MNNG-induced Cell Death Is Controlled by Interactions between PARP-1, Poly(ADP-ribose) Glycohydrolase, and XRCC1*

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PARP-1 (poly(ADP-ribose) polymerases) modifies proteins with poly(ADP-ribose), which is an important signal for genomic stability. ADP-ribose polymers also mediate cell death and are degraded by poly(ADP-ribose) glycohydrolase (PARG). Here we show that the catalytic domain of PARG interacts with the automodification domain of PARP-1. Furthermore, PARG can directly down-regulate PARP-1 activity. PARG also interacts with XRCC1, a DNA repair factor that is recruited by DNA damage-activated PARP-1. We investigated the role of XRCC1 in cell death after treatment with supralethal doses of the alkylating agent MNNG. Only in XRCC1-proficient cells MNNG induced a considerable accumulation of poly(ADP-ribose). Similarly, extracts of XRCC1-deficient cells produced large ADP-ribose polymers if supplemented with XRCC1. Consequently, MNNG triggered in XRCC1-proficient cells the translocation of the apoptosis inducing factor from mitochondria to the nucleus followed by caspase-independent cell death. In XRCC1-deficient cells, the same MNNG treatment caused non-apoptotic cell death without accumulation of poly(ADP-ribose). Thus, XRCC1 seems to be involved in regulating a poly(ADP-ribose)-mediated apoptotic cell death.

Poly(ADP-ribosylation) of proteins is involved in the regulation of basal cellular processes and seems to be crucial for genomic integrity and cell survival. Responsible for the synthesis of poly(ADP-ribose) (PAR) are poly(ADP-ribose) polymerases (PARPs) (1). The most abundant and active PARP enzyme is PARP-1, a predominantly nuclear protein of 113 kDa. PARP-1 rapidly binds to DNA breaks, is thereby activated, and covalently automodifies itself under consumption of NAD⁺. To a lesser extent, some other nuclear proteins are also modified with PAR polymers (2). The primary structure of PARP-1 is well conserved between species. The N-terminal DNA binding domain, which contains two zinc finger motifs, is linked to a nuclear localization signal, the main acceptor sites of automodification are located within the central domain, and the 55-kDa C-terminal domain of the enzyme contains the catalytic site (1). PARP-1 appears to physically interact with multiple proteins involved in DNA metabolism, such as histones, transcription factors, replication factors, and DNA repair enzymes (3). Among DNA repair proteins, PARP-1 interacts with x-ray repair cross-complementing protein 1 (XRCC1) (4). A mutant line of Chinese hamster ovary (CHO) cells, which displays hypersensitivity to a broad range of genotoxins was established and termed EM9 cells (5). It turned out that EM9 cells have a reduced ability to rapidly repair DNA single-strand breaks and are genetically unstable as a consequence of XRCC1 deficiency. Different analyses revealed that XRCC1 physically interacts with several DNA repair enzymes, thereby regulating their corresponding activities (6). XRCC1, a polypeptide of 70 kDa, contains two breast cancer C-terminal domains and a nuclear localization signal but is lacking any known enzymatic activity (6). Notably, recruitment of XRCC1 to single-strand breaks strictly depends on PARP-1 activity (7, 8). PAR polymers are synthesized in response to DNA breaks, which can arise directly or indirectly, for example, after treatment with alkylating agents. Produced PAR modifications are rapidly degraded by poly(ADP-ribose) glycohydrolase (PARG), which cleaves the polymers with high specificity at the glycosidic bonds, generating free ADP-ribose. PARG is the physiological counterpart for all PARP enzymes, encoded by a unique gene (9). Human PARG, encoded by 18 exons is 110 kDa in size (10) and the catalytic domain resides in the C-terminal part of the enzyme (11, 12). Interestingly, recently a 39-kDa protein termed ARH3 has been isolated (13), which possesses a glycohydrolase activity although it is structurally unrelated to the conventional 110-kDa PARG. In addition, several PARG isoforms with different sizes, resulting either from alternative initiation events or from post-translational proteolysis, have been described in mammalians (14). Human PARG contains several putative localization signals: nuclear localization signal, nuclear export signals, and a mitochondrial localization signal (15, 16). Whereas PARG activity is detected predominantly in the cytoplasm, full-length PARG is localized to the nucleus (17, 18). Knockout of the full-length isoform of PARG in mice resulted in an increased sensitivity to genotoxic and endotoxic stress (19) and the loss of PARG activity in Drosophila melanogaster caused progressive neurodegeneration (20). Finally, it was demonstrated that after complete abrogation of PARG expression, murine embryonic
cells were only viable in the presence of PARP inhibitors. After withdrawal of these inhibitors an accumulation of PAR polymers was observed and cells underwent apoptosis (21). Thus, the metabolism of PAR plays a fundamental role for the decision of the cell to survive or die (22).

