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Polyubiquitylation of PARP-1 Through Ubiquitin K48 Is Modulated by Activated DNA, NAD$^+$, and Dipeptides

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Abstract Poly(ADP-ribose) polymerase-1 (PARP-1) is the most abundant and the best-studied isoform of a family of enzymes that catalyze the polymerization of ADP-ribose from NAD$^+$ onto target proteins. PARP-1 is well known to involve in DNA repair, genomic stability maintenance, transcription regulation, apoptosis, and necrosis. Polyubiquitylation targets proteins towards degradation and regulates cell cycle progression, transcription, and apoptosis. Here we report polyubiquitylation of PARP-1 in mouse fibroblasts in the presence of proteasome inhibitor and in full-length recombinant PARP-1 in vitro under standard ubiquitylation assay conditions by immunoprecipitation and immunoblotting. Mutation of ubiquitin K48R but not ubiquitin K63R abolishes polyubiquitylation of PARP-1, indicating that K48 of ubiquitin was used in the formation of polyubiquitin chain and that ubiquitylated PARP-1 is likely destined for degradation. Full-length PARP-1 was ubiquitylated most likely at the N-terminal 24 kDa domain of PARP-1 as suggested by the inhibition of ubiquitylation by activated DNA and the absence of polyubiquitin in the C-terminal 89 kDa PARP-1 derived from caspase-catalyzed cleavage. NAD$^+$ inhibited ubiquitylation of PARP-1, while dipeptides ArgAla and LeuAla enhanced ubiquitylation of PARP-1. ATP inhibited the synthesis of poly(ADP-ribose) by PARP-1 and affinity purified polyubiquitylated PARP-1 was active in PAR synthesis. The results suggest polyubiquitylation of PARP-1 could regulate poly(ADP-ribosyl)ation of nuclear proteins by PARP-1 and consequently apoptosis and PARP-1 regulated cellular processes through ubiquitin-dependent degradation pathways. J. Cell. Biochem. 104: 318–328, 2008.
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Key words: poly(ADP-ribose) polymerase; protein degradation; ubiquitylation; poly(ADP-ribose); apoptosis

Poly(ADP-ribose) polymerase-1 (PARP-1) is the best-studied isoform of the poly(ADP-ribose) polymerase (PARP) family, which catalyzes the poly(ADP-ribosyl)ation of protein acceptors using NAD$^+$ as the substrate [Burzio et al., 1979; Riquelme et al., 1979]. As a highly conserved and abundant nuclear protein, PARP-1 is present in all animals and plants [D'Amours et al., 1999]. PARP-1 plays regulatory roles in DNA repair, transcription, apoptosis, and the maintenance of genome stability mainly through PARP-1 binding to a variety of DNA structures, its catalytic action to attach poly(ADP-ribose) to itself and other nuclear proteins such as histones and transcriptional factors p53 and NFκB, etc., and PARP-1 specific, noncovalent binding to nuclear proteins [for reviews, see D'Amours et al., 1999; Kim et al., 2005; Schreiber et al., 2006]. In addition, PARP-1 itself can serve as a component of positive or negative transcriptional cofactors [Kraus and Lis, 2003]. The subcellular localization of PARP-1 to centromeres and the association of PARP-1 to centrosome indicate a potential role of PARP-1 in cell division as well [Burkle, 2005].

Human PARP-1 functions as a homodimer and contains an N-terminal 42 kDa double zinc-finger DNA-binding domain (DBD), a central

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15 kDa automodification center domain, and a C-terminal 55 kDa catalytic domain. DNA with strand breaks activates the PARP-1 polymerase activity resulting in PARP-1 auto-poly(ADP-ribosyl)ation as well as poly(ADP-ribosyl)ation of more than thirty nuclear protein acceptors [D'Amours et al., 1999].

Specific caspase-catalyzed cleavage of PARP-1 has been extensively studied. The cleavage of PARP-1 into a 24 kDa N-terminal fragment and an 89 kDa C-terminal fragment by particularly caspase-3 or -7 has been widely used as the hallmark for the initiation of apoptosis [Kaufmann et al., 1993]. Upon caspase mediated cleavage, the 24 kDa fragment competes with PARP-1 for DNA binding, while the 89 kDa fragment binds to full length PARP-1 and interrupts the formation of PARP-1 homodimer, a prerequisite for PARP-1 activity. Blockage of PARP-1 activity in the 24 and 89 kDa fragments stops the consumption of NAD^+ and ATP toward poly(ADP-ribosyl)ation and preserves the energy for certain ATP-dependent steps of apoptosis [Virag and Szabo, 2002]. Upon severe cellular assaults such as high level of alkylating agents, necrosis is triggered. PARP-1 is continuously activated in necrosis and results in the depletion of NAD^+ and ATP and leads to cell death.

Besides the caspase cleavage and auto-poly (ADP-ribosyl)ation, relatively little is known on PARP-1 post-translational modifications. Regulation of PARP-1 by acetylation [Hassa et al., 2005] and phosphorylation [Kauppinen et al., 2006] has been reported.

Ubiquitylation plays critical roles in regulating biological processes such as cell cycle progression, DNA repair, apoptosis, and transcription through ubiquitin dependent protein degradation [for reviews, see Pickart, 2001; Pickart and Eddins, 2004; Varshavsky, 2005]. Through three consecutive steps involving the ubiquitin activating enzyme, E1, a ubiquitin conjugating enzyme, E2, and a ubiquitin ligase, E3, acceptor proteins are ubiquitylated. The ε-amino group of a lysine residue in the ubiquitin substrate covalently conjugates to the carboxyl group of the C-terminal glycine 76 in the ubiquitin. Stepwise addition of multiple ubiquitin residues to the conjugated ubiquitin residues produces polyubiquitin. All seven lysine residues in the ubiquitin [Peng et al., 2003; Pickart and Fushman, 2004] have been found to form ubiquitin–ubiquitin linkages in vivo. Lys48-linked polyubiquitin chains are the targeting signal that leads to the recognition and degradation of protein substrates by the 26 S proteasome [Chau et al., 1989]. Rather than targeting a protein substrate for degradation, Lys63 mediated polyubiquitylation allows various roles including DNA repair, endocytosis, and enhancement of translation [Spence et al., 2000].

