In vivo phosphorylation of poly(ADP-ribose) polymerase is independent of its activation

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Abstract Poly(ADP-ribose) polymerase (PARP) is a nuclear enzyme, which is activated by DNA strand breaks. Although PARP is known to be cleaved by the cysteine protease, caspase-3/CPP32, during apoptosis, signal cascade which regulates the PARP activity has not been fully understood. In this study, we investigated post-translational modification of PARP. We found that PARP was phosphorylated by a serine kinase in vivo. PARP was activated temporarily and extensive auto-modification occurred on PARP, possibly by the fragmented DNA during apoptosis induced by etoposide in Jurkat cells. However, the phosphorylation level was not changed for up to 6 h, after PARP cleavage began in apoptosis by the treatment with etoposide. Furthermore, we showed the presence of a PARP-associated kinase in nuclear extracts of the HTLV-I infected T-cell lines but not in uninfected T-cell lines, whereas this kinase did not inhibit the PARP activity even in the presence of ATP. Taken together, in vivo phosphorylation of PARP might be independent of the activation or cleavage of PARP.

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1. Introduction

Poly(ADP-ribose) polymerase (PARP, EC 2.4.2.30) is a DNA repair enzyme, which binds to and is activated by DNA strand breaks generated by ionizing radiation or alkylating agents. PARP catalyzes the transfer of ADP-ribose units from its substrate, nicotinamide adenine dinucleotide (NAD\textsuperscript{+}), to nuclear proteins such as histones and PARP itself [1–3]. PARP has been implicated in DNA repair and genomic stability [4–7].

Several proteases related to interleukin-1β-converting enzyme (ICE) and the product of the \textit{Caenorhabditis elegans} ced-3 are activated and required during apoptosis. PARP is cleaved into an 89-kDa carboxy-terminal fragment containing the auto-modification domain and the NAD-binding domain and an approximately 24-kDa N-terminal fragment containing the DNA binding domain by a cysteine protease, caspase-3/prICE/CPP32/Yama/apopain and Mch3 during apoptosis [8,9]. PARP cleavage can also be mediated by Nedd2, ICE, TX and Mch2, with lower efficiency [10]. Therefore, PARP may be selectively targeted for degradation during apoptosis. However, little is known about the signalling pathway which regulates the PARP activity. Here, we report that PARP was phosphorylated by a serine kinase in vivo. Although PARP was activated temporarily, the phosphorylation level was not changed, even when PARP cleavage began in apoptosis by the treatment with etoposide, suggesting that the phosphorylation of PARP did not coincide with the temporary activation or cleavage of PARP during apoptosis. Furthermore, we found a PARP associated kinase in the HTLV-I infected T-cell lines, whereas this kinase did not inhibit the PARP activity.

2. Materials and methods

2.1. Cell culture and metabolic labelling

JPX-9 cell was a kind gift from Dr. K. Sugamura [11]. Jurkat, JPX-9, MT-2, MT-4 and HUT102 cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FCS) at 37°C in a humidified 5% CO\textsubscript{2} atmosphere. For metabolic labelling, Jurkat cells were incubated in phosphate-free RPMI 1640 medium supplemented with 10% FCS dialyzed against Tris-buffered saline (20 mM Tris-HCl, 0.15 M NaCl, pH 7.5) containing \textsuperscript{32}Porthophosphate (ICN, 18.5 MBq/ml) at 37°C for 8 h. During metabolic labelling, cells were treated with 100 μM etoposide for the indicated time. For the expression of the Tax protein, JPX-9 cells were cultured in the presence of 10 μM CdCl\textsubscript{2} at 37°C for 3 days.

2.2. Immunoblotting analysis

Cells were lysed in the lysis buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 4 mM EDTA, 1% NP-40, 0.1% SDS, 1 mM DTT and 1 mM PMSF. The lysate was subjected to SDS-PAGE, followed by 20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 4 mM EDTA, 1% NP-40, 0.1% SDS, 0.1% sodium deoxycholate, 50 mM NaF, 0.2 mM NaN\textsubscript{3}, 10 mM β-glycerophosphate, 1 mM PMSF and 1 μg/ml aprotinin. The lysates were pre-cleared with a protein G-Sepharose (Pharmacia) coated with normal rabbit IgG and the supernatant was subjected to SADS-PAGE, followed by immunoblotting analysis using polyclonal antibody against PARP or poly(ADP-ribose) antibody.

