Deletion Mutants of Poly(ADP-Ribose) Polymerase Support a Model of Cyclic Association and Dissociation of Enzyme from DNA Ends During DNA Repair†

Mark Smulson,* Nancy Istock, Ruchuang Ding, and Barry Cherney‡

Georgetown University School of Medicine, Department of Biochemistry and Molecular Biology, Basic Science Building, Room 351, 3900 Reservoir Road, N.W., Washington, D.C. 20007

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ABSTRACT: With an *in vitro* DNA repair system, Satoh and Lindah [(1992) *Nature 356*, 356–358] demonstrated that unmodified poly(ADP-ribose) polymerase (PADPRP) binds to radiation-damaged DNA and inhibits repair in the absence of NAD. However, in the presence of NAD, PADPRP undergoes automodification and the DNA is repaired. It was hypothesized that PADPRP cycles between an unmodified form, which protects DNA breaks, and an automodified form, which is released from DNA, thereby allowing access to repair enzymes. We have now tested this model with bacterially expressed mutants of PADPRP with deletions in the three major functional domains of the enzyme [Cherney, B. W., Chaudry, B., Bhatia, K., Butt, T. R., & Smulson, M. E. (1991) *Biochemistry 30*, 10420–10427]. Deletion mutants with an intact amino-terminal DNA-binding domain, and therefore capable of binding to DNA strand breaks in the *in vitro* assay, inhibited repair; however, whether the deletion was in the NAD-binding, active site domain or the automodification domain, the inhibition of repair exerted by these mutant proteins was not alleviated by NAD. A PADPRP mutant with a deletion in the DNA-binding domain did not inhibit DNA repair. Thus, the behavior of these PADPRP deletion mutants is consistent with the model proposed earlier. The model was also supported by experiments with Manley extracts of HeLa cells stably transfected with a PADPRP antisense RNA construct. Extracts of cells induced to express antisense RNA did not markedly inhibit *in vitro* DNA repair, nor did the addition of NAD influence the assay. In contrast, noninduced cell extracts inhibited repair and inhibition was alleviated by NAD. Finally, exposure of nuclei from mid-S phase HeLa cells to NAD increased single deoxynucleotide incorporation as catalyzed by exogenous *Escherichia coli* DNA polymerase. This effect, probably attributable to chromatin restructuring, also may be explained, at least in part, by the proposed model for PADPRP cycling and DNA strand break rejoining.

The poly(ADP-ribosyl)ation of nuclear proteins plays prominent roles in the modulation of chromatin structure adjacent to regions of DNA replication, recombination, and repair (Jacobson & Jacobson, 1989; Poirier & Moreau, 1992). The enzyme poly(ADP-ribose) polymerase (PADPRP) catalyzes this reaction in the presence of DNA and the substrate NAD and modifies specific nuclear proteins (for example, histones and topoisomerases) located adjacent to DNA strand breaks (Thraves et al., 1985). PADPRP also undergoes extensive automodification with the attachment of ADP-ribose polymers via an ester linkage to glutamic acid residues (Cherney et al., 1987; Kawaichi et al., 1981). The various activities of PADPRP are organized into separate functional domains. These domains comprise an amino-terminal DNA-binding domain, which contains two zinc fingers linked through a flexible glycine-rich region and shows no sequence identity to other zinc finger proteins, a central hydrophilic automodification domain containing 15 glutamic acid residues, and a carboxy-terminal NAD-binding domain. It was recently demonstrated that DNA repair is severely limited in eukaryotic cells expressing PADPRP antisense mRNA (Ding et al., 1992) or the partial DNA-binding domain of the enzyme (Molinete et al., 1993).

We have recently expressed a full-length human PADPRP cDNA, as well as various constructs with deletions in the three functional domains, under the control of the heat-inducible lambda P_L promoter in *Escherichia coli* (Cherney et al., 1991). This expression system directed the synthesis of ubiquitin–PADPRP fusion proteins in which ubiquitin was attached to the amino terminus of PADPRP. The full-length fusion protein possessed catalytic properties nearly identical to those of native PADPRP. Deletion of the PADPRP NAD-binding domain abolished catalytic activity; however, partial polymerase activity remained after deletions in the DNA-binding or automodification domains.

