Base Excision Repair Is Impaired in Mammalian Cells Lacking Poly(ADP-ribose) Polymerase-1[†]

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ABSTRACT: In mammalian cells, damaged bases in DNA are corrected by the base excision repair pathway which is divided into two distinct pathways depending on the length of the resynthesized patch, replacement of one nucleotide for short-patch repair, and resynthesis of several nucleotides for long-patch repair. The involvement of poly(ADP-ribose) polymerase-1 (PARP-1) in both pathways has been investigated by using PARP-1-deficient cell extracts to repair single abasic sites derived from uracil or 8-oxoguanine located in a double-stranded circular plasmid. For both lesions, PARP-1-deficient cell extracts were about half as efficient as wild-type cells at the polymerization step of the short-patch repair synthesis, but were highly inefficient at the long-patch repair. We provided evidence that PARP-1 constitutively interacts with DNA polymerase β. Using cell-free extracts from mouse embryonic cells deficient in DNA polymerase β, we demonstrated that DNA polymerase β is involved in the repair of uracil-derived AP sites via both the short and the long-patch repair pathways. When both PARP-1 and DNA polymerase β were absent, the two repair pathways were dramatically affected, indicating that base excision repair was highly inefficient. These results show that PARP-1 is an active player in DNA base excision repair.

Cells have multiple strategies for repairing the various types of damage that constantly alter their DNA. Among these defense mechanisms, the base excision repair (BER)[1] pathway is the major process for the correction of oxidative base damage, multiple forms of alkylation damage, apurinic/apyrimidinic (AP) sites formed by spontaneous loss of bases, and uracil residues in DNA (*1*). Base excision repair is initiated by the action of DNA glycosylases, which catalyze the hydrolysis of the N-glycosyl bond linking damaged bases to the sugar−phosphate backbone generating AP sites (*2*). Natural AP sites are produced by monofunctional DNA glycosylases that remove the altered base without cleaving the phosphodiester bond adjacent to the lesion. One example of such DNA glycosylases is the widespread uracil-DNA glycosylase (UDG) that removes uracil, which usually arises from the deamination of cytosine. These natural AP sites are further processed mainly by the abundant 5′ acting AP endonuclease HAP1 (Ref-1; APE) that hydrolyzes the DNA backbone generating 3′-hydroxyl and 5′-deoxyribose phosphate (dRP) moieties (*2*, *3*). The latter are removed by a DNA deoxyribophosphodiesterase (dRpase) activity, and the resulting nucleotide gaps are filled in by a DNA polymerase and sealed by a DNA ligase. Some DNA glycosylases active on oxidized bases possess an intrinsic AP lyase activity that cleaves the DNA strand at the 3′ side of the resulting abasic site by a β-elimination event, leaving an incised AP site (*3*). Among these bifunctional enzymes, the human 8-oxoguanine DNA glycosylase (hOGG1) removes 8-oxoguanine lesions induced by reactive oxygen species and ionizing radiation (*4*). The blocked 3′ termini generated require further processing by the 3′-phosphatase or 3′ phosphodiesterase accessory activities of HAP1 (*5*).

The entire BER process mainly involves replacement of a single nucleotide residue (short-patch repair, SPR) and has been reconstituted in vitro with the purified human proteins uracil−DNA glycosylase (UDG), AP endonuclease (HAP1), DNA polymerase (pol) β, XRCC1, and either DNA ligase III or DNA ligase I (*6*). However, Stucki et al. (*7*) showed that DNA pol δ and/or ϵ might function as a backup system for DNA pol β in the short-patch repair. Furthermore, longer DNA repair patches of 2−14 nucleotides extending 3′ to the lesion can be generated in a minor, alternative, gap-filling

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[1] Abbreviations: AP, apurinic/apyrimidinic; BER, base excision repair; BRCT, BRCA-1 C-terminus; dRP, deoxyribose phosphate; FEN1, Flap endonuclease 1; LPR, long-patch repair; OGG1, 8-oxoguanine DNA glycosylase 1; PAGE, polyacrylamide gel electrophoresis; PARP, poly(ADP-ribose) polymerase; PCNA, proliferating cell nuclear antigen; pol, polymerase; RPA, replication protein A; SPR, short-patch repair; UDG, Uracil DNA glycosylase.

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procedure (long-patch repair, LPR) involving DNA replication proteins such as DNA pol δ and/or ϵ, the proliferating cell nuclear antigen (PCNA), the structure-specific nuclease Flap endonuclease 1 (FEN1; *8*−*11*) and the replication protein A (RPA) (*12*, *13*). However, the long-patch BER synthesis was shown to be largely dependent on DNA pol β for the repair of AP sites (*14*−*17*). The role of FEN1 was to remove the splayed arm structure produced by excessive gap-filling synthesis by DNA polymerase β; this function was promoted by the addition of PCNA in the repair reaction (*14*). The selection of the BER branch (SPR or LPR) seems to be determined by the type of DNA glycosylase that recognizes the lesion (*18*).

Poly(ADP-ribose) polymerase-1 (PARP-1) is the first described member of a growing family of poly(ADP-ribosyl)-ating enzymes encompassing now PARP-2, a novel DNA-damage activated PARP (*19*), vPARP (PARP-4), a protein from the cytoplasmic vault particles that was also localized to the mitotic spindle (*20*) and tankyrase (PARP-5), a telomeric protein interacting with TRF1, a negative regulator of telomere length (*21*). PARP-1 was proposed to be a DNA-damage signaling enzyme involved in base excision repair (*22*−*24*). Definitive evidence for the involvement of PARP-1 in DNA repair was provided by the generation of PARP-1-deficient mice, which have been found to be extremely sensitive to alkylating agents and to ionizing radiation (*25*−*27*). Moreover, PARP-1-deficient cells displayed a dramatic delay in DNA strand-break rejoining following exposure to alkylating agents (*28*). PARP-1 interacts with XRCC1 (*29*, *30*), a partner of DNA pol β (*6*) and DNA ligase III (*31*) in base excision repair. This association decreases the catalytic activity of PARP-1 in vivo, reinforcing the potential protective function of PARP-1 at DNA breaks (*29*). Therefore, PARP-1 is probably associated with a multifunctional complex including, at least XRCC1, DNA pol β and DNA ligase III acting in the BER pathway.

To address the issue of the role of PARP-1 in BER, the ability of PARP-1deficient cell extracts to repair single abasic sites (AP) present on a covalently closed circular duplex plasmid was tested in a standard in vitro repair assay (*9*). Since the intermediate abasic sites produced by either monofunctional DNA glycosylases (i.e., UDG) or bifunctional DNA glycosylase AP lyases (i.e., OGG1) are repaired in a different way by the BER machinery (*18*), we generated DNA substrate containing either of these abasic sites. We provide evidence that (i) PARP-1-deficient cell extracts show defective base excision repair of AP sites derived from both uracil and 8-oxoguanine; (ii) the defect is moderate in the short patch pathway and dramatic in the long-patch pathway and associated for both pathways to the polymerization step; (ii) the enzymatic activity of PARP-1 is involved in the BER process; (iv) the repair defect in PARP-1$^{-/-}$ cells results in a dramatic loss of cell viability in long-term clonogenic assays. In attempting to identify a link between PARP-1 and the repair polymerase (β, δ, or ϵ), we observed an interaction between PARP-1 and DNA polβ, involving the BRCT domain of PARP-1 and the C-terminal half part of DNA polβ. We performed in vitro repair assays using mouse fibroblast cells rendered deficient in DNA polβ and/or PARP-1 by gene inactivation. We confirmed that DNA polβ is involved in both the short and the long-patch repair pathways of uracil-derived AP sites, and that in the absence of both PARP-1 and DNA polβ, these two BER pathways are dramatically affected. These results clearly demonstrate that PARP-1 is required for efficient base excision repair.

