Camptothenic-sensitive Relaxation of Supercoiled DNA by the Topoisomerase I-like Activity Associated with Poly(ADP-ribose) Polymerase-1*

Tetsu M. C. Yung, Marianne Parent, Erick L. Y. Ho, and Masahiko S. Satoh§

From the DNA Repair Group, Health and Environment Unit, Laval University Medical Center, Centre Hospitalier Universitaire de Québec, Faculty of Medicine, Laval University, Ste-Foy, Quebec G1V 4G2, Canada

Poly(ADP-ribose) polymerase-1 is a highly abundant nuclear enzyme implicated in transcription, DNA replication, and DNA repair through binding of nascent RNA and interactions with various factors. We found that purified fractions of recombinant human poly(ADP-ribose) polymerase-1 expressed in Escherichia coli possess yet another activity, a Mg2+-dependent DNA supercoil relaxation activity. Cleavage of recombinant poly(ADP-ribose) polymerase-1 by caspase-3, an apoptotic protease, reduced this activity, as did the removal of either of the two zinc finger motifs located in the N-terminal DNA-binding domain of poly(ADP-ribose) polymerase-1. In addition, this activity was separated from E. coli topoisomerase I by gel-filtration column chromatography, suggesting that this activity is specifically associated with poly(ADP-ribose) polymerase-1.

Because this relaxation activity did not require ATP and was resistant to VP16, a topoisomerase II inhibitor, this activity is closer to that of topoisomerase I. However, the supercoiled DNA relaxation activity associated with poly(ADP-ribose) polymerase-1 is distinct from that of human or E. coli topoisomerase I, as this activity could not completely remove superhelical tensions from plasmid DNA. Thus, we referred to this activity as topoisomerase I-like activity. This Mg2+-dependent DNA supercoil relaxation activity was found to be sensitive to camptothenic, a mammalian topoisomerase I inhibitor.

Poly(ADP-ribose) polymerase-1 (PARP-1)1 is a highly abundant nuclear enzyme present at ~1 × 10^6 molecules per nucleus (1). This protein has an N-terminal DNA-binding domain containing two zinc finger motifs, a C-terminal NAD+-binding domain that catalyzes the modification of proteins through the addition of multiple ADP-ribose polymers, and an automodification site between the C-terminal and N-terminal domains to which multiple polymers are added upon PARP-1 activation (2, 3). Poly(ADP-ribose)lation is an early cellular response to DNA damage (4, 5). Typically, PARP-1 automodification takes place within 5 min of cellular exposure to DNA-damaging agents (4, 5).

The amount of automodified PARP-1 reaches maximal levels within 20–60 min, and the ADP-ribose polymers are degraded within 60–120 min (4, 5). Cells that have this pathway blocked are more sensitive to induction of cell death by apoptosis following DNA damage (6–8). Because poly(ADP-ribose)lation is activated in response to DNA break formation, studies of PARP-1 have generally been focused on its role in DNA repair (9, 10). However, PARP-1 itself does not possess any DNA repair activity per se (11, 12). More recently, PARP-1 has been proposed to have roles in transcription, DNA replication, and DNA repair through interactions with transcription initiation activators (13), DNA polymerase α (14, 15), and DNA ligase III-XRCC-1 complexes (16–19), respectively. In addition, PARP-1 has been proposed to be functionally linked to topoisomerase I (Topo I) (20).

Topo I plays a critical role in the removal of superhelical tensions generated during transcription and DNA replication (21, 22). A link between PARP-1 and Topo I was first reported in 1983 by Jongstra-Bilen et al. (23). After Ultragel ACA 34, hydroxyapatite, blue dextran-Sepharose, Sephadex G-150, and phosphocellulose chromatography, Jongstra-Bilen et al. still found Topo I activity in purified fractions of PARP-1 prepared from calf thymus (23). Another link between PARP-1 and Topo I was reported by Boothman et al. (24). In cell extracts, Boothman et al. demonstrated that Topo I activity is transiently down-regulated when the extracts are prepared from cells exposed to DNA-damaging agents (24). Interestingly, the down-regulation of Topo I activity is inhibited by treatment of cells with a known PARP-1 inhibitor, 3-amino benzamide (24).

Because modification of a protein by ADP-ribose polymers often inhibits enzymatic activity of the protein (10), Boothman et al. suggested that poly(ADP-ribose)lation of Topo I was responsible for this down-regulation (24). To date, however, no clear evidence for poly(ADP-ribose)lation of Topo I in vivo has been obtained.