Satoh and Lindahl (1992) have described an *in vitro* DNA repair system in which γ-irradiated plasmid DNA containing single-strand breaks can be repaired to closed circular DNA by using Manley extracts of human cells (Manley et al., 1983). DNA repair in this system is stimulated by NAD. Removal of PADPRP from the extract increases the basal level of DNA repair and abolishes NAD sensitivity; the basal level of repair is reduced and NAD sensitivity restored on addition of purified PADPRP to the reaction. These authors proposed a model to explain their results: non-poly(ADP-ribosyl)ated PADPRP molecules present in the extract bind tightly to DNA strand breaks in the plasmid substrate; subsequently, automodification of PADPRP, because of the associated large negative charge, results in release of the enzyme from the 3′-OH of the DNA, thereby allowing access of DNA repair enzymes (Satoh & Lindahl, 1992).

We have now used the previously characterized functional domain mutants of PADPRP , as well as Manley extracts derived from HeLa cells transfected with a PADPRP antisense

* To whom reprints should be addressed.

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‡ Present Address: Laboratory of Immunology, Food and Drug Administration, CBER FDA, 8800 Rockville Pike, Bldg. 29A, Room 2D06, Bethesda, MD 20892.

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RNA construct (Ding et al., 1992) and a chromatin-associated DNA rejoining assay, to further clarify the validity of the model proposed above.

MATERIALS AND METHODS

Cell Culture. HeLa cells adapted for spinner culture were obtained from Norman Cooper at the National Institutes of Health and maintained in MEM Spinner Medium (Quality Biologicals) supplemented with 5% (v/v) horse serum (Gibco-BRL). HeLa cell extracts were prepared according to the protocol of Manley et al. (1983). Whole-cell extracts for pilot experiments were a gift from John Brady and Michael Radonovich at the National Institutes of Health. Extracts were depleted of PADPRP by passage through a column of double-stranded DNA-cellulose (Sigma) (Satoh & Lindahl, 1992). HeLa cells transfected with an antisense RNA construct (Ding et al., 1992) were grown in 150-mm dishes to near confluence and harvested by scraping. They were induced for PADPRP antisense expression for 72 h with 1 μM dexamethasone as described in detail earlier (Ding et al., 1992).

DNA Repair Reactions. γ-Irradiated pBluescript plasmids for use as substrate in the DNA repair reactions were prepared as previously described (Satoh & Lindahl, 1992). Briefly, CsCl-purified closed circular plasmids were exposed to 50 Gy of γ-rays from ^{137}Cs in a Gamma Radiator 100. Complete or PADPRP-depleted extracts were diluted to the desired protein concentration in cell extract resuspension buffer described by Manley et al. (1983). When appropriate, recombinant PADPRP proteins were added to PADPRP-depleted extract and included in the volume of extract added to the reaction mixture. NAD (final concentration 2 mM) was added to the reaction mixture in the form of a 10 mM stock solution dissolved in 20 mM Hepes (pH 7.8). Creatine phosphokinase was dissolved in 0.25 M glycylglycine buffer (pH 7.4) at a concentration of 2.5 mg/mL. The final concentrations of all components of the DNA repair reactions and the overall protocol was essentially similar to that used earlier (Satoh & Lindahl, 1992).

Densitometry. Quantity One Scanning and Analysis software from Pdi (Huntington Station, NY) was used for densitometry of photographic negatives of ethidium bromide stained agarose gels. The percentage DNA repair was calculated by dividing the quantity of plasmid DNA in the repair band (closed circular plasmid), adjusted by a factor of 1.45 to compensate for reduced binding of ethidium bromide, by the total amount of closed circular and open circular DNA (Wood et al., 1988).

PADPRP Activity Assay and Immunoblot Analysis. PADPRP activity was assayed as described previously (Cherney et al., 1991) . Complete or PADPRP-depleted extracts (20–25 μg of protein) were incubated in a final volume 125 μL containing 50 mM Tris-HCl (pH 8.0), 25 mM $MgCl_2$, and 0.11 mM NAD containing 2 μCi of [^{32}P]NAD for 1 min at 25 °C. Exogenous DNA, when added, consisted of 0.1–1.0 μg of sheared, single-stranded salmon sperm DNA. Reactions were terminated by addition of 2 mL of ice-cold 20% (w/v) trichloroacetic acid (TCA). Acid-precipitable material was collected on Whatman GF/C filters, which were then rinsed first with 20% TCA and then with 95% (v/v) ethanol. Incorporation of radioactivity was measured by liquid scintillation counting. The activity of recombinant PADPRP from bacterial extracts was measured in the same manner.

