行政院國家科學委員會專題研究計畫　期中進度報告

DNA-dependent protein kinase 在細胞有絲分裂的功能

(2/3)

計畫類別：個別型計畫
計畫編號：NSC93-2320-B-002-109-
執行期間：93 年 08 月 01 日至 94 年 07 月 31 日
執行單位：國立臺灣大學醫學院分子醫學研究所

計畫主持人：呂勝春

報告類型：精簡報告
報告附件：出席國際會議研究心得報告及發表論文
處理方式：本計畫可公開查詢

中　華　民　國 94 年 5 月 30 日

Cdk-dependent activation of poly(ADP-ribose)polymerase member 10 (PARP10) and its function in cell proliferation

Han-Yi E. Chou[1], Han-Ting Chou[1], and Sheng-Chung Lee[1, 2, 3, *]

Institute of Molecular Medicine[1], and Institute of Clinical Medicine[2],

College of Medicine, National Taiwan University

And

Institute of Biological Chemistry[3], Academia Sinica

Taipei, Taiwan

Key words: PARP10, Cdk, cell cycle progression

Total number of characters: 42275

- Corresponding author: Sheng-Chung Lee
- Institute of Molecular Medicine, College of Medicine
- National Taiwan University, Taipei, Taiwan
- Fax: 886-2-2321-0977
- Email: slee@ntu.edu.tw

Summary

Proteins of the poly(ADP-ribose) polymerase (PARP) family play wide array of functions, covering virtually every aspect of DNA metabolism and function, most notably with the response to DNA damage, transcription and the maintenance of genomic stability. Here we report the identification and characterization of a novel PARP family member, PARP10 (FLJ14464 or hypothetical protein LOC84875). Over-expression of PARP10 results in loss of cell viability, while knockdown by siRNA leads to delayed G1 progression and concomitant cell death. PARP10 exists in both cytoplasm and nucleus, only that in nucleolus is specifically phosphorylated by Cdk through late-G1 to S phase. In an *in vitro* assay, the enzymatic activity of PARP10 depends on the Cdk phosphorylation. The phosphorylation is shut down in G2, and re-appeared from prometaphase to cytokinesis in the nucleolar organizing regions. The phosphoryated PARP10 is completely absent in growth-arrested cells and may serve as a marker for proliferating cells. These results suggest that PARP10 functions in cell proliferation.

Introduction

Poly (ADP-ribose) polymerase (PARP) catalyzes the covalent attachment of ADP-ribose units from NAD^+ to itself and to a number of proteins involved in chromatin architecture (e.g., histones H1, H2B, HMG proteins, lamin B) and DNA metabolism (DNA replication factors), resulting in the loss of their affinity for DNA (1). The catalytic domain of PARP is located in the 40kDa fragment of the carboxyl-terminal region, which shares homology and defines the PARP superfamily (2). PARP1 has been shown to participate in fundamental biological activities including safeguarding genomic integrity, regulating gene transcription, and facilitating DNA repair, and remains as the major consumer of NAD^+ accounting for more than 90% poly (ADP-ribose) production (1, 2, 3). There are seventeen members of PARP family have been defined (38). These include PARP2 (4, 5, 6, 7), PARP3 (7), PARP4 (VPARP) (8), PARP5 (Tankyrase-1) (9), Tankyrase-2 (10), PARP6 (BAB14092), PARP7 (TiPARP; CAB45747) (3), PARP8 (FLJ21308; XP_018395), PARP9 (BAL; NP_113646), PARP10 (FLJ14464; BAB55067), PARP11 (AAF91391), PARP12 (zinc finger CCCH type domain-containing 1; FLJ22693; XP_034821), PARP14 (KIAA1286; XP_291055), PARP15 (XP_093336), PARP16 (XP_113792; FLJ20509). The biological functions of most of these members are completely unknown. PARP2 is also part of the DNA repair pathway (4), moreover, when both