EXPERIMENTAL PROCEDURE

Generation and Culture of Mouse Embryonic Fibroblasts. Mouse embryonic fibroblasts were isolated by microdissection of embryos at day 13.5 of gestation (*28*). Cells lacking DNA polymerase β (pol$\beta^{-/-}$) and the double knockout PARP-1$^{-/-}$pol$\beta^{-/-}$ cells were derived from embryos obtained through the intercrosses of PARP-1$^{+/+}$pol$\beta^{+/-}$ (*32*) or PARP-1$^{+/-}$pol$\beta^{+/-}$ mice. Each embryo was genotyped by Southern blot analysis of neuronal DNA using the polβ gene-specific probe described by Gu et al. (*33*), to screen for the disruption of the polβ allele, and the PARP-1 gene-specific probe, described by Ménissier de Murcia et al. (*25*) to screen for the disruption of the PARP-1 allele. Spontaneously immortalized embryonic fibroblasts (3T3) were maintained in DMEM, 4.5 g/L glucose medium supplemented with 10% fetal bovine serum and 0.5% gentamycin.

DNA Substrates. Plasmids carrying a single abasic site were produced as previously described (*9*). Briefly, pGEM3Zf-(+) single-stranded DNA was annealed with a 30-fold M excess of the oligonucleotides (i) 5′-GATCCTCTAGAG**U**C-GACCTGCA-3′, (contains an uracil), (ii) 5′-GATCCT-CTAGA**8oxoG**TCGACCTGCA-3′ (contains a 8-oxoguanine), or (iii) 5′-GATCCTCTAGAGTCGACCTGCA-3′ (control oligonucleotide). Closed circular double-stranded DNA was obtained using T4 DNA polymerase, T4 gene 32 protein, and T4 DNA ligase (Boehringer Mannheim). The plasmid containing a uracil (pGEM-U), the plasmid containing a 8-oxoguanine (pGEM-8-oxo-G), and the control plasmid (pGEMcontrol) were then purified by cesium chloride equilibrium centrifugation. The abasic site was generated by incubating pGEM-U with *Escherichia coli* uracil DNA glycosylase (provided by S. Boiteux, CEA, Fontenay-aux Roses, France) to give pGEM-AP or by incubating pGEM-8oxoG with *Saccaromyces cerevisiae* 8-oxoguanine DNA glycosylase OGG1 (provided by S. Boiteux) to give pGEM-AP*, respectively.

DNA Repair Reaction. The in vitro base excision repair assay was carried out essentially as described previously (*9*). Briefly, 350 ng of plasmid pGEM-AP (or pGEM-AP*) containing single abasic site or the control substrate pGEM-control, were incubated with 50 μg of protein cell extracts prepared as described (*34*), for the indicated times at 30 °C in 50 μL reaction buffer (Hepes/KOH, 45 mM, pH 7.8; KCl, 60 mM; $MgCl_2$, 7.5 mM; DTT, 0.9 mM; ATP, 2 mM; phosphocreatine, 40 mM; creatine phosphokinase (type I, Sigma), 2.5 μg; bovine serum albumin, 18 μg) containing 20 μM of each dNTP and 5 μCi (3000 Ci/mmol, Amersham) of α-[^{32}P]dTTP (for pGEM-AP and pGEMcontrol) or α-[^{32}P]-dGTP (for pGEM-AP* and pGEMcontrol) to investigate the overall BER, and α-[^{32}P]dCTP to investigate the long-patch repair pathway. After the repair reaction, the DNA product was purified, digested with the appropriate restriction endonucleases, and resolved on denaturing 15% polyacrylamide gel electrophoresis. The BER products were visualized by autoradiography and analyzed on a PhosphorImager for quantification (Molecular Dynamics).

GST-Pull Down and Western Blot Analyses. GST-pull down analysis was performed as described (*35*). Briefly,

Cos-1 cells (10^6 cells) were transfected by calcium-phosphate coprecipitation with 10 μg of recombinant DNA. Forty-eight hours later, cells were lysed in lysis buffer (Tris-HCl, 50 mM, pH 7.8; NaCl, 120 mM; NP-40, 0.1%; PMSF, 0.5 mM). When indicated, 3-aminobenzamide (1 mM, Sigma, St-Louis, MO) was added in the culture medium 2 h before lysis and was maintained in the lysis buffer throughout all the steps. Lysates were cleared by centrifugation and incubated 2 h with gluthatione-Sepharose beads (Pharmacia, Uppsala, Sweden). For DNase I treatment, lysates were incubated 1 h on ice with $MgCl_2$, 6 mM; $CaCl_2$, 2 mM; and 20 units of DNase I (Boehringer Mannheim) before adding the beads. When indicated, ethidium bromide (10 μg/mL) was added to the lysis buffer and maintained throughout all the steps. Beads were washed three times with lysis buffer, and samples were resuspended in Laemmli buffer, boiled for 5 min, and analyzed by 10% SDS−PAGE and immunoblotting.

For western blot analysis, cells were lysed in lysis buffer, and 8 μg of protein was analyzed by 10% SDS−PAGE and immunobloting.

For immunodetection, blots were incubated with anti-PARP-1 (Monte, 1/2000) or anti-DNA polymerase β (1/1000) polyclonal antibodies or with anti-GST monoclonal antibody (1/10000, kindly provided by Y. Lutz, IGBMC, Illkirch, France). Blots where then probed with horseradish peroxidase-coupled secondary antibodies (goat anti rabbit, 1/20000 or sheep anti mouse, 1/20000, Sigma, St-Louis, MO), and immunoreactivity was detected by enhanced chemiluminescence (NEN, Boston, MA) according to the manufacturer.

Cell Survival Assays. Wt, PARP-$1^{-/-}$, pol$\beta^{-/-}$, and PARP-$1^{-/-}$pol$\beta^{-/-}$ immortalized 3T3 mouse embryonic fibroblasts were exposed either to *N*-nitroso-*N*-methylurea (MNU, 1.5 mM, 30 min) or to hydrogen peroxide (H_2O_2, 25 μM, 30 min), washed with PBS, and trypsinized. Cells were seeded in triplicate at a density of 3×10^3 cells/10 cm-diameter dish together with 5×10^5 Mitomycin C (10 μg/mL, 150 min)-treated wild-type mouse embryonic fibroblasts. After 8−9 days of culture, the clones were rinsed with PBS, fixed in ethanol 100%, stained with Giemsa 3% (w/v), and counted.

RESULTS

PARP-1 Is Moderately Involved in Short Patch Repair but Mainly in Long-Patch Repair of Uracil-Derived AP Site. The efficiency of PARP-1-deficient 3T3 fibroblasts to repair a uracil-derived AP site was compared to that of wild-type fibroblasts. A covalently closed double-stranded DNA (pGEM-U) containing a unique uracil in a specific position or a normal T:A base pair has been constructed from the pGEM plasmid, with restriction sites suitable for analysis of DNA repair synthesis (Figure 1A). The DNA substrate containing a uracil was treated with UDG leaving an abasic (AP) site. To demonstrate the presence of an AP-site, an aliquot of DNA incubated with UDG was further treated with *E. coli* endonuclease III that incises the 3′ side of the abasic (AP) residue. The incision in the circular form of the plasmid DNA was visualized on an agarose gel (Figure 1B). The digestion of the substrate DNA with *Sma*I and *Hin*dIII generated a 33 bp fragment containing the lesion. Restriction with *Hin*cII and *Hin*dIII generated a 16 bp fragment that did not contain the lesion, since the *Hin*cII cut is located one bp 3′ to the lesion (Figure 1A). Whole cell extracts of immortalized 3T3 PARP-$1^{+/+}$ (wt) and PARP-$1^{-/-}$ embryonic fibroblasts (*28*) were incubated in standard DNA repair buffer with the DNA substrate and either α-[^{32}P]dTTP to analyze incorporation at the site of the lesion (overall repair = short + long-patch repair), or α-[^{32}P]dCTP to reveal repair 3′ of the lesion (long-patch repair). After repair synthesis, the DNA was purified and digested with the appropriate restriction enzymes. The repaired DNA products were analyzed by denaturing polyacrylamide gel electrophoresis. An example of time course for each repair assay is presented in Figure 1C. Radioactive incorporation increased as a function of time and is maximal at 3 h. These incorporations were lesion dependent since no radioactivity was observed in the $pGEM_{control}$ plasmid.

