Protein−Protein Interaction of the Human Poly(ADP-ribosyl)transferase Depends on the Functional State of the Enzyme[†]

Joachim Griesenbeck,* Shiao Li Oei, Philipp Mayer-Kuckuk, Mathias Ziegler, Gerhard Buchlow, and Manfred Schweiger

Institut für Biochemie, Freie Universität Berlin, Thielallee 63, D-14195 Berlin, Germany

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ABSTRACT: Poly(ADP-ribosyl)transferase (pADPRT) is a nuclear protein which catalyzes the polymerization of ADP-ribose using NAD^+ as substrate, as well as the transfer of ADP-ribose polymers to itself and other protein acceptors. The catalytic activity of pADPRT strictly depends on the presence of DNA single-strand breaks. In this report, protein−protein interaction of pADPRT was found to depend on both the extent of automodification with poly(ADP-ribose) and the presence of DNA. Specific binding of radiolabeled pADPRT to transblotted proteins was first tested in blot overlay experiments. For radiolabeling, use was made of the ability of the enzyme to incorporate [^{32}P]ADP-ribose from [^{32}P]NAD^+. Varying the concentration of NAD^+, two different forms of automodified pADPRT were obtained: oligo(ADP-ribosyl)ated pADPRT with less than 20 ADP-ribose units per chain, and poly(ADP-ribosyl)ated pADPRT with polymer lengths of up to 200 ADP-ribose residues. Interaction of these probes with transblotted HeLa nuclear extracts, purified histones, and distinct regions of recombinant pADPRT was investigated. While the oligo(ADP-ribosyl)ated enzyme associated preferentially with transblotted purified histones, or pADPRT present in HeLa nuclear extracts, poly(ADP-ribosyl)ated pADPRT bound to a variety of transblotted proteins in the nuclear extracts. In the presence of DNA, both the oligo- and the poly(ADP-ribosyl)ated enzymes bound to the transblotted recombinant zinc finger domain of pADPRT even at high salt concentrations. In the absence of DNA, the transblotted automodification domain of pADPRT appeared to be the region involved in self-association. In another set of experiments, unmodified or poly(ADP-ribosyl)ated pADPRT was immobilized on Sepharose. Affinity precipitation of recombinant pADPRT domains confirmed the specific interaction of pADPRT with its zinc finger region and the automodification domain, whereas no interaction was observed with the NAD^+ binding domain. Affinity precipitation of HeLa nuclear extracts with poly(ADP-ribosyl)ated pADPRT−Sepharose led to the enrichment of a number of proteins, whereas nuclear proteins bound to the unmodified pADPRT−Sepharose in a smaller extent. The results suggest that protein−protein interaction of the human pADPRT is governed by its functional state.

Nuclear NAD^+ protein (ADP-ribosyl)transferase polymerizing (pADPRT;[1] EC 2.4.2.30) modifies proteins by forming ADP-ribose chains and seems to be ubiquitous and highly conserved in eukaryotic cells. The posttranslational transfer of ADP-ribose moieties from NAD^+ to proteins is thought to be involved in the regulation of processes such as differentiation, proliferation, and neoplastic transformation [for reviews, see Lindahl et al. (1995) and Oei et al. (1997)]. The main target for this group transfer is pADPRT itself (Ogata et al., 1981). Nevertheless, immunochemical estimation of unmodified and poly(ADP-ribosyl)ated pADPRT in intact cells in culture indicated that only a small fraction of pADPRT was modified with poly(ADP-ribose) (Cole et al., 1991).

The pADPRT enzyme consists of three functional domains (Kameshita et al., 1984). The amino-terminal DNA binding domain, which acts as a molecular nick sensor, encompasses two zinc finger motifs (Gradwohl et al., 1990) and a bipartite nuclear localization signal (Schreiber et al., 1992). The central region of the protein bears the poly(ADP-ribosyl)ation sites and a second DNA binding site for internal DNA structures (Sastry et al., 1989; Sastry & Kun, 1990). The C-terminal part of the enzyme contains the catalytic NAD^+ binding domain. The zinc finger domain of pADPRT exhibits a specific affinity to DNA single-strand breaks. The presence of nicked DNA strongly enhances the catalytic activity.

So far the molecular mechanism of the automodification reaction of pADPRT has not been established. Cross-linking experiments and kinetic studies suggested that self-association (dimerization) of pADPRT is essential for auto-poly(ADP-ribosyl)ation (Bauer et al., 1990; Mendoza-Alvarez & Alvarez-Gonzalez, 1993). Considering the stoichiometry of the automodification reaction, Panzeter and Althaus proposed that it involves a dimer-intermediate (1994). The presence of a leucine zipper motif within the automodification domain of pADPRT from *Drosophila* led to the

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* To whom correspondence should be addressed at the Institut für Biochemie, Freie Universität Berlin, Thielallee 63, D-14195 Berlin, Germany. Fax: +49-30-838 6509. Phone: +49-30-838 2910. E-mail: joe@chemie.fu-berlin.de.

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[1] Abbreviations: aa, amino acid; pADPRT, poly(ADP-ribosyl)transferase; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; IPTG, isopropyl β-D-thiogalactopyranoside; PAG(E), polyacrylamide gel (electrophoresis); PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid.

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assumption that this motif might be responsible for protein−protein interaction between pADPRT and other physiological acceptors of ADP-ribose (Uchida et al., 1993). *In vitro* poly(ADP-ribosyl)ation has been described for several nuclear proteins, such as DNA polymerases α and β, DNA ligase II, topoisomerases I and II, calcium/magnesium-dependent endonuclease, and terminal deoxynucleotidyl transferase [reviewed in Oei et al. (1997)]. The covalent modification of these proteins results in a down-regulation of their enzymatic activities. Minaga et al. have shown that, besides pADPRT itself, histones serve as main acceptors of poly(ADP-ribose) *in vivo* (1979). Recently, domains for self-association and histone binding of pADPRT have been identified by nitrocellulose transblot studies (Buki et al., 1995). It has previously been demonstrated that regions important for pADPRT dimerization are located in the zinc finger region and within the automodification domain (Bauer et al., 1990).

In this report, we show that interaction of the human pADPRT with HeLa proteins is strongly influenced by the functional state of the enzyme. Oligo(ADP-ribosyl)ated pADPRT preferentially bound to pADPRT present in transblotted nuclear extracts, whereas poly(ADP-ribosyl)ated pADPRT specifically interacts with multiple proteins in HeLa nuclear extracts. Moreover, a dependence of self-association on DNA binding was revealed. The presence of DNA is important for interaction of pADPRT with the zinc finger domain, whereas interaction with the automodification domain of the enzyme is not affected.