In addition to the possible role of PARP-1 in the removal of superhelical tensions through interaction with Topo I, PARP-1 alone can be involved in this removal, as we found a supercoiled DNA relaxation activity associated with PARP-1. In this report, we demonstrate that a purified fraction of recombinant human PARP-1 prepared from Escherichia coli lysates was able to relax supercoiled DNA. This activity was reduced either by automodification of PARP-1, upon removal of either of two PARP-1 zinc finger motifs or by treatment of recombinant PARP-1 (rPARP-1) with caspase-3, a protease known to cleave PARP-1 during apoptosis (25–27). Because of the tight

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† Recipient of the Fonds de la Recherche en Santé du Quebec Doctoral Award.
‡ Salary support award recipient from the Canadian Institutes of Health Research and NCIC. To whom correspondence should be addressed. Tel.: 418-656-4141 (ext. 47340); Fax: 418-654-2159; E-mail: Masahiko.sato@crchul.ulaval.ca.
1 The abbreviations used are: PARP-1, poly(ADP-ribose) polymerase-1; rPARP-1, recombinant poly(ADP-ribose) polymerase-1; Topo I, topoisomerase I; CPT, camptothenic; IPTG, isopropyl-β-D-thiogalactoside; ΔZn, zinc finger deletion mutant of PARP-1; PBS, pBlueScript II KS-; ΔZn1 or 2, zinc finger 1 or 2.
association of mammalian Topo I activity with PARP-1 purified from mammalian tissues, the activity of PARP-1 to relax DNA supercoils may have been overlooked. Interestingly, this activity was inhibited by camptothecin (CPT), an inhibitor of mammalian Topo I (28, 29).

MATERIALS AND METHODS

Antibodies and Enzymes—The monoclonal antibodies C-II-10 and F1–23, recognizing the automodification domain and the second zinc finger motif of PARP-1, respectively (30), were kindly provided by Dr. Guy Poirier. Human Topo I and anti-human Topo I antibodies were obtained from Topogen. An anti-E. coli Topo I antibody was raised by immunizing rabbits with a synthetic polypeptide, Lys-Thr-Ala-Lys-Lys-Pro-Lys-Lys-Asp-Glu-Arg-Gly-Ala-Cys–NH₂ buffer containing rPARP-1 and its Mutants—pET3a-PARP-1 and pET3a-24 kDa, expression constructs for the non-histidine-tagged rPARP-1 and the 24-kDa PARP-1 fragment, respectively, were prepared and used for transformation of HMS174 (DE3)pLySE as described previously (31). Expression of rPARP-1 and the 24-kDa PARP-1 fragment was induced with 0.4 mM isopropyl-p-D-thiogalactoside (IPTG) for 3 h at 37 °C. The proteins were then purified by phosphorylulose, double-strand DNA cellulose, and heparin-Sepharose column chromatography (31). pET3a-PARP-1-His, an expression construct for C-terminal histidine-tagged rPARP-1, was provided by Dr. Matsumoto and was used in the preparation of pET3a-89 kDa, pET3a-ZnA1, pET3a-ZnA2, and pET3a-(C125G) for expression of the 89-kDa PARP-1 fragment and pET3a-ZnA1 for expression of the zinc finger 1 deletion mutant of PARP-1 (ΔZn1) were prepared by using PCR to remove the sequences encoding Ala37–Asp114 and Ala41–His96, respectively. To prepare pET3a-Zn2 for expression of the zinc finger 2 deletion mutant of PARP-1 (ΔZn2), the DNA sequence corresponding to His90–Thr96 was removed by restriction digest of the internal XbaI fragment of the PARP-1 gene. To create the construct, pET3a-Zn2 (C125G), the sequence (Cys95-Gly) in zinc finger 2 was replaced by glycine using the Promega site-directed mutagenesis kit to create a point mutation (32). These constructs were used for transformation of HMS174 (DE3)pLySE. Expression of the mutant proteins was then induced by using 0.4 mM IPTG, as described (31), and the E. coli in culture was spun down at 3,500 × g for 10 min. The pellet was washed in phosphate-buffered saline and spun down. The resulting pellet was resuspended in WB buffer (10 ml of 50 mM Tris-HCl, pH 7.0, 10% glycerol, 2 mM NaCl, and 1 mM phenylmethylsulfonyl fluoride) containing 10 mM imidazole, and rPARP-1 or its mutants was extracted by sonication. After a 30-min centrifugation at 35,000 × g, the supernatant was used for purification.

