PARP-2 is required for efficient base excision DNA repair in association with PARP-1 and XRCC1

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Abbreviations

3-AB, 3-aminobenzamide; APE1, apurinic/apyrimidinic endonuclease 1; BER, base excision repair; BRCT, BRCA1 C-terminus; dRP, deoxyribose phosphatase; EST, expressed sequence tags; FEN-1, flap endonuclease-1; GFP, green fluorescent protein; GST, glutathione-S-transferase; h, human; HMG, high mobility group; LPR, long patch repair; m, mouse; MEF(s), mouse embryonic fibroblast(s); MNU, N-nitroso-N-methylurea; PAGE, polyacrylamide gel electrophoresis; PARP, poly(ADP-ribose) polymerase; PNK, polynucleotide kinase; pol, polymerase; SPR, short patch repair; XRRC1, x-ray cross complementing factor 1.
Abstract

The DNA damage-dependency of poly(ADP-ribose) polymerase-2 (PARP-2) activity is suggestive of its implication in genome surveillance and protection. Here we show that the PARP-2 gene, mainly expressed in actively dividing tissues follows, but to a smaller extend, that of PARP-1 during mouse development. We found that PARP-2 and PARP-1 homo- and heterodimerize; the interacting interfaces, sites of reciprocal modification have been mapped. PARP-2 was also found to interact with three other proteins involved in the base excision repair (BER) pathway: XRCC1, DNA polymerase β and DNA ligase III, already known as partners of PARP-1. XRCC1 negatively regulates PARP-2 activity, as it does for PARP-1, while being a polymer acceptor for both PARP-1 and PARP-2. To gain insight into the physiological role of PARP-2 in response to genotoxic stress, we developed by gene disruption mice deficient in PARP-2. Following treatment by the alkylating agent MNU, PARP-2 deficient cells displayed an important delay in DNA strand breaks resealing, similar to that observed in PARP-1 deficient cells, thus confirming that PARP-2 is also an active player in base excision repair despite its low capacity to synthesize ADP-ribose polymers.
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Key words: DNA binding protein/ NAD+ metabolism/ cellular response to DNA damage/ genomic instability/PARP homologues/gene expression.
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**Introduction**

In response to DNA interruptions, PARP-1, the founding member of the poly(ADP-ribose) polymerase superfamily, catalyses the successive covalent addition of ADP-ribose units from NAD to a limited number of nuclear acceptors to form a branched anionic polymer. PARP-1 is a nuclear enzyme involved in the detection and signaling of DNA strand breaks introduced either directly by ionizing radiation, or indirectly following enzymatic incision of a DNA lesion (abasic sites, oxidized or alkylated bases) repaired by the base excision repair (BER) pathway (see for review 1). The discovery of numerous PARP-1 protein partners and/or poly(ADP-ribose) acceptors involved in DNA architecture (histones H1, H2B, lamin B and HMG proteins) or in DNA metabolism (DNA replication factors, DNA repair proteins i.e. XRCC1, transcription factors, topoisomerase, and PARP-1 itself) has shed light onto the implication of PARP-1 in these processes (see for review 1).

The function of PARP-1 in BER has long been assumed, until direct evidence demonstrated the presence of PARP-1 in the BER complex, associated to XRCC1 (2,3) and DNA polymerase (pol) β (4). The polymer produced by PARP-1 upon activation by DNA breaks triggers the recruitment of XRCC1, which shows high affinity for oligo(ADP-ribosyl)ated PARP-1 (3-5). The requirement of PARP-1 in BER was established *in vivo*, since PARP-1 knock-out cells displayed a severe...
defect in strand breaks resealing following genotoxic treatment (6,7). The preferential role of PARP-1 in long patch BER was observed using extracts from these PARP-1 knock-out cells (4). Photoaffinity labeling experiments revealed that PARP-1 binds to BER intermediates (8). In reconstituted in vitro systems containing purified human BER enzymes, PARP-1 was shown to stimulate strand displacement DNA synthesis by DNA polβ, in cooperation with FEN-1, leading to long patch BER (9).

The mouse models in which the PARP-1 gene has been knocked out (10-12) revealed the dual facets of PARP-1 function. In proliferative cells inflicted with sub-lethal doses of DNA damage, PARP-1 as a survival factor participates in DNA damage detection and signaling, leading to cell cycle arrest and DNA repair, in order to avoid deleterious genetic alterations (1). On the other hand, in post-mitotic cells, massive DNA damage as observed in pathological conditions such as cerebellar or cardiac ischemia or septic shock, overactivates PARP-1, triggering energy depletion leading to cell death (see for review 13).

The PARP-1 knock-out mice were at the origin of the discovery of a new DNA damage-dependent PARP protein, named PARP-2, since an unexpected residual poly(ADP-ribose) synthesis could be measured in PARP-1 deficient cells following DNA damage (14,15). In addition to PARP-2 (15-17), several other PARPs were discovered almost simultaneously, all having in common a conserved catalytic domain responsible for poly(ADP-ribose) synthesis: PARP-3 (17), vPARP, a 193 kDa PARP belonging to the vault particles (18), Tankyrase 1 and 2, two
proteins associated to the telomeric protein TRF1 but also found at the Golgi or at nuclear pore complexes (19-22) and the 2,3,7;8-tetrachlorodibenzo-p-dioxin-inducible TiPARP (23). PARP-1 and PARP-2 are the only ones reported to be DNA damage dependent and in Arabidopsis thaliana, both PARP-1 and PARP-2 genes are induced by ionizing radiation (24). The N-terminal part of mammalian PARP-2 contains a nuclear location signal and a functional DNA binding domain (15) distinct from that of PARP-1 (two zinc fingers). The nature of this DNA binding domain has yet to be determined.