Ubiquitin regulatory pathways are known to be coupled in cellular regulation to other post-translational modifications such as phosphorylation in the regulation of ubiquitylation of p53, c-Myc, and I*k*B. Ubiquitylation and poly (ADP-ribosyl)ation regulates several common cellular processes such as transcription, DNA repair, apoptosis and others. While ubiquitylation involves three enzymatic steps and a large number of ubiquitin protein ligases, poly(ADP-ribosyl)ation is carried by several isoforms of PARP. Both ubiquitylation and poly(ADP-ribosyl)ation target signaling proteins in cellular regulation. However, little is known as to the interactions between ubiquitylation and poly (ADP-ribosyl)ation. Ubiquitylation of the 85 kDa C-terminal fragment of PARP-1 but not of the full-length PARP-1 in cells under radiation treatment has been reported [Masdehors et al., 2000]. The interaction of PARP-1 with human ubiquitin conjugating enzyme 9 (UBC9) has been found by the yeast dihybrid analysis [Masson et al., 1997]. However, neither has ubiquitylation of PARP-1 been confirmed nor has sumoylation of PARP-1 been reported. HECT ubiquitin protein ligases and PARP subfamily appear to share the common WWE domain yet the functional characterization of the WWE domain is lacking [Aravind, 2001]. In as much as ubiquitylation mediates the proteasome-dependent protein degradation, ubiquitylation of PARP-1 could play a regulatory role in apoptosis, necrosis, and other PARP-1-regulated cellular processes. PARP-1 degradation through pathways other than caspases appears to have been observed in Syrian hamster embryo cells [Guillouf et al., 2000]. Demonstration of ubiquitylation or caspase-independent degradation of PARP-1 would open up non-caspase dependent regulatory pathways for this important cellular regulatory enzyme. Further studies could lead to a better understanding of factors regulating the cellular processes through PARP-1 post-translational modifications. As a first step in dissecting the

interactions between ubiquitylation and poly (ADP-ribosyl)ation, we demonstrated here the ubiquitylation of PARP-1 in vivo and in vitro and effects of NAD^+ and activated DNA. The results suggest that polyubiquitylation of PARP-1 has unusual features and could play roles in PARP-1 degradation and consequently in apoptosis and necrosis.

MATERIALS AND METHODS

Materials

ALLN (*N*-acetyl-L-leucinyl-L-leucinyl-L-nor-leucinal) was purchased from Calbiochem. ArgAla and LeuAla were purchased from BACHEM. NAD^+ and activated DNA (D4522) were from Sigma. Other chemicals were from standard sources.

Bovine ubiquitin was purchased from Sigma. The preparation of biotin–ubiquitin has been described [Wang, 2003]. QuickChange site-directed mutagenesis kit (Stratagene) was applied to construct plasmids capable of expressing biotin-tagged ubiquitin, biotin-tagged Ub K48R, and biotin-tagged Ub K63R using pET-AB'C [Wang, 2003]. Nucleotide sequences of all ubiquitin mutants were sequenced and verified. Biotin–ubiquitin had similar activity as ubiquitin in ubiquitylation assays and was used in most cases unless specified otherwise [Wang, 2003]. Biotin provided tag for non-radioactive assays of ubiquitylation and purification of ubiquitylated proteins. Ubiquitylation enzyme mixtures containing E1, E2, and E3 (E1/E2/E3) were purified by the standard procedures of the affinity purification of ubiquitylation enzymes E1/E2/E3 from rabbit reticulocytes using ubiquitin–Sepharose column. The DTT/pH 9 eluate was used to carry out ubiquitin conjugation in vitro as previously described [Hershko et al., 1983; Hermida-Matsumoto et al., 1996]. Recombinant full-length human PARP-1 (>95% pure, 6xHis-tagged) was prepared as described [Simbulan-Rosenthal et al., 2001].

Cell Culture and Maintenance

PARP-1 +/+ mouse fibroblasts were cultured in 10 ml of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μg/ml). Cells in 10 cm culture plates were incubated in 37°C incubator with constant 5% CO_2. Every 2 or 3 days, when cells grew to about 90–100% confluency in the culture plate, the cells were trypsinized, collected and pelleted down. The cells were resuspended and passaged 10- or 20-fold with DMEM medium containing fetal bovine serum, penicillin and streptomycin in new culture plates.

Proteasome Inhibitor Treatment

Proteasome inhibitor, ALLN was dissolved in dimethyl sulfoxide (DMSO). For the examination of the accumulation of ubiquitin conjugates, 80% confluent PARP-1 +/+ mouse fibroblasts were incubated with 100 μM ALLN overnight [Gregory and Hann, 2000]. The final concentration of DMSO in the cell culture medium was 0.5%.

The cells were then harvested and lysed. Briefly, the cells were scraped, spun down, washed with PBS, and lysed by sonication 3×10 s at intervals of 1 min between in 1:1 lysis buffer (25 mM Tris-HCl, pH 7.5, 0.5 mM EGTA, 0.25% NP40, 0.125% sodium deoxycholate, 0.5 mM PMSF, 5 μg/ml pepstatin, 10 μg/ml leupeptin, 5 μg/ml aprotinin, 1 mM EDTA, 0.05% CHAPS, and 2.5 mM DTT). Cell lysates were centrifuged at 5,000*g* for 5 min, and lysate supernatants were collected. Total protein concentration of the supernatant was measured at $OD_{705\ nm}$ with protein concentration detection reagents A and B from BioRad (Cat# 500-0113 and # 500-0114). The supernatant was aliquoted and frozen at −80°C. The aliquots were used subsequently in ubiquitylation assays.

Induction of Apoptosis

After overnight treatment of 80% confluent PARP-1 +/+ mouse fibroblasts with 100 μM ALLN, anti-Fas antibody (50 μg/ml) and cycloheximide (1 μg/ml) were added to the cells to induce apoptosis [Simbulan-Rosenthal et al., 1998]. The treated cells were then collected and lysed at 8 and 24 h, respectively.

