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Decreased expression of topoisomerase IIβ in poly(ADP-ribose) polymerase-deficient cells

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ABSTRACT

Recent studies with poly(ADP-ribose) polymerase (PARP)-deficient mice have highlighted the role of this enzyme in genomic stability and response to various genomic insults. In the absence of DNA damaging treatment, we report here that a PARP-deficient cell line (PARP–/–) established from knockout mice displays a decrease in topoisomerase II (topo II) activity as measured by decatenation of kinetoplast DNA. Immunoblotting of whole and nuclear cell extracts showed that reduced activity was associated with decreased amount of the 180 kDa topo IIβ protein but not of the 170 kDa topo IIα. The decreased topo IIβ expression did not stem from transcriptional regulation of gene expression since levels of topo IIβ mRNA were similar in PARP (–/–) compared with the parental PARP (+/+) cells. The decreased topo II activity was associated with cell resistance to VP16, a topo II inhibitor. These observations indicate that PARP may play a role in the stabilization and/or distribution of topo IIβ.

INTRODUCTION

Poly(ADP-ribose) polymerase (PARP) is a 113 kDa nuclear enzyme that participates in the control of DNA integrity (1,2). PARP binds to nicked DNA and utilizes NAD as a substrate to catalyze the formation and binding of poly(ADP-ribose) polymers to nuclear proteins. The occurrence of nicked DNA is either direct, for instance following ionizing radiation treatment, or indirect due to repair enzymes activities (1). Poly(ADP-ribose) is a short-lived polymer *in vivo* since poly(ADP-ribose) glycohydrolase activity rapidly degrades it leading to the concept that poly(ADP-ribosyl)ation is an immediate but transient post-translational modification of nuclear proteins induced by DNA damaging treatments. The acceptor proteins of poly(ADP-ribosyl)ation were mostly identified through *in vitro* experiments and include histones, DNA polymerases, DNA ligase, topoisomerase (topo) I and II and PARP itself (reviewed in 3). In all cases, the resulting post-translational modification has been shown to alter the activities of the acceptor proteins (1,4).

Although PARP is not an essential protein since PARP (–/–) mice are viable (5–7), it can play a physiological role both at the structural and functional level in untreated cells, besides its role in cell response to environmental insults (8). For instance: (i) PARP was found in a multiprotein DNA repair replication complex with polymerase α and δ, topo I and II, ligase and accessory proteins (9,10); (ii) repression of PARP expression caused an alteration in chromatin structure (11); (iii) cell proliferation was slightly altered in knockout PARP(–/–) mice cells (7,12); (iv) a hamster V79 mutant cell line with low PARP expression (13) exhibited as expected an increased sensitivity to various alkylating agents (14) but in addition resistance to VP16 and doxorubicin, two drugs which exert their cytotoxicity through interaction with topo II (15).

Topo II activity is responsible for the modulation of the topological state of DNA by catalyzing the formation of transient DNA double-strand breaks and passage of another double-stranded helix through the break (16). Type II topoisomerases are nuclear enzymes and exist as two isoforms, topo IIα (170 kDa) and topo IIβ (180 kDa), encoded by two genes (17). A relationship between topo II and PARP is reminiscent of the fact that topo II activity decreased upon poly(ADP-ribosyl)ation as measured in an *in vitro* reaction catalyzed by this enzyme (18).

However, in the V79 mutant cell line the mechanism involved in VP16 resistance has been attributed to increased expression of the glucose-related stress protein, GRP78, and not to a defect in PARP expression (19,20). In order to get more information on a potential role of PARP in topo II activity, we decided to use a fibroblastic PARP (–/–) cell line obtained from knockout mice (5). We report here, a decrease in topo II activity in a PARP (–/–) cell line and analyze the origins of this alteration as well as the consequences in terms of cell response to topo II inhibitors.

MATERIALS AND METHODS

Cell lines and culture conditions

The generation of PARP knockout (–/–) mice has been previously described (5). Mouse embryonic fibroblast (MEF) cell lines, designated as PARP (+/+) and PARP (–/–), were isolated from 13.5-day-old embryos from homozygous mutant PARP (–/–) and wild-type PARP (+/+) mice as described (5) and were grown at

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37°C (5% CO_2/95% air) in DMEM medium supplemented with 10% fetal calf serum and penicillin (2×10^5 U/l)/streptomycin (50 mg/l). The mean doubling times were 24 and 36 h for the PARP (+/+) and PARP (–/–) MEF cell lines, respectively (12).