In our attempts to further characterize PARP-2 and compare its biological implication with that of PARP-1 with respect to DNA damage surveillance, we discovered that the expression pattern of PARP-2 and PARP-1 genes follows almost the same tissue distribution. The two proteins homo- and heterodimerize and poly(ADP-ribosyl)ate each other. In addition, PARP-2 was found to interact with the BER proteins XRCC1, DNA polβ and DNA ligase III, all being PARP-1 partners as well. XRCC1 could be heteromodified by PARP-2 and was able to negatively regulate PARP-2 activity as it does for PARP-1. The requirement for PARP-2 in BER was demonstrated in vivo by the COMET assay in mouse embryonic cells lacking PARP-2. Our results showed that PARP-2 is a component of a functional BER complex in vivo, likely through dimerization with PARP-1. This strengthens the role of PARP-2 as a survival factor following genotoxic stress.
Experimental Procedures

Plasmids – The *Sma*I/NotI fragment encoding full length murine PARP-2 (mPARP-2) cDNA was isolate from pVL-mPARP-2 (15), and sub-cloned into *Hpa*I/NotI sites of the pBC vector (25) in frame with GST, allowing the expression of GST-mPARP-2 fusion protein. Truncated forms of mPARP-2 were generated by PCR and cloned in frame with GST in the pBC vector. The XhoI/XhoI PCR product encompassing full length mPARP-2 was ligated into the XhoI site of pEGFP-C3 vector (Clontech, Palo Alto, USA), allowing the expression of GFP-mPARP-2. Complementary oligonucleotides encoding the Flag epitope following a methionine were linked into the EcoRV/EcoRI sites of the pIRES-eGFP vector (Clontech, Palo Alto, USA), allowing the expression of the Flag epitope (F). The cDNA encoding full length human XRCC1 (hXRCC1, kindly given by K. Caldecott) was subcloned into the EcoRI sites of pIRES-Flag vector, allowing the expression of Flag-tagged XRCC1 (F-hXRCC1).

In situ hybridization - In situ hybridization was performed as described in Niederreither and Dollé (26) on serial sections (10 µm) of frozen embryos or mouse adult organs dissected from 2 or 16 weeks CD1 mice and frozen in OCT. A XhoI/PstI fragment from a mouse PARP-1 EST clone (AA032357, Research Genetics, Huntsville, AL), encoding residues 337 to 572, was subcloned into
pBluescript SK(+) and antisense and sense mPARP-1 riboprobes were produced using T3 and T7 RNA polymerases, respectively. The murine PARP-2 probe corresponding to residues 8 to 363 is described in Amé et al (15). Exposure varied from 4 to 6 weeks for PARP-1 and PARP-2 probes.

**Immunoprecipitation, GST-pull down and Western blot analyses** — For immunoprecipitation of purified proteins, 1 µg of purified hPARP-1 and/or mPARP-2 (as indicated) were incubated 2h at 4°C with 20 µl of F1.23 monoclonal anti PARP-1 antibody and 1 µg BSA in 1 ml LSB (20 mM Tris-HCl pH 8, 120 mM NaCl, 0.1% NP40, 0.5 mM PMSF) with protease inhibitors (Complete Mini, Roche, Mannheim, Germany). Protein-G Sepharose beads (Pharmacia, Uppsala, Sweden) were added, and after 30 min incubation at 4°C, bound immune complexes were washed three times with LSB buffer, the pellets were resuspended in Laemmli buffer and heated 3 min at 100°C before analysis by Western blot. For immunoprecipitation of endogenous PARP-1 from HeLa cells, cells were lysed in LSB buffer 20 min at 4°C, scraped and centrifuge 20 min at 13000 rpm at 4°C. After preclearing with protein-G sepharose 30 min at 4°C, 20 µl of F1.23 anti PARP-1 antibody was added and immunoprecipitation was carried on as described above.

GST-pull down analyses were performed in Hela S3 cells as described in Dantzer et al. (4).

For immunodetection, blots were incubated with anti-PARP-1 (Monte 1/2500, 4), anti-PARP2 (Yuc 1/2500, 15), anti-XRCC1 (Roman 1/5000, 3), anti
DNA polß (1/1000, 4) and anti-DNA ligase III (1/250, kindly given by A. Tomkinson, San Antonio, TX) polyclonal antibodies or with anti-GST (1/10000, IGBMC, Illkirch, France) and anti-GFP (1/1000, Roche, Indianapolis, USA) monoclonal antibodies. 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). When indicated, 3-AB (1 mM) was added 2h prior lysis and maintained throughout all the lysis and washing steps.

*Heteromodification of GST-fusion proteins by PARP-2 or PARP-1 – GST pull down* were performed as described above, except that washes were done with HSB (20 mM Tris-HCl pH 8, 500 mM NaCl, 0.5 % NP40, 0.5 mM PMSF). After a last wash with activity buffer (50 mM Tris-HCl pH 8, 4 mM MgCl₂, 0.3 mM DTT), each sample was split onto three, the beads were pelleted (volume of the pellet: ± 20 µl) and resuspended in 300 µl activity buffer containing either 300 pmol of hPARP-1, 600 pmol of mPARP-2 or no PARP. Reaction was started by the addition of 180 µl of activity buffer containing DNase I activated calf thymus DNA, and [³²P]-NAD. Final concentrations were 0.5 µg DNA, 1 µM NAD for control and PARP-2 and 0.1 µM for PARP-1 samples. In addition, each sample contained 1 pmol of [³²P]-NAD (1000Ci/mmmole). After 4 min at 25°C, reaction was stopped by the addition of 500 µl of cold HSB on ice, and beads were washed three times with HSB, resuspended in 12 µl Laemmlli buffer and heated 3 min at 100°C before analysis by Western blot.
Poly ADP-ribosylation of PARP-2 and XRCC1 – Purified mPARP-2 (200 pmol) was incubated with 1 to 8 fold purified hXRCC1 (3) for 2 min at 25°C in 40 µl activity buffer containing 300 ng BSA, 5 µM [32P]-NAD (1000 Ci/mmole) and 100 ng of DNase I activated calf thymus DNA. Reaction was stopped by addition of 15 µl of Laemmli buffer on ice, and reaction products were analyzed by gel electrophoresis on 8 % SDS-PAGE and autoradiography of the Coomassie blue stained and dried gel.

