterial *lacZ* gene, to identify the transfected cells. One day after transfection, cells were treated with 0.5 mM MMS for 30 min, after which the medium was replaced by fresh medium. One hour after drug exposure, cells were fixed and incubated in 5-bromo-4-chloro-3-indolyl β-D-galactoside to visualize cells transiently expressing the *lac-Z* gene. Cell viability was determined as the percentage of flat (presumably living) versus round (presumably dead) *lac-Z*-positive cells (28).

Flow cytometric analysis

Flow cytometric analysis was carried out in a fluorescence-activated cell sorter (Epics Elite, Coulter). To monitor DNA synthesis, incorporation of 5-bromodeoxyuridine (BrdUrd) was measured 24 h after MMS treatment of primary MEFs (29).

Micronucleus assay

Primary MEFs were seeded on coverslips the day before treatment. The cells were then exposed to cytochalasin B (6 µg/ml) (30) and MMS (0.05 mM) for 48 h and subsequently fixed with methanol–acetone (1:1, vol:vol). Nuclei were stained with 0.05 µg/ml of 4′-6-diamidino-2-phenylindol (DAPI) (Sigma). For each sample, micronuclei were scored in 1000 binucleated cells.

Single cell gel electrophoresis (comet) assay

Primary MEFs, mock treated or exposed to 0.15 mM MMS, were suspended in low melting point agarose in DMEM and pipetted onto a frosted glass microscope slide pre-coated with a layer of normal melting point agarose. The slides were then immersed in lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% sodium lauryl sarcosinate, 10% dimethyl sulphoxide, 1% Triton X-100, pH 10.0) at 4°C for 55 min to remove cellular proteins. Slides were then placed in a tank containing 0.03 M NaOH, 1 M NaCl and 1 mM EDTA for 40 min, before electropheresis at 16.5 V for 15 min at room temperature in a buffer containing 0.03 M NaOH and 1 mM EDTA. Following electrophoresis, slides were washed with neutral buffer (400 mM Tris–HCl, pH 7.5) and DNA stained with 20 µg/ml ethidium bromide. The parameters of the 'comets' were quantified with the use of the image analysis software Vision Explorer (Graftek, Mirmande-France). Duplicate slides were processed for each experimental point. One hundred comets were analysed per slide. The tail moment is defined as the product of the percentage of DNA in the tail and the displacement between the head and the tail of the comet (31).

RESULTS

PARP-deficient cells display severe decreased growth rate, viability and G_2/M accumulation following exposure to MMS

Cell growth rate and cell-cycle distribution reflect early events following DNA damage. *PARP*$^{-/-}$ primary MEFs and *PARP*$^{+/+}$ MEFs proliferated in culture, with doubling times of 45 and 49 h, respectively (Fig. 1A). Field *et al*. reported a similar doubling time for wild-type primary MEFs (32). Their plating efficiency was similar (data not shown). However, upon exposure to a sublethal dose of MMS (as low as 0.1 mM) *PARP*$^{-/-}$ MEFs showed a significant decrease in their ability to proliferate (doubling time of 169 h), indicating that *PARP*$^{-/-}$ cells had virtually stopped dividing, compared with wild-type cells (72 h).

Since PARP has been implicated in a checkpoint that monitors the DNA status before entry into mitosis (19), the cell-cycle distribution of asynchronously dividing cells from each genotype, as measured by BrdUrd incorporation, was examined 24 h after increased doses of MMS treatment. The results displayed in Figure 1B show that proliferation of MEFs is not affected by the absence of PARP, as long as no damage is present in DNA. Wild-type MEFs were slightly affected in the cell-cycle distribution following DNA damage. In contrast, mutant MEFs exhibited a marked MMS-dependent accumulation in G_2/M, indicating that PARP-deficient cells failed to resume their progression through the cell cycle after damage.