MATERIALS AND METHODS

Materials. Reagents and histones were purchased from Sigma, unless otherwise noted. DNA restriction enzymes, DNA Klenow polymerase, DNA ligase, DNase I, proteinase K, and IPTG were obtained from Eurogentec, Boehringer Mannheim, or Gibco BRL. Reinforced nitrocellulose membrane (BA-S 85) was purchased from Schleicher & Schuell. [α-^{32}P]NAD$^+$ and [α-^{32}P]dCTP were obtained from ICN. Polyclonal antibodies raised against human pADPRT, developed in goat, were used as described before (Schneider et al., 1987); polyclonal antibodies developed in rabbit, raised against the 40 000 C-terminal domain, were kindly provided by G. de Murcia, Strasbourg, France; secondary antibody alkaline phosphatase conjugates were purchased from Sigma.

Preparation of Nuclear HeLa Extracts. Nuclear HeLa extracts were prepared as described by Dignam et al. (1983).

Recombinant DNA Technology. Standard recombinant techniques were used as described by Sambrook et al. (1989).

Overexpression and Purification of His-Tagged Full-Length pADPRT and Deletion Constructs. The cDNA encoding the wild-type human pADPRT (Schneider et al., 1987) was cloned into the pQE31 vector using the QIAexpress-standard-protocol (Quiagen). After overexpression in *E. coli* cells, the His-tagged pADPRT was purified as described previously (Oei et al., 1996). The obtained protein had the same enzymatic properties as pADPRT of human placenta (Flick, 1994). Parts of the human pADPRT cDNA encoding distinct domains of the enzyme were cloned in frame into the pQE vector system (Quiagen), yielding His-tagged pADPRT-deletion constructs. After overexpression in *E. coli* cells, the deletion constructs were purified using Ni^{2+}−nitrilotriacetic acid−Sepharose.

Overexpression of pADPRT−β-Galactosidase Fusion Constructs. Parts of the human pADPRT cDNA were cloned in frame into the pAX vector (Markmeyer et al., 1990) and transformed into *E. coli* TG 1 cells. The cells were grown for 10 h at 25 °C in 10 g/L casein, 5 g/L yeast extract, 5 g/L NaCl, 2 g/L $MgSO_4$, and 1 g/L casamino acids with 50 mg/L ampicillin. Overexpression was induced by adding 300 mg/L IPTG. The cells were grown for a further 7 h at 25 °C and harvested by centrifugation at 4000*g* at 4 °C. The pellet was resuspended in 10 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1 mM EDTA, and 1 mM β-mercaptoethanol and sonicated. The crude extracts were incubated with sample buffer for 3 h at 25 °C and cleared by centrifugation at 15800*g* before SDS−PAGE. The amount of fusion construct in the crude extracts was determined densitometrically from Coomassie staining in a SDS−PAG using Enhanced Analysis System software (Herolab GmbH, Wiesloch).

ADP-Ribosylation and α-^{32}P-Labeling of pADPRT. Purified His-tagged pADPRT (5 μg) was incubated in a total volume of 100 μL of "binding buffer" (10 mM Tris-HCl, pH 8.0, 7 mM $MgCl_2$, and 50 μM $ZnCl_2$) with 6 nM [α-^{32}P]-NAD$^+$ (0.3 μCi/nmol) and 10 μg of sonicated salmon sperm DNA (Boehringer Mannheim) for 30 min at 25 °C. In the absence of supplementary unlabeled NAD$^+$, the labeling resulted in oligo(ADP-ribosyl)ated enzyme. To obtain the radioactively labeled poly(ADP-ribosyl)ated pADPRT, labeling was carried out as described above for 30 min at 25 °C, and the reaction was subsequently continued with unlabeled NAD$^+$ at a final concentration of 1 mM and incubated for a further 30 min at 25 °C. This procedure resulted in a modified enzyme with the labeled ADP-ribose positioned close to the site of attachment to the protein (Alvarez-Gonzalez, 1988). After radiolabeling, both oligo- and poly(ADP-ribosyl)ated pADPRT had the same specific radioactivity as determined by TCA precipitation of control samples. Residual [α-^{32}P]NAD$^+$ was removed by gel filtration, using a Sephadex G 50 column (Pharmacia). The G 50 gel was saturated with a 10% BSA solution before use. Labeled proteins were analyzed by SDS−polyacrylamide gel electrophoresis and autoradiography. Size analysis of ADP-ribose polymers was performed as described by Panzeter and Althaus (1990). Briefly, the modified protein was precipitated with 10% TCA. ^{32}P-Labeled polymers were detached from protein by incubating the precipitate at 60 °C for 3 h with 10 mM Tris, 1 mM EDTA, pH 12. After phenol/chloroform extraction, the supernatants were lyophilized. Polymers were then resolved in 7 M urea, 25 mM NaCl, 4 mM EDTA (pH 8.0), 0.02% xylene cyanol, and 0.02% bromphenol blue and loaded onto a 20% polyacrylamide sequencing gel in 0.09 M Tris, 0.09 M boric acid, and 2 mM EDTA (pH 8.3). After electrophoresis, the gel was dried and subjected to autoradiography.

Synthesis of α-^{32}P-Labeled Poly(ADP-ribose). Purified His-tagged pADPRT (5 μg) was incubated in a total volume of 100 μL of binding buffer with 6 nM [α-^{32}P]NAD$^+$ (0.3 μCi/nmol) and 10 μg of sonicated salmon sperm DNA (Boehringer Mannheim) for 30 min at 25 °C. Unlabeled NAD$^+$ was then added to a final concentration of 1 mM, and the reaction was continued for 30 min at 25 °C. After TCA precipitation, modified proteins were treated with 100 μg/mL proteinase K in the presence of 0.1% SDS at 37 °C for 1 h. α-^{32}P-Labeled ADP-ribose polymers were extracted with phenol/chloroform, precipitated with ethanol, and dissolved in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA.

Removal of DNA by DNase I Treatment. α-^{32}P-Labeled pADPRT was incubated with 4 μg/mL DNase I (Boehringer Mannheim) for 15 min at 25 °C to obtain DNA-free probes which were then subjected to the blot overlay binding experiments. The efficiency of the DNA digest was verified by agarose gel electrophoresis. DNase I treatment did not affect protein amount or polymer size as established by SDS−PAGE and chain length analysis of ADP-ribose polymers (data not shown). Consequently, digested and undigested samples differed only in the presence of DNA.

α-^{32}P-Labeling of DNA. A M13 sequencing primer was annealed to single-stranded M13 mp19 and filled up with Klenow polymerase in the presence of dNTPs ([α-^{32}P]dCTP, dATP, dGTP, dTTP) to produce double-stranded, nonligated open circular DNA according to standard procedures (Sambrook et al., 1989). To remove remaining dNTPs, labeled DNA was run through a Sephadex G 50 spin column.