Generation and culture of mouse embryonic fibroblasts - Mouse embryonic fibroblasts (MEFs) were isolated by micro-dissection of embryos at day 13.5 of gestation resulting from intercrosses between PARP-2+/- heterozygous mice. Each embryo was genotyped by PCR to screen for the disruption of the PARP-2 allele. The generation of these mice, and the genotyping PCR procedure will be described elsewhere. MEFs were maintained in DMEM, 4.5 g/l glucose medium supplemented with 10% fetal bovine serum and 0.5% gentamycin. For Western blot analysis, 10⁵ cells were resuspended in Laemmli buffer, sonicated and proteins were analyzed by Western blot as described above, using anti-PARP-2 (Yuc) and anti PARP-1 (Monte) polyclonal antibodies. The evaluation of residual poly(ADP-ribose) synthesis in MEF cell extracts was performed as described by Amé et al (15).
Single cell gel electrophoresis (comet) assay – Passage 3 MEFs were thawed 48h prior to harvesting on 60-mm Petri dishes. The following day, cells were either mock treated or exposed 30 min to N-nitroso-N-methylurea (MNU) as indicated. Comet assay was performed as described in Trucco et al, (6). Slides were dried in cold ethanol, and DNA was stained prior scoring with 2 µg/ml ethidium bromide. Fifty comets per slides were observed using a Zeiss Axioplan equipped with a DP50 camera (Olympus) and analyzed using the VisCOMET software (Impuls Bildanalyse GmbH, Gilsching, Germany) to calculate the tail moment as defined by Olive et al (27).

Results

Tissue distribution of PARP-1 and PARP-2 transcripts during embryogenesis and in mouse adult tissues

In situ hybridization experiments were performed to compare the expression patterns of the PARP-1 and PARP-2 genes at various stages of mouse development and in adult tissues. Antisense probes for PARP-1 and PARP-2 yielded specific labelling patterns that appeared similar, although not perfectly identical. During early developmental stages, both genes were expressed throughout the embryo (data not shown). Differential labelling patterns became
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apparent by E12.5. At that stage, both genes were expressed at high levels in the developing liver and kidney (Figure 1, A-C). Only PARP-1, however, was found to be expressed at higher levels in the genital ridge and the spinal ganglia. The signals observed throughout other embryonic regions for both PARP-1 and PARP-2 antisense probes were higher than for the corresponding sense probes (data not shown), indicating an ubiquitous moderate expression of both enzymes.

At E18.5 (Figure 1, D-I), PARP-1 and to a lesser extent PARP-2 were preferentially expressed in the thymus and in regions of the nervous system (see below). Within the developing trunk, preferential expression of PARP-1 and PARP-2 persisted in the liver and became restricted to the cortical region of the kidney, the spleen, adrenal gland and in stomach and intestinal epithelia (Figure 1, G-I, and data not shown). Note that PARP-1 transcripts appeared more restricted than those of PARP-2 towards the base of the intestinal crypts (G-I, insets).

From E14.5 to E18.5, as well as in the adult mouse, both genes were expressed at the highest levels in the thymus (Figure 1, D-F and data not shown). In the adult mouse, PARP-1 and -2 expression was particularly high in the subcapsular zone of the thymus (data not shown), where immature lymphocytes proliferate. Expression decreased as lymphocytes mature and in the medulla. PARP-1, and to a lesser extend, PARP-2 transcripts were detected in the white pulp of the spleen, especially in the germinal centers and in Peyer’s patches in the intestine wall (data not shown) suggesting that high levels of PARP-1 and -2 expression are related to proliferation of immature lymphocytes.
At E18.5, \textit{PARP-1} was preferentially expressed in specific brain regions (see the olfactory bulb, cerebellar and cerebral cortex in Figure 1, D-F) and in the olfactory epithelia. Expression was also higher in the cranial and spinal ganglia. \textit{PARP-2} expression appeared more homogeneous in craniofacial tissues, although it was slightly upregulated in brain and cranial/spinal ganglia. In the adult brain (Figure 1, J-L), both \textit{PARP-1} (28) and \textit{PARP-2} genes showed high expression in neuronal cells forming the stratum granulosum of the dentate gyrus and the stratum pyramidale of the hippocampus (CA 1-3). Weaker expression was detected in cells of the cerebral cortex. Only \textit{PARP-1}, however, was expressed at high levels in the Purkinje cell layer of the cerebellum (data not shown).

It is in testis that the expression pattern of \textit{PARP-1} and \textit{PARP-2} is the most distinct. \textit{PARP-1} is expressed at high levels in the seminiferous tubules of the developing testis (Figure 1, G-I). Expression was particularly strong in the basal layers of the seminiferous epithelium (Figure 1 M-N and 29), while no signal was detected in the luminal layers of the seminiferous epithelium indicating a down regulation of \textit{PARP-1} expression at the haploid stage of meiosis. In contrast, the \textit{PARP-2} signal was weak and rather homogeneous, throughout the seminiferous tubules and the interstitial tissue (Figure 1, O).