Preparation of nuclear and whole cell extracts

Nuclear extracts were prepared as previously described (21). Cells (~5×10^6) were scraped and collected by centrifugation. The cell pellets were washed twice with PBS and resuspended in 1 vol of hypotonic buffer (5 mM KH_2PO_4, pH 7, 2 mM $MgCl_2$, 0.1 mM EDTA, 1 mM PMSF, 1 mM benzamidine, 10 mM 2-mercaptoethanol, 10 µg/ml soybean trypsin inhibitor, 50 µg/ml leupeptin) and incubated for 20 min at 4°C. The swollen cells were disrupted by 25 strokes of a Dounce B homogenizer and the nuclei pelleted (2 min at 10 000 *g*). The pelleted nuclei were resuspended in 1 vol of nuclei extraction buffer (hypotonic buffer with 0.35 M NaCl, 10 µg/ml aprotinin and 10% glycerol) and incubated for 30 min with gentle stirring. After centrifugation at 10 000 *g* (10 min), the nuclear extract (i.e. the supernatant) was removed, immediately frozen and stored at –80°C. Whole cell extracts were prepared as described (22). Protein concentrations were measured by the microassay procedure, using BioRad protein assay dye (BioRad Laboratories). All the experiments were performed with at least three independent preparations of extracts.

Determination of topoisomerase II unknotting activity

Topo II activity was evaluated using the decatenation assay of kinetoplast kDNA (21). The reaction mixture (20 µl) contained 50 mM Tris–HCl, pH 7.4, 120 mM KCl, 10 mM $MgCl_2$, 0.5 mM DTT, 0.5 mM EDTA, 1 mM ATP and 200 ng of kDNA (Topogen Inc.). The reaction was initiated at 30°C by the addition of nuclear extract and stopped by the addition of 2 µl of 10% SDS, 0.5% bromophenol blue, 30% glycerol solution. The samples were electrophoresed in a 1% agarose gel at 6 V/cm for 4 h, in TAE buffer. Photographs of the ethidium bromide stained gels were scanned and processed in a PhosphorImager (Storm System™; Molecular Dynamics) and the amount of liberated minicircles quantified, using the software ImageQuant.

Western blotting

Nuclear and whole cell extracts (100 µg protein) were resolved by electrophoresis in an 8% SDS–PAGE gel and transferred to PVDF membrane (Schleicher & Schuell). The membrane was blocked in TBS-T supplemented with skimmed milk (5% w/v) and then incubated with antibodies directed against topo IIα and topo IIβ. Polyclonal anti-human topo IIα antibody (Topogen Inc.) cross-reacts with the murine protein. Polyclonal anti-murine topo IIβ antibody was from Bio-Trend (Koln, Germany) and anti-GRP78 from Stress Gen (Tebu, France). Antibodies were detected by incubation with horseradish peroxidase-conjugated anti-rabbit IgG. Binding of antibody was revealed using an ECL detection system (Amersham).

Northern blot analysis

Total RNA was isolated from cells using Trizol reagent (Gibco-BRL Life Technologies) according to the manufacturer's instructions. RNA (10 µg) was denatured and electrophoresed on a 1% agarose gel containing formaldehyde and transferred by pressure blotting (Pharmacia) to nylon membranes (Hybond N; Amersham). Blots were hybridized with a topo IIβ probe corresponding to the 1.9 kb *Eco*RI–*Pst*I cDNA insert of SP-12 specific for topo IIβ (23). To assess the equivalence of RNA loading, blots were also probed with the *Pst*I–*Pst*I fragment of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA. The cDNA probes were radiolabeled with [α-^{32}P]dCTP by random priming (Random Primers DNA Labeling System; Gibco-BRL Life Technologies). Membranes were prehybridized at 42°C in hybridization buffer (50% formamide, 4× Denhardt's solution, 5× SSPE, 100 µg/ml herring testis DNA, 1% SDS) for 4–6 h. cDNA probes were hybridized with the blots for 18–20 h at 42°C. Blots were washed for 15 min at room temperature in 1× SSC, 0.1% SDS, followed by three times for 30 min at 52°C in 0.1× SSC, 0.1% SDS. The blots were scanned and processed on a PhosphorImager (Storm System; Molecular Dynamics) and the radioactivity quantified. Experiments were done on three independent preparations of RNA.