Gel Electrophoresis and Electrophoretic Transfer. Proteins were separated on 6 × 10 cm SDS−PAGE minigels according to Laemmli (1970), except that the samples were not boiled. Electrotransfer of proteins onto nitrocellulose sheets and immunostaining were performed at 4 °C for 10 h at 100 mA (Towbin et al., 1979).

Blot Overlay Binding Studies. The overlay binding procedure was performed according to a method previously developed by Otto (1983) and modified by Crawford et al. (1992). Proteins were electrophoretically separated in SDS−PAGs and then transblotted onto nitrocellulose. After being stained with Ponceau S, the blots were renatured and blocked overnight in "overlay buffer" (0.5% BSA, 0.25% gelatin, 0.2% Triton X-100, 50 mM Tris-HCl, pH 7.4, and 5 mM β-mercaptoethanol) at 4 °C. For binding studies with the full-length enzyme, the blots were incubated overnight in 5 mL of overlay buffer at various salt concentrations (100 mM, 250 mM, and 500 mM NaCl) in the presence of 5 μg of [α-^{32}P]ADP-ribose-labeled pADPRT at 4 °C. For binding studies with ^{32}P-labeled DNA (50 000 cpm/mL) or ^{32}P-labeled poly(ADP-ribose) (50 000 cpm/mL), blots were incubated overnight in 5 mL of overlay buffer containing 250 mM NaCl at 4 °C. The blots were washed 3 times for 10 min with "TBST buffer" (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.05% Tween-20), to release nonspecifically bound radioactive material. Autoradiography was performed at −80 °C with X-OMAT film (Eastman Kodak Co.) in the presence of an intensifying screen. To quantify radioactivity bound to the blot, radiolabeled bands were excised and analyzed by Cerenkov counting.

Preparation and ADP-Ribosylation of pADPRT−Sepharose. Purified His-tagged pADPRT was immobilized on CL-4B Sepharose (Pharmacia Biotech) using the cyanogen bromide method as described by March et al. (1974). Briefly, 10 mL of CNBr-activated CL-4B Sepharose was equilibrated with "coupling buffer" (100 mM carbonate, pH 9.5). Fifty milligrams of purified pADPRT in 50 mL of coupling buffer was added and shaked overnight at 4 °C. The gel was washed 5 times with coupling buffer, containing 1 M NaCl to remove noncoupled ligand. The pADPRT−Sepharose was equilibrated with 1 M NaCl, 100 mM Tris-HCl, pH 7.5, 10 mM β-mercaptoethanol, and 1 mM NaN_3 and stored at 4 °C. pADPRT immobilized on the CL-4B Sepharose matrix showed only 10% of the activity of the full-length enzyme in solution, as measured by the incorporation of [α-^{32}P]ADP-ribose from radiolabeled NAD^+ in the presence of DNA. To obtain oligo(ADP-ribosyl)ated pADPRT−Sepharose, 20 μL of the gel was incubated in a total volume of 100 μL of binding buffer with 1 μM NAD^+ and 10 μg of sonicated salmon sperm DNA (Boehringer Mannheim) for 30 min at 25 °C. To obtain poly(ADP-ribosyl)ated pADPRT−Sepharose, 20 μL of the gel was incubated in a total volume of 100 μL of binding buffer with 1 mM NAD^+ and 10 μg of sonicated salmon sperm DNA (Boehringer Mannheim) overnight at 25 °C. Synthesis of ADP-ribose polymers was verified by addition of [α-^{32}P]NAD^+ to the reaction mixture and subsequent chain length analysis according to Panzeter and Althaus (1990). Before use, oligo- or poly(ADP-ribosyl)-ated pADPRT−Sepharose was washed twice with 2.5 M NaCl, 100 mM Tris-HCl, pH 7.5, and 10 mM β-mercaptoethanol and subsequently 5 times with TBST buffer.

Affinity Precipitation with pADPRT−Sepharose. Distinct domains of pADPRT, i.e., His-tagged deletion constructs of pADPRT, were overexpressed in *E. coli* cells and purified as described above. About 20 μg of purified protein in 500 μL of TBST buffer containing 20 μg of BSA was incubated with 25 μL of CL-4B Sepharose for 1 h at 4 °C. After centrifugation, the supernatants were transferred to 25 μL of pADPRT−Sepharose and incubated for 1 h at 4 °C. After centrifugation, the supernatants containing unbound proteins were precipitated for SDS−PAGE analysis as described below. The pADPRT−Sepharose was washed 5 times with TBST buffer. Proteins bound to the pADPRT−Sepharose were eluted with 60 μL of SDS−PAGE sample buffer and separated by electrophoresis. For affinity precipitation of nuclear HeLa extracts, 1 mg of protein in 500 μL of TBST buffer containing 20 μg of BSA was employed and treated the same way.

Protein Precipitation. Protein was precipitated according to the method of Rodriguez-Vico et al. (1989). In brief, 100 μL of the protein solution was mixed with 600 μL of *n*-hexane/2-propanol, 3:2 (v/v). Protein was then sedimentated by centrifugation at 15800*g*, for 15 min at 25 °C. Residual solvent was removed by vacuum evaporation, and the pellet was dissolved in SDS−PAGE sample buffer.

RESULTS

His-tagged recombinant full-length pADPRT (Figure 1) was purified to homogeneity as described previously (Oei et al., 1996). Its enzymatic properties were indistinguishable from those of the enzyme isolated from human placenta (Flick, 1994). For the study of protein−protein interactions, the enzyme was radiolabeled, utilizing the auto(ADP-ribosyl)ation reaction. Two radiolabeled pADPRT forms, oligo- and poly(ADP-ribosyl)ated pADPRT, differing in the extent of automodification were tested for their ability to bind to proteins transblotted onto nitrocellulose. To obtain oligo(ADP-ribosyl)ated pADPRT, purified enzyme was incubated with substoichiometric amounts of [^{32}P]NAD^+ (in a molar ratio of 70:1) in the presence of DNA. As a result, radiolabeled automodified enzyme contained polymers with less than 20 ADP-ribose residues (Figure 2B, lane 1). The labeled protein migrated as a distinct protein band with an apparent molecular weight of about 116 000 in SDS−PAG, as established by Coomassie staining and autoradiography of the gel (Figure 2A, lanes 1). Poly(ADP-ribosyl)ated pADPRT was obtained when incubation with [^{32}P]NAD^+ as described above was continued in the presence of 1 mM unlabeled NAD^+. This led to the formation of polymers with up to 200 ADP-ribose residues per chain (Figure 2B, lane