Apart from testis, the expression pattern of \textit{PARP-2} resembles that of \textit{PARP-1} except that the level of expression of \textit{PARP-2} is weaker.
PARP-2 and PARP-1 homo- and heterodimerize

PARP-1 is known to act as a catalytic dimer (30,31). To investigate possible homodimerization of PARP-2, extracts from Hela cells transfected with a plasmid allowing the overexpression of murine PARP-2 (mPARP-2) in fusion with GST were mixed with extracts from Hela cells transfected with a plasmid allowing the expression of either mPARP-2 fused to GFP, or GFP alone (Figure 2, lanes 2 and 6, respectively). GST-fusion proteins were also generated expressing truncated versions of mPARP-2 (see Figure 2): amino acids 1-69 (Nt, the DNA binding domain), amino-acids 63-202 (domain E) and amino-acids 203-559 (F, the catalytic domain). GST-fused proteins were trapped on glutathione-sepharose beads, and co-purifying GFP-tagged mPARP-2 was assessed by Western blot analysis using anti-GFP antibody. Figure 2 shows that PARP-2 is able to homodimerize (lane 2) through its E domain (lane 4).

To further investigate the possibility that PARP-2 forms heterodimers with PARP-1 and to prevent any cross reaction with PARP-2, we immunoprecipitated PARP-1 from HeLa cell extracts using the F1.23 specific monoclonal antibody raised against the N-terminal part of PARP-1 (32). PARP-2 was coimmunoprecipitated with PARP-1 (Figure 3A, lane 3). A negative control using an unrelated antibody did not trap either of these two proteins (lane 2). The interaction between PARP-2 and PARP-1 was also observed (lane 4 and see figure
5B) in the presence of the PARP inhibitor 3-aminobenzamide (3-AB), indicating that it occurs independently of their polymerizing activity.

The complex between PARP-1 and PARP-2 was reconstituted *in vitro* using purified proteins: mPARP-2 was coimmunoprecipitated with human PARP-1 (hPARP-1) using the F1.23 antibody (Figure 3B, lane 4) demonstrating a direct interaction between PARP-2 and PARP-1.

**Identification of the domains involved in the association of PARP-2 with PARP-1**

To map the interaction domain within PARP-1, GST fusion proteins were generated expressing truncated versions of hPARP-1 (Figure 4A): amino acids 1-371 (A-C, the DNA binding domain), amino-acids 174-366 (B and C), amino-acids 384-524 (D, encompassing the BRCT domain), amino-acids 572-1014 (F, encompassing the catalytic domain) and amino-acids 525-655 (region E). These fusion proteins were overexpressed in Hela cells and GST pull down experiments were performed followed by Western blot analyses. Copurification of endogenous PARP-2 was efficient with constructs containing either the DNA binding domain (lane 2) or the BRCT domain (lane 4). These domains are those involved in the homodimerization of PARP-1 (Figure 4A and 30), as well as in the binding to several partners such as XRCC1 (3), DNA polβ (4), DNA ligase III (Figure 4A),
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histones, hUbc9 and transcription factors such as E47, TEF-1, RXRα, Oct-1 and YY1 (see for review 1), suggesting that the DNA binding and the BRCT domains of PARP-1 are interfaces for protein-protein association.

In reciprocal experiments, full length and truncated versions of mPARP-2 fused to GST were expressed in HeLa cells and affinity purified on glutathione-sepharose beads. Copurification of endogenous PARP-1 was efficient with full length mPARP-2 and with its E domain (Figure 4B, lanes 2 and 4, respectively). These results showed that the E domain of PARP-2 is involved in both the homodimerization of PARP-2 (Figure 2) and the heterodimerization with PARP-1 (Figure 4B).

PARP-2 and PARP-1 poly(ADP-ribosyl)ate each other in vitro

The ability of PARP-1 to poly(ADP-ribosyl)ate PARP-2 was evaluated. Truncated versions of mPARP-2 fused to GST and expressed in HeLa cells were isolated on glutathione-sepharose beads as described above for Figure 4B, except that the washing buffer used contained 0.5 M NaCl and 0.5 % NP40 to prevent the interaction between endogenous PARP-1 and mPARP-2 (data not shown). Trapped proteins on the beads were incubated for 4 min with either hPARP-1 or mPARP-2 or neither, in the presence of [32P]-NAD (0.1 μM for hPARP-1 and 1 μM for mPARP-2 or control) and DNase I activated calf thymus DNA.
Autoradiography revealed that hPARP-1 was able to poly-(ADP-ribosyl)ate the E domain of mPARP-2 (Figure 5A, panel 3), and to a lesser extent the DNA binding domain (panel 2). Automodification of mPARP-2 was weakly detected only on the E domain (panel 3). In the presence of 3-AB, no auto/heteromodification of the E domain of mPARP-2 was observed (panel 5), confirming that the radioactive labelling detected was due to polymer synthesis.

The reciprocal experiment showed that, in the presence of 1 µM [32P]-NAD, mPARP-2 poly(ADP-ribosyl)ates the DNA binding domain and the BRCT domain of hPARP-1 (Figure 5B, panels 1 and 3, respectively). These domains contain most of the polymer acceptor sites in the automodification reaction of PARP-1 (Figure 5B).

These results show that PARP-1 and PARP-2 can associate to form homo- or heterodimers, and can be reciprocally poly(ADP-ribosyl)ated.