Quantification by computerized PhosphorImager analysis of the incorporation of [^{32}P]dTMP in the 33 nucleotides *Sma*I−*Hin*dIII fragment from pGEM-AP revealed that the overall DNA repair was about half efficient in the PARP-1-deficient cell extracts (60% of repair efficiency, Figure 1C and data not shown). By contrast, we observed a dramatic reduction in the long-patch DNA repair capacity of PARP-1-deficient cells extracts (approximately 80−90%) (Figure 1C and data not shown, and see Figure 2, lanes 1 and 2). The contribution of the LPR pathway to the overall BER of natural abasic sites is approximately 20% (*36*), less than the defect observed in the overall repair for PARP-1-deficient cell extracts. This indicates that PARP-1 is absolutely necessary to perform the long-patch repair synthesis, but is also involved, to a lesser extent, in the short-patch repair process. Since maximal incorporations were obtained following 3 h (Figure 1C and data not shown), the following experiments were performed under this condition.

The BER defect in PARP-1-deficient cells resides in the polymerization step in both LPR and SPR pathways rather than in the ligation step, since there was no accumulation of shorter fragments reflecting unligated product. To confirm that the long-patch repair defect was restricted to the polymerization step, we examined the reaction products formed in the presence of α-[^{32}P]dCTP and after digestion with *Sma*I−*Hin*dIII. We observed a similar reduction in the amount of the 33 bp repaired DNA in PARP-$1^{-/-}$ cell extracts, without accumulation of smaller fragments (16−32 bp) reflecting unligated products (data not shown). These results demonstrate that PARP-deficient cells are impaired in the long-patch repair synthesis.

It has been reported that the cell extracts used in these experiments are devoid of any detectable amounts of NAD^+ (*34*, *37*). Therefore, this first set of experiments clearly demonstrates that the PARP-1 protein is required for BER, even without considering its catalytic activity.

Addition of NAD^+ Increases the Efficiency of LPR Synthesis. To address the contribution of PARP-1 activity in BER, wt and PARP-$1^{-/-}$ cell extracts were supplemented with NAD^+ before starting the reaction. The results (Figure 2) showed that the long-patch repair synthesis was stimulated (2.6-fold) by the addition of NAD^+ in the wt cells (compare lane 3 with lane 1) and also in the PARP-$1^{-/-}$ cells (1.8-fold, compare lane 4 with lane 2). Consequently, the stimulation of PARP-1 activity increased the efficiency of the LPR in wt and also in PARP-$1^{-/-}$ cell extracts, in this

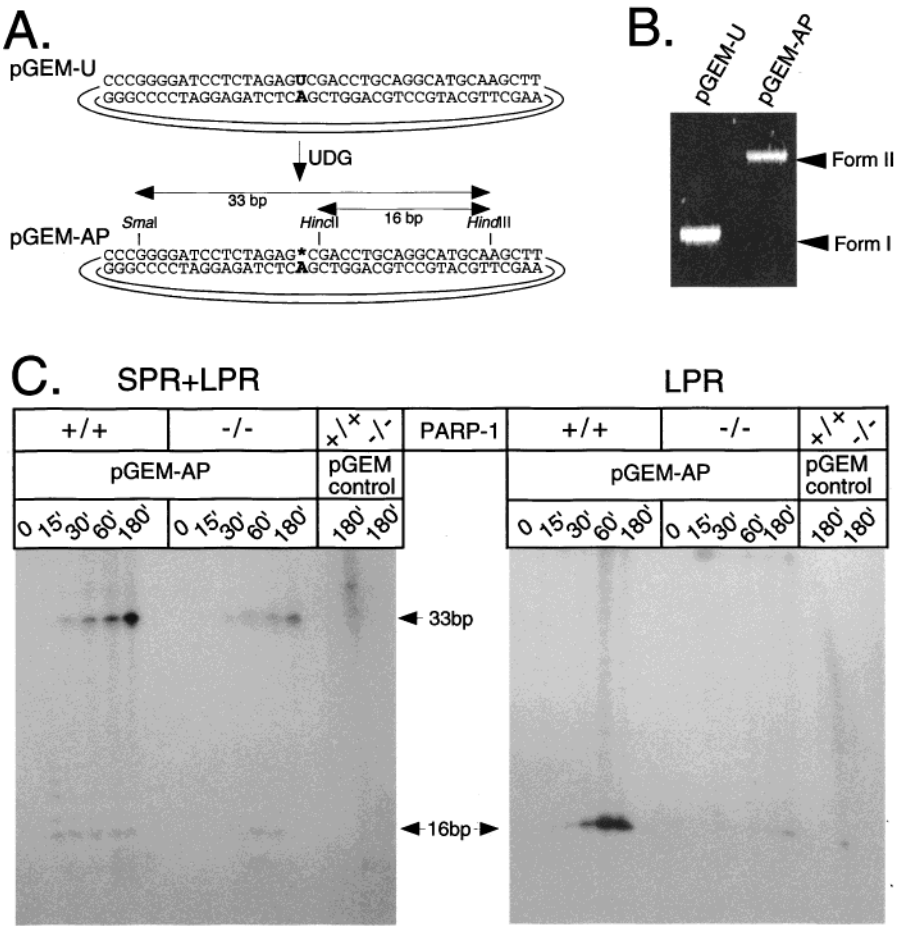

FIGURE 1: Repair of a single natural abasic site by PARP-1-deficient 3T3 cell extracts. (A) The circular plasmid DNA substrate containing a single uracil-induced AP site (pGEM-AP) was obtained by incubation of pGEM-U with *E. coli* uracil-DNA-glycosylase (UDG). The position of the AP site (asterisk) and the restriction sites are indicated. (B) Incubation of pGEM-U (left lane, form I) with *E. coli* uracil-DNA glycosylase. To verify that the AP-site is generated, an aliquot of UDG-treated DNA was further treated with *E. coli* Endonuclease III (right lane, form II) and analyzed on 1% agarose gel. (C) pGEM-AP or pGEMcontrol plasmids were incubated for 15, 30, 60, and 180 min with a cell-free extract of wt (+/+) or PARP-$1^{-/-}$ (−/−) 3T3 cells under standard repair conditions (see Experimental Procedure). To measure the overall repair (SPR + LPR), repair replication was performed in the presence of α-[^{32}P]dTTP and plasmid DNAs were digested with *Sma*I and *Hin*dIII to release the 33-bp fragment (see A). To measure the long-patch repair efficiency (LPR), repair replication was performed in the presence of α-[^{32}P]dCTP and plasmid DNAs were digested with *Hinc*II and *Hin*dIII to release a 16-bp fragment downstream to the lesion (see A). Repair products were analyzed by autoradiography after electrophoretic separation on denaturing 15% polyacrylamide gels.

latter case probably reflecting PARP-2 activity in a possible backup reaction, since we have shown recently that PARP-2 is responsible for 10% of the total nuclear PARP activity in cells (*19*). The effect of NAD+ could be to accelerate the repair process, allowing more lesions to be repaired during the 3 h incubation time. These results indicate that both the PARP-1 protein and its poly(ADP-ribosyl)ation activity are involved in the LPR pathway.