NF2–nitrilotriacetic acid-agarose (Qiagen) was pre-equilibrated with WB buffer containing 10 mM imidazole, and 1 ml of NF2–nitrilotriacetic acid-agarose was added to the lysate. After 30 min of gentle shaking, NF2–nitrilotriacetic acid-agarose was loaded into a 10-ml empty disposable column, washed with WB buffer containing 1 mM imidazole until the absorbance at 280 nm fell below 0.1, and then washed with WB buffer containing 20 mM imidazole until the absorbance fell below 0.05. Before elution of the rPARP-1 mutants was eluted with WB buffer containing 250 mM imidazole and used after dialysis against 50 mM Tris-HCl, pH 7.0, 10% glycerol, 100 mM NaCl, and 2 mM MgCl₂.

Preparation of Recombinant E. coli Topo I—Histidine-tagged E. coli Topo I was prepared by PCR amplification of the E. coli Topo I cDNA from E. coli DH5α, and the amplified product was cloned into pET32a to generate pET32a-E. coli-Topo I-His. The cloned sequence was confirmed by DNA sequencing and was induced and purified as described above.

Protein Purification by Column Chromatography—Non-histidine tagged PARP-1 and the 24-kDa PARP-1 fragment were purified by phosphocellulose column chromatography, followed by double-strand DNA cellulose and heparin-Sepharose column chromatography, as reported (31). The 24-kDa PARP-1 fragment was further purified with a Superdex 75/100 gel filtration column chromatography, as reported (31). The protein liquid chromatography. This fragment was eluted with 50 mM Tris-HCl, pH 8.0, 12% glycerol, and 2 mM MgCl₂ with a flow rate of 0.4 ml/min.

DNA Relaxation Assay: Negative Supercoils—DNA relaxation assays were carried out in a buffer containing 50 mM NaCl, 12.5 μg/ml of pBluescript II KS⁺ (pBS), 5 mM MgCl₂, 10 mM Tris-HCl, pH 7.5, and purified PARP-1 in a 20-μl reaction mixture. The reactions were continued for 20 min at 37 °C and terminated by the addition of 0.5% SDS, 5 mM EDTA, and 250 μg/ml proteinase K. After 30 min of incubation at 37 °C, the DNA was precipitated by ethanol, dissolved in 10 mM Tris-HCl, pH 8.0, and 1 mM EDTA and applied to a 1% agarose gel for separate nicked open circular DNA from closed circular DNA and visualized by UV.

DNA Relaxation Assay: Positive Supercoils—pBS (50 μg/ml) was treated with 2000 units/ml of Topo I (Fermentas) in the buffer provided by the supplier for 60 min at 37 °C. The reactions were terminated by the addition of 0.5% SDS, 5 mM EDTA, and 250 μg/ml proteinase K. After 30 min of incubation at 37 °C, the DNA was precipitated by ethanol, dissolved in 10 mM Tris-HCl, pH 8.0, and 1 mM EDTA. Relaxation reactions were then carried out as described above, except for the addition of 1.5 μM ethidium bromide (33), and were terminated by the addition of phenol/chloroform. After precipitation of DNA by ethanol, the DNA was dissolved in 10 mM Tris-HCl, pH 8.0, and 1 mM EDTA and applied to a 1% agarose gel for analysis. The DNA was then stained with 0.5 μg/ml ethidium bromide and visualized by UV.

Poly(ADP-ribosylation) Reaction—Purified PARP-1 fractions were incubated with 13 μC [32P]NAD⁵⁺, 50 μM NaCl, 1 μM of dX 174 DNA-HaeIII digest (7.5 pmol of DNA breaks/ml), 5 mM MgCl₂, and 10 mM Tris-HCl, pH 7.5, in a 10-μl reaction mixture. Reactions were continued for 15 min at 37 °C and terminated by the addition of SDS-polyacrylamide gel loading buffer. After heating the samples at 95 °C for 5 min, samples were fractionated on SDS-7.5% polyacrylamide gels, and the poly(ADP-ribosyl)ated proteins were visualized by autoradiography after gel drying.

To prepare automated PARP-1, rPARP-1 was incubated in a buffer containing 10 mM Tris-HCl, pH 7.5, and 50 mM NaCl in the presence of DNA breaks (dX 174 DNA-HaeIII digest) and NAD⁺ (0.25 mM) in a 10-μl reaction mixture. The reactions were continued for 10 min at 30 °C, and the relaxation assays (negative supercoils) were carried out as described after the addition of 12.5 μg/ml of pBS, 5 mM MgCl₂, 50 mM NaCl, and 10 mM Tris-HCl, pH 7.5, to a total reaction volume of 20 μl.

Protein Analysis—Western blotting of PARP-1 and E. coli Topo I were carried out following a standard method. Silver staining of protein was carried out by using the Bio-Rad silver staining kit.