**PARP-2 belongs to a BER complex containing XRCC1, PARP-1, DNA polβ and DNA ligase III**

Given that PARP-1 is involved in base excision repair through its association with the scaffold protein XRCC1 (2-4), we examined whether PARP-2 and XRCC1 could also interact. The Western blot used to delineate the region of PARP-2 interacting with PARP-1 (described in Figure 4B) was probed with the
anti-XRCC1 antibody. Results showed that full length mPARP-2 (Figure 4B, lane 2) and its E-domain (lane 4) interacted with endogenous XRCC1, demonstrating that PARP-2 belongs to the BER complex through its interaction with XRCC1. A similar approach was used to identify the region of human XRCC1 (hXRCC1) interacting with PARP-2. Figure 6A shows that only the GST-fusion proteins harboring the central BRCT domain (BRCT1) of human XRCC1 (lanes 3 and 4) could interact with endogenous PARP-2. Neither the second BRCT of hXRCC1 (BRCT2) nor the N-terminal part of hXRCC1 known to interact with DNA polβ were found associated to PARP-2 (lanes 2 and 5, respectively). Therefore, XRCC1 interacts with both PARP-1 and PARP-2 through the same region, the BRCT1 module. The association between PARP-2 and XRCC1 resists stringent conditions (500 mM NaCl), indicating a high affinity of one protein for the other (data not shown).

DNA polβ (4) and DNA ligase III (Figure 4A) are other BER partners that interact with PARP-1. We tested whether these BER factors were associated with mPARP-2 by probing the Western blot described in figure 4B with anti-DNA ligase III and anti-DNA polβ antibodies. Results showed that both DNA ligase III and DNA polβ were trapped with full length mPARP-2 and with its E domain, implying that PARP-2 belongs to a multiprotein BER complex containing at least PARP-1, XRCC1, DNA polβ and DNA ligase III.
To examine whether the interactions between all these repair factors are regulated by poly(ADP-ribosyl)ation, we performed a GST-pull down analysis with mPARP-2 fused to GST expressed in Hela cells in the presence or absence of 1 mM 3-AB (Figure 6B). The interaction between mPARP-2 and either PARP-1 or DNA ligase III was independent on poly(ADP-ribose) synthesis. PARPs inhibition led to a slight decrease in PARP-2/DNA polß interaction and to a significant inhibition of PARP-2/XRCC1 interaction (figure 6B, compare lanes 5 and 6). A reciprocal experiment was performed, with the expression of the Flag-tagged full length hXRCC1 in Hela cells and immunoprecipitation of this recombinant protein in the presence or absence of 1 mM 3-AB (Figure 6C). Results showed that the association between hXRCC1 and DNA ligase III or DNA polß was not significantly affected by PARPs inhibition, whereas the interaction between hXRCC1 and both PARP-1 and PARP-2 was abolished by PARPs inhibition (figure 6C, compare lanes 5 and 6 and 4) indicating that polymer synthesis is a prerequisite for XRCC1 binding to PARP-2 as well as to PARP-1.

XRCC1 negatively regulates PARP-2 activity

XRCC1 was shown both in vitro and in vivo to negatively regulate PARP-1 activity, by limiting PARP-1 automodification (3), forcing it to reside on the damaged DNA. The same effect was observed for PARP-2 in an in vitro poly(ADP-ribosyl)ation assay containing mPARP-2, DNase I activated DNA and [32P]-NAD
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(figure 7A). Increasing the concentration of purified recombinant His-tagged hXRCC1 (3) leads to the inhibition of mPARP-2 activity. This inhibition occurs even though hXRCC1 is a polymer acceptor of mPARP-2, as shown by the radioactive labelling corresponding to poly(ADP-ribosyl)ated hXRCC1. Thus, as for PARP-1, XRCC1 limits PARP-2 automodification.

In order to determine the polymer binding sites on XRCC1, truncated versions of hXRCC1 fused to GST were expressed in HeLa cells and purified on glutathione-sepharose beads at high stringency and in the presence of 3-AB, to avoid copurification of endogenous PARP-1 and PARP-2 (see figure 5B and 3). The beads were incubated for 4 min with either hPARP-1 or mPARP-2 or neither PARP, in the presence of $[^{32}P]$-NAD (1 µM for control and mPARP-2, 0.1 µM for hPARP-1) and DNase I activated calf thymus DNA. The autoradiography shown in figure 7B revealed that polymer binding sites were present in the C-terminal part, lying between residues 314-428 (corresponding to the BRCT1 domain, panels 3 and 4) and to a lesser extend between residues 427-633 (encompassing the BRCT2 domain, panel 5). These polymer binding sites are functional for both mPARP-2 and hPARP-1. These results indicate that hXRCC1 is mainly poly(ADP-ribosyl)ated on the BRCT domain that interacts with PARP-1 and PARP-2. The C-terminal region of hXRCC1 encompassing the BRCT2 domain that interacts with DNA ligase III could also be poly(ADP-ribosyl)ated, in contrast to the N-terminal part that showed no polymer binding sites (panel 2). These results suggest that
poly(ADP-ribosyl)ation of hXRCC1 regulates its interaction with PARP-1 and PARP-2. The interaction between hXRCC1 and DNA ligase III was not affected by the inhibition of PARP activity (see Figure 6C), therefore the function of the poly(ADP-ribosyl)ation of the C-terminal part of hXRCC1 is still unclear. In addition to PARP-1, PARP-2, DNA polβ and DNA ligase III, XRCC1 has been shown to associate other partners in BER such as APE1 (33) and PNK (34). We can hypothesize that poly(ADP-ribosyl)ation of the C-terminal part of XRCC1 may regulate its association with one (or more) of them.