PARP-1-Deficient Cells Are Also Affected in Short but Mainly in Long-Patch Repair Pathways of a Single AP Site Derived from 8-Oxoguanine. Oxidative DNA damage such as 8-oxoguanine is processed by the bifunctional DNA glycosylase AP lyase, hOGG1, that leaves a 3′ incised AP (AP*) site. To determine the involvement of PARP-1 in the repair of this oxidative lesion, we generated a DNA substrate containing a single 3′-incised AP* site (Figure 3A), by treating pGEM plasmid containing 8-oxoguanine with purified OGG1. A complete conversion of the circular (form I) pGEM-8oxo-G to nicked circular (form II) pGEM-AP* was observed (Figure 3B) following treatment with OGG1, indicating that an incised AP-site was present in the plasmid. DNA repair of this AP*-site was measured in triplicate at 3 h (Figure 3C). α-[^{32}P]dGTP and α-[^{32}P]dCTP were used to monitor the overall BER in the 33 bp *Sma*I−*Hin*dIII restriction fragment and the LPR pathway in the 17 bp *Acc*I−*Hin*dIII fragment, respectively (Figure 3A). PARP-$1^{-/-}$ cell extracts showed a 50% decrease in the overall repair synthesis of a single AP* site and a much higher decrease (90%) in the LPR pathway (Figure 3C) compared to the parental cells. In a recent report, Dianov et al. (*38*) attributed 25% of overall repair of 8-oxoguanine to LPR. Therefore, our results indicate that PARP-1 is necessary to achieve complete repair of 8-oxoguanine-induced AP-sites via the long-patch repair pathway and also moderately via the short-patch repair pathway. The absence of short oligonucleotides for both pathways, reflecting unligated products, indicated that the inability of PARP-$1^{-/-}$ cells to repair 8-oxoguanine-induced AP sites is restricted to the polymerization step and not the ligation step. These results, compared to those observed for the repair of an uracil-derived AP site, show that, for both uracil- or 8-oxoguanine -derived AP-sites, cells lacking PARP-1 are half as efficient as wild-type cells in

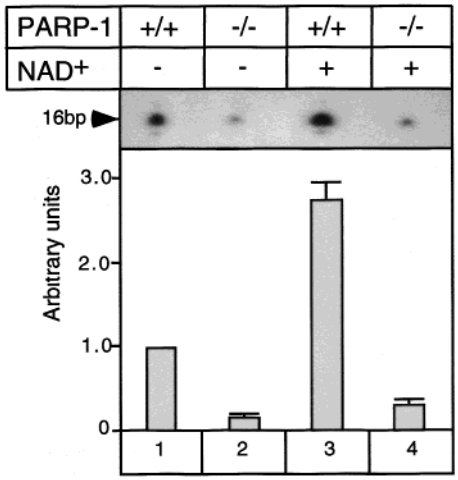

FIGURE 2: Effect of NAD^+ on the LPR pathway. pGEM-AP was incubated with a cell-free extract of wt (+/+, lanes 1 and 3) or PARP-$1^{-/-}$ (−/−, lanes 2 and 4) 3T3 fibroblasts in the presence of α-[^{32}P]dCTP for 180 min, and in the absence (lanes 1 and 2) or in the presence (lanes 3 and 4) of 500 μM NAD^+. Plasmid DNAs were digested with *Hin*cII−*Hin*dIII and reaction products were analyzed by autoradiography after separation on a 15% denaturing polyacrylamide gel. One of three independent experiments giving the same results is illustrated. Spots of all three experiments were quantified on a PhosphorImager, and mean data are depicted in the histogram. Error bars represent standart deviation.

performing overall repair, but are almost completely impaired in the long-patch repair synthesis.

PARP-1 Interacts with DNA polβ. Since PARP-$1^{-/-}$ cells are impaired in the polymerization step of both SPR and LPR, we wondered if PARP-1 could interact with one of the DNA polymerases that are involved in base excision repair. Using immunoprecipitation and GST-pull down assays, we were not able to detect any physical interaction between PARP-1 and DNA polymerases δ or ε (data not shown), both mainly involved in the long-patch repair pathway (*7*). Instead, using GST-pull down analysis, we observed an interaction between PARP-1 and DNA polβ (Figure 4). Truncated versions of human PARP-1 fused to GST (*29*) were expressed in Cos-1 cells and affinity purified on glutathione-sepharose beads. Copurification of endogenous DNA polβ was tested by Western blot (Figure 4A) and was efficient with GST-PARP−D (372−523) (lane 4). There was no DNA polβ association with GST alone, GST-PARP−B+C (202−371), or GST-PARP-E+F (524−1014) (lanes 1, 3, and 5, respectively), whereas association was observed in some experiments with GST-PARP-A−C (1−371) (data not shown). These results indicate that PARP-1 is associated with DNA polβ through its central D-domain, harboring the BRCT motif that is also involved in the interaction between PARP-1 and XRCC1 (*29*).

The same approach was used to identify the region of DNA polβ that mediates the interaction with PARP-1. Truncated versions of rat DNA polβ fused to GST were expressed in Cos-1 cells and affinity purified on glutathione-sepharose beads. Copurification of endogenous PARP-1 was tested by Western blot (Figure 4B) and was efficient with GST-DNA polβ and GST-DNA polβ (1−256) (lanes 2 and 3) and also observed to a lesser extend with GST-DNA polβ (1−150) (lane 4). There was no PARP-1 association with GST alone or GST-DNA polβ (1−124) (lanes 1 and 5). These results indicate that DNA polβ is associated with

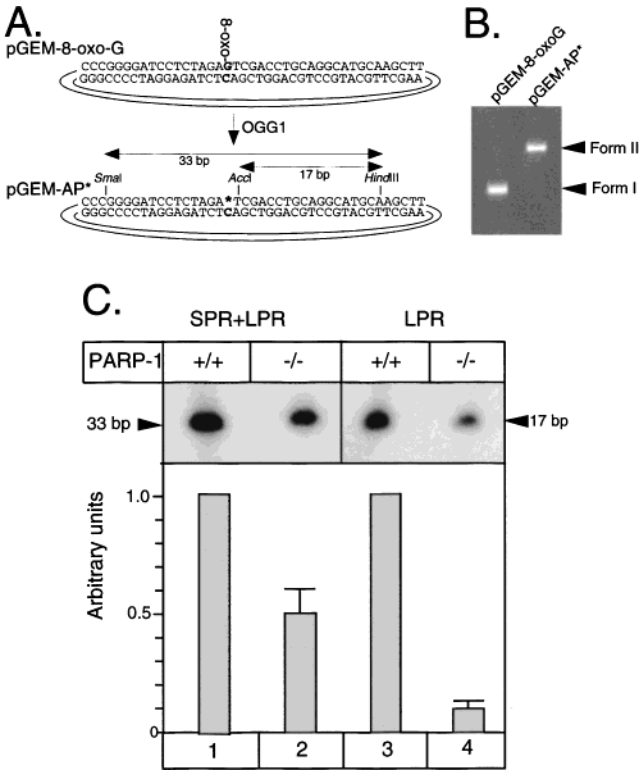

FIGURE 3: Repair of an 8-oxoguanine induced abasic site by PARP-1-deficient 3T3 extracts. (A) The circular plasmid DNA substrate containing a single 8-oxoguanine-induced AP site (pGEM-AP*) was obtained by incubation of pGEM-8oxoG with *S. cerevisiae* 8-oxoguanine-DNA glycosylase. The position of the AP* site (asterisk) and the restriction sites are indicated. (B) Characterization of the single AP* site-containing plasmid. Lane 1: plasmid containing the 8-oxoguanine lesion (form I); lane 2: after incubation with *S. cerevisiae* 8-oxoguanine-DNA glycosylase (form II). (C) pGEM-AP* or pGEMcontrol plasmids (data not shown) were incubated for 180 min with a cell-free extract of wt (+/+) or PARP-$1^{-/-}$ (−/−) 3T3 cells under standard repair conditions. To measure the overall repair (SPR+LPR), repair replication was performed in the presence of α-[^{32}P]dGTP and repaired DNA was digested with *Sma*I and *Hin*dIII to release the 33-bp fragment (see panel A). To measure the long-patch repair pathway (LPR), repair replication was performed in the presence of α-[^{32}P]dCTP and repaired DNA was digested with *Acc*I and *Hin*dIII to release the 17-bp fragment (see panel A). Reaction products were analyzed by autoradiography after separation on a 15% denaturing polyacrylamide gel. One out of three independent experiments giving the same results is illustrated. Spots of all three experiments were quantified on a PhosphorImager, and mean data are depicted in the histogram. Error bars represent standart deviation.