The consequences of DNA damage on cell viability was evaluated by [methyl-^{3}H]thymidine incorporation 3 days after MMS exposure (Fig. 2A). MMS treatment had a minimal effect on the viability of *PARP*$^{+/+}$ MEFs, which were able to recover even at a dose of 0.2 mM, suggesting that the observed delay in

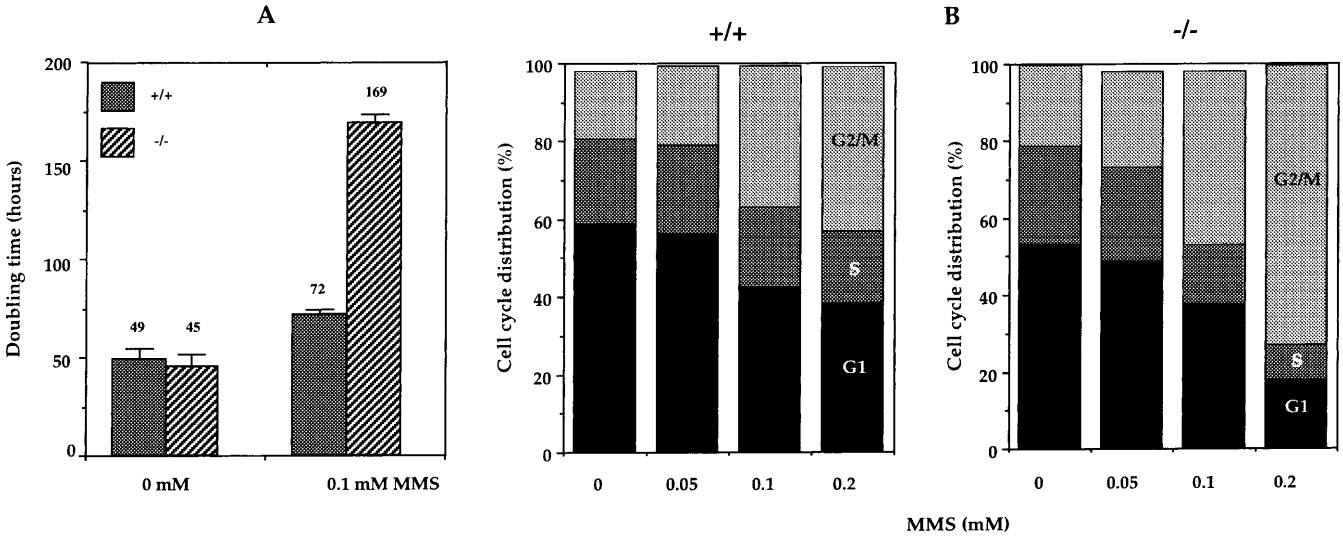

Figure 1. Doubling time and cell-cycle progression in primary MEFs of both +/+ and –/– genotypes. (**A**) Doubling time after mock or MMS treatment for 30 min; cells were counted every day. The results shown are the averages of two experiments, each performed in triplicate. (**B**) Cell-cycle distribution as assessed by flow cytometry of both genotypes following mock or treatment with different doses of MMS.

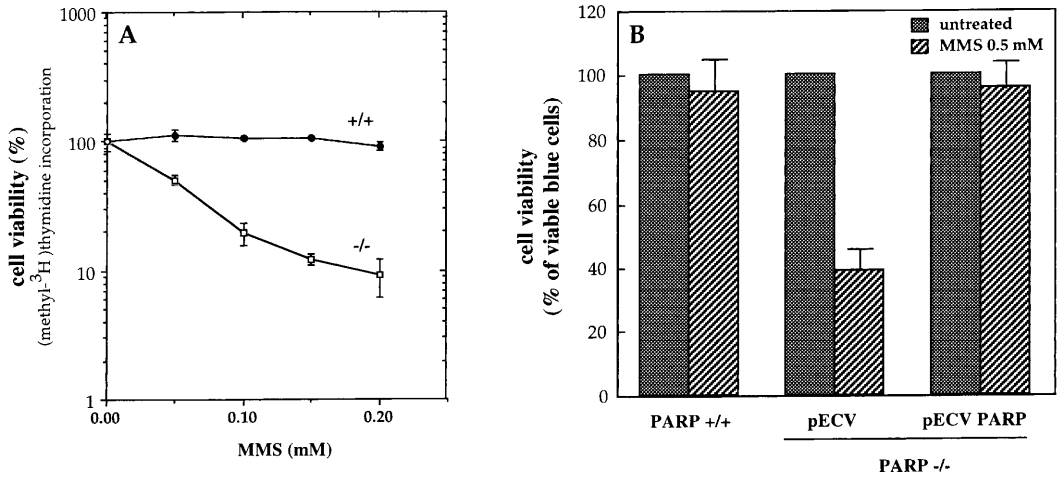

Figure 2. Viability of primary MEFs after MMS treatment. (**A**) Cells were exposed to various doses of MMS for 30 min and the viability was measured 3 days after damage by [methyl-^{3}H]thymidine incorporation. (**B**) Human PARP cDNA was transiently transfected in immortalized *PARP*$^{-/-}$ cells together with a plasmid expressing bacterial β-galactosidase activity. Forty-eight hours after transfection, cell viability was monitored following exposure to 0.5 mM MMS, as the percentage of living versus dead *lac-Z*-positive (blue) cells.

the cell cycle 24 h after DNA damage was only transient. In contrast, the absence of PARP led to hypersensitivity, suggesting that a lethal signal is generated within the damaged mutant cells.