Cytotoxicity studies

Cytotoxicity was measured using the MTT assay. Briefly, cells were seeded into 96-well plates at 3×10^3 cells/well (in 100 µl) and allowed to attach overnight. Cells were exposed to increasing concentrations of VP16 for 2 h, then washed with complete medium and re-incubated for 72 h at 37°C. At this time, MTT (50 µl of 1 mg/ml in PBS) (Sigma) was added and the plates were further incubated for 3 h at 37°C. Plates were centrifuged, the supernatant removed and the formazan crystals were solubilized by addition of DMSO (100 µl/well) and gentle shaking. The plates were scanned at 570 nm using a Titertek Multiskan Plus plate reader. Drug cytotoxicity was expressed as the ratio of the absorbance values of treated compared with those of untreated cells. Dose–response curves were plotted from which the drug IC_{50} (i.e. the concentration that reduces the absorbance value to 50% of the control) was determined. Within each experiment, determinations were done in triplicate. Each value represents the mean ± SEM of three independent experiments.

RESULTS

PARP-deficient cells show decreased topoisomerase II activity

Topo II is one of the nuclear proteins identified as acceptor for the poly(ADP-ribosyl)ation reaction *in vitro* and *in vivo* (24). Topo II activity was determined by a decatenating assay using kDNA as substrate. Decatenating activity was measured either in kinetic experiments or with various amounts of nuclear extract. We observed a decreased decatenating activity in PARP (–/–) nuclear extracts (Fig. 1A) by 2 ± 0.2-fold when compared with PARP (+/+). Kinetics of decatenation showed the same decrease in activity on comparing the value of slope, with PARP (–/–) and PARP (+/+) extracts (Fig. 1B) equal to 1.2 and 2.3, respectively ($P > 0.99$).

Extracts from PARP-deficient cells show decreased topoisomerase IIβ amounts

To examine the basis for the decreased topo II activity in PARP (–/–) cells, the amounts of topo IIα and IIβ were determined in cell extracts by western blotting. Since the decreased topo II activity might result from a differential localization of the enzyme (nucleus versus cytoplasm), immunoblots were performed both with whole and nuclear cell extracts. Consistent with the decreased activity observed in PARP (–/–) cell extracts, we found 2.8- and

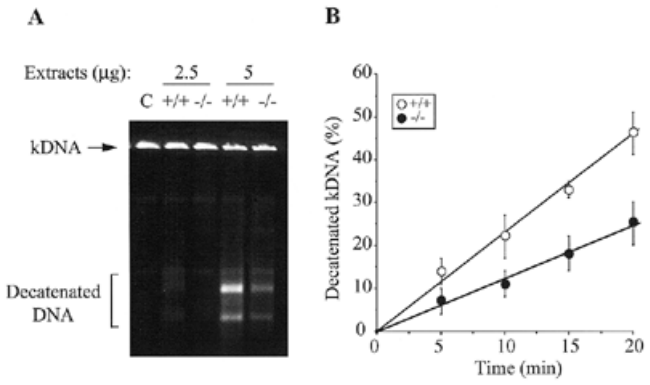

Figure 1. Decatenation activity of topo II. Topo II activity of nuclear extracts from PARP (+/+) and PARP (–/–) cell lines was determined by decatenation of kDNA as described in Materials and Methods. (**A**) A representative experiment with various amounts of nuclear extract incubated for 15 min. (**B**) Kinetics of decatenation. Each value represents the mean (± SD) of three independent preparations of extract.