parp-1 and parp-2 genes are disrupted, mouse embryo dies early in development at the onset of gastrulation. A specific female embryonic lethality is observed when parp-1 null and parp-2 heterozygous mice were created, associated with specific X-chromosome instability (11). PARP3 is present in the centrosome throughout the cell cycle, and is always localized to the daughter centriole. Over-expression of PARP3 prevents centriole duplication and causes a G1/S cell cycle block (12). VPARP (PARP4) is a component of cytoplasmic vault particle (13). VPARP is found both in the cytoplasm and in the nucleus and during mitosis it co-localizes with the mitotic spindle. Together with TEP1, VPARP is also found in the telomeres (14). Tankyrase 1 associates with TRF1 and regulates telomere length (15). It is found also in the centrosomes and at nuclear pores. Tankyrase 2 is a cytoplasmic protein. Both tankyrase 1 and tankyrase 2 share about 30% sequence homology with PARP1 in their catalytic domain. TCDD-induced PARP (TiPARP) (16), BAL protein (17), hypothetical proteins of mouse, BAC25913, XP_291055, and NP_766481, rat, XP_216963, and XP_221401, and human FLJ14464 (NP_116178) and NP_073587 (18) belong to a closely related subfamily of PARP. Another TiPARP-related protein, human ZAP (NP_073587) may involve in the resistance to viral infection. Studies of the rat homolog indicate that the protein may primarily function to inhibit viral gene expression and induce an innate immunity to viral infection (19).

Compartmentalization has long been known to have a key role in regulation of cellular processes. By keeping enzymes and regulatory complexes in compartments where the delivery of substrate or exit of product is controlled, competing reactions can occur simultaneously in different parts of the cell. Moreover, spatial confinement facilitates the working of molecules participating in reaction chains and is crucial for coupling unfavorable with energetically favorable chemical reactions. Although in many cases intracellular compartmentalization relies on boundaries imposed by membranes, several non-membrane-bounded compartments exist in eukaryotic cells. One of these, the nucleolus, has recently attracted much attention. The emerging view is that molecular confinement in the nucleolus actively contributes to the control of cellular survival and proliferation (20, 21, 22, 23). In higher eukaryotic cells, the nucleolus is a nuclear compartment assembled at the beginning of interphase, maintained during interphase, and disorganized during mitosis. Even if its structural organization appears to not be dissociable from its function in ribosome biogenesis, the mechanisms that govern the formation and maintenance of the nucleolus have not been elucidated. Inhibition of cdk1-cyclin B during mitosis leads to resumption of rDNA transcription, but is not sufficient to induce proper processing of the pre-rRNA and total re-localization of the processing machinery into rDNA transcription sites. Similarly, at the exit from mitosis, both translocation of the late processing machinery and

pre-rRNA processing are impaired in a reversible manner by CDK inhibitors. Therefore, CDK activity seems indispensable for the building of functional nucleoli. Furthermore, inhibition of CDKs of cells in interphase also hampered proper pre-rRNA processing and induced a dramatic disorganization of the nucleolus. The mechanisms governing both formation and maintenance of functional nucleoli involve CDK activities and couple the cell cycle to ribosome biogenesis (24). (These results are relevant to the CDK-regulated PARP activity and its involvement in G1/S progression!)

PARP10 was discovered serendipitously during our investigation of functions of CDK-phosphorylated Eg5 protein. The phosphorylation of Threonine residue 927 (T927) of Eg5 by Cdk1 has been shown important for targeting to the microtubule spindle and for the functions of Eg5 in mitosis (25, 26). To further study the functions of Eg5 by T927 phosphorylation, we have developed phospho-specific antibodies to peptide with sequence surrounding phospho-T927. In addition to the phospho-Eg5 detected during mitosis, we observed a cross-reactive species with a slightly higher molecular weight appeared during G1/S and M phase. We identify and functionally characterize the polypeptide as a newly defined member of the PARP superfamily, PARP10 (also known as FLJ14464) (). It localizes both in cytoplasm and nucleus, with a subpopulation that concentrates in the nucleolus during late G1/S and acquires

cdk2-dependent phosphorylation at the threonine residue 101 (T101). Phosphorylated PARP10 associates with RNA pol I modules in a DNA-dependent and differentiation stage-regulated manner. Downregulation by siRNA and overexpression experiments indicate that maintaining the level of PARP10 is required for proper G1/S progression and cell viability. Together, our results suggest that PARP10 plays crucial functions in cell proliferation.

Experimental Procedures

Cell culture

HeLa and Ptk2 cells were cultured in Dulbecco's modified Eagle's medium and 脉-modified Eagle's medium, respectively. Both cultures were supplemented with 1mM L-glutamine, 100 units/ml penicillin, 100贡g/ml streptomycin and 10% (v/v) fetal bovine serum (Hyclone), in a humidified atmosphere of 5% CO_2 at 37°C. HaCaT cells were grown in Defined Keratinocyte medium at 37°C in 5% CO_2 with the additional supplements supplied by the manufacturer. All the above-mentioned reagents, with the exception of fetal calf serum, were purchased from Invitrogen.