In Vitro Ubiquitylation Assays

In the presence of recombinant PARP-1, biotin–ubiquitin or bovine ubiquitin was incubated with 0.1–0.3 μg/μl affinity purified E1/E2/E3 fraction from rabbit reticulocytes in the presence of 50 mM HEPES, pH 7.5, 1 mM EGTA/EDTA/DTT, 5 mM $MgCl_2$, 10% glycerol, 2.5 mM ATP, and 0.05 unit/μl of inorganic pyrophosphatase at 37°C for 1 h. The reaction was stopped by addition of SDS sample buffer, boiled, and subjected to immunoprecipitation

(IP), or directly to SDS–PAGE and Western blot.

Immunoprecipitation

Equal amounts of cell extracts or reaction mixtures were incubated for 30 min at 4°C in a final volume of 200 μl EBC buffer (50 mM Tris–HCl, pH 8.0, 120 mM NaCl, 0.5% NP-40, aprotinin (0.1 TIU/ml), 1 mM PMSF), 1 μg of control mouse (as appropriate) immunoglobulin G (IgG, Pharmacia), and 20 μl of protein A/G-agarose beads (Pharmacia). After removal of the beads by centrifugation, the supernatant was incubated for 4 h to overnight at 4°C with 500 μl NET-N buffer (20 mM Tris–HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% NP-40) and 1 μg of mouse anti-PARP-1 monoclonal antibody (PharMingen). 20 μl of fresh protein A/G-agarose beads were then added and incubated with the mixtures overnight at 4°C. When cell extracts were used, either no antibody or normal IgG was added instead of PARP-1 antibody as controls. The beads were separated by centrifugation at 200*g* for 5 min at 4°C and washed 5 times with NET-N buffer, after which bound proteins were subjected to immunoblotting (IB) analysis with mouse monoclonal antibody against human ubiquitin (Santa Cruz) or horseradish peroxidase (HRP) conjugated streptavidin (Amersham Pharmacia Biotech) for the detection of ubiquitin or biotin–ubiquitin.

Western Blot/Immunoblot Analysis

The cell lysates or reaction mixtures were run on SDS–PAGE. Proteins were then transferred onto nitrocellulose membrane. Ponceau S stain was applied after transfer to confirm the equal loading in each lane. Different procedures were followed depending on different probes that were used for Western blots.

With HRP-conjugated streptavidin. The nitrocellulose membrane was incubated with blocking buffer (5% nonfat milk in PBST (1× PBS pH 7.4, 0.05% Tween-20)) for 1 h. The membrane was then consequently incubated with streptavidin-HRP conjugate solution (1: 1,000 in blocking buffer) for 1–3 h and washed with PBST 3 times for 15, 5, and 5 min, respectively. SuperSignal West Dura extended duration HRP substrate (Pierce) was added to and incubated with the membrane for 5 min. The signals on the membrane were detected with Kodak Biomax MR films.

With antibodies. The nitrocellulose membrane was incubated with blocking buffer (5% nonfat milk in PBST) for 1 h at room temperature. The blocked membrane was incubated with primary antibody solution (primary antibody diluted in blocking buffer) for overnight at 4°C or for 2–4 h at room temperature. The membrane was then washed with PBST 3 times for 15, 5, and 5 min, respectively. Then secondary antibody solution (HRP conjugated sheep-anti-mouse antibody diluted 3,000 fold in blocking buffer) was applied to the membrane at room temperature for 1 h. The membrane was washed again with PBST 3 times for 15, 5, and 5 min, respectively. HRP substrate was incubated with the membrane for 5 min at room temperature. Signals on the membrane were detected with Kodak Biomax MR films.

RESULTS

Polyubiquitylation of PARP-1 In Vivo

Mouse immortalized PARP-1 +/+ fibroblasts were first incubated in the presence and absence of the proteasome inhibitor, ALLN, for 24 h. Ubiquitylation of PARP-1 was examined by IP of the cell extracts with mouse anti-PARP-1 antibody followed by IB using mouse anti-ubiquitin antibody. Polyubiquitylation of PARP-1 was clearly observed in cells pretreated with the proteasome inhibitor ALLN, but not in control cells (Fig. 1A). A small fraction of the ubiquitylated proteins appeared below 113 kDa, the size of full-length PARP-1, probably resulted from the partially degradation or cleavage of ubiquitylated PARP-1 or ubiquitylation of PARP-1 fragments.

Since PARP-1 is cleaved by caspase-3 during apoptosis, apoptosis of mouse fibroblasts was induced by anti-Fas to ascertain the polyubiquitylation of PARP-1 under different cellular conditions. Apoptosis was induced in PARP-1 +/+ mouse fibroblasts by anti-Fas and the ubiquitylation of PARP-1 was monitored during apoptosis. With anti-Fas treatment, PARP-1 was gradually converted into the 89 kDa fragment as probed by anti-PARP-1. The longer the treatment, the more PARP-1 was cleaved into 89 kDa (Fig. 1B). After the induction of apoptosis (8 and 24 h), poly(ADP-ribose) was synthesized by PARP-1 as probed by anti-poly(ADP-ribose) (anti-PAR) antibody (Fig. 1C). Extensive polyubiquitylation of PARP-1 was observed before the induction of apoptosis and in the early hours