Cleavage of rPARP-1 by Caspase—3—rPARP-1 (0.5 pmol) was incubated with 0.4 μg of caspase-3 (PharMingen) for 30 min at 37 °C in a buffer containing 5 mM MgCl₂, 10 mM Tris-HCl, pH 7.5, and 50 mM NaCl in a 10-μl reaction mixture. DNA relaxation assays (negative supercoils) were then carried out as described above after the addition of 12.5 μg/ml of pBS, 5 mM MgCl₂, 50 mM NaCl, and 10 mM Tris-HCl, pH 7.5, to a total reaction volume of 20 μl.

RESULTS

Relaxation of Negatively Supercoiled DNA by PARP-1—The C-terminal histidine-tagged rPARP-1 was expressed in E. coli, HMS174 (DE3)pLySE/pET3a-PARP-1-His, and purified by Ni²⁺–nitrilotriacetic acid-agarose chromatography. As shown in Fig. 1A (Silver staining, lane 4), 116- and 89-kDa proteins were found in the purified fraction derived from the E. coli lysates. However, no apparent induction of protein was found in the equivalent fraction derived from E. coli, HMS174 (DE3)pLySE, lysates (Fig. 1A, lane 2), even after treatment with IPTG, suggesting that the presence of the 116- and 89-kDa proteins was dependent upon the expression of PARP-1 cDNA. These two products were recognized by C-II-10 monclonal antibody against PARP-1 (Fig. 1A, Western blotting, lane 8) and were found to be poly(ADP-ribosyl)ated after incubation of the purified fraction with DNA breaks and [32P]NAD⁺ (Fig. 1A, Poly(ADP-ribosylation, lane 12). These data allow us to identify the 116- and 89-kDa proteins as enzymatically active full-length rPARP-1 and C-terminal fragments of rPARP-1, respectively. We then tested these purified fractions for their ability to relax supercoiled DNA. As shown in Fig. 1B, lane 5, we found that the rPARP-1 fraction was able to resolve negative supercoils.

Previously, we used a three-step column chromatography procedure, including phosphocellulose, DNA cellulose, and heparin-Sepharose, to purify non-histidine-tagged rPARP-1 to over
95% homogeneity (31). In the final heparin-Sepharose purification step, rPARP-1 was eluted with a linear gradient of NaCl and was recovered in fractions near 1.6M NaCl. The DNA supercoil relaxation activity was found only in the fractions containing rPARP-1 (data not shown). The peak fractions were then pooled and, as shown in Fig. 2A, over 99% of the proteins in the pooled fraction were full-length rPARP-1 when analyzed by silver staining and Western blotting with the C-II-10 monoclonal antibody against PARP-1. This pooled fraction contained an Mg$^{2+}$-dependent DNA supercoil relaxation activity (Fig. 2B, lanes 5-8 with Mg$^{2+}$ versus lanes 9-12 with EDTA). The addition of 0.03 μg of protein (0.25 pmol of rPARP-1) eluted from the heparin-Sepharose column to the reaction (Fig. 2B, lane 8) was sufficient to observe a relaxation activity, whereas 0.24 μg and 0.36 μg of protein in the eluate from the double-strand DNA cellulose and phosphocellulose columns, respectively, were required to obtain a similar level of activity, suggesting that the specific activity increased after purification. On the other hand, when corresponding fractions prepared from E. coli, HMS174 (DE3)pLysE, were used, no relaxation activity
was found (Fig. 2B, lane 2). Thus, the Mg$^{2+}$-dependent DNA supercoil relaxation activity was only found in fractions where rPARP-1 is present. Because the relaxation activity does not require ATP, an essential cofactor for type II topoisomerases (21, 22), the relaxation activity present in rPARP-1 fraction may be more similar to that of type I topoisomerases, including Topo I and topoisomerase III. *E. coli* topoisomerase III shows a higher DNA supercoil relaxation activity at 56 °C than at 30 °C (34). However, as the relaxation activity present in rPARP-1 was not promoted at 56 °C (data not shown), this activity is likely closer to that of Topo I. As shown in Fig. 2B, both human Topo I (lanes 13–16) and *E. coli* Topo I (lanes 17–20) were capable of converting supercoiled DNA into its relaxed form. On the other hand, a significantly reduced amount of plasmid DNA in its relaxed form was produced by the activity associated with rPARP-1 (Fig. 2B, lanes 5–8) and plasmids with linking numbers ranging from 1 to 5 were found instead. These plasmids were produced as early as after 2.5 min of incubation, and the amount of plasmids increased as more supercoiled DNA were relaxed (Fig. 2C). Furthermore, this pattern was not changed by the addition of increased amounts of rPARP-1 (Fig 2B, lanes 5 and 6). Thus, the relaxation activity present in the rPARP-1 fraction is apparently distinct from that of *E. coli* Topo I. As no detectable *E. coli* Topo I was found in the rPARP-1 fraction (Fig. 1A, lane 17), we concluded that the unique supercoiled DNA relaxation activity is specifically associated with the purified rPARP-1.