Antibodies and Western blot analysis

Rabbit anti-phosphorylated hEg5 (T927) antibody shares the sequence specificity with PARP10 (T101). Monoclonal antibody to PARP10 was obtained by fusion FO myeloma cells with BALB/c splenocytes immunized with recombinant protein corresponding to the N-terminal 144 amino acid region. Protein lysates were subjected to SDS-PAGE electrophoresis and blotted onto polyvinylidene difluoride membrane (Millipore). Membranes were blocked with 1%BSA in TBS/0.1% Tween 20 for one hour at room temperature and hybridized with the primary antibody overnight at 4^oC. After three times of wash with TBS/0.1% Tween 20, the membrane was incubated with the corresponding HRP conjugated secondary antibody diluted in TBS/0.1% Tween 20 for 1hr at room temperature. The blots were visualized using the enhanced chemiluminescence system (ECL, DuPont-NEN). Rabbit

antibodies to Nopp140, monoclonal antibodies to PCNA and α-tubulin, were used in various experiments.

Immunofluorescence staining

Unless specified in the text, cells were typically seeded at 50% confluency on acid-treated glass coverslips. Cells were fixed with 4% paraformaldehyde in PBS for 10 minutes, permeabilized for 5 minutes with 0.5% Triton X-100, then blocked in PBS containing 0.1% BSA and 0.1% Triton X-100 for 30 minutes, then incubated with the indicated antibodies for 1 hour. Cells were washed three times with PBS and then incubated with goat anti-mouse IgG or goat anti-rabbit IgG secondary antibody coupled to Alexa® 488, Alexa® 594 (Molecular Probes) or Texas red-conjugated goat anti-human IgG (Jackson Laboratory) for 40 minutes. Cells were counter stained with DAPI to highlight the nuclei. Cells were examined under Leica DM RD immunofluorescence microscope and images were captured with a cool CCD, processed by CoolSNAP software (Roper Scientific Inc.).

Isolation of nucleolar fraction

Nucleoli were prepared from exponentially growing HeLa cells, 0.5 x 10^9 cells were collected and washed with cold PBS by centrifugation at 800xg for 8 minutes. Cell pellet was resuspended in 20 volumes of RSB-8 buffer (10mM Tris, 10mM NaCl, 8mM MgOAc, pH 7.4). After 30 minutes on ice, the swollen cells were centrifuged at 1000xg for 8 minutes, resuspended in RSB buffer (10mM Tris, 10mM NaCl, 1.5mM MgOAc, 0.5% NP-40, pH 7.2) and dounce homogenized 40 strokes using a

loose pestle. The nuclei free of cytoplasmic tags were collected by centrifugation at 800xg for 8 minutes, resuspended in 0.88M sucrose/5.0mM MgOAc (20ml/ gram of cells), and centrifuged at 2500xg for 20 minutes. The clean, pelleted nuclei were resuspended in 3 ml, 0.34M sucrose/ 0.5mM MgOAc, and sonicated for 6 x 10 seconds. The sonicated sample was checked using phase contrast microscopy, ensuring that there were no intact cells and that the nucleoli were readily observed as dense, refractile bodies. The sample was then layered over 4 x volumes of 0.88M sucrose, and centrifuged at 3000xg for 20 minutes at 4°C. The pellet contained the nucleoli, while the supernatant consisted of the nucleoplasmic fraction. The nucleolar fraction was snap frozen in liquid nitrogen and stored at -80°C. All buffers were supplemented with 5mM sodium fluoride, 2mM sodium vanadate, 10μg/ml leupeptin, 10μg/ml aprotinin and 2mM PMSF.

In vitro **enzymatic assays**

In vitro phosphorylations of recombinant protein were performed by incubating 100 ng of purified protein in a total volume of 20 μl with recombinant kinase. Incubations were carried out for 30 min at 30^{o}C in kinase buffer (50 mM Tris [pH 7.4], 10 mM $MgCl_2$, 6 mM EGTA, and 10 μM ATP). *In vitro* activated cdks were purified from insect Sf21 cells by co-infection of recombinant baculoviruses encoding human cdks and His-tagged cyclins using standard Nickel column chromatography.