Nevertheless, the induction of PARP-1 activity by irreparable amounts of DNA breaks can deplete the cell of NAD and ATP, finally leading to cell death (23). For example, PARP-1-dependent necrosis can be triggered by treatment with 1 mM H$_2$O$_2$ (24). Yu et al. (25) described yet another cell death program depending on PARP-1 activity. In response to an exposure to 0.5 mM N-methyl-N-nitro-N-nitrosoguanidine (MNNG), PAR polymers accumulate, instantly provoking the translocation of apoptosis inducing factor (AIF) from mitochondria to the nucleus. This death stimulus then induces nuclear shrinkage and finally caspase-independent cell death (25, 22). How an accumulation of PAR is accomplished, whether by overactivation of PARP-1 or by repression of PARP activity, is not known. Therefore, the analysis of the relationship between PARP-1 and PARG is necessary to unravel the role of PAR metabolism in cell death.

Previously we reported that PARG interacts with human PARP-1 from HeLa cell extracts (26). In line with our findings, an affinity of PARP for PARG was shown in a recent proteomic approach (27). Here we characterize the functional relationship between PARP-1 and PARG. Both enzymes interact directly and PARG has the ability to modulate PARP-1 activity. In addition, PARG also interacts with XRCC1. Above all, we provide evidence that the interplay between PARP-1 and PARG activity [32P]PAR was synthesized in vitro as described earlier (33) and incubated with purified proteins or nuclear extracts (20 μg of proteins per 30 μl reactions) in phosphate buffer for 30 min at 30 °C. Reactions were stopped by precipitation with acetone. Precipitated nucleotides were dissolved in TE buffer. Samples containing equal amounts of radioactivity were applied to cellulose-coated plates. Thin layer chromatography was performed using the solvent system 0.3 M LiCl, 1 M acetic acid. After separation, dried cellulose plates were subjected to autoradiography and quantified using a phosphorimager or by Cerenkov counting of excised thin layer slices. Protein modifications with [α-32P]ADP-ribose were analyzed by PAGE and autoradiography. Alternatively, reactions were stopped by precipitation with acetone and nucleotides were separated by thin layer chromatography.

**EXPERIMENTAL PROCEDURES**

**Cells and Extracts—**HeLa S3 cells (ATCC) were grown in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal calf serum (Biochrom, Berlin, Germany). CHO cell lines AA8, EM9 (28), EM9-V, and EM9-XH (29) were kindly provided by Dr. K. W. Caldecott and cultured in α-minimal essential medium (Invitrogen) as described by Loizou et al. (30). Nuclear extracts were prepared by the method of Schreiber et al. (31).

**Cloning and Purification of Proteins—**The cDNA encoding amino acids 378–976 of human PARG (accession number DQ867088) was cloned as a SalI fragment into the vector pGEX-5X-3 (Amersham Biosciences). Expression of glutathione S-transferase (GST) or the GST-PARG65 construct was performed in *Escherichia coli* BL21 Codon Plus-RIL cells (Stratagene), which additionally were transfected with the pREP4 plasmid (Qiagen). Cells were cultured in LB broth overnight at 25 °C and overexpression was induced by adding 0.1 mM isopropyl-1-thio-β-d-galactopyranoside for 2 h. GST-PARG$_{65}$ was bound to glutathione-Sepharose (Amersham Biosciences) in phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na$_2$HPO$_4$ (7 H$_2$O), 1.4 mM KH$_2$PO$_4$), supplemented with 1% Triton X-100, 5 mM dithiothreitol. After washing, proteins were eluted with phosphate-buffered saline supplemented with 2% N-octyl-β-d-glucoside, 40 mM glutathione, pH 8.0. Eluted GST-PARG$_{65}$ was dialyzed in phosphate-buffered saline with 1 mM dithiothreitol. XRCC1, Lig III, and PARP-1 constructs were expressed and purified as described before (32, 33).

**PAR Metabolism—**[α-32P]NAD$^+$ was obtained from Amer sham Biosciences and galloptannins were from Sigma. Poly(ADP-riboseylation) activities were determined as described previously (26) with minor modifications. Purified recombinant proteins as specified in the legends to the figures or 20 μg of nuclear proteins were incubated in 25 μl of phosphate buffer (50 mM potassium phosphate, pH 7.2, 200 μM EDTA, 10 mM β-mercaptoethanol, 100 μg/ml bovine serum albumin) with 10 μM [α-32P]NAD$^+$ and 10 μg/ml nicked DNA at 30 °C for 20 min. All reactions were performed in the presence of 200 μM EDTA and absence of Mg$^{2+}$ ions to reduce endogenous phosphodiesterase and/or ADP-ribose pyrophosphatase activities that convert PAR and ADPR to AMP (34). Reactions were stopped by trichloroacetic acid precipitation and incorporation of [32P]PAR was determined. For determination of relative PARG activity [32P]PAR was synthesized in vitro as described earlier (33) and incubated with purified proteins or nuclear extracts (20 μg of proteins per 30 μl reactions) in phosphate buffer for 30 min at 30 °C. Reactions were stopped by precipitation with acetone. Precipitated nucleotides were dissolved in TE buffer. Samples containing equal amounts of radioactivity were applied to cellulose-coated plates. Thin layer chromatography was performed using the solvent system 0.3 M LiCl, 1 M acetic acid. After separation, dried cellulose plates were subjected to autoradiography and quantified using a phosphorimager or by Cerenkov counting of excised thin layer slices. Protein modifications with [α-32P]ADP-ribose were analyzed by PAGE and autoradiography. Alternatively, reactions were stopped by precipitation with acetone and nucleotides were separated by thin layer chromatography.