**Fig. 3. Reduced relaxation activity of supercoiled DNA by caspase-3 cleavage of rPARP-1.** A, histidine-tagged rPARP-1 (0.5 pmol) was incubated with 0.4 μg of caspase-3 for 30 min at 37 °C. Cleaved products were visualized with the C-II-10 monoclonal antibody, which recognizes the automodification domain of PARP-1, and with the F1–23 monoclonal antibody, which recognizes the second zinc finger motif of PARP-1. Under these conditions, nearly 90% of rPARP-1 was cleaved. *E. coli* Topo I was also treated with caspase-3 and used for Western blotting with anti-*E. coli* Topo I antibody. B, relaxation assays of supercoiled DNA were carried out by using caspase-3-cleaved rPARP-1.

Reduced DNA Relaxation Activity After Treatment of rPARP-1 with Caspase-3—Caspase-3 is a protease activated during apoptosis (25). PARP-1 is known to be a substrate of this protease that requires the peptide sequence Asp-Glu-Val-Asp for cleavage (25, 26). This cleavage sequence is not present in the catalytic domain (26, 27). When 0.5 pmol of rPARP-1 was incubated with 0.4 μg of caspase-3, the 89-kDa and 24-kDa PARP-1 fragments could be visualized by Western blotting with the C-II-10 monoclonal antibody (Fig. 3A, lane 9 versus 10). The caspase-3 cleavage sequence of PARP-1 is located between the second zinc finger motif and the automodification site (see Fig. 4A for illustration, and Refs. 26, 27). Thus, cleavage of PARP-1 by caspase-3 results in the formation of a 24-kDa N-terminal fragment, containing the two zinc finger motifs, and an 89-kDa C-terminal fragment, composed of the automodification site and the catalytic domain (26, 27). When 0.5 pmol of rPARP-1 was incubated with 0.4 μg of caspase-3, the 89-kDa and 24-kDa PARP-1 fragments could be visualized by Western blotting with the C-II-10 monoclonal antibody (Fig. 3A, lane 4) that recognizes the automodification domain of PARP-1 (30) and the F1–23 monoclonal antibody (Fig. 3A, lane 8) that recognizes the second zinc finger motif of PARP-1 (30). Based on these results, we determined that ~90% of rPARP-1 (0.45 pmol) was cleaved under our caspase-3 cleavage conditions. These cleaved products were then used in relaxation assays. As shown in Fig. 3B, negligible relaxation activity was detected in reactions containing 0.5 pmol of recombinant 24-kDa or 89-kDa PARP-1 fragments, in contrast to full-length rPARP-1, which showed a significant relaxation activity. Therefore, if PARP-1 is directly involved in the relaxation of supercoiled DNA, conversion of rPARP-1 to its 24-kDa and 89-kDa fragments by caspase-3 should reduce its relaxation activity. In fact, a significant reduction in relaxation activity was observed when rPARP-1 was treated with caspase-3 (Fig. 3B), indicating that PARP-1 is involved in the relaxation of supercoiled DNA.
Reduced DNA Relaxation Activity After Removal of the Zinc Finger Motifs of PARP-1—To identify the domain in PARP-1 involved in relaxation of supercoiled DNA, we tested the ability of the 24-kDa or the 89-kDa PARP-1 fragments to relax supercoiled DNA (Fig. 4A). As described previously, these fragments are found during apoptosis (26, 27). As shown in Fig. 3B, 0.5 pmol of the 24-kDa and 89-kDa rPARP-1 fragments had a negligible amount of relaxation activity. Although concentrations of up to 10 pmol of the 89-kDa rPARP-1 fragment continued to show no detectable relaxation activity, the use of 2.5 pmol of the 24-kDa rPARP-1 fragment resulted in measurable relaxation activity (Fig. 4B). Previously, we demonstrated that the 24-kDa rPARP-1 fragment is capable of binding DNA breaks and RNA, although its affinity for DNA breaks and RNA is reduced by 75 and 85%, respectively, comparable with the affinity of full-length rPARP-1 for these nucleic acids (31). Thus, the reduced relaxation activity of the 24-kDa rPARP-1 fragment (about 5-fold lower than that of full-length rPARP-1) may be due to its reduced binding affinity for nucleic acids. Taken together, these results suggest that the DNA-binding domain of PARP-1 is involved in the relaxation of supercoiled DNA. Therefore, we prepared rPARP-1 deletion mutants in which either the zinc finger 1 (ΔZn1) or zinc finger 2 (ΔZn2) was deleted (Fig. 4A). Both ΔZn1 and ΔZn2 retained the ability to relax DNA supercoils (Fig. 4C), although their relaxation activity was lower than wild-type rPARP-1 (Fig. 4C). In addition, when ΔZn2 was disrupted by replacement of a Cys with Gly at amino acid 125 (32), this relaxation activity was still found (Fig. 4C), indicating that the presence of one zinc finger alone was sufficient to provide a relaxation activity.