For immunoblot analysis, proteins were resolved by SDS–polyacrylamide gel electrophoresis on 7.5% gels and transferred to nitrocellulose. Antiserum to human PADPRP was used to probe blots together with the ProtoBlot alkaline phosphatase system (Bio-Rad).

Table 1: PADPRP Activity in Complete and PADPRP-Depleted HeLa Cell Extracts and in Extracts of *E. coli* Expressing Recombinant Full-Length PADPRP[a]

extract	DNA	benzamide	PADPRP activity (%)[b]	relative PADPRP activity (cpm)[c]
complete HeLa	+	–	100	7.5×10^4
	–	–	4	
	+	+	6	
PADPRP-depleted HeLa	+	–	7	
induced *E. coli*	+	–		3.5×10^4
	+	+		0.5×10^4

[a] Extracts were assayed for PADPRP activity as described under Materials and Methods. [b] PADPRP activity expressed as percentage of that observed with the complete HeLa extract in the presence of exogenous DNA. Results represent the means of four experiments. [c] PADPRP activity of portions of complete HeLa cell (20 μg of protein) and *E. coli* (1 μg of protein) extracts containing approximately equivalent amounts of PADPRP protein as judged by immunoblot analysis. Results are from a representative experiment.

Recombinant PADPRP Deletion Mutants. PADPRP and deletion mutants PM-1, PM-4, PM-5, and PM-6 were expressed as ubiquitin fusion proteins as described in detail by Cherney et al. (1991). After induction of *E. coli* and sonication in polymerase buffer, the disrupted cells were separated by centrifugation and the cells resuspended in polymerase buffer containing 0.5 M KCl. The pellet was centrifuged again, and the pellet was reextracted with 0.5 M KCl. This was recentrifuged and the supernatant used as source of polymerase. When appropriate, 1.0 μg of protein from these extracts was included with PADPRP-depleted extract in DNA repair reactions.

RESULTS

PADPRP Depletion of HeLa Cell Extracts. HeLa cell extracts were prepared according to the protocol of Manley et al. (1983), that is, under conditions that should exclude chromosomal DNA. The poly(ADP-ribosyl)ation activity of such extracts was virtually completely dependent on the addition of sonicated, single-stranded salmon sperm DNA, which comprised fragments ~600 nucleotides in length (Table 1). PADPRP activity was abolished in the presence of benzamide, an inhibitor of the enzyme. We prepared PADPRP-depleted Manley extracts by chromatography on double-stranded DNA–cellulose in 0.4 M NaCl (Slattery et al., 1983). An intact PADPRP protein band was not detectable in PADPRP-depleted extracts by immunoblot analysis with a rabbit antiserum to human PADPRP (Figure 1, panels A and B). A minor immunologically reactive band (approximately 100 kDa) smaller than PADPRP was occasionally observed in PADPRP-depleted extracts (Figure 1 B); if this band represents a fragment of PADPRP, it would appear to lack a large portion of the DNA-binding domain because it was retained by the DNA–cellulose column in barely detectable amounts (Figure 1C). In contrast, an immunoreactive 116-kDa band corresponding to PADPRP was eluted from the DNA–cellulose column by 1 M NaCl (Figure 1C). The PADPRP-depleted extract was essentially devoid of PADPRP activity (Table 1).

For the experiments planned with PADPRP deletion mutants, it was important that the amount of recombinant full-length PADPRP added back to PADPRP-depleted extracts be approximately equivalent to the amount of native

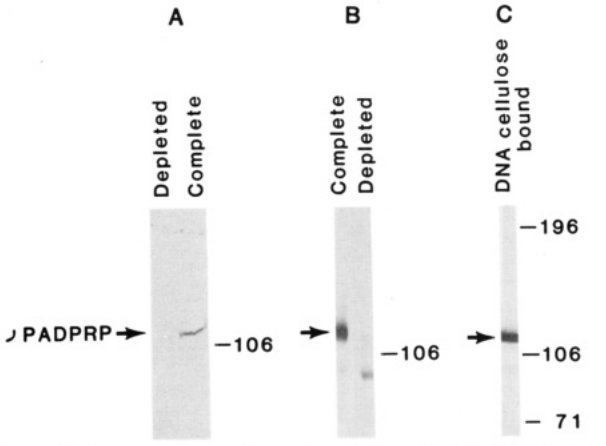


FIGURE 1: Immunoblot analysis of complete and PADPRP-depleted HeLa cell extracts. (A and B) Complete or PADPRP-depleted HeLa cell extracts (20 μg of protein) subjected to immunoblot analysis with a rabbit antiserum to human PADPRP. (C) Protein (<1 μg) eluted with 1 M NaCl from a double-stranded DNA–cellulose column after application of a HeLa cell extract (Satoh & Lindahl, 1992). The positions of molecular size markers (in kilodaltons) are indicated. Arrows mark the position of PADPRP.