**Antibodies and Immunostaining Analyses—**Antibodies against PARG were raised in rabbits immunized with the purified His-tagged catalytic domain of PARG (26). These PARG antibodies recognize the catalytic 65-kDa PARG fragment and to a lesser extent also the human full-length PARG. Anti-pentahistidine antibodies were obtained from Qiagen. Antibodies directed against XRCC1 (H-300, from rabbit), AIF (H-300, from rabbit), GST (Z-5, from rabbit), PARP-1 (A-20, from goat), and YY 1 (C-20, from rabbit) were from Santa Cruz. Monoclonal α-tubulin antibodies (DM1A, from mouse) were from Sigma (Taufkirchen, Germany), polyclonal anti-PAR antibodies were from Alexis (96-10—04 from rabbit). For immunofluorescence analyses, cells were fixed with 3% formaldehyde, 0.25% Triton X-100, and antibodies against PAR or AIF were used and visualized using Alexa Fluor-conjugated secondary antibodies (Invitrogen). Nuclei were counterstained with DAPI (3 μM, Invitrogen). Necrotic cells were stained by incubating fixed cells with 100 μg/ml propidium iodide (Molecular Probes) in BBS (3.1 mM KCl, 134 mM NaCl, 1.2 mM CaCl$_2$, 1.2 mM MgSO$_4$, 0.25 mM KH$_2$PO$_4$, 15.7 mM NaHCO$_3$, 2 mM glucose, pH 7.2) for 30 min at 37 °C and analyzed immediately using a fluorescence microscope. Photomicrographs were obtained at room temperature with a microscope (Leica, Wetzlar, Germany) equipped with a digital camera. The relative amount of PAR accumulation was quantified using imaging software (Matrix Vision, Leica Application Suite).
Oppenweiler, Germany). Localization of AIF in cells and nuclear shrinkage of cells were estimated by visual inspection. At least 300 cells were counted for each sample and all experiments were repeated three times.

**Affinity Precipitation and Pulldown Assays**—For GST pulldown experiments, GST or GST-PARG<sub>65</sub> (7.5 μg) together with recombinant PARP-1 or XRCC1 constructs (20 μg) were incubated with glutathione-Sepharose (50 μl) in 0.5 ml of BP (10 mM Tris/HCl, 7 mM MgCl<sub>2</sub>, 150 mM NaCl, 50 μM ZnCl<sub>2</sub>, 0.05% (v/v) Nonidet P-40, 1 mM dithiothreitol, pH 8.0) by head over head rotation at 4 °C for 45 min. After 5 washing steps with BP, bound proteins were extracted with SDS gel loading buffer and subjected to Western blot analysis. Precipitation of XRCC1-bound proteins from EM9-XH cells was performed as described by Caldecott et al. (35). For immunoprecipitation, 40 μl of nuclear extracts from HeLa S3 cells (75 μg of protein) were diluted with 120 μl of immunoprecipitation buffer (20 mM HEPES, 1 mM EGTA, 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, pH 7.5) and pre-cleared by incubation with 50 μl of protein A-Sepharose (Sigma), covalently coupled with control goat antibodies, at 4 °C for 30 min. Thereafter, the unbound fraction was incubated with 10 μg of goat anti-PARP-1 antibodies (PARP-1 (A-20), Santa Cruz) covalently coupled to 35 μl of protein A-Sepharose at 4 °C for 30 min. The Sepharose beads were washed three times with immunoprecipitation buffer including 100 mM NaCl, and bound proteins were extracted subsequently with SDS gel loading buffer and subjected to Western blot analysis.

**Yeast Two-hybrid Analysis**—Human full-length PARG was cloned into the two-hybrid vector pGADT7 (Clontech). pAS-XRCC1 (36) was kindly provided by K. W. Caldecott. Plasmids were transformed into the yeast strain Pj69-4A (37) and diploids were selected using synthetic medium lacking leucine and tryptophan. For validation of protein-protein interaction, colonies were transferred to histidine- or adenine-free medium. The cellular NAD level was determined from perchloric acid extracts as described by Jacobson and Jacobson (38).