PARP-1 and that the central to C-terminal part (124−335) of DNA polβ is necessary for this interaction. Residues 200−206 and 301−310 of DNA polβ were shown in NMR studies to contact the N-terminal domain of XRCC1 (*39*); therefore, DNA pol β presumably interacts with XRCC1 and PARP-1 through the same domain.

Regulation of the Interaction between PARP-1 and DNA polβ. The next step was to examine the conditions under which this interaction could occur. We asked two questions about the PARP-1/DNA polβ and PARP-1/XRCC1 interactions: (i) are they regulated by poly(ADP-ribosyl)ation and (ii) do they required the presence of DNA? To address these questions, we overexpressed in Cos-1 cells the GST-PARP-D (372−523) (Figure 5A) and the GST-XRCC1 (170−428) (Figure 5B) fusion proteins, the latter containing the domain of XRCC1 that interacts with PARP-1. To answer the first

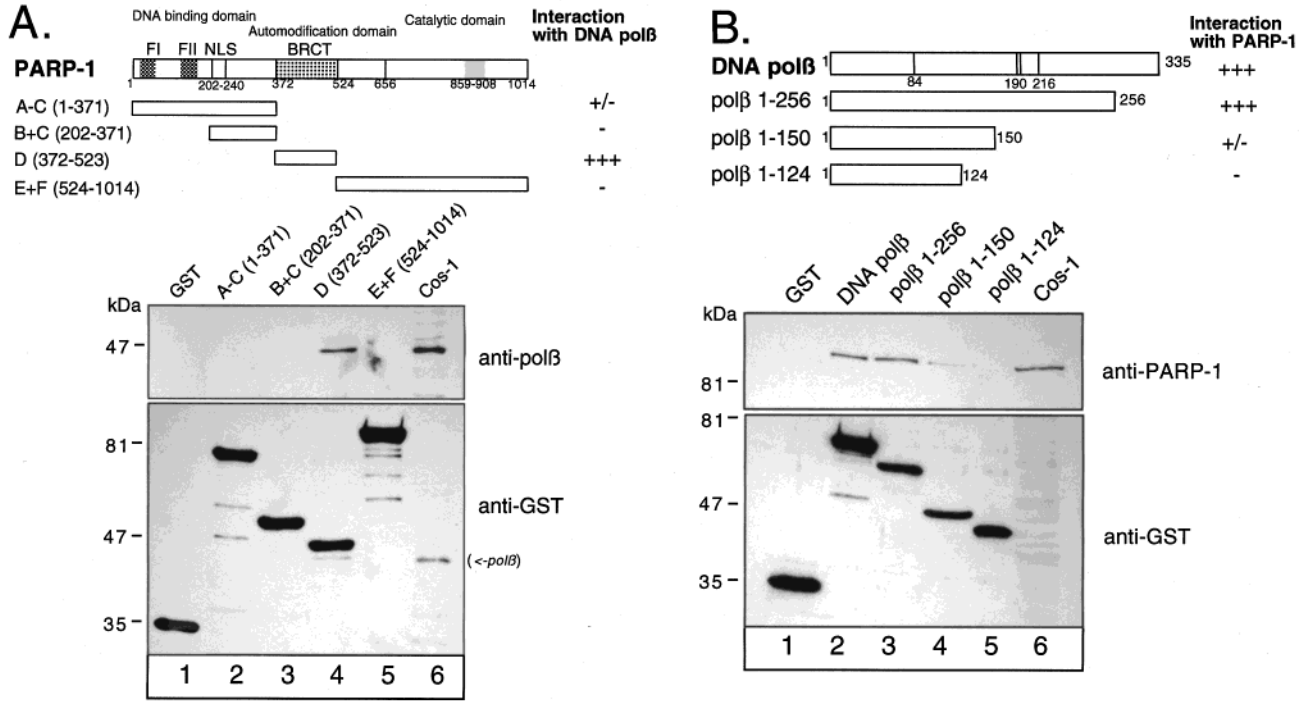

FIGURE 4: Interaction between PARP-1 and DNA polβ: mapping of the interacting domains. The GST-tagged deletion mutants of PARP-1 (panel A) or DNA polβ (panel B) are schematically represented. GST (panels A and B, lane 1) and GST-tagged deletion mutants of PARP-1 (panel A, lanes 2−5) or DNA polβ (panel B, lanes 2−5) were expressed in Cos-1 and interacting endogenous proteins were selectively extracted by GST-pull down and analyzed by Western blotting as described in the Experimental Procedure, using anti DNA polβ (panel A, top) or anti-PARP-1 (panel B, top), respectively. Blots were subsequently probed with anti-GST antibody (panels A and B, bottom). Lane 6: Cos-1 cell extract. In panel A, the arrow shows the DNA polβ that is still detectable in the GST immunodetection.

question, the cells were then either preincubated for 2 h with 3-aminobenzamide (1 mM) to inhibit PARP-1 activity or treated with H_2O_2 (10 mM) for 10 min to stimulate PARP-1 activity, or both, or left untreated. Treatment with H_2O_2 had no effect on the interaction between PARP-1 and either DNA polβ or XRCC1 (Figure 5A, lane 3, and B, lane 2), possibly because the poly(ADP-ribose) that is produced in the cell following H_2O_2 treatment may not be totally preserved from degradation by poly(ADP-ribose) glycohydrolase during the GST-pull down procedure. However, when PARP-1 activity was inhibited with 3-aminobenzamide (Figure 5B, lanes 3 and 4), the interaction between GST-XRCC1 (170−428) and PARP-1 was abolished, indicating that this interaction was dependent on poly(ADP-ribosyl)ation. This observation confirmed our previous results showing that automodified PARP-1 protein was preferentially associated with XRCC1 (*29*). The interaction between GST-PARP-D (372−523) and DNA polβ was not diminished when poly(ADP-ribosyl)ation was inhibited (Figure 5A, lanes 4 and 5). This difference suggests that, even if XRCC1 and DNA polβ share the same interacting domain on PARP-1, they are likely not interacting with PARP-1 simultaneously.

To answer the second question, ethidium bromide was added to the lysis buffer to eliminate the eventual protein−DNA interactions (Figure 5, panels A, lane 6, and B, lane 5). Alternatively, cell extracts were treated with DNAse I to remove genomic DNA eventually present in the cell lysate, leading to the same results (data not shown). We observed that DNA was not involved in the interaction between PARP-1 and XRCC1 (Figure 5B, lane 5). On the opposite, DNA was shown to be required for the interaction between PARP-1 and DNA polβ, since the treatment of cell extract with ethidium bromide or DNAse I (data not shown) abolished the interaction between GST-PARP-D (372−523) and DNA polβ (Figure 5A, lane 6). Since the BRCT domain present in the D-domain of PARP-1 is not able to bind DNA by itself (*40*), DNA does not bridge the two DNA-binding proteins. This suggests that DNA could either be necessary for the correct folding of DNA polβ or the interaction between PARP-1 or DNA polβ occurs only in the vicinity of damaged DNA (see Discussion).

In conclusion, PARP-1 interacts with XRCC1 and with DNA polβ via its central D-domain, encompassing the BRCT motif.

Repair Efficiency of Cell Extracts from Mouse Embryonic Fibroblasts Lacking both PARP-1 and DNA polβ. We then questioned the functional relationship between PARP-1 and DNA polβ in vivo, toward base excision repair. We generated immortalized 3T3 fibroblasts from 13.5 d.p.c embryos deficient in either PARP-1, polβ, or both genes obtained by intercrosses between PARP-$1^{+/+}$pol$\beta^{+/-}$ (*32*) or PARP-$1^{+/-}$-pol$\beta^{+/-}$ mice. A western blot analysis was performed on cell lysates from wt, PARP-$1^{-/-}$, pol$\beta^{-/-}$, and PARP-$1^{-/-}$pol$\beta^{-/-}$ 3T3 cell lines (Figure 6A), using the anti-PARP-1 and anti-DNA polβ antibodies. As expected, PARP-1 was absent in PARP-$1^{-/-}$ and PARP-$1^{-/-}$pol$\beta^{-/-}$ extracts (lanes 2 and 4), whereas DNA polβ was absent in DNA pol$\beta^{-/-}$ and PARP-$1^{-/-}$pol$\beta^{-/-}$ cell extracts (lanes 3 and 4), confirming the inactivation of the corresponding genes in these cell lines.