PADPRP present in normal Manley extracts. It had previously been shown that the K_m for NAD and the activity of bacterially expressed PADPRP were nearly identical to those of the native enzyme (Cherney et al., 1991). We estimated the approximate amount of the 116-kDa PADPRP protein in both Manley HeLa extracts and extracts from induced bacteria by immunoblot analysis. The Manley extract contained a larger number of proteins than the bacterial extract and probably also contained other poly(ADP-ribose)-metabolizing enzymes, for example, poly(ADP-ribose) glycohydrolase. Thus, we calculated that 1 μg of bacterial extract should yield approximately the same PADPRP activity as 20 μg of a Manley extract. This equivalence ratio proved to be approximately correct (Table 1), and so in later experiments we added 1 μg of bacterial extract containing recombinant PADPRP to approximately 20 μg of PADPRP-depleted extract.

Conditions for DNA Repair. We determined the time course of DNA repair with the Manley extract prepared in our laboratory (Figure 2). The amounts of open circular plasmid DNA (substrate) and closed circular DNA (product) recovered from each reaction were determined by densitometry of photographic negatives from agarose gels and the percentage repair calculated. Essentially no repair was observed after 2 min of incubation. However, after 10 min, a band of closed circular plasmid DNA was detected. After 30 min, the maximal amount of DNA repair appeared to have occurred, and NAD stimulation of the reaction was apparent.

Different preparations of Manley extracts varied in their relative efficiencies (5–60%) in catalyzing the conversion of open circular to closed circular plasmid DNA, perhaps attributable to limitations or ranges of concentrations of the repair enzymes in the extracts of HeLa cells grown under our experimental conditions. It had previously been observed that the stimulation of *in vitro* DNA repair by NAD is dependent on the amount of extract used (Satoh & Lindahl, 1992). To determine the optimum extract concentration for the repair assay, we incubated various amounts (1.5–100 μg) of protein extract in the presence or absence of NAD and measured the conversion of open circular to closed circular plasmid DNA. The extent of repair (5–60%) increased with the amount of extract (data not shown). In each instance, the addition of NAD to the assay stimulated DNA repair, consistent with the earlier data (Satoh & Lindahl, 1992). On the basis of these results, we subsequently used 20–25 μg of protein extract per assay, an amount that appeared to produce the most consistent data and resulted in up to 50% repair in the presence of NAD.

Effect of NAD on the Accessibility of 3′-OH Primer Sites-In Nuclei. We reinvestigated (Roberts et al., 1974) the effect of NAD on single deoxynucleotide incorporation into the chromatin of nuclei from mid-S phase HeLa cells. A 2–3-fold increase in single *deoxynucleotide* incorporation was observed in nuclei that had been preincubated with NAD (4 mM) (Figure 3). Similar results had been obtained earlier in nuclei probed with endogenous, rather than exogenous, DNA polymerase (Roberts et al., 1974). This effect of NAD was shown to be attributable to nuclear poly(ADP-ribosyl)-ation because it was prevented by the PADPRP inhibitor nicotinamide (Roberts et al., 1974). In view of the Satoh and Lindahl model for the contribution of PADPRP to DNA repair, as well as data presented below, it is probable that the NAD-induced enhanced primer accessibility to DNA polymerase is attributable, at least in part, to the automodification of PADPRP and its dissociation from DNA strand breaks in the nuclear preparations. Although considerable data from several laboratories have indicated that the modification of