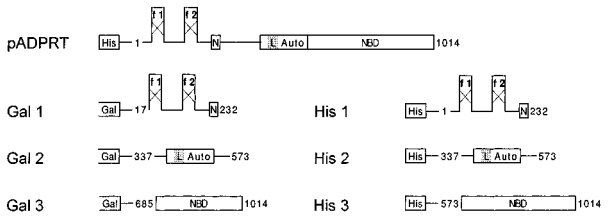

FIGURE 1: Schematic representation of recombinant pADPRT constructs. pADPRT, recombinant His-tagged full-length pADPRT. Gal 1−3, recombinant pADPRT−β-galactosidase fusion constructs. His 1−3, recombinant His-tagged pADPRT-deletion constructs. His, N-terminal fused 6-fold His-tag. Gal, N-terminal fused β-galactosidase. f1 and f2 represent Zn^{2+}-coordinated finger motifs. N, nuclear localization signal. Auto, automodification domain within an internal putative leucine zipper region (L). NBD, catalytic NAD^+ binding domain. The numbers indicate relative amino acid positions of the human pADPRT enzyme.

2). The poly(ADP-ribosyl)ated enzyme migrated as a high-weight smear in SDS−PAG, not detectable by Coomassie staining (Figure 2A, upper panel, lane 2). A significant proportion of the modified protein hardly entered the gel, as visualized by autoradiography (Figure 2A, bottom panel, lane 2).

Self-Association of pADPRT. To characterize the pADPRT domains involved in self-association, recombinant deletion constructs were created. Parts of the cDNA of the human pADPRT gene were cloned into the pAX vector system. This system allowed the overexpression of distinct pADPRT domains as β-galactosidase fusion proteins (Figure 1, Gal 1−3) in *E. coli*. In this study, three different constructs were used: Gal 1 bears the zinc finger domain up to aa 232, including both zinc fingers and the nuclear localization signal. Gal 2 consists of the automodification domain (aa 337−573), containing the major acceptor sites for ADP-ribose polymers. Gal 3, starting with aa 685, encompasses the NAD^+ binding domain of pADPRT. In Figure 3A, a Coomassie-stained SDS−PAG of purified full-length pADPRT and crude *E. coli* extracts expressing the different β-galactosidase fusion constructs is shown. The fusion proteins migrated with an apparent molecular weight from 140 000 to 160 000. An identical gel was transblotted onto nitrocellulose and subsequently immunostained with anti-pADPRT antibodies (Figure 3B). Blot overlay experiments were performed with radiolabeled oligo- or poly(ADP-ribosyl)ated enzyme (see Materials and Methods). Labeled pADPRT associated with transblotted proteins was visualized by autoradiography.

At low salt concentrations (up to 100 mM NaCl), oligo-(ADP-ribosyl)ated pADPRT bound to the full-length enzyme and the constructs Gal 1 and 2, bearing the zinc finger domain and the automodification domain, respectively (Figure 3C, lanes 1−3). No interaction was observed with Gal 3, encompassing the NAD^+ binding domain (Figure 3C, lane 4). The radiolabeled protein bands were excised from the blot, and the amount of pADPRT bound to the transblotted proteins was determined by Cerenkov counting. At 100 mM NaCl, about 10 ng of oligo(ADP-ribosyl)ated pADPRT bound per microgram of transblotted full-length enzyme, whereas only 5 and 3 ng bound per microgram of fusion construct Gal 1 and Gal 2, respectively. This might be attributed to the degree of renaturation of the proteins immobilized on the blot. In a similar experiment, the influence of ionic strength on self-association of the full-length pADPRT with distinct regions of the enzyme was

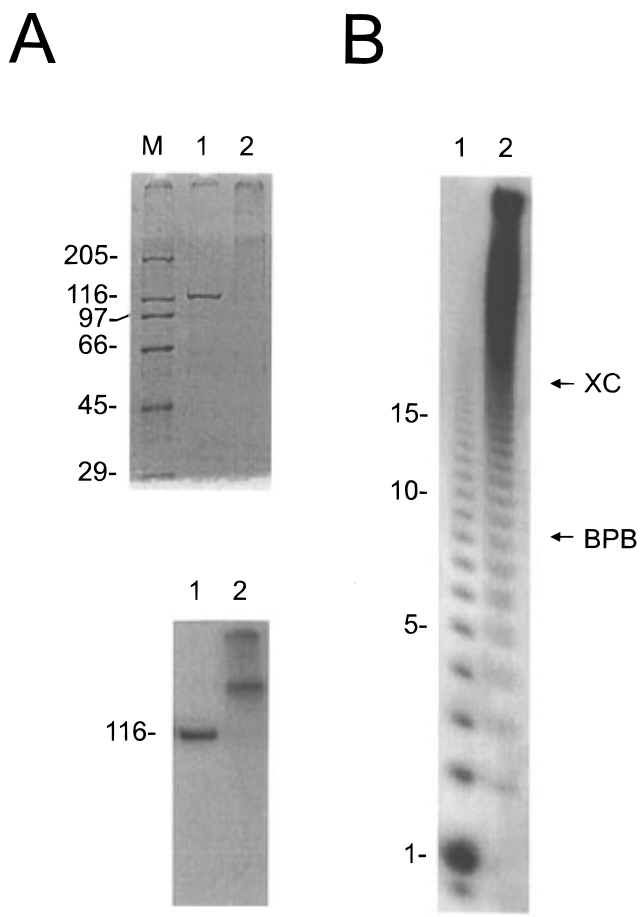

FIGURE 2: Automodification of pADPRT with oligo- or poly(ADP-ribose) chains. Full-length pADPRT was labeled with [^{32}P]NAD^+ as described under Materials and Methods to give rise to oligo-(ADP-ribosyl)ated pADPRT or poly(ADP-ribosyl)ated pADPRT. (A) 8% SDS−PAG of 1 μg of oligo- (lane 1) and poly(ADP-ribosyl)ated pADPRT (lane 2). Upper panel, staining with Coomassie Blue. Relative molecular sizes of marker proteins, lane M, are indicated ($M_r \times 10^{-3}$). Bottom panel, autoradiogram of the gel (the uppermost radioactive band in lane 2 represents protein aggregates that just entered the stacking gel, which was not removed from this gel). (B) Size analysis of polymers detached from oligo-(ADP-ribosyl)ated pADPRT (lane 1) or poly(ADP-ribosyl)ated pADPRT (lane 2). ^{32}P-Labeled ADP-ribose polymers were detached from pADPRT by alkaline treatment as described under Materials and Methods. The isolated polymers were then separated by 20% PAGE. The autoradiogram of the gel is shown. Positions of bromphenol blue (BPB) and xylene cyanol (XC) and numbers of ADP-ribose units are indicated.