**PARP-2 is required for efficient DNA repair of alkylated DNA in vivo**

The presence of PARP-2 in a BER complex containing at least PARP-1, XRCC1, DNA polβ, DNA ligase III strongly supports a role of PARP-2 in this DNA repair pathway. We have generated mice deficient in PARP-2 by inactivation of exon 9 of the PARP-2 gene by homologous recombination (Ménissier-de Murcia et al., in preparation). Mouse embryonic fibroblasts (MEFs) were prepared from 13.5 d.p.c. embryos. Western blot analyses of crude extracts from these MEFs at passage 2 were performed using several polyclonal anti-PARP-2 antibodies raised against full length mPARP-2 or its catalytic domain. These antibodies recognized PARP-2 in PARP-2+/+ and PARP-2+/- cells, but failed to detect any PARP-2 or truncated fragment of it in PARP-2−/− cells (Figure 8A, lower panel and data not shown). The same blot was probed with the anti-PARP-1 antibody (Figure 8A,
upper panel), showing the presence of PARP-1 at comparable levels in MEFs from any genotype, indicating no deregulation of PARP-1 expression in the PARP-2 deficient cells.

To evaluate the contribution of PARP-2 to PARP activity, we measured the polymer formation in whole cell extracts from PARP-2+/+, PARP-2-/- and PARP-1-/- passage 3 MEFs (Figure 8B). Results showed that in vitro poly(ADP-ribose) synthesis stimulated by DNA strand breaks was only moderately affected in PARP-2-/- cells compared to PARP-2+/+ cells, as opposed to the severe inhibition of polymer synthesis in PARP-1-/- cells (15). Immunofluorescence analyzes using the 10H monoclonal antibody raised against poly(ADP-ribose) showed no evident decrease in polymer synthesis in PARP-2-/- cells treated with 1 mM H2O2 or 2 mM MNU compare to PARP-2+/+ cells (data not shown). These observations demonstrate that the absence of PARP-2 has only a weak effect on the total PARP activity stimulated by DNA breaks.

The capacity of PARP-2-/- cells to repair DNA lesions induced by alkylating agents was evaluated in vivo using the single-cell gel electrophoresis assay (COMET assay) and compared to that of PARP-2+/+, PARP-1+/+ and PARP-1-/- cells. Passage 3 MEFs of the four genotypes were exposed to MNU for 30 min as indicated in Figure 8C, or mock-treated. Measurement of the COMET tail moment reflecting the level of DNA fragmentation (27) revealed that DNA breakage varied in a linear manner with increasing doses of MNU in the range of 0-1 mM for each
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genotype (data not shown). A repair assay performed with 1 mM MNU showed that PARP-2−/− cells displayed a considerably slower rejoining kinetic (a two-hour delay in DNA strand breaks resealing) compared to PARP-2+/+ and PARP-1+/+ cells, but similar to that observed for PARP-1−/− cells (Figure 8C). These results unambiguously show that in spite of the presence of PARP-1, PARP-2−/− cells are defective in BER, demonstrating the requirement of PARP-2 for efficient DNA strand breaks resealing.
Similar expression pattern of PARP-1 and PARP2 genes

In this study, we showed that the expression of the PARP-1 and PARP-2 genes were almost similar, both being ubiquitously expressed at all stages of mouse development and in adult tissues, with variable levels and with a generally weaker intensity for PARP-2 compared to PARP-1. Expression of both transcripts seemed to be correlated with proliferation, with higher levels occurring during early fetal development and organogenesis, and in the highly proliferative cell compartments of adult mice. It is conceivable that cells undergoing intensive division need functional DNA damage sensing and repair factors in order to avoid inherited genomic alterations. Interestingly, we observed that murine tumors also displayed high expression of both PARP-1 and PARP-2 compared to normal tissue (data not shown). PARP-1 and -2 cannot be exclusively considered as genes expressed in highly proliferating cells since high expression of both genes was also detected in the post-mitotic neurons of cranial and spinal ganglia, in hippocampal pyramidal cell layers and in the dentate gyrus of the brain, although the two latter are known to contain progenitor neuronal cells (35). Several genes involved in DNA damage sensing and repair have been reported to be expressed in neuronal cells, such as ATM (36), p53 (37), T:G mismatch-specific thymidine-DNA glycosylase
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(38) and APE1 (39) because these cells need to be efficiently protected from DNA injury.

**PARP-1 and PARP-2 heterodimerize**

The almost similar tissue distribution of PARP-1 and PARP-2 raise the question of why eukarotic cells need simultaneously two DNA damage-dependent PARPs. The requirement of a functional PARP-1/PARP-2 heterodimer could be a plausible hypothesis. In this study, we demonstrated that PARP-2 and PARP-1 homo- or heterodimerize and heteromodify each other. The PARP-2 E domain appears to act as a protein/protein interface regulated by auto- or heteromodification. Interestingly this domain is enriched in glutamate residues that are potential automodification sites. Despite a significant amino-acid sequence conservation (38% identity, 47% similarity), the PARP-1 E domain displays none of these properties, unlike the neighboring BRCT motif. It seems likely that the E domain of PARP-2 combines the properties of the D and E domains of PARP-1.

At the cellular level, both enzymes reside into the nucleus and colocalize partially into the nucleolus (40 and Amé, Schreiber *et al*, in preparation). This distribution suggests the need for heterodimers in the nucleolar compartment where repetitive sequences (rDNA) need to be particularly protected.
Clearly, the biological significance of PARP-1/PARP-2 heterodimerization needs to be further elucidated. Does PARP-2 (endowed with a low specific activity) need PARP-1 (the most active member of the family) at a DNA lesion in order to amplify the cellular response to DNA damage? Interestingly, a direct interaction between PARP-1 and PARP-3, in the centrosome compartment, has also recently been found (Augustin, Spenlehauer et al. in preparation) suggesting a possible generalization of this observation to other PARP homologues. This type of organization of PARPs in physiological complexes would increase the number of possible partners, that in turn may adapt the responses of the cell to the nature of the injury and to the local environment.