Expression of PARP was restored in immortalized *PARP*$^{-/-}$ cells to ascertain the cellular responses to MMS exposure in the same cell line. Transient expression of PARP was achieved by transfecting a plasmid encoding the human PARP (pECV PARP); the empty vector (pECV) was used as a control. To score the transfected cells, a plasmid expressing the *lac-Z* protein was cotransfected. Cell viability was determined following treatment with 0.5 mM MMS, as a percentage of flat (presumably living) versus round (presumably dead) *lac-Z*-positive cells. The results presented in Figure 2B show that MEFs lacking PARP, transfected with the empty plasmid pECV were much more sensitive to MMS than *PARP*$^{+/+}$ cells. Reintroduction of wild-type PARP cDNA in cells transfected by pECV PARP completely restored cell viability of *PARP*$^{-/-}$ cells after MMS treatment (Fig. 2B), thus demonstrating that the absence of PARP was responsible for the sensitization of cells to DNA alkylation. Therefore, the cytotoxic effects observed in *PARP*$^{-/-}$ cells treated with MMS are comparable with those obtained with cell lines exposed to various alkylating agents, in which PARP inhibition was achieved either by the use of chemical inhibitors (33) or *trans*-dominant-negative mutant (16,18), and in *PARP*$^{-/-}$ MEFs exposed to MNU (19).

Table 1. Induction of micronuclei in MEFs after MMS treatment

MMS (mM)	Binucleated cells scored	Binucleated cells with micronuclei	Total micronuclei	Binucleated cells with 0–6 micronuclei						
				0	1	2	3	4	5	6
PARP$^{+/+}$										
0	1000	64	76	936	52	12	0	0	0	0
0.05	1000	127	156	873	103	19	5	0	0	0
PARP$^{-/-}$										
0	1000	113	152	887	86	18	7	0	0	0
0.05	1000	328	493	672	223	69	19	10	7	0

Chromosome aberrations in PARP-deficient cells

Susceptibility to the induction of chromosome damage often correlates with a susceptibility to cell mortality and mutation. The effect of MMS on chromosome stability of *PARP*$^{-/-}$ fibroblasts was therefore determined. The clastogen effect was determined by analysing the induction of micronuclei in binucleated cells treated with cytochalasin B. Since micronuclei represent chromatin fragments that are not incorporated into the nucleus during mitosis, they are considered to be a simple indicator of chromosomal damage. Table 1 shows the frequency of induction of micronuclei and the total number of micronuclei for each cell mock-exposed and exposed to 0.05 mM MMS. Spontaneously, *PARP*$^{-/-}$ cells exhibited a 2-fold increase in the total number of micronuclei, in the absence of DNA damage; 24 h after MMS treatment, the frequency of micronuclei per cell in *PARP*$^{-/-}$ cells was increased 3.1-fold in comparison with the wild-type cells (Table 1), thus demonstrating the sensitivity of PARP-deficient cells to alkylation-induced chromosome damage.

Cells lacking PARP display a severe base excision repair deficiency

Cells expressing the anti-sense mRNA (14), or cells exposed to 3-aminobenzamide to inhibit PARP activity, have a reduced capacity to repair base-damaged DNA, as evidenced by the nucleoid technique (34) or by the alkaline elution method (35). We chose to monitor *in vivo* DNA repair in primary MEFs using the comet assay (36) after MMS treatment. At the site of a methylated base on DNA, the sequential action of DNA glycosylases and apurinic/apyrimidinic endonucleases leads to the formation of gaps ranging from one to several nucleotides (11,37–39). When cellular DNA contains breaks, single-cell gel electrophoresis under alkaline conditions results in the streaming of cellular DNA towards the anode, giving the appearance of a comet. The product of the percent of DNA in the tail and the mean distance of migration in the tail is taken as a measure of the extent of DNA breakage, named tail moment. This parameter, which is now considered as the most sensitive indicator of DNA breakage (31), was found to vary in a linear manner with increasing doses of MMS in the range of 0–0.30 mM for each genotype (Fig. 3A).

