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An enzyme-linked immunosorbent assay-based system for determining the physiological level of poly(ADP-ribose) in cultured cells

Chieri Ida [a, b], Sachiko Yamashita [a], Masaki Tsukada [a], Teruaki Sato [a], Takayuki Eguchi [a], Masakazu Tanaka [c], Shin Ogata [d], Takahiro Fujii [a], Yoshisuke Nishi [a], Susumu Ikegami [a], Joel Moss [e], Masanao Miwa [a, *]

[a] Faculty of Bioscience, Nagahama Institute of Bio-Science and Technology, Nagahama, Shiga 526-0829, Japan
[b] Department of Applied Life Studies, College of Nagoya Women's University, Nagoya-shi, Aichi 467-8610, Japan
[c] Department of Microbiology, Kansai Medical University, Hirakata City, Osaka 573-1010, Japan
[d] Laboratory of Molecular and Cellular Biology, Graduate School of Bioresources, Mie University, Tsu, Mie 514-8507, Japan
[e] Cardiovascular and Pulmonary Branch, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD 20892, USA

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ABSTRACT

PolyADP-ribosylation is mediated by poly(ADP-ribose) (PAR) polymerases (PARPs) and may be involved in various cellular events, including chromosomal stability, DNA repair, transcription, cell death, and differentiation. The physiological level of PAR is difficult to determine in intact cells because of the rapid synthesis of PAR by PARPs and the breakdown of PAR by PAR-degrading enzymes, including poly(ADP-ribose) glycohydrolase (PARG) and ADP-ribosylhydrolase 3. Artifactual synthesis and/or degradation of PAR likely occurs during lysis of cells in culture. We developed a sensitive enzyme-linked immunosorbent assay (ELISA) to measure the physiological levels of PAR in cultured cells. We immediately inactivated enzymes that catalyze the synthesis and degradation of PAR. We validated that trichloroacetic acid is suitable for inactivating PARPs, PARG, and other enzymes involved in metabolizing PAR in cultured cells during cell lysis. The PAR level in cells harvested with the standard radioimmunoprecipitation assay buffer was increased by 450-fold compared with trichloroacetic acid for lysis, presumably because of activation of PARPs by DNA damage that occurred during cell lysis. This ELISA can be used to analyze the biological functions of polyADP-ribosylation under various physiological conditions in cultured cells.

PolyADP-ribosylation, a post-translational modification of proteins in eukaryotic cells, is mediated by poly(ADP-ribose) (PAR) polymerases (PARPs) and ADP-ribosyltransferase 2 (ART2). Using NAD^+as the substrate, these enzymes catalyze formation of linear and branched ADP-ribose polymers that are attached to acceptor proteins. PolyADP-ribosylation is involved in various biological functions, including carcinogenesis, differentiation, and cell death [1]. Among 17 PARPs that have been identified and characterized, PARP-1 and PARP-2 are activated by DNA strand breaks [2–5]. The roles of PARPs in DNA repair have been studied extensively [1,6].

Many proteins that are polyADP-ribosylated by PARPs have been identified, including histones [7], p53 [8], and PARP-1 itself [9]. These acceptor proteins contain a PAR-binding consensus motif [10]. PARP-1 (EC 2.4.2.30), one of the major PARPs, is found mainly in nuclei and is activated by binding to DNA strand breaks via zinc-finger motifs [1,6,11]. However, polyADP-ribosylation also occurs in the absence of intracellular DNA damage [12–15]. PolyADP-ribosylation may be involved in various biological functions, including not only DNA repair but also differentiation and transcription [16,17]. Various biological functions may be regulated physiologically by PAR through gene regulation in the absence of

Abbreviations used: PAR, poly(ADP-ribose); PARP, poly(ADP-ribose) polymerase; ART2, ADP-ribosyltransferase 2; PARG, poly(ADP-ribose) glycohydrolase; ELISA, enzyme-linked immunosorbent assay; RIPA, radioimmunoprecipitation assay; TCA, trichloroacetic acid; HRP, horseradish peroxidase; FBS, fetal bovine serum; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; T-PBS, Tween 20 in phosphate-buffered saline; 3-AB, 3-aminobenzamide; DMSO, dimethyl sulfoxide; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

* Corresponding author.
E-mail address: m_miwa@nagahama-i-bio.ac.jp (M. Miwa).