2.3. Immunoprecipitation

\textsuperscript{32}P-labelled Jurkat cells were lysed in the special RIPA buffer containing 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 2 mM EDTA, 1% NP-40, 0.1% SDS, 0.1% sodium deoxycholate, 50 mM NaF, 0.2 mM NaN\textsubscript{3}, 10 mM β-glycerophosphate, 1 mM PMSF and 1 μg/ml aprotinin. The lysates were pre-cleared with a protein G-Sepharose (Pharmacia) coated with normal rabbit IgG and the supernatant was incubated with anti-PARP polyclonal antibody for 60 min at 4°C. The precipitates were washed four times with the RIPA buffer. After elution of protein in 1 × Laemmli sample buffer containing 50 mM Tris-HCl (pH 6.8), 2% SDS, 0.1% bromophenol blue (BBP), 10% glycerol and 5% 2-mercaptoethanol with boiling for 5 min, samples were fractionated on a 7% SDS-polyacrylamide gel and detected by autoradiography.

2.4. Induction of apoptosis

Jurkat cells were induced to undergo apoptosis by exposure to...
100 μM etoposide. For analysis of DNA fragmentation, Jurkat cells treated with 100 μM etoposide were collected at the indicated time, washed with ice-cold PBS, and lysed in 500 μl of a lysis buffer containing 5 mM Tris-HCl (pH 8.0), 10 mM EDTA, and 0.5% Triton X-100. After 10 min on ice, the lysates were centrifuged (13,000 g) for 10 min at 4°C to separate the soluble fragmented DNA from insoluble intact chromatin. The supernatant was treated with proteinase K (200 μg/ml) in the presence of 1% SDS overnight at 37°C. Then the lysates were extracted with phenol-chloroform-isooamyl alcohol (24:1), and the aqueous phase was made to 300 mM NaCl and 2 volumes of ethanol were added. The pellet was rinsed with 70% ethanol, air dried, and dissolved in 15 μl of the buffer containing 10 mM Tris-HCl, 1 mM EDTA (pH 8.0) and RNase A (0.6 mg/ml). After incubation at 37°C for 30 min, the samples were electrophoresed on a 2% agarose gel. This procedure could isolate the low molecular weight degraded DNA without high molecular weight intact DNA.

2.5. Phospho-amino acid analysis

Agarose gel. This procedure could isolate the low molecular weight degraded DNA without high molecular weight intact DNA. 

little is known about the in vivo phosphorylation of PARP. 

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NaCl, 10 mM MgCl₂, 2 mM EGTA, 50 mM NaF, 10 mM pH 3.5 bufer (pyridine/acetic acid/water, 1:10:189 (v/v)) at 4°C. 

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Phosphorylation was detected by autoradiography. 

Figure 1. In vivo phosphorylation of PARP. 

b: Phospho-amino acid analysis of PARP. 

Phospho-amino acid standards were identified by autoradiography and phospho-amino acid standards were identified by spraying the plate with ninhydrin.

2.6. In vitro kinase and PARP assay

Nuclear extracts were immunoprecipitated with anti-PARP antibody in the lysis buffer containing 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 2 mM EGTA, 50 mM NaF, 10 mM β-glycerophosphate, 10 μg/ml aprotinin, 1 mM PMSF, 1 mM dithiothreitol (DTT) and 1% NP-40. The anti-PARP immunoprecipitates were incubated with [γ-32P]ATP with or without poly(dI-dC)poly(dI-dC) (0.2 μg, Pharmacia) in the assay buffer containing 10 mM HEPES (pH 7.9), 50 mM NaCl, 10 mM MgCl₂, 5% glycerol, 1 mM EGTA and 1 mM dithiothreitol (DTT) in a final reaction volume of 30 μl. After incubation at 30°C for 10 min, the reaction mixture was subjected to SDS-PAGE. Phosphorylation was detected by autoradiography.

For the PARP assay, the anti-PARP immunoprecipitates were incubated with [γ-32P]ATP with or without poly(dI-dC)poly(dI-dC) (0.2 μg) in the assay buffer containing 10 mM HEPES (pH 7.9), 50 mM NaCl, 10 mM MgCl₂, 5% glycerol, 1 mM EGTA and 1 mM dithiothreitol (DTT) in a final reaction volume of 30 μl. After incubation at 30°C for 10 min, the reaction mixture was subjected to SDS-PAGE, and poly-(ADP-ribose)ylation was detected by autoradiography. To study the effect of the phosphorylation on the PARP activity, the reaction mixture was also incubated with unlabelled 0.2 mM ATP.

3. Results

3.1. In vivo phosphorylation of PARP

Previously Tanaka et al. [12] reported that PARP was phosphorylated by protein kinase C (PKC) in vitro [12], however, little is known about the in vivo phosphorylation of PARP. 