Association of DNA Supercoil Relaxation Activity with the 24-kDa PARP-1 Fragment—To further study whether the DNA supercoil relaxation activity is associated with the DNA-binding domain that contains two zinc fingers, the 24-kDa PARP-1 fragment was size-fractionated using Superose 12 HR 10/30 gel filtration column chromatography. As shown in Fig. 5A, the 24-kDa PARP-1 fragment migrates around 30 kDa (31).
PARP-1 fragment was eluted from the column 15.8 min after elution of the blue dextran 2000. Under these purification conditions, E. coli Topo I was eluted 11.4 min after blue dextran 2000 and 4.5 min earlier than the 24-kDa PARP-1 fragment, allowing the separation of the 24-kDa PARP-1 fragment from E. coli Topo I (Fig. 5A). When the 24-kDa PARP-1 fragment, purified to over 99% homogeneity (Fig. 5C and Ref. 31), was used for fractionation (Fig. 5C), no detectable amount of activity to relax DNA supercoils was found in the fractions in which E. coli Topo I was expected to be eluted (Fig. 5B fraction 6 for E. coli Topo I versus Fig. 5C, fraction 6 for the 24-kDa PARP-1 fragment), suggesting that the purified fractions of the 24-kDa PARP-1 fragment do not contain detectable amounts of E. coli Topo I activity. We then measured the activity to relax DNA supercoils in fractions containing the 24-kDa PARP-1 fragment. As shown in Fig. 5C, the majority of fragments was eluted in fraction 8, and we found a DNA supercoil relaxation activity in the same fraction. Thus, these results suggest that the relaxation activity present in the 24-kDa PARP-1 fragment is not due to the contamination of E. coli Topo I and that the activity is indeed associated with the 24-kDa PARP-1 fragment.

**Suppression of DNA Relaxation Activity After Automodification of PARP-1**—The binding affinity of the zinc finger motifs for DNA breaks and RNA is reduced by automodification of PARP-1. We then studied the effect of automodification on the activity to relax DNA supercoils. Incubation of rPARP-1 with 75 fmol of DNA breaks at a low concentration of NAD\(^+\) (2 nM of \[^{32}\text{P}]\text{NAD}^+\) resulted in the automodification of rPARP-1 with short ADP-ribose polymers (Fig. 6, Poly(ADP-ribosyl)-ation). Under identical PARP-1 automodification reaction conditions, E. coli Topo I appeared not to be a substrate of PARP-1, as poly(ADP-ribosyl)ated E. coli Topo I was not found (Fig. 6, Poly(ADP-ribosyl)-ation), even after the addition of a 1000-fold excess of the E. coli Topo I (4 μg; Fig. 6, Silver staining), an amount required to completely relax 250 ng of pBS (Fig. 2B). We then tested the effect of poly(ADP-ribosyl)ation on the ability of the purified rPARP-1 to relax supercoiled DNA. In the presence of 150 fmol or more of DNA breaks, the DNA supercoil relaxation activity was reduced (Fig. 7, lane 3), possibly because of competition between supercoiled DNA and an excess of DNA breaks for rPARP-1 binding. Thus, we used 75 fmol of DNA breaks, which did not appear to affect the supercoil relaxation activity of the purified rPARP-1 fraction (Fig. 7, lane 4), for our analysis. Addition of NAD\(^+\) (0.25 mM) together with 75 fmol of DNA breaks strongly suppressed the relaxation activity (Fig. 7, lane 6), whereas even in the presence of DNA breaks and NAD\(^+\), inhibition of rPARP-1 automodification by 3-aminobenzamide restored the relaxation activity (Fig. 7, lane 7). Thus, these results again suggest that the DNA supercoil relaxation activity is associated with PARP-1, and PARP-1 automodification inhibits this activity.