We compared the efficiency of cell extracts from these four cell lines in repairing DNA substrates containing a single uracil-derived AP-site (Figure 1A). First, we examined the overall repair process and found that this repair pathway was about half as efficient in the PARP-1-deficient cell extracts, as observed in Figure 1 (60% repair efficiency, Figure 6B,

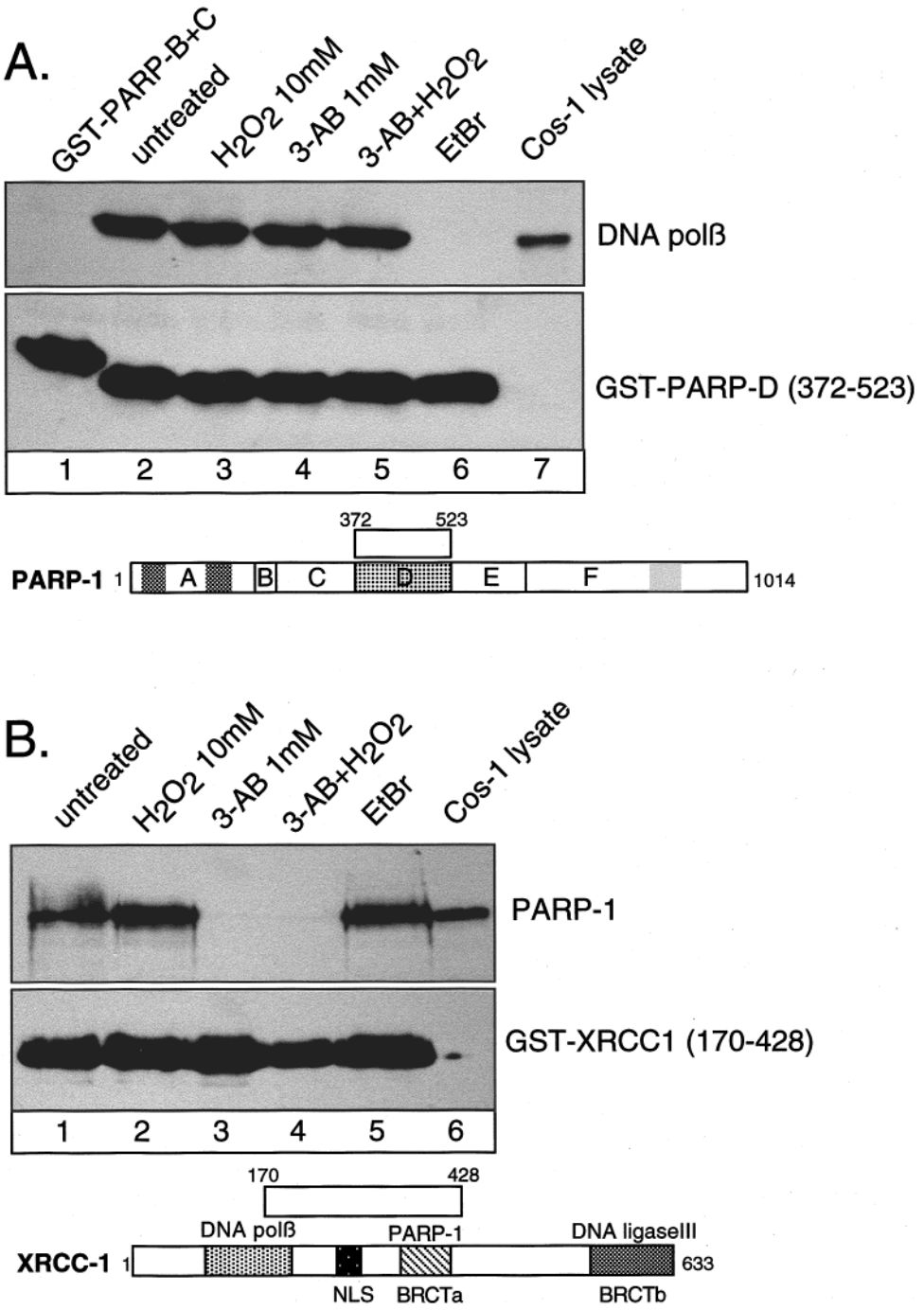

FIGURE 5: Conditions regulating the interactions between PARP-1, DNA polβ, and XRCC1. (A) Interaction between GST-PARP-D (372−523) fusion protein with endogenous DNA polβ in Cos-1 cells either untreated (lane 2) or treated with 10 mM H_2O_2 (lanes 3 and 5), with 3-aminobenzamide (lanes 4 and 5), with ethidium bromide (EtBr, lane 6) as described in the Experimental Procedure. GST-pull down analysis was performed as in Figure 4, and blots were probed successively with anti-DNA polβ (top) and anti-GST (bottom) antibodies. Lane 1: the GST-PARP−B+C(202−371) fusion protein expressed in untreated Cos-1 cells was used as a negative control. Lane 7: Cos-1 cell extract. (B) Interaction between GST-XRCC1 (170−428) fusion protein with endogenous PARP-1 in Cos-1 cells either untreated (lane 1) or treated with 10mM H_2O_2 (lanes 2, and 4), with 3-aminobenzamide (lanes 3 and 4), with ethidium bromide (EtBr, lane 5) as described in the Experimental Procedure. GST-pull down analysis was performed as in Figure 4, and blots were probed successively with anti-PARP-1 (top) and anti-GST (bottom) antibodies. Lane 6: Cos-1 cell extract.

lane 2). Cells deficient in DNA polβ were also affected in their overall repair efficiency (Figure 6B, lane 3), as previously reported (*32*, *36*); however, the remaining 40% of overall repair in these cells reinforce the hypothesis that other DNA polymerases (δ and/or ϵ) can substitute DNA polβ to perform short-patch repair (*8*, *36*). Interestingly, the absence of both PARP-1 and DNA polβ strongly decreased the overall repair efficiency (only 17% residual repair, Figure 6B, lane 4). Therefore, whereas cells can tolerate the absence of either PARP-1 or DNA polβ, the absence of both proteins leads to dramatic overall repair deficiency.

We then examined the capacity of these four cell extracts to perform long-patch DNA repair. Again, we observed a dramatic reduction in the long-patch DNA repair capacity of PARP-1-deficient cell extracts (17% residual repair, Figure 6B, lane 6), confirming the results reported in Figure 2. Cells