**RESULTS**

**PARG Interacts with PARP-1**—Previously, we demonstrated an interaction between full-length PARG immobilized to tannin-Sepharose and endogenous PARP-1 from HeLa cell extracts (26). We also detected interaction of PARP-1 with the immobilized 65-kDa C-terminal fragment of PARG (amino acids 378–976), in the following termed PARG<sub>65</sub>, which contains the catalytic activity (data not shown). To further characterize the interaction between PARP-1 and PARG we performed GST pulldown experiments with purified recombinant constructs (Fig. 1A). We expressed catalytically active GST-PARG<sub>65</sub> in *E. coli*, purified it, and incubated it with His-tagged full-length PARP-1. Fractions from GST pull-down experiments were separated by SDS-PAGE and subjected to Western blot analysis using anti-polyhistidine and anti-GST antibodies, respectively (Fig. 1A). As evident, after GST pulldown full-length PARP-1 was detected only in fractions eluted from GST-PARG<sub>65</sub> beads (Fig. 1A, *uppermost panels, fourth lane*). If His-tagged constructs of isolated domains of PARP (33) were incubated, only the automodification domain showed an affinity to GST-PARG<sub>65</sub>, whereas no interaction was observed with the other PARP domains (Fig. 1A). These experiments were performed in the absence of NAD<sup>+</sup> or PAR, thus demonstrating a direct protein-protein interaction between catalytically active PARG<sub>65</sub> and the automodification domain of PARP-1. Next, purified full-length PARP-1 and GST-PARG<sub>65</sub> were incubated as described before and the elution fraction from the GST pull-down was analyzed for PARP activity by incubation with [α-<sup>32</sup>P]NAD and nicked DNA. Eluted PARP-1 was catalytically active (Fig. 1B, *second lane*), indicating that native PARP-1 bound to the GST-PARG<sub>65</sub> beads. Menard et al. (39) reported that PARP was no acceptor for PAR modifications *in vitro*, whereas [<sup>32</sup>P]ADP-riboylation of PARG<sub>65</sub> was only detectable if GST-PARG<sub>65</sub> was added in excess (data not shown).

To study the impact of the interaction between PARP-1 and PARP *in vivo*, we performed co-immunoprecipitation experiments. First, Western blot analyses showed that PAR modifications were virtually absent in extracts of untreated HeLa cells (Fig. 1C). PARG<sub>65</sub> was detected after co-immunoprecipitation of these extracts with anti-PARP-1 antibodies (Fig. 1D). Thus, a preformed complex of PARG and PARP-1 might exist in the nucleus of HeLa cells, independent of PARP-1 activation. To further study the interplay of these enzymes, we supplemented nuclear extracts of HeLa cells with increasing amounts of recombinant GST-PARG<sub>65</sub> and incubated the extracts with [α-<sup>32</sup>P]NAD and nicked DNA. After reaction for 20 min, modifications with [<sup>32</sup>P]poly(ADP-ribose) were visualized in SDS-PAGE (Fig. 2B). Amounts of released [<sup>32</sup>P]ADP-ribose were determined by evaluation of thin layer chromatograms (Fig. 2A), and polymer chain lengths were analyzed by gel electrophoresis (Fig. 2C). As expected, the addition of increasing amounts of catalytically active GST-PARG<sub>65</sub> enhanced the levels of released [<sup>32</sup>P]ADP-ribose (Fig. 2A, *bars 1–4*), whereas the sizes of the produced [<sup>32</sup>P]PAR polymers were decreased (Fig. 2C, *lanes 1–4*).

Previously we presented evidence that tannins elevate the level of PAR in HeLa cell extracts by inhibition of PARG (26). In the presence of nuclear proteins 150 μM tannins had no modulating influence on PARP-1 activity (26). If similar experiments as shown in Fig. 2, *bars/lanes 1–4*, were performed in the presence of 150 μM tannins, the levels of [<sup>32</sup>P]ADP-ribose were not affected by the addition of PARG<sub>65</sub> (Fig. 2A, *bars 5–8*), but, notably, the sizes of the polymers were significantly reduced (Fig. 2, B and C, *lanes 5–8*). In conclusion, even inde-
dependent of its hydrolyzing activity PARG₆₅ may affect the catalytic activity of PARP-1 by direct protein-protein interaction, resulting in the synthesis of only short polymers.