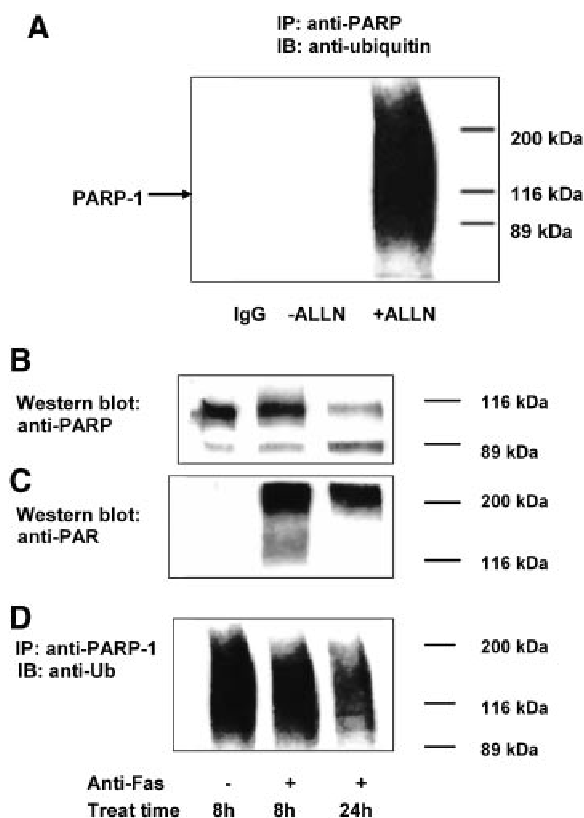

Fig. 1. Ubiquitylation of PARP-1 in vivo. **A**: PARP-1 ubiquitylation in vivo in the presence of proteasome inhibitor. Mouse fibroblast immortalized PARP-1 +/+ cells grew to 80% confluency. Cells were treated with 100 μM ALLN in DMSO while control cells were treated with the same amount of DMSO but no ALLN. 24 h later the cells were harvested and lysed by sonication. Thirty micrograms of cell lysates were used in immunoprecipitation (IP). The normal mouse IgG control sample (**left**), the cell lysates without ALLN treatment (**center**), and the cell lysates with ALLN treatment (**right**) are shown after IP with anti-PARP and immunoblotting (IB) with anti-ubiquitin. Arrow indicates the expected PARP-1 molecular weight. **B**–**D**: Polyubiquitylation of PARP-1 in anti-FAS induced apoptotic mouse fibroblasts. Mouse PARP-1 +/+ fibroblasts were treated with proteasome inhibitor ALLN overnight. Cycloheximide and anti-Fas antibody were added to induce apoptosis. Cells were harvest at 8 and 24 h after induction. Control cells, 8 h (−), without anti-Fas and cycloheximide treatment were harvested at 8 h. Equal amounts of proteins from each cell lysate were analyzed by Western blot with anti-PARP (**B**) or anti-PAR (**C**), or IP with anti-PARP followed by IB with anti-ubiquitin (**D**). Chemiluminescence signals were detected with sheep anti-mouse secondary antibody conjugated with HRP.

after the induction of apoptosis (Fig. 1D). However, as more PARP-1 was cleaved into the 89 kDa fragment, less polyubiquitylation was observed. As the anti-PARP antibody can recognize both full-length and the C-terminal 89 kDa PARP-1, the decreased polyubiquitylation of PARP-1 suggest that the 89 kDa was not ubiquitylated. The anti-PARP antibody did not provide sufficient sensitivity to detect the smear of poly-ubiquitylated PARP-1 above PARP-1 in the anti-PARP-1 immunoprecipitate. Nonetheless, the appearance of the 89 kDa PARP-1 and the disappearance of polyubiquitylated PARP-1 in apoptosis were consistent with the polyubiquitylation of PARP-1 in vivo.

Polyubiquitylation of PARP-1 In Vitro

Recombinant full-length human PARP-1 was then used to confirm the ubiquitylation of PARP-1 in vitro. PARP-1 was incubated with biotin–ubiquitin and affinity purified E1/E2/E3 from rabbit reticulocytes under standard ubiquitylation assay conditions. Biotin–ubiquitin is a recombinant ubiquitin fusion protein with a 23-amino acid-residue peptide fused to ubiquitin N-terminus. Biotin was attached to the 23-amino-acid residue peptide. The readiness of biotin–ubiquitin for in vitro ubiquitylation assays has been established [Wang, 2003]. Polyubiquitylation of PARP-1 was examined by IP using anti-PARP-1 followed by IB with streptavidin-HRP. As shown in Figure 2A, time dependent increase in the polyubiquitylation of PARP-1 was observed and the ubiquitylation of PARP-1 required the presence of ubiquitin, E1/E2/E3, and PARP-1. Similar results were obtained using unmodified ubiquitin and probing with anti-ubiquitin antibody (data not shown).

Ubiquitin mutants were then used to determine the conjugation site in ubiquitin since conjugation of PARP-1 through ubiquitin Lys48 or Lys63 could suggest different functions for the ubiquitylation of PARP-1. Ubiquitin mutants, UbK48R or UbK63R, were used in the in vitro ubiquitylation of PARP-1 under standard assay conditions. As shown in Figure 2B, polyubiquitylation of PARP-1 was observed in reactions using ubiquitin or ubiquitin K63R but not using ubiquitin K48R. Mutation of Lys48 residue of ubiquitin completely abolished the polyubiquitylation of PARP-1. The results suggest that Lys48 of ubiquitin is conjugated to PARP-1, which in turn suggests that polyubiquitylation of PARP-1 is likely destined to the degradation by the 26 S proteasome. The ubiquitylation of PARP-1 through ubiquitin K48 is consistent with the earlier results that PARP-1 polyubiquitylation in vivo was observed in the presence but not in

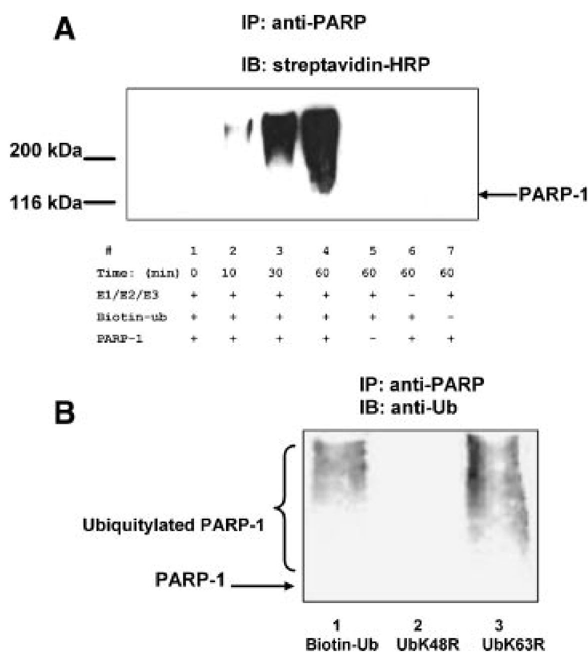

Fig. 2. **A**: PARP-1 ubiquitylation in vitro. Recombinant PARP-1 was incubated at 37°C with recombinant biotin-tagged ubiquitin and affinity purified E1/E2/E3 under in vitro ubiquitylation assay conditions. Aliquots of reaction mixtures were immunoprecipitated with anti-PARP and immunoblotted with streptavidin-peroxidase. Normal mouse IgG was used as the control. **B**: Polyubiquitylation of PARP-1 through ubiquitin K48 linkage. Biotin-tagged ubiquitin, or biotin-tagged ubiquitin K48R or K63R was expressed in *E. coli* BL21 (DE3), and purified with Softlink monomeric avidin affinity chromatography. Biotin–ubiquitin and mutant proteins were incubated with recombinant PARP-1 under standard ubiquitylation assay conditions and analyzed by IP and IB as in Figure 1B.

the absence of the proteasome inhibitor (Fig. 1A).