**CPT Sensitivity of the Supercoiled DNA Relaxation Activity Associated with rPARP-1**—As shown in Fig. 8A, 0.2 units of human Topo I activity was significantly inhibited by 0.25 mM of CPT, an inhibitor of mammalian Topo I (28, 29). The relaxation activity associated with the purified rPARP-1 (0.5 pmol of rPARP-1) was similarly inhibited by 0.25 mM of CPT. In addition, Topotecan, another inhibitor of Topo I (35, 36), also inhibited this relaxation activity (data not shown). On the other hand, VP16, an inhibitor of topoisomerase II (28, 35), had no effect on the relaxation activity (Fig. 8A). These data suggest that PARP-1 is a target for Topo I inhibitors. In the case of mammalian Topo I, CPT stabilizes intermediate DNA-Topo I complexes that result from the covalent interaction between the active site Tyr of Topo I (Tyr\(^{235}\) in the case of human Topo I) and the 5′ ends of DNA nicks (37). Thus, denaturation or proteolytic digestion of mammalian Topo I after incubation with supercoiled DNA in the presence of CPT results in the production of nicked open circular DNA (29). Using 20× the amount of human Topo I used in the relaxation assay (4 units instead of 0.2 units) in the presence of CPT, an increase in nicked open circular DNA was indeed observed (Fig. 8B). By contrast, a 20-fold increase in rPARP-1 (10 pmol) resulted in no increase in open circular DNA (Fig. 8B). Even the addition of up to 60 pmol of rPARP-1 did not increase the amount of open circular DNA observed (data not shown). Therefore, we have thus far obtained no evidence that PARP-1 forms covalent complexes with DNA ends in the presence of CPT.

**DISCUSSION**

In this report, we have demonstrated that a purified fraction of rPARP-1 prepared from E. coli lysates contains the activity to relax negatively supercoiled DNA. This relaxation activity requires Mg\(^{2+}\), in a manner similar to that of E. coli Topo I (21, 22). However, the relaxation activity associated with rPARP-1 fractions is not due to a contamination by E. coli Topo I, as no detectable amount of E. coli Topo I was found in the rPARP-1 fraction (Fig. 1A). In addition, the specific cleavage of rPARP-1 by caspase-3 resulted in a reduction of the relaxation activity (Fig. 3B), and rPARP-1 automodification reduced this activity (Fig. 7). Because the relaxation of DNA supercoils occurred in the absence of ATP, a topoisomerase II cofactor (Fig. 2B), and the activity was not sensitive to a topoisomerase II inhibitor, VP16 (Fig. 8A), the supercoiled DNA relaxation activity associated with PARP-1 is closer to that of topoisomerase I. However, this activity is distinct from that of human and E. coli Topo I, as PARP-1 was incapable of converting supercoiled DNA to relaxed supercoiled DNA.
DNA into its relaxed form (Fig. 2B). Thus, we refer to this activity as Topo I-like activity.

**Possible Mechanism of Superoiled DNA Relaxation Mediated by PARP-1**—During the relaxation assay of DNA supercoils by rPARP-1, plasmids with linking numbers ranging from 1 to 5 were produced (Fig. 2B). These plasmids were not converted to a relaxed form even after the addition of saturating amounts of rPARP-1 (Fig. 2B). Furthermore, these plasmids were produced as soon as after 2.5 min of incubation, and the pattern did not change after longer incubation times (Fig. 2C). Because human and *E. coli* Topo I are capable of converting supercoiled plasmid DNA into their relaxed form, the mechanism of supercoiled DNA relaxation by PARP-1 may be distinct from human and *E. coli* Topo I. Human and *E. coli* Topo I relax DNA supercoils through the formation of covalent complexes with either the 3′ (human Topo I) or 5′ (*E. coli* Topo I) DNA ends by means of tyrosine residues (21, 22). In the 24-kDa PARP-1 fragment, six tyrosine residues (amino acids 2, 6, 45, 109, 150, and 168) are present. However, Tyr<sub>168</sub> is not conserved across species, and removal of Tyr<sup>2</sup> and Tyr<sup>6</sup> (ΔZN1), or Tyr<sup>45</sup>, Tyr<sup>109</sup> and Tyr<sup>150</sup> (ΔZN2) did not completely eliminate the relaxation activity associated with the rPARP-1 fraction (Fig. 4). Thus, none of the tyrosine residues seems to be a prominent candidate for the formation of a covalent complex with DNA ends. Although it is plausible that PARP-1 resolves DNA supercoils through covalent DNA complex formation with other amino acid residues, PARP-1 may unwind supercoils without forming covalent complexes. In this regard, the mechanism of supercoiled DNA relaxation by rPARP-1 may be explained by a DNA nick induction followed by sealing of this nick. As shown in Fig. 2B, nicked circular plasmids were produced by a treatment of supercoiled DNA with a limited amount of DNase I (lane 3). Then, the nicked circular plasmids DNA were converted into closed circular plasmids with linking numbers ranging from 1 to 5 after DNA nick sealing with T4 DNA ligase (lane 4). Interestingly, the DNA ladder pattern produced by DNase I and T4 DNA ligase treatment resembled the one produced by rPARP-1 (Fig. 2B, lane 4 versus 5). If PARP-1 can induce DNA nicks and the sealing of nicks, the production of these plasmids with linking numbers ranging from 1 to 5 by rPARP-1 can be explained as well as can the fact that the ladder pattern does not change during the supercoiled DNA relaxation assay with rPARP-1 (Fig. 2B) or by the addition of saturating amounts of PARP-1 (Fig. 2B). PARP-1 is an enzyme with an affinity for DNA and RNA (38, 39). This binding perhaps requires the formation of various interactions between the amino acid residues of the zinc finger and the DNA. During these interactions, DNA nicking and nick sealing might occur, and thus, PARP-1 could relax DNA supercoils.