**What is the function of PARP-2 in BER?**

It is more likely that both PARPs are required simultaneously to act in the same macromolecular base excision DNA repair complex. We and others have demonstrated the requirement of XRCC1, PARP-1 and DNA polβ for both short patch (SPR) and long patch (LPR) BER pathways (2,4,9,41-45). This new link between XRCC1 and PARP-2, observed only in the presence of polymer synthesis (as for the PARP-1/XRCC1 interaction) strongly suggests a concerted role of the PARP-1/PARP-2 heterodimer during base excision repair, most probably at the recruitment step of XRCC1 at damaged sites. The phenotype of embryonic
fibroblasts derived from PARP-2 knockout mice displaying a severe delay in strand breaks resealing after MNU treatment, supports this point of view. Interestingly, the absence of PARP-2 is as dramatic as the absence of PARP-1. This observation was quite unexpected, since PARP-2 activity in response to DNA damage is about 10 times less than PARP-1 activity. However, if we assume that PARP-1 and PARP-2 have to act as an heterodimer in base excision repair, then the absence of one of each would have the same consequence on repair efficiency.

Although more work is necessary to unravel the relative function of PARP-1 and PARP-2 in BER, the implication of the former in this pathway is becoming more evident. Two characteristic properties of PARP-1 place this enzyme at early steps of the repair process, most probably downstream from the action of DNA glycosylases and/or APE1: (i) detection and binding to the sugar-phosphate backbone interruption; (ii) bending of the nicked substrate by 100° (46) that generates a distorted structure, in turn, recognized by the next enzyme in the pathway (47). Both the ability of XRCC1 to bind the inside bend of DNA (48) and its increased affinity for oligo(ADP-ribosyl)ated PARP-1 and/or PARP-2 or polymers (this study and 3,5) may contribute to organize a protein platform at the DNA break for additional BER enzymes: PNK, DNA polβ, and finally DNA ligase III (see for review 49). Additionally, we have shown that the polymerization step of LPR was mainly affected in PARP-1 deficient cells (4). Lavrik et al (8) showed that PARP-1, associated to DNA polβ, efficiently binds to the repair intermediates
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containing a flap 5′-abasic site that are formed before sub-pathway choice leading to either SPR or LPR. The same group demonstrated that PARP-1, together with FEN-1, stimulates strand displacement synthesis by DNA polβ (9) leading to LPR. The authors proposed that the dRP group might serve as a sensor for the recruitment of PARP-1 onto BER intermediates, then PARP-1 would activate long patch BER by recruiting other long patch repair proteins. It remains an open question whether PARP-2 is also bound to this dRP-containing repair intermediate along with PARP-1. It is also possible that PARP-2, either alone or together with PARP-1, is involved in a distinct step of the repair process. Since PARP-1 and PARP-2 DNA binding domains differ totally, we can assume that they may have distinct DNA targets. The elucidation of the specific DNA structures (repair intermediates) recognized by PARP-2 will undoubtedly help to elucidate at which step(s) of BER it is involved.

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Legends to figures

Figure 1. Comparative \textit{in situ} analysis of PARP-1 and PARP-2 transcript distributions in mouse embryos and adults organs.

Each row consists of dark-field views of PARP-1 (middle) and PARP-2 (right) \textit{in situ} hybridization signals (white dots) on adjacent sections, and one of the corresponding bright-field views (left) to show histological details. Sagittal sections through the trunk region of an E12.5 embryo (A-C), the head and neck of an E18.5 fetus (D-F) and the abdominal cavity of an E18.5 fetus (G-I). The insets show an enlargement of one of the intestinal loops. Frontal sections of an adult (16 week-old) mouse brain (J-L). Sections through the testis of a 16 week-old male (M-O). Abbreviations: ad: adrenal gland; cb: cerebellum; cg: cranial ganglia; cx: cortex; dg: dentate gyrus; gr: genital ridge; hp: hippocampus; ht: heart; in: intestinal epithelium (G) or interstitial tissue (M); ki: kidney; li: liver; lu: lung; ob: olfactory bulb; sg: spinal ganglia; st: seminiferous tubules; te: testis; th, thymus.

Figure 2. PARP-2 homodimerizes.

Top: Schematic representation of mPARP-2. DBD, DNA binding domain. Bottom: Extracts from Hela cells expressing GST (lane 1) and GST-tagged mPARP-2 (lanes 2 and 6) or deletion mutants of mPARP-2 (lanes 3-5) were mixed with extracts from Hela cells expressing GFP (lane 6) or GFP-mPARP-2 (lanes 1-5). Interacting proteins were analysed by GST-pull down and Western blot with anti-GFP.
antibody (top). Blot was subsequently probed with anti-GST antibody (bottom).
Lane 7 (input): 1/50 of the total cell extract of Hela cells transfected with GFP-mPARP-2.

Figure 3. PARP-2 interacts with PARP-1 in vitro and in vivo.

A. Co-immunoprecipitation of PARP-2 with PARP-1 in HeLa cell extracts. Extracts from untreated (lanes 2 and 3) or 1 mM 3-AB treated (lane 4) HeLa cells were incubated with the F1.23 mouse monoclonal anti-PARP-1 antibody (lanes 3 and 4) or with anti β-galactosidase antibody (lane 2). Bound immune complexes were analyzed by Western blot with a mixture of anti-PARP-1 and anti-PARP-2 polyclonal antibodies. Lane 1: control immunoprecipitation without HeLa extract.