Effects of Activated DNA and Caspase-3 on PARP-1 Ubiquitylation

DNA with strand breaks exhibits high affinity to PARP-1 and activates the poly(ADP)-ribosylation activity of PARP-1 [Juarez-Salinas et al., 1979]. Activated DNA activates PARP-1 by binding to the N-terminal DNA binding domain in PARP-1 [Gradwohl et al., 1987; Weinfeld et al., 1997; D'Silva et al., 1999]. PARP-1 ubiquitylation was carried out in vitro with and without preincubation of PARP-1 with activated DNA to assess the effect of DNA binding on the PARP-1 ubiquitylation. Activated DNA treated recombinant PARP-1 was incubated with ubiquitin and affinity purified E1/E2/E3 under the standard ubiquitylation assay conditions. While PARP-1 without pretreatment with activated DNA was extensively ubiquitylated (Fig. 3A), the ubiquitylation of PARP-1 was completely abolished after preincubation of PARP-1 with activated DNA as shown in Figure 3A, right lane. The strong inhibition of PARP-1 ubiquitylation by activated DNA suggests possible overlapping of the ubiquitylation site and the DNA binding domain in PARP-1 or indirect inhibition of ubiquitylation by activated DNA.

Caspase-3 specifically cleaves PARP-1 at residues DEVD↓G in the PARP-1 DNA binding domain into an N-terminal 24 kDa fragment and a C-terminal 89 kDa fragment prior to apoptosis [Lazebnik et al., 1994; Casiano et al., 1996; Greidinger et al., 1996]. Caspase-3 was used to cleave ubiquitylated PARP-1 in attempt to locate the ubiquitylation site in PARP-1. PARP-1 was first incubated with ubiquitin and affinity purified E1/E2/E3 to form ubiquitylated PARP-1, and then caspase-3 was added to cleave PARP-1. As shown in Figure 3B, the intensity of the polyubiquitylated PARP-1 was greatly reduced after caspase-3 treatment. Considering that mouse anti-PARP antibody (PharMingen) recognized the C-terminal catalytic domain in PARP-1, the greatly reduced level of polyubiquitylated PARP-1 is consistent with the suggestion that the N-terminal 24 kDa fragment which resides in the DNA binding domain being the polyubiquitylation site in PARP-1. Whether the 89 kDa fragment can be polyubiquitylated was examined by fully digesting PARP-1 with caspase-3 followed by ubiquitylation under standard conditions. The complete digestion of PARP-1 to 89 kDa was first confirmed using anti-PARP-1 (data not shown). As shown in Figure 3C, no polyubiquitylation of the 89 kDa fragment was detected. Whether the 24 kDa fragment can be polyubiquitylated could not be determined directly at present, since the antibodies recognized the catalytic domain and attempts of producing the PARP-1 24 kDa fragment in *Escherichia coli* were unsuccessful.

Effects of NAD^+, ArgAla, and LeuAla on PARP-1 Ubiquitylation

While activated DNA binds to the C-terminus of PARP-1, NAD^+ binds to the N-terminal catalytic domain of activated PARP-1. Ubiquitylation of PARP-1 was then carried out in the presence and absence of NAD^+ and activated DNA using ubiquitin and affinity purified E1/E2/E3 under ubiquitylation assay conditions.