Cells were exposed to 0.15 mM MMS or to a mock treatment for 30 min. The repair kinetics displayed in Figure 3B show that, while at 24 h virtually all the DNA breaks resulting from exposure to MMS were resealed in the two cell lines, *PARP*$^{-/-}$ cells display considerably slower rejoining kinetics compared with *PARP*$^{+/+}$ cells. For example, 6 h after treatment with 0.15 mM MMS, almost all (95%) strand breaks were repaired in wild-type cells, whereas in *PARP*$^{-/-}$ cells only 36% of strand breaks were repaired. MMS-induced DNA strand-breaks with half-lives of ~1 and 5 h were measured for *PARP*$^{+/+}$ and *PARP*$^{-/-}$ cells, respectively.

The same experiment was performed with EM9 cells bearing a functional mutation in the XRCC1 gene (40) as well as with the parental line AA8. A delay in the kinetics of DNA resealing was also observed with EM9 compared with AA8 cells, although the time course was not comparable (data not shown). A similar delay in strand-break rejoining has already been observed in XRCC1-deficient EM-C-11 cells treated with monofunctional-alkylating agents (41). Taken together, these results demonstrate unambiguously that the absence of PARP dramatically reduces the base excision repair capacity of mammalian cells injured with alkylating agents; no sensitization could be observed following UV-C exposure (data not shown).

DISCUSSION

A key question of long standing has been the implication of PARP in DNA repair. Several studies during the past decade have shown that the inhibition of PARP activity using either chemical analogues of NAD^+ (33), random mutated cells (42), overexpression of a dominant-negative mutant (16–18,43) or anti-sense RNA (14), resulted in multiple cellular responses including decrease of cell viability, cell-cycle arrest at the G_2/M border and finally cell death when cells were exposed to sublethal doses of alkylating agents (18). The recent generation of PARP KO mice by homologous recombination has permitted the re-evaluation of the *in vivo* role of PARP, both at the cellular level and at the whole animal level. Interestingly, PARP-deficient cell lines recapitulate most of the phenotypes observed up to now with the chemical inhibitors and the various genetic and molecular approaches mentioned above. The disruption of the *PARP* mouse gene totally abolishes the expression of the first four exons, as detected by northern blotting, or by western blotting with a polyclonal antibody against the first or the second zinc finger (19 and data not shown). In the absence of any residual DNA-binding activity, which could exert a dominant-negative effect, the loss of PARP is, therefore, responsible for the sensitization of PARP-deficient cell lines to monofunctional-alkylating agents and γ-radiation (Favaudon *et al.*, in preparation). The restoration of cell viability by the ectopic expression of the PARP cDNA confirmed this conclusion.

Following MMS treatment, the prolonged delay observed in DNA strand-break resealing demonstrates that *PARP*$^{-/-}$ cells are severely affected in the BER pathway; no sensitization was