Chromosome spreads

HeLa cells were treated with 0.5 μg/ml colcemide for 60 min, harvested by trypsinization, hypotonically swollen in 10 mM Tris-HCl (pH 7.4), 10 mM NaCl, and 5 mM $MgCl_2$, and sedimented onto cover slips by centrifugation for 15 s at 900x g. Chromosomes were swollen for an additional 15 min in 25 % PBS, fixed in 3.7 % formaldehyde in 25 % (v/v) PBS for 10 min, and permeabilized with 0.5 % NP-40 in 25 % PBS for 10 min. Samples were blocked with 1% BSA in PBS, and then processed as described in immunofluorescence staining.

Nuclease treatment

All reactions were performed at room temperature in the presence of complete proteinase inhibitors (Roche) in all buffers. Cells were grown for 48 hrs as monolayers on cover slips. The cells were washed once in PBS, pH 7.4 followed by NtB buffer (10 mM PIPES pH 7.0; 100 mM NaCl; 300 mM sucrose; 3 mM $MgCl_2$; 1 mM EGTA for RNA and 1 mM $CaCl_2$ for DNA) containing 0.5 % Triton X-100 for 5 min. After a brief rinse in PBS buffer, cells were treated with nucleases (0.1 mg/ml of RNase or 1unit/ml of DNase) in NtB buffer for 15 minutes. A final PBS wash is followed by procedures for immunofluorescence analysis as described.

In situ **run-on transcription assay**

Cells grown as monolayers on cover slips were washed twice with PB buffer (22 mM NaCl, 1 mM $MgCl_2$, 8 mM KCl, 11mM K_2HPO_4, 100 mM CH_3COOK, 1 mM ATP, 1 mM dithiothreitol, 15% glycerol, 0.2 mM phenylmethylsulfonyl fluoride [pH 7.4] supplemented with digitonin) for 4 min on ice. Then the cover slips were washed once with PB buffer and incubated on ice for 10 min with PB buffer supplemented

with α-amanitin (100 μg/ml; Sigma). Subsequently, transcription mix (10x concentrate) was added to give final concentrations of 2 mM ATP, 0.1 mM CTP, 0.1 mM GTP, 0.2 mM 5-bromouridine 5'-triphosphate (Br-UTP; Sigma), and 2 mM $MgCl_2$. The run-on transcription was carried out at for 20 min and was terminated by rinsing the cover slips in ice-cold PBS. Cells were then processed for immunofluorescence analysis.

PARP activity assay

The cDNA sequence encoding the catalytic domain (aa 823-1025) of hNuPARP was amplified by PCR and inserted into the pRSET B expression vector. The His-tagged protein was purified on a Nickel column. Poly (ADP-ribose) activity assays were carried out as described previously (9). Reactions contained 0.5贡g of purified recombinant protein, incubated at 25^{o}C for 30 min in 50μl assay buffer (50mM Tris-HCl, pH 8.0, 4mM $MgCl_2$, 0.2mM DTT, and 1 mM NAD^{+}). Some assays contained the PARP inhibitor 3-aminobenzamide (3AB) at 1 mM final concentration. Reactions were stopped by the addition of SDS sample buffer, and analyzed by Western Blot.

Please give me the PARP assays!

SiRNA (I thought that you have 2 siRNAs)

DNA sequence corresponding to hNuPARP nucleotides 215 to 227 was subcloned into the RNAi-ready pSIREN-RetroQ vector (BD Biosciences) and transfected into HeLa cells using Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions. Suppression of PARP10 after specific time points was analyzed by western blotting as described previously, and by RT-PCR using AccessQuick RT-PCR

system (Promega). For PARP10, the specific primers are 5'-ATGGTTGCAATGGCGGAGGCAGA-3' and 5'-TCCAGGTACAACTCCAGCAACA-3'; for hGAPDH, the primers are 5'-CATCACTGCCACCCAGAAGCTGTGGA-3' and 5'- TACTCCTTGGAGGCCATGTAGGCCATG-3'.

Flow Cytometry

Transfected HeLa cells were harvested at the time indicated, stained with propidium iodide (Boehringer) and analyzed by Epics Elite flow cytometer (Coulter). The multicycle (M cycle) program for cell cycle distribution (Phoenix Flow Systems, Inc.) was used to analyze the data.