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substantial DNA damage [18]. Inhibitors of PARPs lead to centrosome amplification in CHO-K1 cells in the absence of apparent DNA damage [19].

The physiological level of PAR in the absence of DNA damage is not well characterized because of the low levels and rapid turnover of PAR in intact cells. PAR metabolism is regulated not only through the activity of PARPs but also through the activity of poly(ADP-ribose) glycohydrolase (PARG) and ADP-ribosylhydrolase 3 [20]. In our previous study, we showed that PARG knockout *Drosophila melanogaster* accumulate high levels of PAR in brain cells, cannot fly because of neuronal cell death, and die within 2 weeks [21]. Similarly, PARG knockout mice show embryonic lethality [22]. PAR binds apoptosis-inducing factors in mitochondria and is involved in neuronal cell death by a process termed parthanatos [23]. Thus, further investigation of the physiological functions of polyADP-ribosylation is necessary and requires accurate measurement of PAR levels.

The purpose of the current study was to measure the physiological levels of PAR, which are quite low, in cultured cells. Various methods have been reported for this purpose, including radioimmunoassay [24], radioisotope labeling methods [25,26], the use of nonisotopic compounds [27,28], enzyme-linked immunosorbent assay (ELISA) [29,30], and stable isotope dilution mass spectrometry [31]. However, some current assays have low sensitivity for measuring PAR in intact cells, are not amenable to high-throughput measurements, or need expensive instrumentation. In addition, the level of PAR is difficult to determine under similar experimental conditions because of the rapid turnover of PAR by PARPs and PAR-degrading enzymes. In particular, artifactual synthesis and/or degradation of PAR frequently occur during lysis of cells in culture. Therefore, establishing more sensitive and suitable methods for measuring the physiological level of rapidly turned-over PAR is necessary. In this study, we developed a sensitive ELISA to measure the physiological levels of PAR in cultured cells.

Materials and methods

Materials

Radioimmunoprecipitation assay (RIPA) buffer, DNase I, and RNase A were purchased from Nacalai Tesque. Nuclease P1 was obtained from Yamasa (Japan). Trichloroacetic acid (TCA), skim milk, *o*-phenylenediamine, and hydrogen peroxide were obtained from Wako Pure Chemical Industries. Proteinase K was purchased from Merck Millipore. Sheared salmon sperm DNA was obtained from BioDynamics Laboratory. RNA from baker's yeast was purchased from Worthington Biochemical.

PAR antibody (10H, IgG3 kappa) secreted from hybridoma cells was purified using a Protein A Sepharose Fast Flow column (GE Healthcare) [32]. Anti-PAR polyclonal antibody was produced in a rabbit by injecting PAR mixed with methylated bovine serum albumin and was purified with a Protein A Sepharose Fast Flow column [33]. Goat anti-rabbit IgG (H&L) conjugated to horseradish peroxidase (HRP) was obtained from Rockland Immunochemicals. Mouse monoclonal anti-tubulin antibody was purchased from Sigma–Aldrich. Mouse monoclonal anti-human PARP1 antibody (F2) and HRP-conjugated goat anti-mouse immunoglobulin antibody and goat anti-rabbit immunoglobulin antibody were obtained from Santa Cruz Biotechnology.

PAR was prepared and purified as described previously [34], but commercially available PAR (Trevigen) showed similar immunoreactivity and was also used in these experiments.