**PARG Interacts with XRCC1**—PARG₆₅ interacts with the automodification domain of PARP-1 (Fig. 1A), which contains a breast cancer C-terminal domain and is known to be the region important for interactions with other proteins (33) such as for instance XRCC1 (4). To analyze a potential direct connection between PARG and XRCC1, we investigated a direct interaction of these proteins using the following set of experiments. First of all, human full-length PARG was cloned into the yeast two-hybrid reporter plasmid pGADT7 and interaction with an appropriate construct containing XRCC1 (pAS-XRCC1; Ref. 36) was studied in yeast cells (Fig. 3A). Indeed, only in the presence of both PARG and XRCC1 was a supplementation of histidine biosynthesis observed, whereas controls using empty vectors were negative (Fig. 3A). The interaction between PARG₆₅ and XRCC1 was further verified by in vitro GST pulldown experiments. For that purpose, His-tagged XRCC1 was purified from E. coli and incubated with purified GST-PARG₆₅. After GST pulldown, fractions were analyzed by Western blot and radiolabeled PARP-1 was visualized by autoradiography. C. nuclear extracts, prepared from untreated or MNNG-treated (0.5 mM MNNG; 10 min) HeLa cells were subjected to Western blot analysis with anti-PAR and anti-PARP-1 antibodies as indicated. The positions of full-length PARP-1 and proteolysis fragments (⁎) are indicated. D, immunoprecipitation of HeLa nuclear extracts (NE) with anti-PARP-1 or control antibodies coupled to protein A-Sepharose. Precipitated proteins were subjected to Western blot analysis with anti-PARP-1 and anti-PARG antibodies as indicated. The anti-PARG antibodies recognize the full-length PARG and the 65-kDa form of PARG, which in part is generated by proteolysis during the procedure. B–D, relative molecular weights of marker proteins and the positions of PARP-1 and PARG are indicated. IB, immunoblot.
Cell Death Is Controlled by PARP-1, PARG, and XRCC1

![Diagram](image)

FIGURE 2. The catalytic activity of PARP-1 is influenced by the interaction with PARG. Purified GST-PARG65 was added to nuclear extracts of HeLa cells in the absence (−) or presence (+) of 150 μM tannins. GST-PARG65 concentrations were without PARG (1 and 2) at 150 (2 and 6), 300 (3 and 7), or 600 nM (4 and 8). 10 μM [32P]NAD and nicked DNA were added and reactions were stopped after 20 min. A, reactions were precipitated with acetone and [32P]ADP-ribose intermediates were separated by thin layer chromatography. The evaluation of [32P]ADP-ribose release is shown. B, in parallel, reactions were subjected to SDS-PAGE (8%) and autoradiography. Relative molecular weights of marker proteins and the position of PARP-1 are indicated. C, the same reactions as in A were precipitated with trichloroacetic acid, polymers were detached by alkaline lysis and subjected to polymer chain length analysis using a 20% polyacrylamide gel. The autoradiogram of the gel is shown and the numbers of ADP-ribose units are indicated.

Malignant cell death is controlled by PARP-1, PARG, and XRCC1. Cell death involves a cell activation process and is realized in various cell lines. PARP-1 and PARG are involved in this process through poly(ADP-ribosylation). The results show that PARP-1 activity is increased by XRCC1, whereas PARG activity is decreased by XRCC1. These findings indicate that PARP-1 and PARG activities are regulated by XRCC1.

XRCC1 Enhances the Synthesis of Poly(ADP-ribose) in MNNG-treated Cells—XRCC1 interacts with PARP-1 (4) and also with PARG (cf. Fig. 3). Thus, XRCC1 might have the capacity to regulate cellular PAR metabolism. Therefore, in the following experiments we addressed the impact of XRCC1 on PAR synthesis using EM9 cells. First, EM9-V and EM9-XH cells were treated with 0.5 mM MNNG, a supralethal dose to induce poly(ADP-ribosylation). Interestingly, immunofluorescence analyses clearly showed that higher amounts of PAR polymers were produced in XRCC1-containing EM9-XH cells compared with XRCC1-deficient EM9-V cells (Fig. 4A). Evaluation of quantified PAR signals revealed that in EM9-XH cells, PAR accumulation was increased about 3–4-fold compared with the accumulation observed in EM9-V cells (Fig. 4, B and C). Western blot analyses of cell extracts confirmed this (Fig. 4D). Even 30 min after MNNG treatment, considerably higher amounts of PAR and larger PAR modifications were still detectable in EM9-XH cells, whereas only lower amounts and smaller sizes of PAR modifications remained in EM9-V cells (compare lanes 3 with 6 in Fig. 4D). Hence, after treatment with toxic MNNG doses XRCC1 influences PAR metabolism in living CHO cells.

To further elucidate the regulation of PAR metabolism by XRCC1, PARP and PARG activities were analyzed and compared in different CHO lines deficient or proficient in XRCC1. Western blot analyses revealed no significant differences in PARP-1, or PARG110/PARG65 levels, whereas levels of DNA ligase III (Lig III) were considerably reduced in XRCC1-deficient cells (data not shown). Consequently, whereas Lig III is stabilized by XRCC1 (35), cellular XRCC1 does not appear to be essential for the stability of PARP-1 or PARG. Previously, Masson et al. (4) proposed that XRCC1 down-regulates PARP-1 activity. Accordingly, we observed an inhibition of poly(ADP-ribosylation) in a reconstituted system with recombinant proteins, when XRCC1 was incubated with PARP-1 in a molar ratio of 8:1 (Fig. 5A). Furthermore, we found that PARG activity was unaltered, even if an excess of XRCC1/Lig III was added to GST-PARG65 (Fig. 5B). Analysing relative PARP and PARG activities in nuclear extracts of different CHO lines deficient or proficient in XRCC1 resulted in comparable PARP and PARG activities in all cell lines (Fig. 5, C and D). Similarly, it has been reported that cellular NAD content and relative PARG activity appeared normal in EM9 cells (40). These seemingly contradictory observations are explainable because in living cells the level of XRCC1 is not higher than the level of PARP-1. A direct decreasing effect on PARP-1 activity was only observed if an excess of XRCC1 was added to isolated PARP-1 (Fig. 5A).