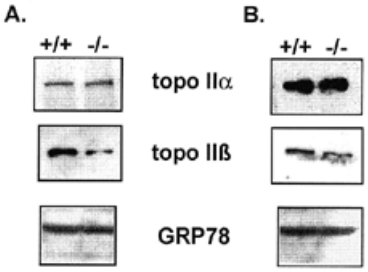

Figure 2. Amount of topo IIα and IIβ and GRP78 proteins in PARP (+/+) and PARP (–/–) cell extracts. Western blot analysis was performed on nuclear (**A**) and whole cell extracts (**B**) (100 µg protein/lane). One representative experiment from two separate preparations of nuclear and whole cell extracts with identical results is shown.

2.1-fold decreases in topo IIβ protein content in nuclear and whole cell extracts, respectively, when compared with extracts from PARP (+/+) cells (Fig. 2). In contrast, levels of topo IIα protein were similar in PARP (–/–) and PARP (+/+) extracts (Fig. 2).

PARP-deficient cells show no difference in topoisomerase IIβ mRNA expression

The expression of topo IIβ mRNA was next evaluated by northern blot analysis. No difference in topo IIβ mRNA levels was noted when normalized with the control GAPDH mRNA (Fig. 3). Consistent data were obtained from an additional two sets of independent RNA preparations.

PARP-deficient cells are resistant to VP16

Topo II is commonly used as the target for antineoplastic agents which act by stabilizing covalent DNA–topo complexes. Since the level of expression of topo II influences cellular sensitivity to

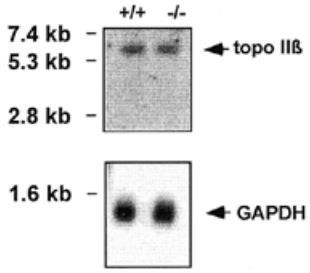

Figure 3. Expression of topo IIβ mRNA in PARP (+/+) and (–/–) cell lines. Northern blot analysis was performed on 10 µg of total RNA as described in Materials and Methods.

topo II inhibitors, we determined the profile of VP16 sensitivity of the fibroblastic PARP (–/–) cell line. Often, high levels of topo II activity induce a sensitive phenotype to topo II inhibitors, whereas low levels of activity induce a resistant phenotype. Cell survival was determined by a colorimetric assay using the MTT dye. The concentration of VP16 that provokes a decrease of 50% in survival (IC_{50}) was determined for PARP (–/–) and PARP (+/+) cells. As expected, the IC_{50} values for VP16 were 52 ± 9 and 15 ± 5 µM for PARP (–/–) and PARP (+/+) cells, respectively (Fig. 4). Thus, the PARP (–/–) cells were ~3.5-fold more resistant than the PARP (+/+) cells. Resistance was also observed with doxorubicin, another topo II inhibitor, although the difference was less pronounced (~2-fold; data not shown). Since VP16 resistance in PARP-deficient cells has been previously associated with an increased expression of the GRP78 protein (19,20), its expression was also determined. However, no difference in GRP78 expression was observed in PARP (–/–) and PARP (+/+) whole and nuclear cell extracts (Fig. 2).

DISCUSSION

We report here that PARP (–/–) cells derived from mutant mice completely devoid of PARP, obtained by homologous recombination in embryonic stem cells (5), exhibited decreased topo II activity. The decreased activity is likely due to reduced levels of topo IIβ protein amount, as we determined by western blot analysis. The lower level of expression was not due to an artifact of nuclear extract preparation since it was also observed in whole cell extracts. Despite the fact that topo II represents one of the substrates for poly(ADP-ribosyl)ation *in vitro* (18), this is the first report, to our knowledge, that shows that the amount of topo IIβ is dependent upon the presence of PARP. Moreover, the lack of difference between topo IIβ mRNA in PARP (–/–) and (+/+) cells suggests that PARP may regulate expression of topo IIβ at the post-transcriptional level.