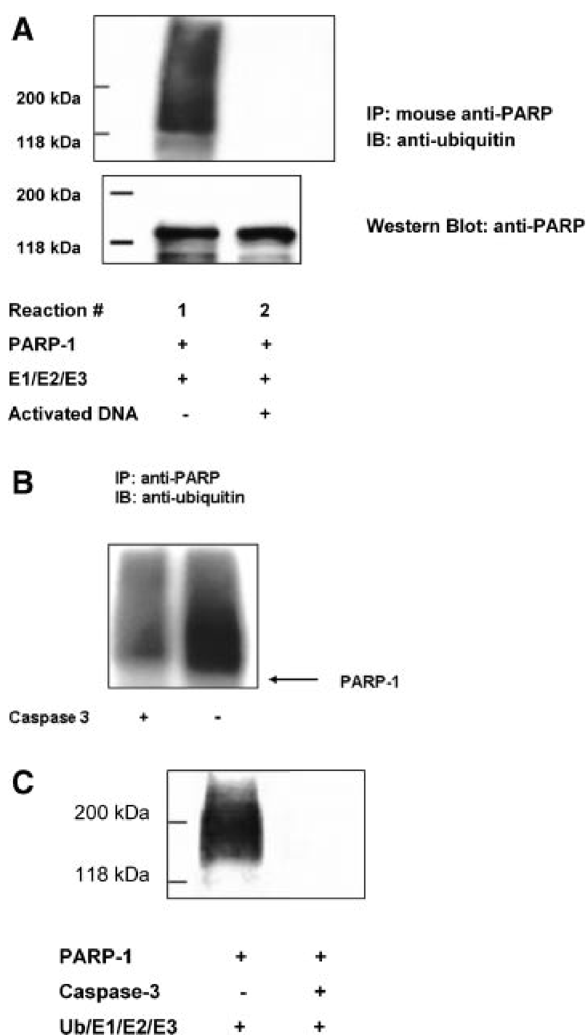

Fig. 3. Effects of activated DNA, caspase 3 on ubiquitylation of PARP-1. **A**: Inhibition of PARP-1 ubiquitylation by activated DNA. The ubiquitylation assays were performed at 37°C for 1 h with recombinant PARP-1, affinity purified E1/E2/E3, and bovine ubiquitin under in vitro ubiquitylation assay conditions in the absence (**Lane 1**) and the presence of 80 μg/μl activated DNA (**Lane 2**). **B**: Casapse-3 treatment of ubiquitylated PARP-1. After ubiquitylation of PARP-1 reaction under in vitro ubiquitylation assay conditions, caspase-3 was added to the reaction mixtures and then incubated at 37°C for 1 more h (**Lane 1**). The control reaction was incubated in the absence of caspase 3 (**Lane 2**). The reaction mixtures were subjected to IP with mouse anti-PARP and IB with mouse anti-ubiquitin followed by anti-mouse secondary antibody conjugated with HRP. **C**: PARP-1 C-terminal 89 kDa was not ubiquitylated. Recombinant PARP-1 was mixed with (**right lane**) or without (**left lane**) caspase-3 in the presence of 50 mM HEPES (pH7.5), 5 mM $MgCl_2$, 5 mM ATP, 0.05 unit/μl inorganic pyrophosphatase, 1 mM DTT/EDTA/EGTA, and 10% glycerol. The mixtures were incubated at 37°C for 1 h. Then affinity purified E1/E2/E3 and ubiquitin were added into the mixtures and incubation at 37°C was extended for 1 h more. The reaction mixtures was subjected to immunoprecipitation (IP) with anti-PARP followed by immunoblot (IB) with anti-ubiquitin to check PARP-1 ubiquitylation. PARP antibody recognizing catalytic domain of PARP-1 was used in the experiments.

Polyubiquitylated PARP-1 was examined using IP with mouse anti-PARP-1 and IB with mouse anti-ubiquitin. As shown in Figure 4A, NAD^+ reduced ubiquitylation of PARP-1 using affinity purified E1/E2/E3 in the absence of activated DNA (Lane 5 compared to Lane 4 in Fig. 4A). It is noteworthy that complete inhibition of PARP-1 ubiquitylation by activated DNA was observed (Lane 6 in Fig. 4A). In the presence of both NAD^+ and activated DNA, activated PARP-1 synthesized poly(ADP-ribose) and ubiquitylation of PARP-1 was completely blocked (Lane 7, Fig. 4A). Due to the inhibition of PARP-1

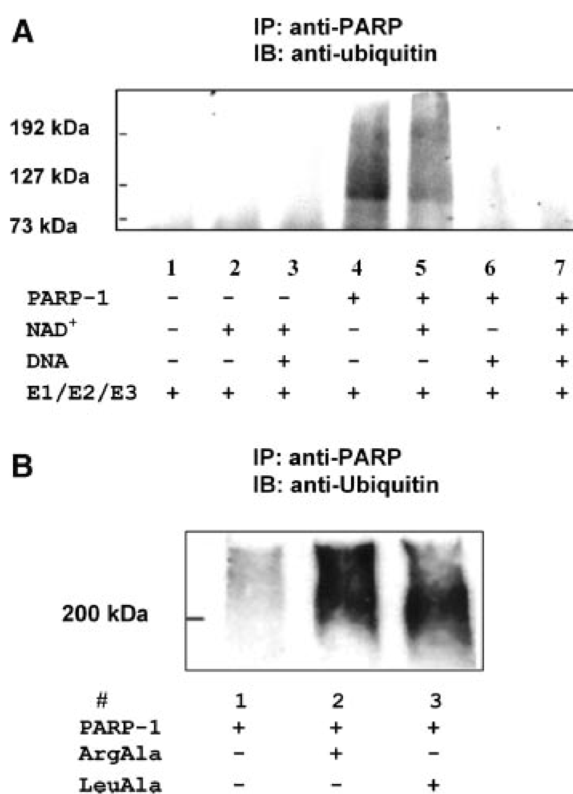

Fig. 4. Effects of NAD^+, ArgAla, and LeuAla on ubiquitylation of PARP-1. **A**: NAD^+ inhibition of ubiquitylation of PARP-1. PARP-1 was incubated with 60 μM bovine ubiquitin and 0.1 μg/μl affinity purified E1/E2/E3 in the presence and absence of 100 μM NAD^+, and the presence and absence of 100 μg/μl activated DNA under in vitro ubiquitylation assay conditions. Negative control reactions were performed in the absence of PARP-1 (**Lanes 1–3**). The reaction products were subjected to IP with anti-PARP antibody followed by IB with anti-ubiquitin antibody. **B**: ArgAla and LeuAla enhancement of ubiquitylation of PARP-1. Recombinant PARP-1 was incubated with recombinant 0.2 μg/μl biotin–ubiquitin and 0.1 μg/μl affinity purified E1/E2/E3 in the absence (Lane 1) and presence of 1.3 mM ArgAla (Lane 2) or 1.3 mM LeuAla (Lane 3) under in vitro ubiquitylation assay conditions. The reaction mixtures were subjected to IP with anti-PARP antibody recognizing the catalytic domain of PARP-1 followed by SDS–PAGE and IB with anti-ubiquitin antibody.

ubiquitylation by activated DNA, the effect of poly(ADP-ribose) on ubiquitylation of PARP-1 could not be determined.

Dipeptides ArgAla and LeuAla are known as N-end rule E3 inhibitors [de Groot et al., 1991; Obin et al., 1999]. ArgAla inhibits type I N-end rule E3s (basic) and LeuAla inhibits type II N-end rule E3s (hydrophobic). These dipeptides were used to examine whether E3 for PARP-1 ubiquitylation is mediated an N-end rule ligase. Recombinant PARP-1 was incubated with affinity purified E1/E2/E3 and biotin–ubiquitin in the absence and presence of ArgAla or LeuAla under the ubiquitylation assay conditions. As shown in Figure 4B, the dipeptide inhibitors did not inhibit the ubiquitylation of PARP-1. On contrary, polyubiquitylation of PARP-1 actually was stimulated by ArgAla and LeuAla.