Cell culture

HeLa cells and HEK 293T cells, human embryonic kidney cells having SV40 T antigen, were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml). HepG2 cells, a human hepatocellular carcinoma cell line, were cultured in RPMI 1640 containing 10% FBS, 2 mM L-glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml). The above cells (5×10^5) were cultured in 100-mm Petri dishes for 48 h without replacing the culture medium at 37 °C in a humidified cell culture incubator containing 5% CO_2.

Sample preparation for ELISA

HeLa cells were washed with ice-cold phosphate-buffered saline (PBS), and then the cells were fixed immediately by the addition of ice-cold 20% TCA, followed by incubation on ice for 20 min. The fixed cells were collected by scraping with a rubber policeman. The cell suspension in 20% TCA was centrifuged at 800 *g* for 10 min at 4 °C, and the cell pellet was washed two times with ethyl ether and then centrifuged at 800 *g* for 10 min at 4 °C. The cell pellets were resuspended in 0.1 N NaOH.

To prepare RIPA buffer samples, HeLa cells were washed with PBS, harvested by scraping with a rubber policeman, suspended in ice-cold RIPA buffer (50 mM Tris–HCl [pH 7.6], 150 mM NaCl, 1% Nonidet P40, 0.5% sodium deoxycholate, protease inhibitor cocktail, and 0.1% sodium dodecyl sulfate [SDS]), and incubated on ice for 20 min. Then, 0.5 N NaOH was added to bring the final concentration to 0.1 N.

The samples treated with TCA or RIPA buffer were sonicated for 30 min and then incubated for 1 h at 37 °C in a water bath to completely solubilize the cell pellets and hydrolyze the ester bonds between PAR and acceptor proteins in alkaline conditions [35,36]. The reaction was stopped by adding equal amounts of a mixture of 0.5 N HCl and 1 M Tris–HCl (pH 7.2). For extensive digestion of DNA and RNA in the samples, DNase I (20 μg/ml), RNase A (20 μg/ml), and nuclease P1 (1 U/ml) were added to the samples, which were incubated overnight at 37 °C in the presence of 5 mM $MgCl_2$ [34]. The samples were incubated with proteinase K (200 μg/ml) overnight at 50 °C to digest all of the proteins in cell extract, including DNase I, RNase A, and nuclease P1, and then were boiled for 5 min at 100 °C to inactivate proteinase K. Then, 50 μl of the samples was transferred to the ELISA plate.

ELISA

Sandwich ELISA was performed as follows. Microtiter plates with 96 wells (Thermo Fisher Scientific, 442404) were coated (16 h at 4 °C) with a mouse monoclonal antibody against PAR (10H) as the PAR-capturing antibody (10 μg/ml) in 50 μl of coating buffer (15 mM Na_2CO_3 and 37 mM $NaHCO_3$, pH 9.6) [32]. The plates were blocked with 200 μl of 5% skim milk in 0.1% Tween 20 in PBS (0.1% T-PBS) for 1 h at 25 °C. The blocking solution was removed, and the plates were washed three times with 0.05% Tween 20 in phosphate buffer (0.05% T-PBS). Samples or PAR standards were diluted with buffer (100 mM mannitol, 100 mM Tris–HCl, and 0.1 M NaCl, pH 9.0), and a 50-μl sample or standard (purified PAR) solution containing 0, 5, 10, 25, 50, and 75 pg of PAR was added to each well and incubated for 2 h at 25 °C. The plates were washed three times with 0.05% T-PBS. A 50-μl sample of rabbit anti-PAR polyclonal antibody (the detection antibody), diluted to 10 μg/ml with 2% skim milk in 0.1% T-PBS, was added to each well [33]. The plates were incubated for 2 h at 25 °C and washed three times with 0.05% T-PBS. A 50-μl sample of goat anti-rabbit IgG polyclonal antibody conjugated to HRP, diluted to 0.08 μg/ml, was added to each well, and the plates were incubated for 1.5 h at 25 °C. The plates were then washed three times with 200 μl of 0.05% T-PBS.