In response to treatment with genotoxic agents, XRCC1 is recruited to DNA single-strand breaks in a PARP-1-dependent fashion (7). In comparison to PARP-1, the cellular concentration of XRCC1 is lower (41). Therefore, recruitment might represent an increase of the local concentration of a limited factor at sites of recruitment. Because neither PARP-1 nor PARG activities are directly regulated by XRCC1 (Fig. 5, C and D), XRCC1 recruitment might be required for the observed PAR accumulation in MNNG-treated XRCC1-proficient cells (Fig. 4). Therefore, in the next experiment, we reconstituted XRCC1 recruitment in vitro by supplementation of XRCC1-deficient nuclear extracts with an excess of recombinant XRCC1. For that purpose, nuclear extracts from XRCC1-deficient EM9 cells were complemented with recombinant XRCC1 during monitoring of PARP activity for a period of 20 min using nicked DNA and [α-32P]NAD+. [32P]Poly(ADP-ribosylation) was visualized by SDS-PAGE (Fig. 6A) and polymer chain lengths were analyzed in parallel (Fig. 6B). Indeed, we observed that PAR syn-
thesis was dramatically enhanced after supplementation with XRCC1 (Fig. 6). Both, the amounts and the sizes of formed polymers increased significantly after supplementing XRCC1 to EM9 extracts (Fig. 6, A and B, right lanes). Large and branched PAR polymers, which were unable to enter the gel, were only produced when XRCC1 was present (Fig. 6, A and B, top of right lanes). The fractions of \[^{32}P\]poly(ADP-ribosylated) proteins with large modifications were quantified as insoluble fractions after acetone precipitation (PAR\(^*\) in Fig. 6C). Soluble \[^{32}P\]ADP-ribose metabolites PAR, ADP-ribose, AMP, and NAD\(^{+}\) were separated by thin layer chromatography and relative ratios of all \[^{32}P\]ADP-ribose metabolites were determined (Fig. 6C). The evaluation of the overall \[^{32}P\]ADP-ribose metabolism revealed that in the presence of XRCC1 a significant and reproducible accumulation of large \[^{32}P\]PAR modifica-
tions (PAR\(^*\)) was achieved (Fig. 6C). Moreover, the levels of \[^{32}P\]ADP-ribose decreased when XRCC1 was added (Fig. 6C). Thus, the observed enhanced formation of \[^{32}P\]PAR polymers was partly caused by a suppression of PARG activity. Neverthe-

![FIGURE 3. PAR interacts with XRCC1.](image)

**A**. pGADT7-PARG110, pAS-XRCC1, and control plasmids as indicated were transformed into yeast cells and diploids selected using medium lacking leucine and tryptophan (+His). For validation of protein-protein interaction, colonies were transferred to histidine-free plates (−His). B. His-tagged XRCC1 was incubated with GST or GST-PARG\(_{65}\) and glutathione-Sepharose. After the washing steps, bound proteins were eluted and subjected to Western blot analysis with anti-polyhistidine or anti-GST antibodies as indicated. 1, input; 2, unbound proteins; 3, wash fractions; 4, eluted proteins. C. Whole cell extracts of EM9-V or EM9-XH cells were incubated with Ni-NTA-Sepharose. Proteins bound to the Sepharose beads were eluted with 80 mM imidazole and subjected to Western blot analysis with anti-XRCC1 or anti-PARG antibodies as indicated. 1, input; 2, unbound proteins; 3, eluted proteins.

![FIGURE 4. MNNG treatment triggers increased PAR synthesis in EM9-XH cells.](image)

**A**. Nonconfluent growing EM9-V and EM9-XH cells were treated with 0.5 mM MNNG in the absence or presence of 1 mM 3-ABA as indicated. 10 min thereafter, cells were fixed and immunostained with anti-PAR antibodies (left panels) and counterstained with DAPI (right panels). Representative images are shown. B. Diagrams of the fluorescence signals from the single cells marked with an asterisk in A. C. Quantification of PAR accumulation in MNNG-treated cells in the absence or presence of 3-ABA as indicated. D. EM9-V or EM9-XH cells were not treated or treated with 0.5 mM MNNG and whole cell extracts were prepared and subjected to Western blot analysis with anti-PAR or anti-α-tubulin antibodies, respectively. For lanes 1 and 4, extracts were prepared from untreated cells, whereas the cells represented by lanes 2 and 5 were MNNG treated for 12 min. For lanes 3 and 6, cells were treated with MNNG for 10 min and thereafter incubated in drug-free medium for a further 20 min. Relative molecular sizes of marker proteins are indicated.