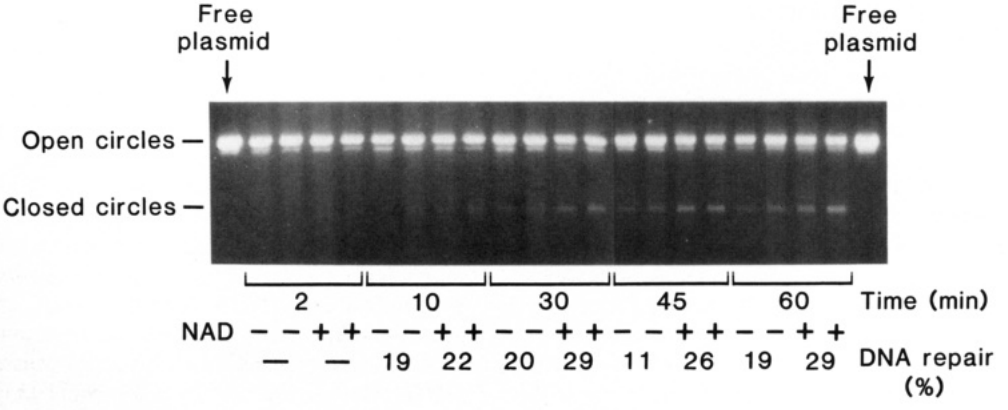


FIGURE 2: Time course of DNA repair. Complete HeLa cell of whole-cell extract (12 μg of protein) and 0.3 μg of irradiated plasmid were incubated in the absence or presence of NAD (2 mM). Paired duplicate reactions were terminated at the times indicated. A print from a photographic negative of a 1.0% agarose gel stained with ethidium bromide is shown. Lanes marked free plasmid contained 0.3 μg of plasmid. The open circular DNA contained single-strand breaks introduced by γ irradiation and represents the substrate for repair. The band immediately below the open circular plasmid this band is an artifact of plasmid purification and was not included when calculating DNA repair. The band was useful, however, as an indication that the total plasmid recovered from each reaction was reasonably constant. DNA repair was assessed by densitometry and calculated as the amount of closed circular DNA expressed as a percentage of the total DNA in both bands.

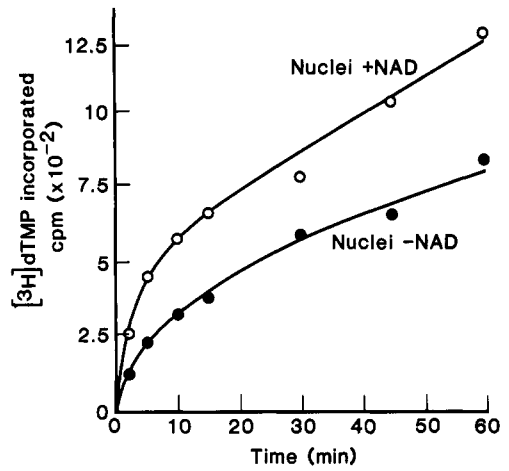


FIGURE 3: Effect of NAD on the accessibility of 3'-OH primer sites in intact HeLa cell mid-S phase nuclei to *E. coli* DNA polymerase I. Nuclei (8×10^6) were incubated in 1 mL of the PADPRP assay mixture containing either no NAD or 4 mM unlabeled NAD for 15 min at room temperature. Five milliliters of 0.25 M sucrose containing 2 mM $MgCl_2$ and 40 mM nicotinamide were then added, and the nuclei were collected by centrifugation and resuspended in twice-concentrated DNA polymerase mixture containing 5 units of *E. coli* DNA polymerase I and [^{3}H]dTTP but no other deoxynucleoside triphosphates by procedures described in detail previously (Roberts et al., 1974). At the indicated times, samples (10 mL) were removed for determination of [^{3}H]dTMP incorporation into nuclei preincubated in the absence (closed circles) or presence (open circles) of NAD.

nuclear proteins, such as histones and other nucleosomal proteins, in the vicinity of DNA strand breaks also plays an important role in chromatin exposure (Althaus et al., 1992; Malik et al., 1983; Satoh & Lindahl, 1992; Thraves et al., 1985), such proteins are not present in the *in vitro* DNA repair assay and therefore cannot contribute to the observed NAD-induced increase in DNA repair. The nuclear assay thus offers a possibility to assess the contributions of *both* processes on replication/repair.

DNA Repair in PADPRP-Depleted HeLa Cell Extracts. We measured the ability of several complete and PADPRP-depleted HeLa cell extracts to facilitate DNA repair and obtained mean percentage repair values of 9.9 ± 4.9 (mean ± SD, $n = 17$) and 26.9 ± 14.0 for complete extracts in the absence and presence of NAD, respectively; and 30.7 ± 17.4 ($n = 10$) and 29.5 ± 18.3 for PADPRP-depleted extracts, in the absence and presence of NAD, respectively. Thus, in the absence of NAD, the extent of DNA repair in PADPRP-depleted extracts was approximately three times that in the complete extracts, presumably because of the loss of PADPRP binding to the strand breaks of the plasmid. Futhermore, whereas DNA repair in the presence of complete Manley extracts was stimulated approximately 3-fold by NAD, repair in the PADPRP-depleted extracts was not affected by NAD.