tested. Figure 4A summarizes results obtained for the binding of oligo(ADP-ribosyl)ated pADPRT to the fusion constructs. High salt concentrations (up to 500 mM NaCl) did not appear to affect the binding of oligo(ADP-ribosyl)-ated pADPRT to Gal 1, whereas interaction with Gal 2 was significantly reduced. Interaction with the transblotted full-length enzyme was observed at all salt concentrations (not shown). Overlay experiments with the poly(ADP-ribosyl)-ated enzyme yielded similar results (not shown). However, the observed binding of the poly(ADP-ribosyl)ated pADPRT to the zinc finger domain was weaker as compared to the oligo(ADP-ribosyl)ated pADPRT [only 1−2 ng of poly-(ADP-ribosyl)ated pADPRT bound per microgram of fusion construct Gal 1 in comparison to 4 ng of the oligo(ADP-ribosyl)ated pADPRT at 250 mM NaCl]. The inhibition of binding of radiolabeled pADPRT to the automodification domain with increasing concentrations of NaCl suggests an ionic interaction with this region.

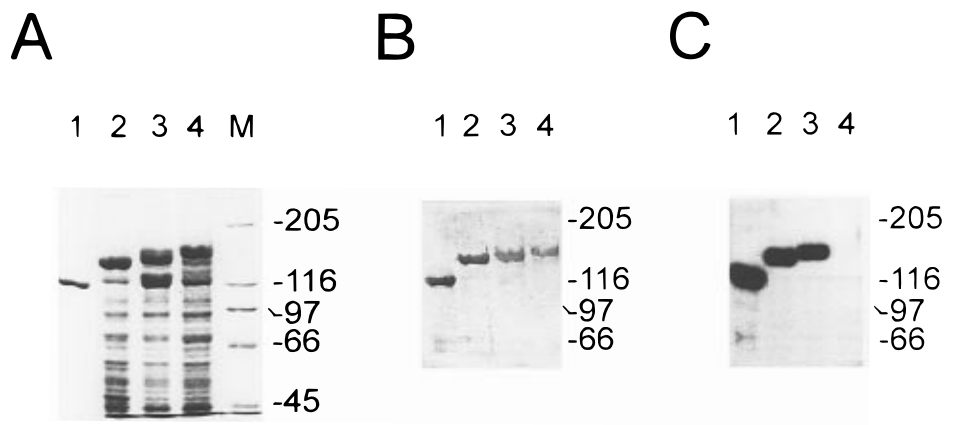

FIGURE 3: Binding of radiolabeled pADPRT to pADPRT−β-galactosidase fusion constructs. 2 μg of purified recombinant full-length pADPRT (lane 1) and crude extracts of *E. coli* cells expressing the pADPRT−β-galactosidase fusion constructs Gal 1−3 (lanes 2−4), each containing 4 μg of the fusion construct, were separated in a 6% SDS−PAG. (A) Staining with Coomassie Blue. Relative molecular sizes of marker proteins, lane M, are indicated ($M_r \times 10^{-3}$). (B and C) Identical gels were transblotted onto nitrocellulose. (B) Immunostaining with anti-pADPRT antibodies. (C) Autoradiogram of a blot after incubation with 5 μg of ^{32}P-labeled oligo(ADP-ribosyl)ated pADPRT (50 000 cpm/μg; 50 000 cpm/mL) in 5 mL of overlay buffer containing 100 mM NaCl as described under Materials and Methods.

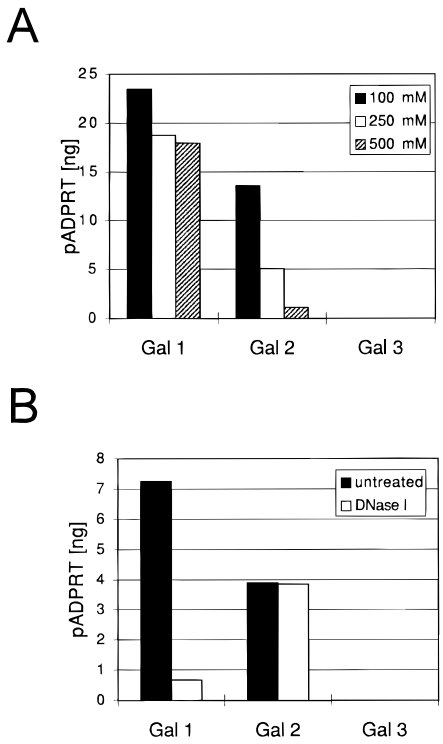

FIGURE 4: Influence of salt concentration and DNA on pADPRT self-association. Crude extracts of *E. coli* cells expressing the pADPRT−β-galactosidase fusion constructs, each containing 4 μg of the fusion construct, were separated in 6% SDS−PAGs and transblotted onto nitrocellulose, as shown in Figure 3. After the blot overlay, radiolabeled bands were excised from the blot and analyzed by Cerenkov counting. The blot overlay experiments were carried out with 5 μg of labeled enzyme (50 000 cpm/μg; 50 000 cpm/mL) in 5 mL of overlay buffer. (A) Binding of oligo(ADP-ribosyl)ated pADPRT to transblotted pADPRT−β-galactosidase fusion constructs at various NaCl concentrations (as indicated). (B) Binding of poly(ADP-ribosyl)ated pADPRT to transblotted pADPRT−β-galactosidase fusion constructs in 5 mL of overlay buffer containing 250 mM NaCl as indicated in the presence of 10 μg of DNA or after DNase I treatment (see Materials and Methods).

Since automodified pADPRT used for the blot overlay experiments was obtained in the presence of DNA, residual DNA was still present during the blot overlay experiment. To investigate the influence of DNA binding on the observed protein−protein interaction, first the DNA binding ability of transblotted proteins was tested. Blot overlay was carried out with ^{32}P-labeled DNA. Both the full-length enzyme and Gal 1, bearing the zinc finger domain, were able to bind DNA, as detected by autoradiography (not shown). If the labeled DNA was subjected to DNase I digestion before the overlay experiment, no binding to any of the transblotted proteins was observed (not shown). DNase I was added to ^{32}P-labeled pADPRT before incubation of the blot. As shown in Figure 4B, binding of pADPRT to Gal 1 was eliminated in the absence of DNA, whereas the interaction with Gal 2 was unaffected. Thus, while self-interaction of pADPRT with the automodification domain was not dependent, interaction with the zinc finger domain was dependent on the presence of DNA.