**Cell Death Is Controlled by PARP-1, PARG, and XRCC1**
Cell Death Is Controlled by PARP-1, PARG, and XRCC1

less, XRCC1 stimulated PARP activity because the consumption of [32P]NAD+ was significantly increased in reactions supplemented with XRCC1 (Fig. 6C). Similarly, supplementation of nuclear extracts from HeLa cells with recombinant XRCC1 resulted in increased poly(ADP-ribosylation) (data not shown). A recruitment of or supplementation with XRCC1 appears to be necessary for efficient PAR accumulation. One possible explanation for this is that XRCC1 displaces PARG from binding PAR or from associations with other proteins. However, because PARG and PARP-1 interact (cf. Fig. 1) and PARG thereby down-regulates PAR syntheses (cf. Fig. 2), it is conceivable that the observed accumulation of PAR polymers is a result of alternating interactions between PARG, PARP-1, and XRCC1.

XRCC1-induced Accumulation of PAR Leads to Apoptotic Cell Death—It is known that an accumulation of PAR in response to high doses of MNNG triggers several apoptotic events, including translocation of AIF from mitochondria to the nucleus and nuclear shrinkage within a few hours (25). Because we observed increased PAR accumulation in EM9-XH cells compared with EM9-V cells after treatment with supralethal MNNG doses (cf. Fig. 4), in the next experiments we monitored translocation of AIF and nuclear shrinkage in CHO cells, 3 or 6 h after MNNG treatment (Fig. 7). The cellular content of AIF was comparable in EM9-V and EM9-XH cells (Fig. 7C). Translocation of AIF after MNNG treatment could be detected in only a few EM9-V cells and in most EM9-XH cells, but not in the presence of the PARP inhibitor 3-ABA (Fig. 7, A and B). Because XRCC1 increases the amount of PAR formation (Figs. 4 and 6), it appears that XRCC1 might as well regulate PARP-1-dependent translocation of AIF from mitochondria to the nucleus. Notably, 6 h after MNNG treatment, shrunken nuclei were detected in more than 80% of EM9-XH cells but only in 10% of EM9-V cells (Fig. 7D). When the XRCC1 proficient parental cell line AA8 was treated with MNNG, similar apoptotic features as obtained with the EM9-XH cells were observed (data not shown). The caspase inhibitor Z-VA-D(OMe)-fmk (100 μM) failed to block MNNG-induced nuclear shrinkage in EM9-XH cells (Fig. 7D). In a control experiment,
the impact of XRCC1 on induction of apoptosis was analyzed, using staurosporine, another trigger of cell death (Fig. 8). Staurosporine has two effects on nuclear structure, either causing caspase-independent partial nuclear condensation (stage I) or caspase-dependent advanced nuclear condensation and fragmentation (stage II) (42). We observed that AIF translocation and nuclear condensation induced by staurosporine were comparable in EM9-V and EM9-XH cells (Fig. 8). Furthermore, Z-VAD(OMe)-fmk did not prevent AIF translocation but effectively blocked the formation of shrunken nuclei (stage II) in both cell lines (Fig. 8). Thus, the impact of XRCC1 on apoptotic events appears to be restricted to MNNG-induced caspase-independent cell death.

DISCUSSION

The investigation presented here suggests a new level of coordination of MNNG-induced cell death regulated by interactions between PARP-1, PARG, and XRCC1. Several conclusions can be drawn from our study. First of all, PARG interacts with PARP-1 and XRCC1. Second, PARG can regulate PARP-1 activity. Third, XRCC1 regulates PAR-mediated apoptotic cell death induced by supralethal doses of MNNG.

The creation of different PARG knockout models revealed the importance of PAR polymers for cellular survival. For example, genetic PARG inactivation in D. melanogaster resulted in a severe PAR accumulation in neuronal cells and lethality at the larval stage (20) and mouse cells lacking PARG showed an accumulation of PAR leading to cell death by apoptosis (21). Thus, PAR can induce apoptosis and PAR signaling appears to play an important role in embryonic development.

In a recent proteomic approach, localization of a fraction of PARG to messenger ribonucleoparticles and an interaction of PARG with Fragile-X-related protein was discovered (27). Given the fact that PARG activity is involved in many different cellular events these observed interactions could be important also for the regulation of the activities of PARP enzymes other...
than PARP-1. All current models regarding the functions of poly-(ADP-ribosylation) are based on the interplay between PARP-1 and PARG, but a direct interaction between both enzymes has not been described before. In the study presented here we show for the first time that PARP-1 interacts with PARG, even independently of the substrates NAD or PAR. In addition, we characterize XRCC1 as another interaction partner of PARG. Remarkably, phosphorylation of XRCC1 by protein kinase CK2 influences its interaction with the DNA repair protein polynucleotide kinase, as recently shown (30). Furthermore, in a large scale characterization of nuclear phosphoproteins from HeLa cells, phosphorylated PARG was detected (43). Thus, it is tempting to speculate that PARG activity or its interaction with other proteins might be regulated by phosphorylation. Nevertheless, functional analyses of how PARG activity is modulated in living cells are lacking yet.