Effect of PADPRP Antisense RNA Expression on DNA Repair. We have recently characterized a HeLa cell line stably transfected with an inducible PADPRP antisense RNA expression plasmid (Ding et al., 1992). The establishment of this cell line has provided a new experimental system for elucidating the role of PADPRP and its mechanism of action in chromatin repair. In the current study, we examined the ability of Manley extracts of these antisense cells to mediate DNA repair in the *in vitro* assay. Manley extracts were thus prepared from HeLa PADPRP-as cells (Ding et al., 1992) that had been grown in the presence or absence of dexamethasone, the inducer of antisense RNA expression in this cell system. In the presence of extracts prepared from cells

Table 2: NAD Does Not Affect DNA Repair in Nuclear Extracts Derived from Cells Induced To Express PADPRP Antisense mRNA[a]

antisense induction (cell extract)	DNA Repair (%)	
	-NAD	+NAD
-	13.9	20.8
+	21.5	24.6

[a] DNA repair was measured as described in the legend of Figure 2 with equivalent (20 μg) of the two cell extracts.

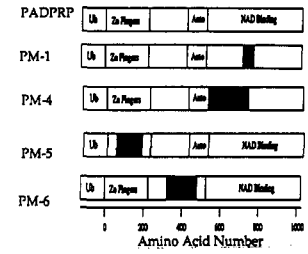
incubated in the absence of dexamethasone, DNA repair was 13.9% and 20.8% in the absence and presence of NAD, respectively; for extracts of cells incubated in the presence of inducer, DNA repair was 21.5% and 24.6% in the absence and presence of NAD, respectively (Table 2). Comparable to the results obtained with the PADPRP-depleted HeLa cell extracts, in the absence of NAD, the extent of DNA repair mediated by the extracts of antisense induced cells was greater than that mediated by extracts of noninduced cells. Furthermore, stimulation of DNA repair by NAD was more marked with extracts of noninduced antisense cells than with extracts of induced cells. Both PADPRP activity measurements and immunoblot analysis (data not shown) indicated that dexamethasone treatment of the intact antisense cells decreased the amount of PADPRP in Manley extracts to approximately the same extent as reported earlier (Ding et al., 1992). The fact that DNA repair is higher when using antisense induced PADPRP-depleted extracts may appear to contradict earlier observations that DNA repair is reduced in antisense-induced cells (Ding et al., 1992). However, in the former case, the increase in cell-free repair with a plasmid substrate in the absence of chromatin is due to the relative absence in the Manley extract of a DNA strand break "blocker" (i.e., PADPRP). In contrast, in the latter case, in whole cells with high NAD concentrations, either by depletion of PADPRP by antisense expression, or by the use of chemical inhibitors of PADPRP, it was concluded that the process of poly(ADP-ribosyl)ation of various nuclear proteins itself plays an important role in DNA repair.

DNA Repair and Deletion Mutants of PADPRP. Addition of recombinant, full-length PADPRP to the depleted HeLa cell extract inhibited DNA repair in the *in vitro* assay (Table 3). This result, using a bacterially expressed enzyme, is consistent with earlier observations (Satoh & Lindahl, 1992) that PADPRP, purified from a eukaryotic cell line, inhibited DNA repair when added to a PADPRP-depleted extract. The further addition of NAD partly overcame the inhibition of DNA strand break rejoining by full-length PADPRP, presumably as a result of PADPRP automodification and dissociation from the DNA. One microgram of bacterial extract was expected to be equivalent to ~20 μg of complete HeLa cell extract in terms of PADPRP protein content and activity (Table 1); however, the repair data suggest that the recombinant PADPRP may be more active in inhibiting repair or more abundant in the bacterial extract than predicted.