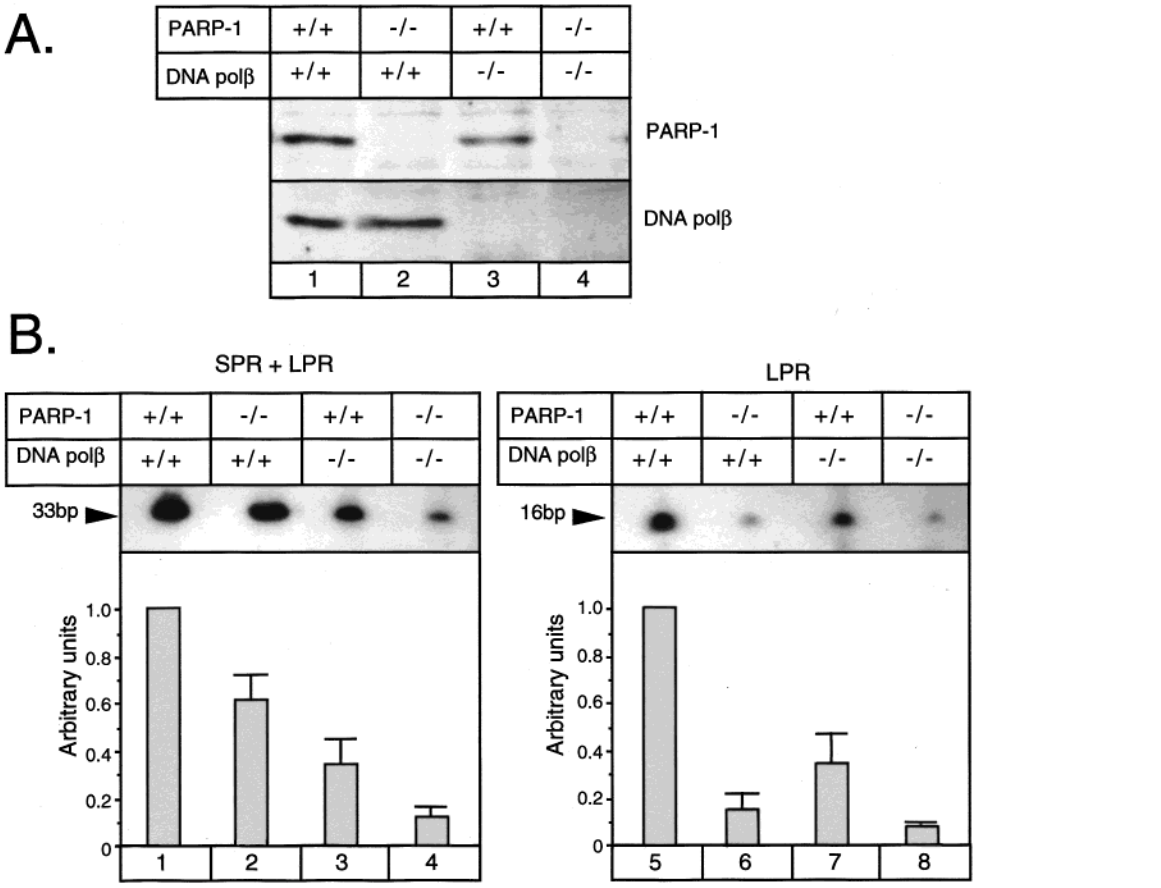

FIGURE 6: (A) Western blot analysis of PARP-1 and DNA polβ in wt, PARP-1$^{-/-}$, pol$\beta^{-/-}$, and PARP-1$^{-/-}$pol$\beta^{-/-}$ 3T3 cells. Eight μg of total protein from cell lysates from the four cell lines were analyzed by Western blot using the anti-PARP-1 (top) and anti-DNA polβ (bottom) antibodies. (B) Repair of an uracil-derived abasic site by PARP-1 and/or DNA polβ deficient 3T3 extracts. pGEM-AP or pGEMcontrol plasmids (see Figure 1A) were incubated for 180 min with a cell-free extract of wt, PARP-1$^{-/-}$, pol$\beta^{-/-}$, and PARP-1$^{-/-}$pol$\beta^{-/-}$ 3T3 fibroblasts under standard repair conditions as described in Figure 1C. Reaction products were analyzed by autoradiography after separation in a 15% denaturing polyacrylamide gel. One out of three independent experiments giving the same results is illustrated (top). Spots of all three experiments were quantified on a PhosphorImager, and mean data are depicted in the histogram (bottom). Error bars represent standart deviation.

lacking DNA polβ were also affected to perform long-patch repair (37% of residual repair, Figure 6B, lane 7), indicating that DNA polβ is involved in this process, as previously noticed (*14*, *15*). In the PARP-1$^{-/-}$pol$\beta^{-/-}$ double mutant cell extracts, the residual repair was very low (8%, Figure 6B, lane 8).

Therefore, the simultaneous absence of PARP-1 and DNA polβ almost totally abolished the two pathways of base excision repair.

Survival Assays. To investigate the long-term consequences of non- or misrepair of genomic DNA in PARP-1$^{-/-}$, pol$\beta^{-/-}$, and PARP-1$^{-/-}$pol$\beta^{-/-}$ 3T3 cell lines, compared to a wt cell line, we performed clonogenic assays following treatment with either the monofunctional alkylating agent *N*-nitroso-*N*-methylurea (MNU) or hydrogen peroxide (H_2O_2). As illustrated in Figure 7A (and data not shown), PARP-1$^{-/-}$, pol$\beta^{-/-}$, and PARP-1$^{-/-}$pol$\beta^{-/-}$ 3T3 cell lines were almost identically 3-fold more sensitive to 1.5 mM MNU than wt cells. With regard to killing by 25 μM H_2O_2, PARP-1$^{-/-}$ cells were 7-fold more sensitive than PARP-1$^{+/+}$ cells (Figure 7B). These results highlight the toxic effect of a deficiency in base excision repair of both alkylated damage and oxidative damage in PARP-1-deficient cells. In contrast, whereas DNA polβ-deficient cells were sensitive to alkylating agents, no significant increased sensitivity toward H_2O_2

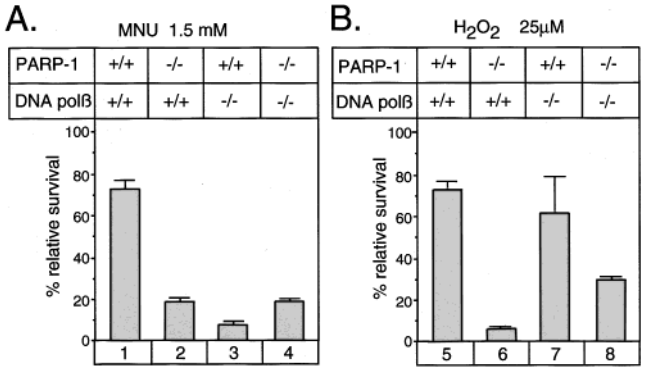

FIGURE 7: Colony forming ability of wt, PARP-1$^{-/-}$, pol$\beta^{-/-}$, and PARP-1$^{-/-}$pol$\beta^{-/-}$ 3T3 cells after treatment with MNU (1.5 mM) or H_2O_2 (25 μM). The cloning efficiencies were determined as described in the Experimental Procedure. Results were determined as the number of clones formed by the treated cells relative to the clones formed by untreated cells grown under identical conditions (% of relative survival). One out of three independent experiments giving the same result is shown. Error bars represent standart deviation.

was observed, as previously reported (*32*). PARP-1$^{-/-}$pol$\beta^{-/-}$ cells showed an intermediate sensitivity to H_2O_2 compared to PARP-1$^{-/-}$ and pol$\beta^{-/-}$ cells. These results suggest that other excision repair systems acting on oxidative damage may be induced in cells lacking DNA polβ.

DISCUSSION

Although extensively debated over the past decade, the recent generation of PARP-1-deficient mice by homologous recombination has permitted the reevaluation of the in vivo role of PARP-1 in DNA repair. These mice are exquisitely sensitive to MNU injection and to whole body γ-irradiation (*25*−*27*). PARP-1-deficient cell lines exhibit a dramatic delay in strand break rejoining (*28*) and, as a consequence, are hypersensitive to agents whose lesions are repaired by base excision. Instead, these cell lines are as sensitive as wild-type cells to chemicals that do not activate this pathway (*41*, *42*). Thus, PARP-deficient cell lines recapitulate the phenotypes observed with chemical inhibitors (*43*), overexpression of a dominant negative mutant (*22*, *23*, *44*), or antisense oligonucleotides (*24*).

To further determine the involvment of PARP-1 in the two well-documented pathways of BER, we have examined the processing of two structurally different AP sites—arising from either uracil or 8-oxoguanine—in PARP-1-deficient cell extracts. We have previously indicated the requirement of PARP-1 in long-patch repair of uracil-derived AP-site (*45*). In this work, we now demonstrate that the absence of PARP-1 has dramatic consequences on the LPR for repairing both uracil- or 8-oxoguanine- derived AP-sites, whereas the SPR is moderately affected. For both pathways, the repair defect observed in PARP-$1^{-/-}$ cells was associated with the polymerization step, leading us to search for a direct link between PARP-1 and the repair DNA polymerases. We found no connection between PARP-1 and DNA polymerases δ or ϵ, but a physical interaction with DNA polymerase β. DNA polymerase β was first considered to be involved solely in short-patch repair; however, it is now becoming clear that this polymerase is also able to perform the long-patch repair process (*14*−*17*, and this study), conducting strand displacement and controlling the size of the released dRP-oligonucleotide (*15*) in cooperation with FEN-1 (*17*). XRCC1 was also considered first as a short-patch repair pathway specific factor (*6*, *46*), although Cappelli et al. (*46*) noticed that the LPR in the XRCC1-deficient EM9 cell line was only half efficient as in wild-type cells. We were able to confirm this result (data not shown). All these data indicate that some connection between the two pathways can occur.