By what mechanism is the amount of topo IIβ protein reduced in the PARP (–/–) cell line? It is worth mentioning that PARP and topo II share some characteristics and perhaps some common cellular functions that may shed light on the present results. First, both proteins are structural components of the nuclear matrix (25,26) and have regions of distribution that overlap within the nucleus such as the nucleolus (27,28). Second, they also have in common a high affinity for supercoiled DNA substrates and they

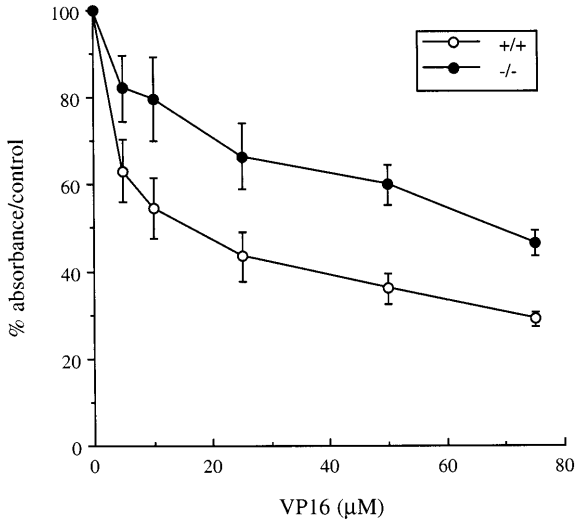

Figure 4. Effect of VP16 in PARP (+/+) and PARP (–/–) cell lines. Cells were treated with the indicated drug concentrations for 2 h at 37°C and toxicity was measured as described in Materials and Methods. Each data point represents the mean (± SEM) of results obtained from three independent experiments.

preferentially interact with DNA crossovers (29,30), which may be essential during mitosis and meiosis. Third, down-regulation of topo II activity by poly(ADP-ribosyl)ation causes alterations in chromatin structure (11) that, in turn, may affect topo IIβ stability. Fourth, despite the fact that mice lacking PARP develop normally, a deficiency in cell proliferation could be noticed (7) which may have links with the lower topo II activity. Fifth, lower topo II expression in untreated cells may influence the cell response to DNA damaging treatments which may constitute a mitotic block preventing cell division with damaged chromosomes.

It is therefore quite plausible that the absence of PARP may directly or indirectly influence the half-life of topo IIβ, thus leading to a drug-resistant phenotype. This decrease in amount of topo IIβ is probably the result of a shorter half-life of the protein. These results further indicate that, although the two topo II isoforms have a different drug sensitivity, the selective loss of the less drug sensitive isoform (topo IIβ) is sufficient to confer drug resistance to the cells.

Resistance to topo II inhibitors such as VP16 is generally associated with a mutation or decreased expression of the enzyme (16). This result is reminiscent of that obtained with a hamster cell line DC3F-9OHE, resistant to the intercalating agent 9-OH ellipticine (31). DC3F-9OHE cells are deficient in topo IIβ expression and their resistance could be reversed by transfection of topo IIβ cDNA, resulting in similar levels of topo IIβ in the resistant and parental cell lines (32). However, in contrast to DC3F-9OHE cells, we showed that the lower expression of topo IIβ protein in the PARP (–/–) cells did not result from decreased levels of the corresponding mRNA.

On the other hand, the VP16-resistant phenotype is reminiscent of the reports from Chatterjee *et al*. that found that V79 mutant cells with low PARP activity (13) exhibited VP16 resistance associated with an increased expression of the glucose-related stress protein, GRP78 (19,20). However, our model differs from the latter (20) since no difference in GRP78 expression was found in PARP (+/+) and PARP (–/–) cell extracts (Fig. 2). Moreover, the V79 PARP-deficient cell lines were sensitive to UV light and topo I inhibitors (14,33) whereas PARP (–/–) cells were not (data not shown). The different behavior of the PARP (–/–) cells compared with the V79 cell lines may be due to the residual (4–10%) PARP activity in V79 mutant cells (13,34).

Because a large number of cancer chemotherapeutic agents are known to exert their cytotoxicity through their interaction with topo II, the control of the activity of this enzyme through poly(ADP-ribosyl)ation may have implications for the search for new combinations of therapeutic drugs. Interestingly, a correlation has been found between a low PARP activity and VP16 resistance in a panel of lung cancer cell lines (35). If PARP inhibitors sensitize cells to ionizing radiation or monoalkylating agents they should not be used in combination with topo II inhibitors if our results with PARP (–/–) cells can be generalized.

Finally, why PARP affects levels of topo IIβ but not topo IIα expression remains to be determined. Ongoing studies on the mechanism of topo IIβ expression in PARP (–/–) cells should provide new insights into the differential regulation of the two isoforms of this important drug target.

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