Results

The T927-101 antibody recognizes both phosphorylated Eg5 and PARP10

In an attempt to explore the functional implications of cdk phosphorylation of the Thr927 residue of human Eg5, we raised a rabbit polyclonal antibody using synthetic phosphopeptide as antigen (Fig. 1A, upper panel). The affinity-purified T927 antibody recognized both Eg5 and a protein with an apparent molecular weight of 130 kDa in Western blot assays using direct cell lysates from HeLa cells (Fig.1A, lower left panel, AS: asynchronous, M: mitosis, I: interphase). The level of Eg5 protein was shown by Western blot assay using a monoclonal anti-Eg5 antibody (Fig. 1A, lower middle panel). The Eg5 protein is indeed phosphorylated during M pahse. The protein level showed minimal fluctuations in AS, M-, and I-phase cells. We reasoned that the cross-reactive species (i.e., the 130 kDa protein) could possess a sequence context similar to that of the original peptide used for generating the antibody and thus conducted a search of public database for proteins with similar molecular weight and sequence surrounding the immunizing peptide. A single candidate FLJ14464 emerged. FLJ14464 has recently been defined as PARP10 (). To verify if PARP10 is responsible for the 130 kDa signal, we generated a monoclonal antibody (clone 19F6) against the N-terminal 144 amino acid region of PARP10, and performed Western blot assays (Fig. 1A, lower right panel). Immunofluorescent

staining of HeLa cells demonstrated that there is partial co-locolization of 19F9 with the rabbit antibody at subnucleolar structures of mitotic cells and a fraction of cells in G1 phase (Fig. 1B, late G1 and S phase). T927-101 and 19F7 were co-stained in the kinetochore regions of condensed chromosomes during metaphase and from anaphase to telophase. Together, these data support that the rabbit antibody displays dual specificity towards phosphorylated Eg5 and the novel PARP10 protein, at threonine residues located at 927 and 101 amino acids, respectively. We refer this rabbit antibody as T927-101.

Cdk- and cell cycle-dependent phosphorylation of T101 of PARP10

We next set to investigate the nature of T101 phosphorylation on PARP10 and its functional consequences. Using nocodazole-arrested and subsequently released HeLa cells, we found that the level of PARP10 protein remains unchanged throughout the cell cycle. However, the pattern of T101 phosphorylation displays significant alterations, being maximal from late G1 phase to the S phase and during mitosis (Fig. 1B). Parallelly, indirect immunofluorescence staining of HeLa cells using 19F6 mAb revealed the distribution of PARP10 throughout the entire cell in all stages of the cell cycle, with a significant co-staining with T927-101 in the nucleolus during interphase. PARP10 acquires T101 phosphorylation during mid to late G1, and persists in this state

until S/G2, when the T927-101 signal is abruptly shut down. During the transition from G2 to prometaphase, PARP10 re-emereged as T101 phosphorylated protein and appeared as a new dotted pattern associated with mitotic chromosomes, and gradually disappears during cytokinesis. PARP10 phosphorylation at T101 is completely absent in early G1 phase cells, though most cells regain the nucleolar dotted T927-102 staining pattern in late G1 phase (Fig. 1B). In light of this tightly regulated, cell cycle dependent pattern of phosphorylation recognized by T927-101 antibody, we tested whether Cdk activity could be responsible for the PARP phosphorylation at T101. To avoid the secondary effects that may emerge from prolonged incubation with the Cdk inhibitors, roscovitin and olomoucin, immunofluorescence staining was performed with synchronized interphase cells to restrict the study on T101 phosphorylation acquired during late G1 phase. The phosphorylation of nucleolar PARP10 is undetectable when the cells were treated with CDK inhibitors (Fig. 2A, T927-101). Contrary, the staining pattern of another nucleolar phosphoprotein, Nopp140, is not affected (Fig. 2A, hNopp140). Furthermore, GST-fusion recombinant PARP10 protein containing the T101 sequence was tested for its ability to get phosphorylated by recombinant baculovirus-derived cdks. When equal amounts of cdk1/cyclinB and cdk2/cyclinE were used in kinase reactions, only the cdk2/cyclinE complex renders GST-PARP10 to be detectable by T927-101 (Fig. 2B). Taking into

account that the cdk2/cyclinE complex acquires maximal activity from late G1 to the S phase, our results indicate that a fraction of PARP10 localizes to the nucleolus gets phosphorylated by cdk2/cyclin E.