To determine whether PARP was phosphorylated in vivo, Jurkat cells were metabolically radio-labelled with [32P]orthophosphate for 8 h. PARP phosphorylation was analyzed by SDS-PAGE of anti-PARP immunoprecipitates. We observed that PARP was phosphorylated in vivo (Fig. 1a). To identify the phosphorylated amino acids in PARP, [32P]-labelled PARP protein from Jurkat cells as shown in Fig. 1a was eluted from a gel section and subjected to acid hydrolysis. [32P]Phospho-amino acids were separated by high voltage electrophoresis and detected by autoradiography. We found that the PARP protein was phosphorylated mainly at the serine residue (Fig. 1b), suggesting that PARP was phosphorylated by a serine kinase in vivo.

4.2. Phospho-amino acid analysis

32P-labelled PARP protein was eluted from acrylamide gel sections in the elution buffer containing 50 mM NH₄HCO₃ and 0.1% SDS. Recovered protein sample was hydrolyzed in 6 N HCl at 110°C for 90 min. Resulting phospho-amino acid samples were mixed with standards (phospho-serine, phospho-threonine and phospho-tyrosine; 2 μg each) and applied onto a cellulose thin layer glass plate (20×20 cm, Merck, Art. 5716). High voltage electrophoresis was performed in pH 3.5 buffer (pyridine/acetic acid/water, 1:10:189 (v/v)) at 4°C. 

Phospho-amino acid standards were identified by spraying the plate with ninhydrin.
etoposide for the time indicated in Fig. 2d, during the metabolic labelling. PARP phosphorylation was analyzed by SDSPAGE of anti-PARP immunoprecipitates. We observed that PARP was phosphorylated with (2, 4 and 6 h) or without 100 μM etoposide (0 h) (Fig. 2d). The phosphorylation level of PARP was not changed for up to 6 h of treatment with etoposide. And the levels of the 32P-labelled bands were slightly declined at 6 h, suggesting that the phosphorylation of PARP did not coincide with the temporary activation or cleavage of PARP during apoptosis.

Fig. 2. Temporary activation and phosphorylation of PARP during apoptosis induced by etoposide in Jurkat cells. a: Time course of cleavage of PARP after treatment of Jurkat cells with etoposide (100 μM). PARP was detected by immunoblotting with antibody against PARP. Asterisk indicates the cleaved 89-kDa fragment. b: Time course of PARP activation after treatment of Jurkat cells with etoposide (100 μM). Auto-modification of PARP was detected by immunoblotting with polyclonal antibody against poly(ADP-ribose). c: Time course of DNA fragmentation after treatment of Jurkat cells with etoposide (100 μM). JH and M indicate bacteriophage λDNA digested by HindIII and φX174 DNA digested by HindIII used as size markers of DNA, respectively. d: Time course of the in vivo phosphorylation of PARP after treatment of Jurkat cells with etoposide (100 μM). Jurkat cells were labelled with [32P]orthophosphate for 8 h. During the metabolic labelling, cells were also treated without (0 h) or with 100 μM etoposide for the indicated time (2, 4 and 6 h). PARP phosphorylation was analyzed by SDS-PAGE of anti-PARP immunoprecipitates and detected by autoradiography.

3.3. PARP associated kinase is independent of the activation of PARP

To further confirm the in vivo phosphorylation of PARP, immunoprecipitates of the nuclear extracts of several unlabelled human T-cell lines with antibody against PARP were incubated with [γ-32P]ATP without dsDNA, and then the reaction mixture was subjected to SDS-PAGE. Phosphorylated PARP was detected by autoradiography. b and c: The anti-PARP immunoprecipitates of Jurkat (b) or MT-2 cells (c) were incubated with [γ-32P]ATP with or without poly-(dl-dC)poly(dl-dC). The reaction mixture was subjected to SDS-PAGE. Phosphorylation was detected by autoradiography (b and c, lanes 1 and 2). For the PARP assay, the anti-PARP immunoprecipitates were incubated with [32P]NAD with or without poly(dI-dC)poly(dI-dC). The reaction mixture was subjected to SDS-PAGE. Phosphorylation was detected by autoradiography (b and c, lanes 3–5). To study the effect of the phosphorylation on PARP activity, the reaction mixture was also incubated with unlabelled 0.2 mM ATP (b and c, lane 5).
also observed that the phosphorylation of PARP is sensitive to calf intestine alkaline phosphatase (data not shown). However, we could not detect the kinase in the CdCl₂ treated JPX-9 cells, which expresses the Tax oncoprotein (Fig. 3a, lane 3), suggesting that the HTLV-I Tax protein could not directly induce the kinase. Furthermore, the effect of the phosphorylation of PARP by the PARP-associated kinase on the PARP activity was examined. Anti-PARP immunoprecipitates from nuclear extracts of Jurkat or MT-2 cells were incubated with or without dsDNA in the presence of [³²P]NAD, and then the total mixture was subjected to SDS-PAGE, followed by autoradiography. PARP was strongly activated to produce poly(ADP-ribose) in the presence of dsDNA (Fig. 3b, c, lanes 3 and 4). This PARP activity was not inhibited by the kinase even in the presence of unlabelled ATP (Fig. 3, lane 5), suggesting that the phosphorylation of PARP by the PARP-associated kinase does not interfere with the activation of PARP.