Inhibition of DNA supercoil relaxation activity by CPT. A, relaxation reactions were carried out in the presence of either CPT or VP16 with rPARP-1 (0.5 pmol) or human Topo I (0.2 units) under assay conditions (described under "Materials and Methods"), except for the presence of 1% dimethyl sulfoxide. B, relaxation reactions were carried out with 10 pmol rPARP-1 or 4 units of human Topo I. After termination of reactions, the proteins were digested with proteinase K and precipitated by ethanol. Then, the purified DNA was fractionated using agarose gel electrophoresis in the presence of ethidium bromide to separate nicked circular DNA from covalently closed circular DNA.

**Human Topo I and PARP-1**—In vivo superhelical tensions are produced during transcription and DNA replication (21, 22). By using live-cell analysis with confocal microscopy, we showed recently that PARP-1 is concentrated into actively transcribed and replicated regions, such as the nucleolus and nucleoplasm in G<sub>1</sub> phase cells, and S-phase foci in S-phase cells. This concentration possibly occurs because of the binding of PARP-1 to nascent RNA or DNA loops (31, 39, 40) through the zinc finger motifs of PARP-1, as N-terminal PARP-1 domain, lacking zinc fingers, cannot localize properly. By concentrating these regions, PARP-1 may allow the removal of superhelical tensions created in these regions. However, because PARP-1 cannot completely remove the superhelical tensions from plasmid DNA, as described, PARP-1 alone may not be sufficient for the removal of such tensions, and thus other topoisomerases must also be required. Interestingly, by using live-cell analysis, we also found that PARP-1 tightly co-localizes with Topo I throughout the cell cycle. Thus, Topo I can be concentrated into actively transcribed and replicated regions through interactions with PARP-1. In addition, it has been demonstrated that the activity of Topo I is promoted by an interaction with PARP-1 (20). Thus, the concentration of Topo I in actively transcribed and replicated regions and the promotion of Topo I activity by PARP-1 may increase the superhelical tension removal efficiency. Taken together, this accumulated evidence suggests that PARP-1 is an enzyme involved in the removal of DNA superhelical tensions through its Topo I-like activity and its cooperation with Topo I.

PARP-1 as a Target of Topo I Inhibitors—Inhibitors of Topo I, developed for the treatment of cancer (28), also seem to target PARP-1 (Fig. 8A). Topo I inhibitors exert their cytotoxic effects by stabilizing Topo I-DNA complexes, thereby disrupting DNA replication and transcription (41, 42). Because relaxation assays using purified rPARP-1 and CPT revealed no such complexes (Fig. 8B), inhibition of the Topo I-like activity of PARP-1 may not disrupt DNA replication and transcription in a manner similar to that of Topo I inhibition. However, if the Topo I-like activity of PARP-1 is involved in the relaxation of supercoiled DNA in vivo, inhibition of this activity may also lead to cytotoxicity because of inhibition of transcription and DNA replication. At this stage, drugs that specifically target either Topo I or the Topo I-like activity of PARP-1 have not yet been identified. The development of such drugs may provide more evidence suggests that PARP-1 is an enzyme involved in the removal of DNA superhelical tensions through its Topo I-like activity and its cooperation with Topo I.
effective cancer treatments by allowing a better control over the cytotoxic effects of anticancer drugs.

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Topo I-like Activity in PARP-1
Camptothecin-sensitive Relaxation of Supercoiled DNA by the Topoisomerase I-like Activity Associated with Poly(ADP-ribose) Polymerase-1
Tetsu M. C. Yung, Marianne Parent, Erick L. Y. Ho and Masahiko S. Satoh

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