Two recent reports described the in vitro reconstitution of a PCNA-dependent long-patch base excision repair pathway, with six purified enzymes: HAP-1, PCNA, RF−C, polδ/ϵ, FEN1, and DNA ligase I (*10*, *11*). These analyses, however, could not exclude some alternative long-patch repair pathways. In vivo analyses are unavoidable in assessing the exact contribution of each participant in the BER processes. Using an in vitro cell-free repair assay, we have demonstrated the requirement of PARP-1 for efficient BER, particularly in the LPR pathway. We have shown that the absence of PARP-1 had dramatical repercussion on long-term cell survival after MNU and H_2O_2 treatment. Comet assays had already indicated a severe delay in the global repair reaction in PARP-1-deficient cells (*28*). Therefore, both our in vivo and in vitro analyses clearly demonstrate the involvement of PARP-1 in BER.

Nucleotide excision repair was shown to be efficient in repairing oxidative lesions (thymines glycol and 8-oxoguanines), both in human cell extracts and in vitro, using purified nucleotide excision repair factors (*47*). The NER of oxidative nonbulky adducts is thought to be initiated by the adduct, and not the AP-site that is generated (*47*); therefore, since the DNA substrate used in our cell-free extract BER assay is an AP site, the NER pathway is probably not induced. This could explain why cells lacking DNA polβ are highly affected in BER but they can survive oxidative damage, if NER can substitute to repair that lesion. The apparent gain of survival that is observed in the PARP-$1^{-/-}$pol$\beta^{-/-}$ double mutant cell line compared to the single PARP-$1^{-/-}$ mutant cell line could be due to better access of NER factors to the lesion when both PARP-1 and DNA polβ are simultaneously absent.

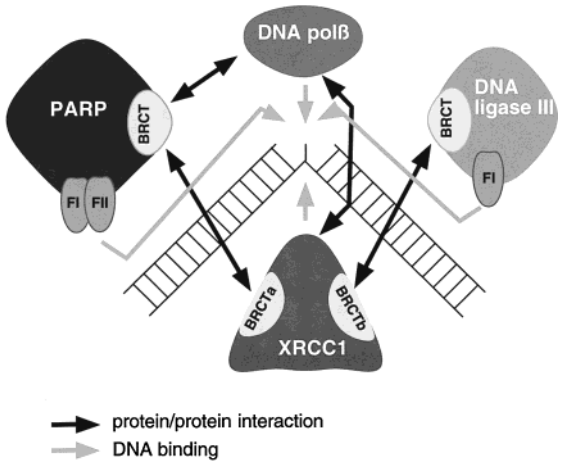

FIGURE 8: Cartoon depicting the protein/protein (dark arrows) and protein/DNA (gray arrows) interactions involving PARP-1, XRCC1, DNA polβ, and DNA ligase III.

Several DNA replication proteins were shown to take part in base excision repair, through the LPR pathway. This was demonstrated for DNA polymerases δ/ϵ, PCNA, and RPA (*7*−*12*). In addition, the base excision repair protein UNG2 was found to accumulate in replicative foci where it interacts with PCNA and RPA (*48*). These data link the replication machinery to the BER machinery. Interestingly, PARP-1 is also associated with the replication machinery, since it was shown to interact with DNA polymerase α-primase, to modify DNA polymerase α, topoisomerase I, and PCNA within the multiprotein DNA replication complex (MRC) and was proposed to have a role in the assembly of a functional DNA synthesome (*49*, *50*).

By which mechanism is PARP-1 involved in BER? PARP-1 has high affinity for XRCC1 (*29*, *30*), both proteins interacting, respectively, via their BRCT modules, a widespread motif in DNA repair and DNA damage-responsive cell cycle checkpoint proteins (*51*, *52*). We show here that the PARP-1 BRCT domain also interacts with the C-terminal domain of DNA polβ, that was shown to be the interface with XRCC1 (*6*, *30*). In addition, XRCC1 interacts with DNA ligase III (*31*). Therefore, even if XRCC1, PARP-1, DNA polβ, and DNA ligase III could be considered as a core BER complex (Figure 8), the interactions between these proteins are likely to be sequential rather than concomitant, since some of the interacting domains of these proteins are overlapping. How are these interactions regulated? XRCC1 interacts preferentially with automodified PARP-1, whereas DNA polβ interacts with PARP-1 regardless of its poly(ADP-ribosyl)ated status. Since the addition of NAD stimulates the

LPR process, we can hypothezise that poly(ADP-ribose) synthesis by PARP-1 regulates the BER process, possibly in a cyclic manner, by regulating the association/dissociation of proteins from the BER core complex.

In addition to their mutual interactions in a BER core complex, PARP-1, XRCC1, DNA polβ, and DNA ligase III bind to nicked DNA (Figure 8). This was recently demonstrated for XRCC1, which can bind the inside of the bent-nicked DNA while DNA polymerase β recognizes the outside of the bend (*39*). PARP-1 was shown to bind to the apex of the V-shape conformation of nicked DNA (*53*). The DNA ligase III harbors a zinc-finger like those of PARP-1, that specifically recognizes single-stranded breaks independently of the surrounding sequence (*54*), suggesting a competition for the two proteins for the same site on DNA. It has now to be determined in what order these proteins bind to the incised DNA and if XRCC1 binds the inside of the bend in conjunction with PARP-1 and DNA ligase III as it does with DNA polβ (*39*).

It is tempting to speculate about the in vivo function of PARP-1 in BER. PARP-1 could take part in several processes: (i) Facilitating or accelerating the repair process, either by recruiting the next repair enzyme (i.e., DNA polβ) and/or by acting directly on their activities [XRCC1 and DNA polβ are poly-ADP-ribosylated in vitro (*29*, *55*)]. DNA pol β was shown to be required for the repair of AP sites via LPR by conducting strand displacement and controlling the size of the released dRP-oligonucleotide (*15*) in cooperation with FEN-1 (*17*). The fact that LPR is mainly affected in the absence of PARP-1 suggests that the protein could also be involved in strand displacement. We know that PARP-1 binds to DNA nicks, gaps, and overhangs (unpublished results), but it remains to be verified if dRP extremity is by itself a target for PARP-1. (ii) Remodeling of chromatin, to allow the access at the damaged site of the repair enzymes and therefore facilitating the repair process (*56*); this hypothesis cannot be examined in our in vitro repair system which uses naked plasmid substrates. (iii) Protection of DNA from accidental recombination events, a role supported by the genomic instability observed in mice and cells deficient in PARP-1 (*25*, *26*, *28*). (iv) Signaling of either the DNA break itself or the startup of the repair process, to inform the cell about the level of injury. The cell would thus decide between repair with or without cell cycle arrest or death via apoptosis. This signal could be mediated by poly(ADP-ribose), either via heteromodification or via noncovalent binding to some target proteins (*57*, *58*). However, we have to keep in mind that other DNA damage signaling proteins, such as ATM and DNA-PK, could also be implicated.

The contribution (if any) of PARP-2 (*19*) in DNA repair has to be questioned. If PARP-2 could serve as a backup system for PARP-1, this backup is efficient in dealing only with a small amount of DNA damage (spontaneous lesions), because PARP-1$^{-/-}$ mice though viable and fertile are highly sensitive to exogenous genotoxic stress (*25*, *28*). This backup could be responsible for the residual long-patch repair that we measured in PARP-1-deficient cell extracts. The generation of mice and cells lacking PARP-2 and both PARP-1 and PARP-2 will undoubtedly help to elucidate the role of poly(ADP-ribosyl)ation in BER.

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