Several control experiments verified binding of labeled enzyme and not just residual [^{32}P]NAD$^+$ or [^{32}P]ADP-ribose. Incubation of the blots with [^{32}P]NAD$^+$ led only to radiolabeling of transblotted full-length enzyme (not shown), presumably due to its ability to incorporate [^{32}P]ADP-ribose. In addition, the presence of pADPRT itself in the radiolabeled bands on the blot was proven by immunostaining following the blot overlay experiments. Antibodies used were directed against the 40 kDa C-terminal domain of pADPRT, encompassing the NAD$^+$ binding domain. Consequently, only pADPRT and Gal 3 were immunoreactive. If immunostaining was performed after blot overlay with oligo- or poly(ADP-ribosyl)ated pADPRT, an additional staining of Gal 1 and 2 could be observed, corresponding to the signals obtained in the autoradiograms of the blots (not shown). Therefore, radiosignals obtained after blot overlay experiments indeed represented association of the radiolabeled enzyme with transblotted proteins.

The specific self-association of pADPRT with distinct regions within the full-length enzyme could also be demonstrated by affinity precipitation with pADPRT covalentely linked to Sepharose (see Materials and Methods). For this purpose, parts of the cDNA of the human pADPRT gene were cloned into the pQE vector system. In this system, distinct pADPRT domains corresponding to the β-galactosidase fusion proteins used in the blot overlay experiments were expressed in *E. coli* as His-tagged deletion constructs (Figure 1, His 1−3). The His-tag enabled a one-step purification of the deletion constructs as described under Materials and Methods. Construct His 1 includes the N-terminal zinc finger region (aa 1−232); His 2 bears the

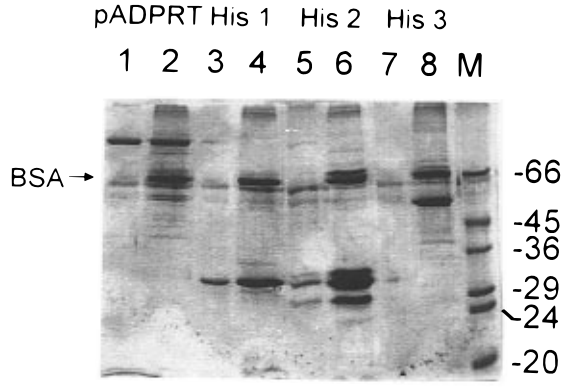

FIGURE 5: Affinity precipitation of His-tagged pADPRT constructs with pADPRT−Sepharose. 20 μg of either purified pADPRT (lanes 1 and 2) or pADPRT-deletion constructs His 1 (lanes 3 and 4), His 2 (lanes 5 and 6), or His 3 (lanes 7 and 8) in 500 μL of TBST buffer containing 20 μg of BSA was incubated with 25 μL of oligo(ADP-ribosyl)ated pADPRT−Sepharose for 1 h at 4 °C. After centrifugation, unbound proteins in the supernatants were precipitated and separated in a 12% SDS−PAG (lanes 2, 4, 6, and 8). After five washings, proteins bound to the oligo(ADP-ribosyl)ated pADPRT−Sepharose were eluted with SDS−PAGE sample buffer for 10 min at 25 °C and separated by electrophoresis (lanes 1, 3, 5, and 7). The gel was stained with Coomassie Blue. Relative molecular sizes of marker proteins, lane M ($M_r \times 10^{-3}$), and the position of BSA present in the supernatants are indicated.

automodification domain (aa 337−573). For this construct, fragmentation could be observed (Figure 5, lanes 5 and 6). However, all three protein bands, migrating with different mobilities in SDS−PAG, were His-tagged as they bind to Ni^{2+}−nitrilotriacetic acid−Sepharose, and interacted with anti-pADPRT antibodies. Construct His 3 encompasses the NAD^+ binding domain of pADPRT (aa 573−1014). In blot overlay experiments, radiolabeled oligo- and poly(ADP-ribosyl)ated pADPRT bound to constructs His 1 and 2, but not to His 3 (not shown), confirming the results obtained using the β-galactosidase fusion constructs.

For affinity precipitation, either 20 μg of purified full-length pADPRT or 20 μg of purified His-tagged deletion constructs was incubated with oligo(ADP-ribosyl)ated pADPRT−Sepharose in the presence of 20 μg of BSA. After centrifugation, unbound proteins in the supernatants were precipitated according to Rodriguez-Vico et al. (1989) and analyzed by SDS−PAGE (Figure 5, lanes 2, 4, 6, and 8). After five washings, proteins bound to the immobilized oligo(ADP-ribosyl)ated enzyme were eluted with SDS−PAGE sample buffer and separated by electrophoresis (Figure 5, lanes 1, 3, 5, and 7). Full-length pADPRT (Figure 5, lane 1) and constructs His 1 (Figure 5, lane 3) and His 2 (Figure 5, lane 5) could be recovered in the eluates. Although the same amounts of the constructs were used for incubation, construct His 2 appeared to bind more weakly to pADPRT−Sepharose as compared to His 1 (Figure 5, compare lanes 5 and 3). Construct His 3 could only be detected in the supernatant (Figure 5, compare lanes 7 and 8). BSA did not bind to the oligo(ADP-ribosyl)ated pADPRT−Sepharose and was, therefore, only present in the supernatants (Figure 5, lanes 2, 4, 6, and 8). These results confirm the observations of the blot overlay system indicating the presence of at least two distinct sites of self-association located in the N-terminal zinc finger region and the automodification domain of pADPRT.

Hetero-Association with HeLa Proteins and Histones. Since the blot overlay method and the affinity precipitation