In experiments with the PADPRP deletion mutants, 1 μg of bacterial extract was also added to PADPRP-depleted HeLa cell extracts. Mutant PM-1, which has a 45 amino acid deletion in the NAD-binding domain but contains an intact DNA-binding domain (Cherney et al., 1991), inhibited repair by ~40% relative to the Manley extract depleted of PADPRP (Table 3). Although PM-1 contains an intact automodification region, it should be incapable of dissociating from the 3'-OH break of the plasmid substrate because of its active site deletion; indeed the extent of DNA repair in the presence of this mutant was not affected by NAD. Mutant PM-4, which

Table 3: Effect of PADPRP Deletion Mutants on DNA Repair *in Vitro*[a]

extract	supplement	DNA repair (%) –NAD	DNA repair (%) +NAD
complete HeLa cell		13.7 ± 0.28	38.0 ± 0.35
PADPRP-depleted HeLa cell		45.3 ± 3.74	44.3 ± 2.43
PADPRP-depleted HeLa cell	PADPRP	8.7 ± 2.41	22.0 ± 3.8
PADPRP-depleted HeLa cell	PM-1	27.3 ± 2.29	26.4 ± 2.67
PADPRP-depleted HeLa cell	PM-4	18.9 ± 0.62	19.3 ± 4.25
PADPRP-depleted HeLa cell	PM-5	42.5 ± 2.20	42.3 ± 0.86
PADPRP-depleted Hela cell	PM-6	31.6 ± 6.52	30.3 ± 5.94



[a] Complete HeLa cell extract (20 μg of protein) or PADPRP-depleted HeLa cell extract (20 μg of protein) supplemented with bacterial extracts containing various PADPRP constructs (1 μg of protein) were incubated in the *in vitro* DNA repair assay in the absence or presence of NAD (2 mM). Results are means ± SD from three independent experiments. Ub, ubiquitin.

contains a larger deletion in the active site region of the enzyme than PM-1 (Cherney et al., 1991), behaved similarly to PM-1 in the DNA repair assay. In contrast, mutant PM-5 has a 106 amino acid deletion in the DNA-binding domain of the enzyme (Cherney et al., 1991) and did not significantly affect the extent of DNA repair observed with the PADPRP-depleted extract. NAD also had no effect on repair in the presence this deletion mutant of PADPRP. Finally, mutant PM-6 which contains functional DNA-binding and active site domains but has a deletion in the automodification domain inhibited DNA repair but was insensitive to NAD. We have recently shown that although this mutant is incapable of automodification, it catalyzes the transfer of ADP-ribose to other acceptor proteins (unpublished observation).

DISCUSSION

Our results showing the effects of previously characterized PADPRP mutants add support to a recent proposed model concerning the mechanism of PADPRP action in DNA strand break rejoining (Satoh & Lindahl, 1992). Our data may also clarify earlier studies of poly(ADP-ribose)-dependent release of template restriction for DNA polymerase in nuclei and chromatin (Roberts et al., 1974; Smulson et al., 1975).

A number of observations have suggested that chemical inhibition of PADPRP enhances the cytotoxic effects of DNA damaging agents (Jacobson & Jacobson, 1989; Poirier & Moreau, 1992). Futhermore, DNA repair *in vivo* was recently shown to be inhibited in cells in which the concentration of PADPRP was markedly reduced as a result of PADPRP antisense RNA expression (Ding et al., 1992). DNA repair was also inhibited by *in vivo* expression of the DNA-binding domain of PADPRP (Molinete et al., 1993). Thus, the poly(ADP-ribosyl)ation of nuclear proteins associated with nucleosomal chomatin regions near DNA strand breaks appears to play an important role in DNA repair. More recent data have demonstrated both enhanced cytotoxicity and gene amplification in cells depleted of PADPRP as a result of PADPRP antisense expression (unpublished observations). We have shown that Manley extracts prepared from HeLa cells induced to express PADPRP antisense RNA allowed a higher degree of *in vitro* DNA repair, in the absence of NAD than noninduced cell extracts, presumably because the cell-free extracts contain a reduced amount of PADPRP and no histones or other nucleosomal proteins (Manley et al., 1983). This is in contrast to what occurs in the intact cell which contains chromatin.

It has been suggested that one biological role for PADPRP is association with a nuclear matrix that forms during DNA replication or cellular differentiation. In this regard, PADPRP was recently observed to be induced during the early stage of differentiation of 3T3-L1 preadipocytes (Kang et al., 1993). Moreover, the several rounds of DNA replication that are required for differentiation of these cells were inhibited in 3T3-L1 cells depleted of PADPRP as a result of PADPRP antisense RNA expression (Kang et al., 1993). Thus, by protecting 3′-OH breaks, PADPRP may help to prevent DNA synthesis in regions of damaged DNA until adequate DNA repair has taken place. Data obtained from the early sequence analysis of PADPRP (Cherney et al., 1987), together with more recent DNA-footprinting studies with recombinant PADPRP, have shown that the enzyme is a sequence-nonspecific DNA-binding protein (Menissier-de Murcia et al., 1989). de Murcia and colleagues (Gradwohl et al., 1990) have shown that the first zinc finger of PADPRP participates in the binding of the enzyme to single-strand breaks, whereas both zinc fingers mediate binding of the enzyme to double-strand DNA breaks. Deoxyribonuclease 1 footprinting techniques have also shown that the enzyme interacts with ~20 bp of DNA when tested with a 66-bp double-stranded DNA oligomer containing a single-strand break at position 33 (Menissier et al., 1989). Futhermore, experiments from a number of laboratories have shown that the automodification of PADPRP decreases its affinity for DNA; other acceptor molecules such as histones also bind less tenaciously to DNA when poly(ADP-ribosyl)ated (Jacobson & Jacobson, 1989; Satoh & Lindahl, 1992).