Ubiquitylated PARP-1 Is Active in Poly(ADP-ribose) Synthesis

Whether ubiquitylation of PARP-1 abolishes the PAR synthesis activity was next examined. PARP-1 was first ubiquitylated using biotin–ubiquitin under standard ubiquitylation conditions, then NAD^+ and activated DNA were added for the poly(ADP-ribose) (PAR) activity assay. The control reaction contained all the components for ubiquitylation except ATP. The effects of ATP on PAR synthesis were first examined. As shown in Figure 5A, ATP clearly inhibited PARP-1 activity. The PAR synthesis was initiated by adding activated DNA and NAD^+ immediately following ubiquitylation of PARP-1 under the standard ubiquitylation assay conditions using biotin–ubiquitin. Ubiquitylated PARP-1 was separated from unmodified PARP-1 after ubiquitylation by affinity chromatography using monovalent avidin that bound to biotin–ubiquitin and bound ubiquitylated PARP-1 was eluted with biotin under native conditions. The amounts of PAR were determined in the total reaction mixtures and the eluates of bound ubiquitylated PARP-1 from the avidin affinity columns by Western blots using mouse anti-PAR antibody [Kawamitsu et al., 1984]. As shown in Figure 5B, the PAR amount was lower in the presence of ATP (Lane 1, Fig. 5B) than that in the absence of ATP (Lane 2, Fig. 5B) due to the inhibition of PARP-1 activity by ATP [Kun et al., 2004]. As shown in Figure 5B, Lane 3, the ubiquitylated PARP-1 purified by affinity chromatography also contained PAR. The small amount of PAR was detected in the control (Lane 4, Fig. 5B) due to the nonspecific binding of PAR to the avidin column. These results are consistent with that ubiquitylated PARP-1 was active in PAR synthesis.

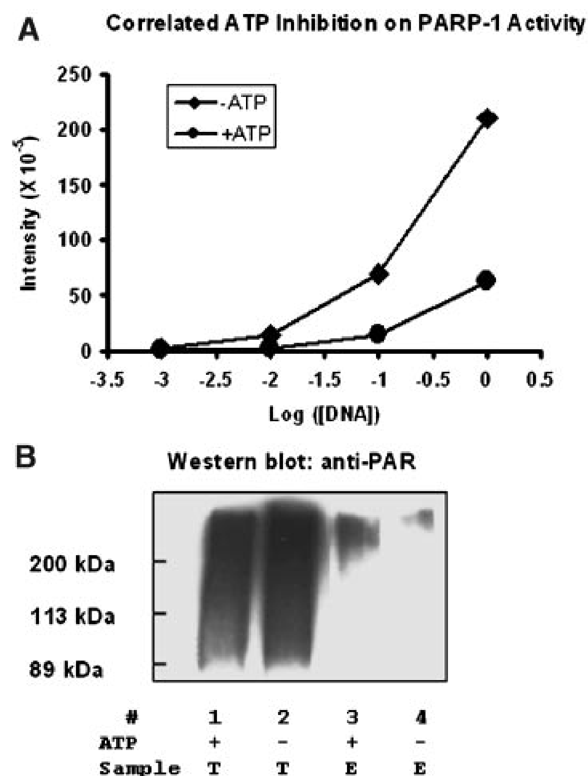

Fig. 5. Effect of ubiquitylation on the polymerase activity of PARP-1. **A**: ATP inhibition of poly(ADP-ribosyl)ation. Recombinant PARP-1 was incubated at 37°C for 1 h in the presence (circle) or absence (square) of 2.5 mM ATP under standard PAR assay conditions with NAD^+ (200 μM) and varying concentrations of activated DNA (40, 4, 0.4, and 0.04 μg/ml). Small aliquots of the reaction mixtures were subjected to SDS–PAGE followed by Western blot analysis with mouse anti-PAR antibody. The image correlated intensities were plotted against logarithmic DNA concentrations. **B**: Poly(ADP-ribosyl)ation of ubiquitylated PARP-1. Recombinant PARP-1 was first pre-incubated for ubiquitylation of PARP-1 at 37°C with 0.2 μg/μl biotin–ubiquitin and 0.1 μg/μl affinity purified E1/E2/E3 in the presence (**Lanes 1** and **3**) or in the absence (**Lanes 2** and **4**) of 2.5 mM ATP under standard ubiquitylation conditions. After 1 h ubiquitylation reaction, 250 μM NAD^+ and 5 μg/μl activated DNA were then added and incubated at 37°C for poly(ADP-ribose) synthesis for an additional h. Aliquots of the reaction mixtures (T) were analyzed for PAR synthesis using anti-PAR antibodies after SDS gel electrophoresis. The remaining fractions of the reaction mixtures were applied to monomeric avidin affinity column to isolate the ubiquitylated PARP by affinity chromatography on monomeric avidin. The flow-through and the eluate using 5 mM biotin elution buffer were collected. The aliquots of the reaction mixtures (T) and the eluate (E) were examined by SDS–PAGE followed by Western blot analysis with mouse anti-PAR [Kawamitsu et al., 1984].

DISCUSSION

Polyubiquitylation of full length PARP-1 was clearly shown in mouse fibroblasts in vivo and in recombinant PARP-1 in vitro through IP by anti-PARP-1 and IB using anti-ubiquitin. Polyubiquitylation of PARP-1 was further supported by the disappearance of observed polyubiquitylation of PARP-1 by caspase-3 treatment, the inhibition of polyubiquitylation by activated DNA and by the PARP-1 substrate, NAD^+. The reduction of polyubiquitylation of PARP-1 during apoptosis in vivo was consistent with the abolished ubiquitylation of 89 kDa PARP-1 after caspase treatment. Additionally, PAR was present in the affinity purified ubiquitylated PARP-1. We were unable to detect poly-ubiquitylated PARP-1 directly by IB with anti-PARP-1 in the anti-PARP-1 immunoprecipitate, likely due to the insufficient sensitivity of the anti-PARP-1 antibody or the inability of anti-PARP-1 to bind tightly to polyubiquitylated PARP-1. Nonetheless, the responses of ubiquitylated PARP-1 to caspase-3, activated DNA, NAD^+, and co-purification of PAR with ubiquitylated PARP-1 were consistent with the ubiquitylation of PARP-1. Our results suggest, for the first time, that full-length PARP-1 can be polyubiquitylated in vivo and in vitro.