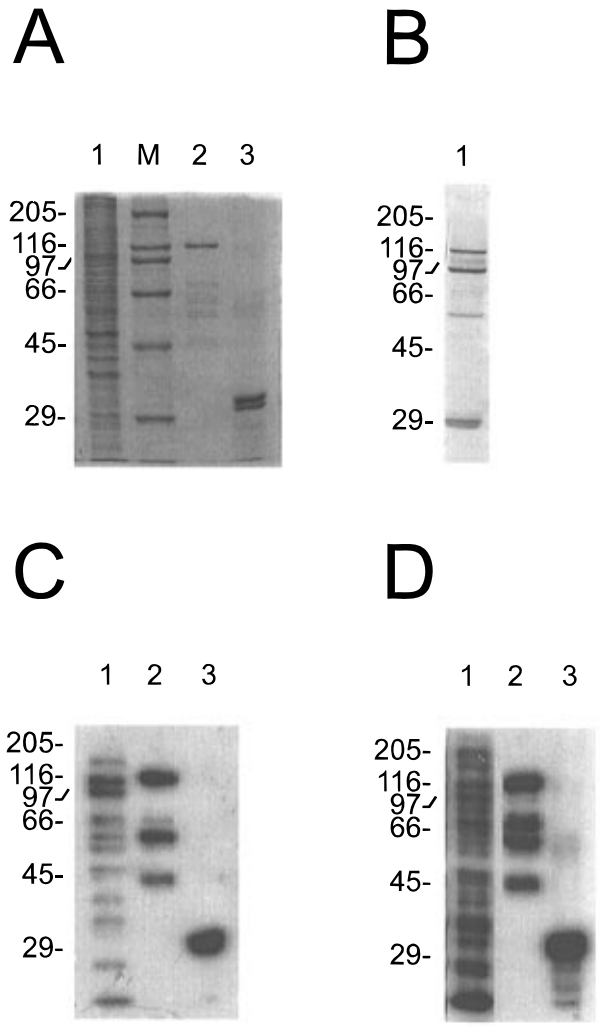

FIGURE 6: Hetero-association of pADPRT with proteins in HeLa nuclear extracts or isolated histones. Nuclear HeLa extracts containing 100 μg of protein (lane 1), 1 μg of purified recombinant full-length pADPRT (lane 2), and 2 μg of purified histone H1 (lane 3) were separated by 10% SDS−PAGE. Relative molecular sizes of marker proteins, lane M, are indicated ($M_r \times 10^{-3}$). (A) Staining with Coomassie Blue. (B, C, and D) Identical gels were transblotted onto nitrocellulose. (B) Immunostaining of HeLa nuclear extract with anti-pADPRT antibodies. (C) Autoradiogram of a blot after incubation with 5 μg of ^{32}P-labeled oligo(ADP-ribosyl)ated pADPRT (50 000 cpm/μg; 50 000 cpm/mL) in 5 mL of overlay buffer containing 250 mM NaCl. (D) Autoradiogram of a blot after incubation with 5 μg of ^{32}P-labeled poly(ADP-ribosyl)ated pADPRT (50 000 cpm/μg; 50 000 cpm/mL) in 5 mL of overlay buffer containing 250 mM NaCl.

with pADPRT−Sepharose exhibited a high specificity, it was also suitable to test the interaction of pADPRT with potential partner proteins. Nuclear HeLa extract (Figure 6A, lane 1) and also purified histones (Figure 6A, lane 3) and purified His-tagged pADPRT as a control (Figure 6A, lane 2) were separated by SDS−PAGE. Western blotting and immunostaining with anti-pADPRT antibodies revealed pADPRT as well as some of its degradation products in the nuclear HeLa extract (Figure 6B, lane 1). Blots, identical to the SDS−PAG presented in Figure 6A, were incubated with radiolabeled oligo- or poly(ADP-ribosyl)ated pADPRT (Figure 6C,D). Both oligo- and poly(ADP-ribosyl)ated pADPRT interacted with nuclear proteins from HeLa extracts, purified pADPRT and some of its degradation products, and transblotted histones (Figure 6C,D, lanes 1−3). Since equal amounts of enzyme carrying similar amounts of radioactivity

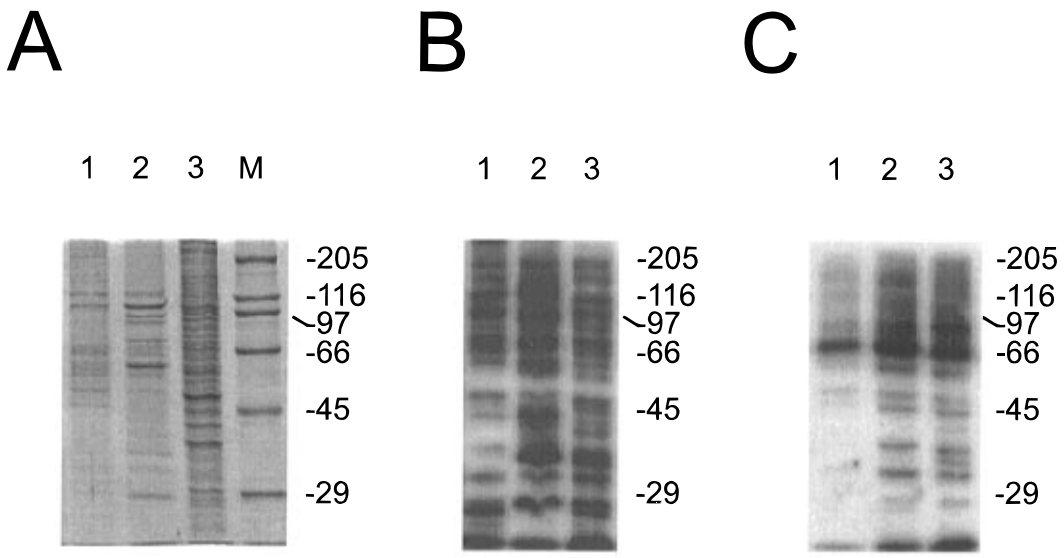

FIGURE 7: Affinity precipitation of HeLa nuclear extracts with pADPRT−Sepharose. HeLa nuclear extracts (1 mg) in 500 μL of TBST buffer containing 20 μg of BSA were incubated with 25 μL of either unmodified pADPRT−Sepharose or poly(ADP-ribosyl)ated pADPRT−Sepharose for 1 h at 4 °C. After five washings, proteins bound to the pADPRT−Sepharose were eluted with SDS−PAGE sample buffer for 10 min at 25 °C and separated by electrophoresis. (A) 10% SDS−PAG of the eluate from unmodified pADPRT−Sepharose (lane 1), the eluate from poly(ADP-ribosyl)ated pADPRT−Sepharose (lane 2), and untreated HeLa nuclear extract containing 100 μg of protein (lane 3) stained with Coomassie Blue. Relative molecular sizes of marker proteins ($M_r \times 10^{-3}$) are indicated. (B and C) Identical gels were transblotted onto nitrocellulose. (B) Autoradiogram of a blot after incubation with 5 μg of ^{32}P-labeled poly(ADP-ribosyl)ated pADPRT (50 000 cpm/μg; 50 000 cpm/mL) in 5 mL of overlay buffer containing 250 mM NaCl. (C) Autoradiogram of a blot after incubation with isolated ^{32}P-labeled ADP-ribose polymers (50 000 cpm/mL) in 5 mL of overlay buffer containing 250 mM NaCl. The autoradiogram was obtained after a 5 times longer exposure of the film as compared to panel B.

were used in the blot overlay experiments (see Materials and Methods), it is obvious that the oligo(ADP-ribosyl)ated pADPRT bound less efficiently to the transblotted proteins as compared to the poly(ADP-ribosyl)ated enzyme (Figure 6C,D, compare lanes 1−3). Moreover, the poly(ADP-ribosyl)ated pADPRT appeared to interact with more proteins in transblotted nuclear HeLa extracts as compared to the oligo(ADP-ribosyl)ated enzyme (Figure 6C,D, compare lanes 1). After incubation with the oligo(ADP-ribosyl)ated pADPRT, two protein bands with apparent molecular masses of about 116 000 and 90 000 Da were strongly radiolabeled. They comigrated with pADPRT and one of its main fragments (Figure 6C and Figure 6B, compare lanes 1), supporting the suggestion that the enzyme preferred self-association in this enzymatic state. If the blot overlay experiment was carried out in the presence of 1 mg/mL histone in the overlay buffer, interaction of radiolabeled oligo- and poly(ADP-ribosyl)ated pADPRT, respectively was inhibited (not shown).