The biological significance of PARP-1 activity has been the subject of numerous publications but it is poorly understood how this catalytic activity is modulated (1–3). As it is known, PARP-1 activity is induced by DNA breaks, introduced either directly or indirectly, and depends on the availability of NAD. Among the proteins interacting with PARP-1, XRCC1 and DNA-dependent protein kinase have been reported to negatively regulate PARP-1 activity (4, 44). On the other hand, we characterized transcription factors that have the ability to directly stimulate PARP-1 activity (45–47). Furthermore, an allosteric activation of PARP-1 auto-modification by Mg$^{2+}$, Ca$^{2+}$, histones H1 and H2, and polyamines has been demonstrated (48). Here, with PARG we present another PARP-1-interacting protein that may function as a negative regulator. In line with these findings, Cortes et al. (19) reported that when the full-length isoform of PARG was knocked-out in mice, residual PARG$_{60}$ severely reduced the automodification activity...
of PARP-1 in vivo. Thus, it is feasible that PARG, when interacting with PARP-1, inhibits the automodification reaction and thereby guarantees that large PAR modifications are not generated. This inhibition of PARP-1 activity might be achieved directly or indirectly by displacement of PARP-1 activators as histones or transcription factors. Thus, PARG appears to have a dual function in PAR metabolism, degradation of PAR and down-regulation of PAR synthesis. In contrast to PARG, PARP-1 is highly abundant in the nucleus (14). In situations of low levels of DNA damage, PAR synthesis and degradation are balanced and no accumulation of PAR occurs. It has therefore been assumed that the high specific activity of PARG compensates for its low cellular concentration (39). Based on our findings it can be speculated that at low levels of DNA damage, only a minor fraction of nuclear PARP-1 is catalytically activated and PAR synthesis and degradation are induced. In EM9-XH cells (right) XRCC1 is recruited by automodified PARP-1, resulting in a displacement of PARG. As a consequence, the extent and length of PAR polymers increase. Accumulated PAR polymers then trigger AIF release, nuclear shrinkage, and apoptotic cell death. In XRCC1-deficient EM9-V cells (left) activation of PARP-1 and PARG cause depletion of NAD and ATP leading to cell death.

**FIGURE 10.** XRCC1 regulates MNNG-induced cell death by coordinating the activities of PARP-1 and PARG. Toxic doses of MNNG introduce irreparable DNA lesions. PARP-1 interacts with PARG and both enzymes are catalytically activated, and PAR synthesis and degradation are induced. In EM9-XH cells (right) XRCC1 is recruited by automodified PARP-1, resulting in a displacement of PARG. As a consequence, the extent and length of PAR polymers increase. Accumulated PAR polymers then trigger AIF release, nuclear shrinkage, and apoptotic cell death. In XRCC1-deficient EM9-V cells (left) activation of PARP-1 and PARG cause depletion of NAD and ATP leading to cell death.
tial amounts of PAR are still detectable 30 min after the treatment (55). Equal amounts of PAR foci triggered by 20 mM H$_2$O$_2$ were detected in EM9-V and EM9-XH cells (55). Consequently, these PAR foci seem to arise independent of the presence of XRCC1. Presumably, H$_2$O$_2$-induced PAR foci persist because of the limiting amount of PAR molecules compared with the amount of damage-activated PARP-1 molecules. In line with this, we observed nuclear condensation also of most of EM9-V cells in response to a treatment with 1 mM H$_2$O$_2$ (data not shown). Thus, the impact of XRCC1 on PAR accumulation and cell death depends on the type and amount of DNA lesions introduced and obviously seems to be specific for treatment with toxic MNNG doses.

When a cell is damaged, the cell has two options: repair or die. If the damages are too extensive the cell must also decide which cell death pathway to follow. Several ATP-dependent steps are required for apoptotic signal transduction and an excessive NAD/ATP depletion below 50% is believed to induce cell death by necrosis (56). Apparently, PARP-1 overactivation leads to necrotic cell death (24). Moreover, PARP-1-mediated necrotic cell death after treatment with 0.5 mM MNNG was detected in mice fibroblasts (24). Moreover, PARP-1-mediated necrotic cell death after treatment with 0.5 mM MNNG was detected in mice fibroblasts by HMGB1 exclusion (57), whereas AIF localization was not monitored in that study. On the other hand, accumulating PAR polymers appear to be an effective apoptotic stimulus signaling AIF release (25, 22). Additionally, studies with cortical neurons revealed that an accumulation of PAR and not excessive NAD consumption was responsible for initiation of apoptosis (22, 58).

Efficient progression of both DNA repair and apoptosis are essential for genome integrity. Poly(ADP-riboseylation) plays relevant roles in DNA damage sensing/repair and apoptosis. From our study we suggest that XRCC1 might be another important determinant regulating both processes.

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