The requirement of proteasome inhibitor to observe polyubiquitylation of PARP-1 in vivo suggests that polyubiquitylated PARP-1 is susceptible to degradation by the 26 S proteasome. This is consistent with the finding using ubiquitin mutants which revealed that only UbK48R failed to polyubiquitylated PARP-1 (Fig. 2B). The Lys48 linked polyubiquitylation has been well established as a signal to target proteins for proteasome catalyzed degradation.

Since PARP-1 is activated by DNA, inhibition of ubiquitylation of PARP-1 by activated DNA implies that the DNA activated PARP-1 is protected from ubiquitylation-mediated degradation by proteasome. With the protection, it would allow PARP-1 to catalyze poly(ADP-ribosyl)ation of nuclear proteins to actively participate in nuclear pathways such as DNA repair. Inhibition of PARP-1 polyubiquitylation by activated DNA, and absence of ubiquitylated chain in the 89 kDa fragment after caspase-3 treatment in vitro and after apoptosis induction in vivo are consistent with the suggestion that PARP-1 was polyubiquitylated at the N-terminal DNA binding domain. The observation that polyubiquitylation of PARP-1 did not completely block the PAR synthesis by PARP-1 suggests that polyubiquitylation site may be away from the catalytic domain or the automodification site in PARP-1.

Recognition and regulatory signals of PARP-1 ubiquitylation could be located in the C-terminal catalytic domain of PARP-1 as suggested by the inhibition of PARP-1 polyubiquitylation by NAD^+. The inhibition could be derived from the NAD^+ induced conformational changes in PARP-1 that in turn inhibited the recognition of PARP-1 by its ubiquitin protein ligase. Single-point mutation at Lys893 within the catalytic domain of PARP-1 did not abolish the ubiquitylation of PARP-1 (data not shown), which suggests that catalytic activity is not required for the ubiquitylation of PARP-1.

PARP-1 is known to be cleaved by caspases 3 and/or 7 during apoptosis. Cleavage of PARP-1 by caspases inactivates the enzyme, which is the prerequisite of apoptosis [Earnshaw, 1995]. Ubiquitylation of PARP-1 was unable to protect PARP-1 from caspase-3 catalyzed cleavage. Therefore, ubiquitylation of PARP-1 will not rescue the cell from apoptosis. Upon caspase-3 treatment, ubiquitylated PARP-1 was cleaved and nondetectable by PARP-1 antibody recognizing the 89 kDa fragment. Our present findings that C-terminal 89 kDa of PARP-1 was not ubiquitylated in vitro differ from the in vivo results reported by Masdehors et al. [2000] where they detected the ubiquitylation of C-terminal 85 kDa fragment of PARP-1 but not the full-length PARP-1 was detected during radiation-induced apoptosis from human lymphocytes. Furthermore, according to Masdehor's report, the monoubiquitylation of the 85 kDa fragment was inhibited by N-end rule E3 inhibitors, that is, the monoubiquitylation of 85 kDa fragment required an N-end rule E3, while our results suggested that polyubiquitylation of full-length PARP-1 may be carried out by an E3 other than N-end rule E3s (Fig. 4B). Whether the cell lines, culture and the use of recombinant PARP-1 contributed to the differences is yet to be determined. It should be noted there are numerous ubiquitin protein ligases and PARP-1 could subject to ubiquitylation by multiple ligases in vivo.

The enhancement of ubiquitylation by N-end rule E3 inhibitors was unexpected but was similarly observed in Cup9 ubiquitylation [Turner et al., 2000]. It was thought that some

E3s contain binding sites both for dipeptides (or a substrate with similar N-terminal dipeptides) and for proteins such as Cup9 (and PARP-1 here). Addition of the dipeptides or N-end rule substrates blocks the corresponding binding sites on E3 and leaves the binding site for Cup9 open. The ubiquitylation is thus increased. It will be interesting to see if ubiquitylation of PARP-1 and ubiquitylation of N-end rule E3 regulate each other. The present study cannot exclude the possibility that other ubiquitin protein ligases may modify PARP-1.

The fundamental studies on the ubiquitylation of PARP-1 lead us to question the physiological roles of PARP-1 ubiquitylation in the regulation of PARP-1 in the cell. As PARP-1 is an abundant protein in the cell, it is hard to imagine the role of ubiquitylation on PARP-1 is solely involved in targeting PARP-1 for degradation. The present results suggest that the polyubiquitylation of PARP-1 has features that imply the physiological importance of the modification. The polyubiquitylation could be modulated by activated DNA and the substrate NAD^+. While the site of modification appears to be at the N-terminal DNA binding domain, the C-terminal catalytic domain may affect polyubiquitylation. Additionally, the caspase cleaved 89 kDa PARP-1 could not be polyubiquitylated. PARP-1 is known to regulate the expression of various proteins at the transcription level [Kraus and Lis, 2003]. A possible role of PARP-1 in transcription is indicated by findings that report frequent association of PARP-1 with transcriptionally active proteins. In recent years it has become evident that one of the ways in which cells meet this kind of regulatory challenge is to make extensive use of the ubiquitin-proteasome system [Muratani and Tansey, 2003]. The regulation of PARP-1 in transcription could be regulated by polyubiquitylation of PARP-1. By controlling the PARP-1 turnover, ubiquitylation may affect the translocation, the quantity, and activity of PARP-1 and thus regulate gene expression. Ubiquitylation of PARP-1 could similarly regulate other cellular processes in vivo such as DNA repair to narrow the time and space windows of the activation of PARP-1.

In summary, polyubiquitylation of full-length PARP-1 may regulate PARP-1 by irreversible degradation of PARP-1. Regulation of PARP-1 ubiquitylation by activated DNA, NAD^+, and poly(ADP-ribose) and regulation of PARP-1 activity through PARP-1 ubiquitylation suggest polyubiquitylation of PARP-1 may subject to tight regulation and warrant further studies to better understand the interactions of polyubiquitylation and poly(ADP-ribosyl)ation pathways. Similar approach and regulation may be applicable to other isoforms in the PARP family. Pharmacological inhibitors of PARP-1 have advanced to produce protective agents of cardiovascular diseases and therapeutics of cancer treatments. Ubiquitylation pathways of PARP-1 degradation could open another drug design approach in modulating PARP-1.

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