Cdk2/cyclin E-dependent activation of PARP10

Sequence comparison revealed that the C-terminal of PARP10 contains a conserved PARP catalytic domain (Fig. 3A). To determine whether PARP10 possess PARP activity, the PARP-catalytic domain was expressed in *E. coli* as a His-tagged fusion protein. An *in vitro* assay for PARP activity detects the addition of ADP-ribose to a recombinant protein containing the N-terminal region 250 amino acid of PARP10 with NAD^+ as substrate was carried out. Western blot using anti-His antibody shows that similar amounts of recombinant proteins were loaded in each assay mixture (Fig. 3B, α-His). Like PARP, the catalytic domain of PARP10 could (ADP-ribosy)late itself when NAD^+ is supplemented, as a ladder of lower mobility bands reacted with the anti-poly (ADP-ribose) antibody (α-PAR) were evident, consistent with the formation of ADP-ribose polymers (Fig. 3A and Fig. 3B, lane 1). To confirm that this ADP-ribosylation reaction was analogous to PARP-catalyzed poly ADP-ribosylation, the PARP-specific inhibitor 3AB was included in the reaction (Fig. 3A, and Fig. 3B, lane 2). The ADP-ribosylation reaction was blocked. These results indicate that

PARP10 is a *bona fide* member of PARP family.

We next evaluated the PARP activity of endogenous PARP10 and its possible relationship with T101 phosphorylation status. When the endogenous PARP10 was isolated from the HeLa cell extracts by immunoprecipitation with monoclonal antibody 19F6, no detectable PARP activity can be seen (Fig. 3C, lanes 5 and 8). However, when the isolated PARP10 was incubated with recombinant Cdk2/cyclin E, the PARP activity is highly stimulated (Fig. 3C, lanes 4 and 7). Again, the PARP activity can be inhibited by 3AB (Fig. 3C. lanes 6 and 9). Together, these results indicate that the PARP10 is a *bona fide* PARP and its enzymatic activity depends on the phosphorylation by Cdk *in vitro*. The coincident phosphorylation of PARP10 at T101 during late G1/S suggests that the Cdk2/cyclin E may also responsible for the activation of PARP10 *in vivo*.

Downregulation of PARP10 by siRNA results in retarded cell cycle progression and leads to decreased cell viability

To further elucidate the cellular role of PARP10, we performed siRNA knockdown experiments. Transient transfection of siRNA construct resulted in a time-dependent decrease in levels of PARP10 protein and RNA (Fig. 4A. mRNA, upper panel, Western blot, lower panel). Flow cytometry analysis of various cell cultures

transfected with siRNA was performed with an initial gating with an arbitrary intensity of GFP, a control of transfected cells with the intensity of fluorescence reflects the level of siRNA expressed. By measuring the forward scatter, we observed a gradual decrease in positively transfected cell number from day two (Fig. 4B). By day 5, most cells died and became fragmented. Propidium iodide staining also revealed a concomitant accumulation of 2N cells through day 2 to 5, suggesting that cell cycle progression is relatively stalled at the G1/S boundary (Fig. 4C). These results show that in HeLa cells, normal protein level of PARP10 is required for the maintenance of proper cell cycle progression. Knockdown the level of PARP10 could lead to the accumulation of cells in the G1/S and loss of viability.

DNA-dependent association of PARP10 and Pol I modules in proliferating cells

We next aim to identify the immunofluorescence staining dotted structure containing phosphorylated PARP10. Active rDNA transcription foci are also arranged in a string of dot-like structures resembling T927-101 staining, and can be readily detected by probing the structural component of Nopp140. In most interphase cells, double immunofluorescence staining demonstrated that phosphorylated PARP10 completely colocolizes with Nopp140 foci. However, in mitotic cells, phosphorylated PARP10 remained appeared as distinct dots associated with condensed chromosomes, while

Nopp140 is dispersed throughout the extra chromosomal region (Fig. 5A, upper panel). Since Nopp140 is known to interact with the RNA polymerase I (RNA Pol I) machinery that remains associated with mitotic chromosomes, we performed chromosome spread assays and observed complete colocalization of phosphorylated PARP10 with RNA Pol I in the nucleolar organizing regions (NORs) of prometaphase HeLa cells (Fig. 5A, lower panel).

The dot-like structures containing Nopp140 and RNA Pol I represent the fibrillar components (FC) of the nucleolus. It is known that blocking rDNA transcription by actinomycin D leads to segregation of the fibrillar components from the granular components of the nucleolus. Whether phosphorylated PARP10 remains associated with the FC in the altered nucleoli was then examined. After actinomycin D treatment, Nopp140 was redistributed as crescent-shaped structures in the segregated nucleoli, or the so called “beads on a necklace” pattern, while phosphorylated PARP10 was restricted to the “beads” area (Fig. 5B), that are reported to be the region where RNA Pol I is concentrated. (You may have to mention that the IF pattern of 19F6 in this preparation!)