4. Discussion

The signal cascade which regulates PARP activity has not been fully understood. In this study, we have first demonstrated that PARP was phosphorylated by an unidentified serine kinase in vivo. Previously other groups reported that PARP is phosphorylated by protein kinase C (PKC) in vitro [12] and PKC inhibits the DNA binding activity of PARP [14]. Recently, we have found that DNA-PK phosphorylates PARP in a dsDNA-dependent manner in vitro (Ariumi et al., unpublished manuscript). Moreover, we have found a PARP-associated kinase in anti-PARP immunoprecipitates of nuclear extracts of HTLV-I infected T-cell lines. Although we demonstrated in vivo phosphorylation of PARP in Jurkat cells, we did not observe the PARP-associated kinase, suggesting that several kinases phosphorylate PARP. While the PARP-associated kinase phosphorylated PARP in a dsDNA-independent manner, the PARP activity was not inhibited by this kinase, suggesting that the kinase might not be conventional PKC (cPKC) or DNA-PK. In this regard, we could not completely exclude the possibility in this study that the apparent enzyme activity of PARP might not be inhibited if the unphosphorylated PARP is present and shows enough activity. However, we observed auto-modification of recombinant PARP protein expressed in *E. coli*, suggesting that the phosphorylation of PARP is dispensable in auto-modification (data not shown). Interestingly, we have found the PARP-associated kinase in the HTLV-I infected T-cell lines but not in uninfected T-cell lines, suggesting that HTLV-I infection induces this kinase. However, we could not detect the kinase in the CdCl₂ treated JPX-9 cells, which expresses the Tax oncoprotein, suggesting that the HTLV-I Tax protein could not directly induce the kinase. We are currently studying the biological significance of the kinase in the HTLV-I infection.

Recent studies have demonstrated that the ICE protease family plays an important role in apoptosis. The proteins shown to be in vitro targets for an identified ICE-like protease are two DNA repair enzymes, i.e. PARP [8-10] and DNA-PK [13,15]. We have also observed that both DNA-PKcs and PARP cleavage began at the same time (2.5 h) when apoptosis started by etoposide in Jurkat cells (data not shown). Furthermore, we have found that PARP was activated and extensive auto-modification occurred on PARP, paralleled with the progress of DNA fragmentation, suggesting activation of PARP by the fragmented DNA. For each ADP-ribosylation conducted by PARP, one molecule of NAD is consumed and an equivalent of four molecules of adenosine triphosphate (ATP) is required to regenerate NAD from nicotinamide. Thus, the extensive synthesis of poly(ADP-ribose) in response to DNA damage causes a temporary reduction of the cellular NAD pool. In this regard, Zhang et al. [16] suggested a model in nitric oxide (NO) induced neurotoxicity, NO, a free radical, triggers DNA damage which in turn activates PARP, which ultimately depletes energy sources from the cell and leads to cell death [16]. Similarly, the rapid PARP activation during apoptosis may deplete the NAD pool in the cell and may participate in the cell death. Furthermore, Vaziri et al. [17] reported that inhibition of PARP activity leads to extension of cellular life span [17]. PARP associates with p53 and activates it post-translationally. Inhibition of PARP activity leads to abrogation of p21 and mdm2 expression in response to DNA damage. Recently, Wang et al. [7] reported that PARP is important for genomic stability but dispensable in apoptosis using the primary PARP⁻/⁻ cells, suggesting that PARP is not directly involved in apoptosis [7]. In contrast, Simbulan-Rosenthal et al. [18] demonstrated that the immortalized PARP⁻/⁻ fibroblasts exhibited neither any of the biochemical nor morphological changes characteristic of apoptosis when exposed to anti-Fas and cycloheximide and stable transfection of PARP⁻/⁻ fibroblasts with wild-type PARP rendered the cells sensitive to Fas-mediated apoptosis, suggesting that PARP may trigger key steps in the apoptotic program [18].

Taken together, PARP was identified as a phosphoprotein in vivo and in vitro; however, in vivo phosphorylation of PARP was independent of the activation of PARP. It remains to clarify the biological significance of in vivo phosphorylation of PARP. Further identification of the PARP kinase would provide a clue to understanding how PARP is involved in DNA repair and apoptosis.

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