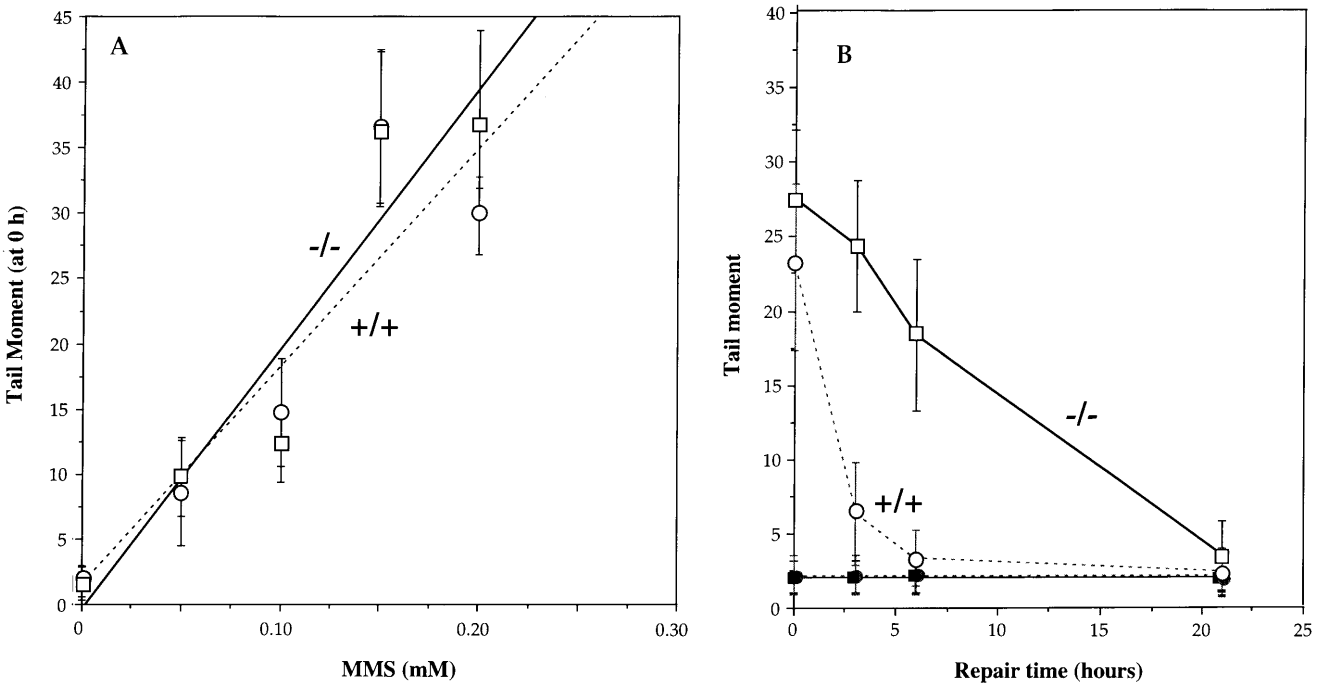

Figure 3. (**A**) Dose-effect relationship of primary embryonic fibroblasts of both +/+ and –/– genotypes as assessed by the single-cell gel electrophoresis assay, after treatment with MMS. Distribution of the tail moment of *PARP*$^{-/-}$ cells (circles) and *PARP*$^{+/+}$ cells (squares) at different doses is shown. (**B**) DNA repair capacity of primary embryonic fibroblasts of both +/+ and –/– genotypes as assessed by the single cell gel electrophoresis assay. Distribution of the tail moment of *PARP*$^{-/-}$ cells (squares) and *PARP*$^{+/+}$ cells (circles) as a function of time after 0.15 mM MMS treatment (open symbols) or mock treatment (filled symbols). The data are the mean of the tail moments of one hundred cells measured for each time point. The results shown are one out of three experiments performed.

observed following UV-C treatment. Although the slower rate of repair, as measured by the comet assay, reflects an apparent ligation defect, it is now necessary to examine how repair synthesis might have been affected by the loss of PARP. In any case, the present results are in full agreement with those obtained with the antisense RNA expression approach (14) and with recent findings from our group indicating that PARP interacts with XRCC1 (8), a protein supposedly serving as an adaptor during the BER reaction through its interaction with DNA polymerase β (11) and DNA ligase III (44). These data, however, underline the difficulty in forming definitive conclusions exclusively from *in vitro* DNA repair assays (45,46).

Although a detailed scenario of the complete multistep BER reaction is still pending (47), it is likely that the incision of the phosphodiester backbone by an AP endonuclease constitutes an entry site for the nick sensor function of PARP, which in turn may rapidly recruit XRCC1 and two of its identified partners, DNA polymerase β (11) and DNA ligase III (44), at the immediate vicinity of the DNA interruption. Interestingly, some of these enzymes and factors involved in BER behave as multimodular polypeptides capable of various combinations through protein–protein contacts. These interactions, mediated by small specific domains, such as the BRCT motif (48) present in PARP (8), XRCC1 (10) and DNA ligase III (49), presumably ensure a rapid recruitment and coordination of the different players of the BER, thus permitting an optimal response to DNA damage. The absence of one of the constituents may drastically reduce the rate of lesion removal and hence the efficiency of the overall pathway. In the case of *PARP*$^{-/-}$ cells, the slower rate of repair, reflected by an increased persistence of strand-breaks following DNA base damage, seems to be the primary cause of the observed cytotoxicity of alkylating agents leading to chromosome instability, G_2/M block and p53 accumulation, finally leading to cell death (19).

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