To address the functional relationship between PARP10 and the RNA Pol I machinery, we investigated the possibility of PARP10 to be involved in initiation of rDNA transcription by *in situ* run-on assays. In this assay system, the activity of RNA Pol I

was promoted by supplementation with α-amanitin to suppress transcriptions from RNA polymerases II and III. We observed that, although phosphorylated PARP10 retained a nucleolar dotted pattern, it appeared in a more diffused manner than in mock-treated samples that did not coincide with sites of Br-UTP uptake (Fig. 5C). These results suggest that PARP10 phosphorylation is likely not required for the initiation of RNA Pol I mediated transcription. This conclusion is further strengthened by the observation that PARP10 phosphorylation is completely suppressed in growth arrested/differentiated HaCaT keratinocyte cells (Fig. 5D), a model in which basal ribosome biogenesis remains intact, as shown by the compact staining pattern of the anti-nucleolin antibody. Another important feature of the Pol I modules involves the participation in rDNA architecture, which is important for determining the maximal throughput of ribosome biogenesis, and is also closely related to the aging process and senescence status of the cell. Therefore, we examined whether the association of phosphorylated PARP10 with Pol I modules requires intact DNA or RNA. After incubation of Triton X-100-permeabilized HeLa cells with RnaseA, phosphorylated PARP10 remained in the nucleoli, although the staining seems weaker and more diffuse than the untreated cells. On the contrary, DNaseI treatment caused a major loss of T927-101 signal (Fig. 5E), indicating that PARP10 may be associated with the RNA Pol I foci in a DNA dependent manner.

Taken together, these data suggest that PARP10 may participate in modulating rDNA architecture and nucleoli biogenesis during the exit of mitosis that is important in actively proliferating cells. Furthermore, these data also suggest that the potential application of the T927-101 antibody as a novel proliferation marker.

Discussion

PARP10 is a novel poly(ADP-ribose) polymerase that belongs to a subfamily ofTiPARP

In this report, we detailed the characterization of a novel member of the PARP superfamily, PARP10, whose suppression induces delayed G1 progression and loss of cell viability. The gene encoding PARP10 is localized to the long arm of chromosome 8q24.3. By sequence comparison, we identified a PARP domain in the C-terminus of PARP10, and show that it catalyzes auto-poly(ADP-ribosy)lation in vitro using the N-terminus 144 amino acid portion as substrate. When histone H3 was employed as substrate, it was not modified by PARP10 (data not shown). The endogenous substrates of PARP10 remain to be identified.

Members of the PARP family show a similarity of 29-60% between their PARP domains, these proteins in general do not resemble each other outside the catalytic domain, suggesting diverse cellular functions other than the well-known role of caretakers of the genome. This is found exactly in members of the TiPARP subfamily to which PARP belongs (Fig. 2A). Members of this emerging subfamily display strikingly divergent cellular functions. This divergence is underscored by the unique primary structure of each subfamily member exemplified by TiPARP, BAL protein, and PARP10. Although each subfamily member possesses

poly(ADP-ribose) polymerase activity, their endogenous substrates under various physiological conditions remain to be identified. Apart from the C-terminal PARP domain, PARP10 does not contain other known domains and the mechanism of its activation is yet to be defined.

Other members closely related to PARP10 include a 960 amino acid mouse protein (BAC25913) and a 985 amino acid rat protein (XP_216963) of unknown functions. These two proteins share significant overall sequence homology (50% identity) with PARP10. Members of the TiPARP subfamily include TiPARP, BAL protein, ZAP protein, and a handful of hypothetical proteins. TiPARP may have functions in the induction of long-term potentiation (LTP) (16, 27) and in T cells from progressing tumors (28). Another member of the TiPARP protein contains a CCCH zinc finger, a PARP and two WWE domains may function as RNA processing protein (18). BAL protein may involve in malignant B lymphoma migration. In addition to the PARP, BAL contains hismacro and SEC14 domains that may function in the chromatin structure and dynamics and transcription. ZAP protein has been shown to have anti-viral activity (19). Together, members of the TiPARP subfamily have functions as diverse as that of PARP1 family.