B. Co-immunoprecipitation of purified mPARP-2 with purified hPARP-1. One µg of hPARP-1 (lanes 1, 2 and 4) was incubated without (lanes 2 and 3) or with 1 µg of mPARP-2 (lanes 1 and 4) and with either F1.23 anti PARP-1 (lanes 2, 3 and 4) or with anti-lamin (lane 1) monoclonal antibodies. Bound immune complexes were analyzed by Western blot as in A. Inputs: hPARP-1 (40ng) and mPARP-2 (20ng).

Figure 4. Interaction between PARP-2 and PARP-1: mapping of the interface domains.

A: schematic representation of hPARP-1. GST (A and B, lane 1) and GST-tagged deletion mutants of hPARP-1 (A, lanes 2-6) or mPARP-2 (B, lanes 2-5) were expressed in HeLa cells and interacting endogenous proteins were extracted by
GST-pull down and analyzed by Western blot, using the indicated antibodies. Blots were subsequently probed with anti-GST antibody (A and B, bottom: one representative GST immunodetection). Panels A, lane 7 and B, lane 6: Crude extract of \(4 \times 10^5\) HeLa cells. In panel A, the stars show the immunodetection of PARP-2 that was carried out before GST immunodetection.

**Figure 5. Heteromodification of PARP-1 and PARP-2.** The GST-tagged deletion mutants of mPARP-2 (A) or hPARP-1 (B) were expressed in HeLa cells, extracted by GST pull down and incubated 4 min at 25°C in activity buffer with or without purified hPARP-1 or mPARP-2 as indicated in the presence of \([^{32}\text{P}]-\text{NAD}\) (1 µM for control and mPARP-2, 0.1 µM for hPARP-1) and DNase I activated DNA. Samples were analyzed by Western blot with anti-GST antibody (left panels) and autoradiography (18 h exposure at \(-80^\circ\text{C}\), right panels). A, bottom panel: 1 mM 3-AB was present throughout the experiment.

**Figure 6. PARP-2 interacts with XRCC1.**

A. Proteins interacting with GST (lane 1) or GST-tagged deletion mutants of hXRCC1 (lanes 2-5) were extracted from Hela cells by GST-pull down and analyzed by Western blot with anti-PARP-2 and anti-PARP-1 antibodies (top: overlay of the two immunodetection signals. The star indicates a cross reaction of anti-PARP-2 antibody with the GST-XRCC1\(_{170-428}\) fusion protein). Blot was subsequently probed with anti-GST antibody (bottom). Lane 6: Crude extract of
2.10^5 HeLa cells and lane 7: 10 ng of purified mPARP-2. B. GST (lanes 1 and 4) or GST-mPARP-2 (lanes 2, 3, 5 and 6) were expressed in HeLa cells and interacting proteins were selectively extracted by GST-pull down and analyzed by Western blot, using successively the indicated antibodies. Blot was subsequently probed with anti-GST antibody (bold). Lanes 3 and 6: 1 mM 3-AB was present throughout the experiment. Input corresponds to 1/50 of the total cell extract used for the GST pull down experiment. C. Control Flag (lanes 1 and 4) or Flag-hXRCC1 (lanes 2, 3, 5 and 6) were expressed in HeLa cells and immunoprecipitated with anti-Flag antibody. Interacting proteins were analyzed by Western blot using successively the indicated antibodies. Blot was subsequently probed with anti-XRCC1 antibody (bold) to detect the immunoprecipitated Flag-hXRCC1 protein. Lanes 3 and 6: 1 mM 3-AB was present throughout the experiment. Input corresponds to 1/50 of the total cell extract used for the immunoprecipitation.

Figure 7. Poly(ADP-ribosyl)ation of XRCC1 and negative regulation of PARP-2
A. Purified mPARP-2 (200 pmol, lanes 1-5) were incubated with 0 (lane 1), 200 (lane 2), 400 (lane 3), 800 (lane 4) or 1600 (lanes 5 and 6) pmol of purified hXRCC1 for 2 min at 25°C as described in Material and Methods. Reaction products were analyzed by 8 % gel electrophoresis and autoradiography of the Coomasie blue stained and dried gel. B. The GST-tagged deletion mutants of hXRCC1 were expressed in Hela cells and analysed as described in Figure 5.
Figure 8. DNA repair capacity of PARP-2^{+/+}, PARP-2^{-/-}, PARP-1^{+/+} and PARP-1^{-/-} MEFs treated with MNU as assessed by the Comet assay. A. Western blot analysis of total cell extract from passage 2 primary MEFs derived from 13.5 d.p.c embryos resulting from intercrosses between PARP-2^{+/+} mice. The blot was sequentially probed with anti-PARP-2 (lower panel) and anti-PARP-1 (upper panel) polyclonal antibodies. B. Relative PARP activity in PARP-2^{+/+}, PARP-2^{-/-} and PARP-1^{-/-} passage 3 primary MEFs. Cell extracts were incubated in standard conditions with [^{32}P]-NAD and DNase I activated DNA for 10 min at 25°C. Activity is expressed as the percentage of the radioactivity of acid-insoluble material produced by cell extracts compared to PARP-2^{+/+} cell extract. C. Kinetic of religation of DNA breaks induced by treatment of passage 4 MEFs cells (PARP-1^{+/+}, squares; PARP-2^{+/+}, circles; PARP-2^{-/-}, triangles and PARP-1^{-/-}, diamonds) with 1 mM MNU for 30 min. The distribution of the tail moment as a function of repair time is indicated. The results shown are one out of three experiments.
(Figure 3, Schreiber et al 2002)
(Figure 4, Schreiber et al 2002)
(Figure 5, Schreiber et al 2002)
(Figure 6, Schreiber et al, 2002)
(Figure 8 Schreiber et al 2002)
PARP-2 is required for efficient base excision DNA repair in association with PARP-1 and XRCC1
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