Hetero-association of poly(ADP-ribosyl)ated pADPRT appeared to be decreased by the presence of DNA. If DNase I digest was performed prior to the blot overlay experiment, the interaction of poly(ADP-ribosyl)ated pADPRT with transblotted HeLa proteins was enhanced (not shown). A large number of transblotted proteins in the nuclear extract as well as histones and pADPRT bound ^{32}P-labeled DNA (not shown). However, this binding was highly unspecific and different from the pattern of distinct radiolabeled bands obtained with the automodified pADPRT. These results indicate that hetero-association of pADPRT with other partner proteins depended on the extent of automodification and that poly(ADP-ribosyl)ation enhanced interaction of pADPRT with these proteins.

These conclusions have been supported by another series of experiments. pADPRT−Sepharose and poly(ADP-ribosyl)ated pADPRT−Sepharose (prepared as described under Materials and Methods) were incubated with purified recombinant pADPRT, purified histones, or nuclear HeLa extracts in the presence of BSA. After five washings, proteins bound to the immobilized unmodified or poly(ADP-ribosyl)ated enzyme were eluted with SDS−PAGE sample buffer and separated by electrophoresis (Figure 7A). Purified His-tagged pADPRT and purified histones bound to unmodified pADPRT−Sepharose, but to an appreciably higher extent to poly(ADP-ribosyl)ated pADPRT−Sepharose (not shown). A variety of nuclear proteins bound to the poly(ADP-ribosyl)ated pADPRT (Figure 7A, lane 2) whereas a smaller amount of protein eluted from the Sepharose carrying the unmodified enzyme (Figure 7A, lane 1). The protein pattern of nuclear proteins eluted from the pADPRT−Sepharose (Figure 7A, lanes 1 and 2) indicates enrichment of specific proteins from the nuclear HeLa extract used for the incubation (Figure 7A, lane 3).

Identical gels to that presented in Figure 7A were transblotted onto nitrocellulose and incubated with ^{32}P-labeled poly(ADP-ribosyl)ated pADPRT (Figure 7B) or ^{32}P-labeled poly(ADP-ribose) detached from the enzyme molecule (Figure 7C). Figure 7B demonstrates that the proteins from crude nuclear HeLa extracts which were enriched by affinity precipitation with pADPRT−Sepharose were specifically detected by the blot overlay assay using the ^{32}P-labeled poly(ADP-ribosyl)ated enzyme (Figure 7B, compare lanes 1 and 2 to lane 3). Therefore, binding of radiolabeled pADPRT to transblotted proteins appears to be representative for the native state of those proteins. Deproteinized ^{32}P-labeled poly(ADP-ribose) detached from the enzyme molecule interacted more weakly with transblotted proteins as compared to blot overlay experiments with ^{32}P-labeled poly(ADP-ribosyl)ated pADPRT. Although the amount and specific radioactivity of isolated polymers were identical to those incorporated in the poly(ADP-ribosyl)ated pADPRT, radiosignals shown in Figure 7C could only be detected after

a 5 times longer exposure of the film. However, some of the radiolabeled bands obtained with isolated poly(ADP-ribose) coincided with those obtained with the poly(ADP-ribosyl)ated enzyme (compare Figure 7C and Figure 7B). Therefore, it cannot be ruled out that the observed interaction of the poly(ADP-ribosyl)ated pADPRT is at least to some extent due to its poly(ADP-ribose) chains.

DISCUSSION

The present study demonstrates that protein−protein interaction of human pADPRT may be highly regulated by the functional state of the enzyme. Protein interaction of pADPRT depends on both the extent of automodification and the presence of DNA. While the poly(ADP-ribosyl)-ated form of pADPRT interacts preferentially with other partner proteins, interaction of the oligo(ADP-ribosyl)ated enzyme with nuclear proteins is significantly weaker than self-association. Self-association with the zinc finger domain appears to be mediated by DNA.

The relevance of the two forms of pADPRT used in this study has been shown for ADP-ribose turnover *in vitro* (Satoh et al., 1994) as well as for *in vivo* systems (Alvarez-Gonzalez & Jacobson, 1987). Poly(ADP-ribosyl)ation of pADPRT has been shown to inhibit the enzymatic activity, because the automodified enzyme dissociates from activating DNA due to electrostatic repulsion (Ohgushi et al., 1980; Zahradka & Ebisuzaki, 1982). Nevertheless, it is shown here that poly(ADP-ribosyl)ated pADPRT interacts specifically with various proteins in HeLa nuclear extracts. It appears that poly(ADP-ribosyl)ation of pADPRT may be required for the specific interaction with at least some nuclear proteins. In contrast, the oligo(ADP-ribosyl)ated enzyme preferentially recognizes itself in transblotted nuclear extracts. Although some nuclear proteins appeared to bind deproteinized ADP-ribose polymers, it is obvious from our experiments that this binding is substantially enhanced when these polymers are bound to pADPRT (Figure 7). Both oligo- and poly(ADP-ribosyl)ated pADPRT interact with histones, known targets of covalent modification with poly(ADP-ribose) *in vitro* and *in vivo* (Boulikas, 1990), as well as partners for protein−protein interaction *in vitro* (Buki et al., 1995).

The finding of two different binding sites within the enzyme molecule is consistent with the results of Bauer et al. (1990). Here we show that this self-interaction is regulated by the presence of DNA. In presence of DNA, pADPRT interacts specifically with the zinc finger domain. In the absence of DNA, interaction with the zinc finger domain is inhibited, whereas interaction with the automodification domain is unaffected. In addition, binding of pADPRT to the automodification domain is inhibited by increasing salt concentrations. These observations indicate a hydrophobic interaction of pADPRT with its zinc finger region, while binding of pADPRT to the automodification domain is instead due to an ionic interaction.

The results suggest a mechanism including the initial binding of pADPRT to a single-strand DNA break followed by the formation of an activated homodimeric complex. Subsequent automodification would lead to a release of poly-(ADP-ribosyl)ated pADPRT molecules from DNA. Poly-(ADP-ribosyl)ated pADPRT may then specifically interact with nuclear partner proteins.

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