Our data with the PADPRP deletion mutants showed that those mutants (PM-1, PM-4, and PM-6) that are capable of binding to single-strand breaks, because they possess an intact DNA-binding domain, inhibited DNA repair when added to a PADPRP-depleted HeLa cell extract. However, because of deletions in the automodification region (PM-6) or the NAD-binding region (PM-1 and PM-4), the inhibition exerted by these mutants was not alleviated by NAD. Biochemical characterization of the deletion mutants has demonstrated the importance of an intact NAD-binding domain for polymerase activity (Cherney et al., 1991). Deletion of the second zinc finger and the region between the two zinc fingers (PM-5) eliminates most, but not all, of the enzymatic activity. PM-5 is not activated by DNA strand breaks, and Gradwohl et al. (1990) have shown that the region deleted in PM-5 plays a key role in the recognition of single-strand DNA.

PM-5 did not inhibit DNA repair in the *in vitro* assay.

The Manley extracts used in the current study contain no histones. Accordingly, chromatin modification cannot account for the effects of PADPRP on DNA repair in the simplified *in vitro* assay. However, a number of studies with intact cells, isolated nuclei, or isolated chromatin have demonstrated that modification of several specific nuclear proteins [including topoisomerases (Kasid et al., 1989), histones (Stone et al., 1977), and herpes virus proteins (Blaho et al., 1992)] that contribute to chromatin structure is an important function of PADPRP. Data have also been presented that suggest that chromatin decondensation (Thibeault et al., 1992) as well as condensation (Butt et al., 1980) are triggered by poly(ADP-ribosyl)ation of chromatin proteins. Histone H1, in particular, has been demonstrated as a key target for poly(ADP-ribosyl)-ation (Wong et al., 1983). Chromatin depleted of this histone is not condensed *in vitro*; reconstitution with histone restores chromatin condensation (Wong et al., 1982). Immunochromatography with antibodies to poly(ADP-ribose) has revealed that chromatin regions adjacent to DNA strand breaks in HeLa cells as well as in polyoma- and SV40-infected cells possess a high concentration of poly(ADP-ribosyl)ated proteins (Baksi et al., 1987; Thraves et al., 1985). In this regard, Mathis and Althaus (1990) demonstrated an influence of chromatin structure on DNA repair while studying the inhibition of repair with chemical inhibitors of PADPRP. An open chromatin structure became apparent during the repair of bulky alkylation adducts. The adducts could not be excised and tended to accumulate in presumably nonnucleosomal domains of chromatin when poly(ADP-ribosyl)ation was inhibited.

These observations, together with the data from our current study (Figures 2 and 3), may begin to clarify earlier observations showing a significant increase in the accessibility of 3′-OH primer sites to DNA polymerase in nuclei preincubated with NAD (Mathis & Althaus, 1990; Roberts et al., 1974). The ability of NAD to release this template restriction and expose DNA primer sites in chromatin has been observed to vary during the cell cycle (Roberts et al., 1974). It was further concluded that there are major alterations in the structure of nucleosomes adjacent to single-strand breaks, a conclusion consistent with later studies on the influence of poly(ADP-ribosyl)ation on chromatin structure. It is tempting to speculate that these observations, at least in part, may additionally be a result of the cycling of endogenous PADPRP from 3′-OH primer sites in chromatin by a mechanism similar to that proposed by Satoh and Lindahl and more recently extended to DNA damage by neocarzinostatin and bleomycin (Satoh et al., 1993) and supported by our results with the PADPRP deletion mutants in the *in vitro* DNA repair assay. In the future it is hoped that the various PADPRP mutants, described in this present study, can be incorporated *in vitro* into nuclei, derived from antisense cells, to test this hypothesis directly.

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