PARP10 functions in cell cycle progression

The exclusive appearance of T101 phosphorylated PARP10 in the nucleolus makes it unique among the superfamily members. Our present results suggest that cdk2 may be responsible for the alteration of specific functions of PARP10 involving DNA-architetural modulations of the nucleolar RNA pol I modules. We performed site directed mutagenesis assays and observed that cdk2 phosphorylation on the T101 residue is not required for nucleolar localization of PARP10 (data not shown). Over-expression of T101A mutant as well as the wild-type of PARP10 caused the cell death. At this moment, it is difficult to assess the effect of T101A mutant on cell cycle progression. The facts that PARP10 acquires T101 phosphorylation in late G1/S and its activity can be stimulated by cdk2 phosphorylation *in vitro* suggest that Cdk2 plays pivotal roles in regulation of the function of PARP10. The physiological significance of PARP10 activation by T101 phosphorylation by Cdk2/cyclin E will be clarified when the endogenous targets of PARP10 are identified.

Downregulation of PARP10 by siRNA causes delayed G1 progression and cell death (Fig. 4), a result that remains consistent using different siRNA targets as well as double knockdowns (data not shown). This is quite different from the knockout phenotypes of *parp-1*$^{-/-}$ and *parp-2*$^{-/-}$ (1, 29), VPARP$^{-/-}$ (14) or knockdown of tankyrase (29). Targeted disruption of either PARP-1 or PARP-2 causes defective base excision repair (BER). Embryonic lethal phenotype was observed when both

PARP-1 and PARP-2 are knocked out (29). Knockdown of tankyrase 1 caused mitotic arrest. Chromosomes aligned normally on the metaphase plate but were unable to segregate. Sister chromatids separated at centromeres and arms but remained associated at telomeres. Telomeres may require a unique tankyrase 1-dependent mechanism for sister chromatid resolution before anaphase (29). Recent studies have shown that PARP-1 plays important roles in transcription regulation (30). PARP-1 modifies histones thereby promoting the de-condensation of higher-order chromatin structures. PARP-1 also acts as a component of enhancer/promoter regulatory complexes. VPARP can ADP-ribosylate major vault protein (MVP) and itself (8). Whether there are other substrates for VPARP is presently unknown.

Tankyrase was found in cytoplasm (31), on nuclear membrane (15) and in Golgi and membrane GLUT4 vesicles, storage of insulin-dependent glucose transporter (32). Another protein poly(ADP-ribose) acceptor is an insulin responsive amino peptidase, IRAP. Tankyrase was shown to bind a protein component of cortical actin cytoskeleton, TAB182 (33), as well as a nuclear/mitotic apparatus protein NuMA during mitosis (34). Tankyrase is now considered a signaling molecule targeted to several subcellular compartments (35). Together with the diverse subcellular localizations of other PARP family members, these results indicate that each enzyme

possesses multiple functions. The cytoplasmic and nuclear existence of PARP10 suggests that it plays pleiotropic functions as well.

PARP10 could be involved in the ribosomal DNA architecture and dynamics that ultimately affects the cell cycle progression.

The most unique properties of PARP10 are its phosphorylation by cdk2 at T101 in the nucleolus during interphase, and its DNA-dependent association with RNA polymerase I. The phosphorylation at T101 is absent in quiescent or differentiated cells. These results suggest that T101 phosphorylated PARP10 may link the ribosomal DNA *replication/integrity* to cell proliferation. The most intriguing aspect of this study is the functions of PARP10 being a component of NORs. During mitosis, cell nuclei undergo extensive structural changes and concomitantly arrest transcriptional activity. Nucleolar structures change dramatically, Pol I transcriptional activity ceases almost completely. Although most nucleolar proteins disperse throughout the mitotic cell, all known basal factors required for transcription initiation are maintained on metaphase chromosomes (22). The selective retention of the Pol I transcription apparatus could be a regulatory mechanism that marks these genes for rapid assembly into the pre-initiation complexes when cells re-enter the G1 phase of the cell cycle. Although we showed that PARP10 does not directly

participate in the initiation of Pol I transcription, its DNA-dependent association to the NORs suggests a possible role in the modulation of rDNA architecture, which is implicated in both regulation of transcriptional throughput, as well as differentiation stage specific reorganization of rDNA loci. Both processes require tight regulation in concert to the progression of the cell cycle. Alternatively, PARP10 could directly modulate Pol I activity by altering its affinity to the rDNA, resulting in down regulation the rRNA transcription. However, the bi-phasic phosphorylation of PARP10 during mid-G1 and mitosis, as well as its complete suppression in non-proliferating/differentiated cells, suggest that the nucleolar function of PARP10 may have nothing to do with nucleologenesis, rather, it plays important roles in DNA-conformation dependent functions, including maintaining the nucleolar integrity and proper functioning.

PARP10 is likely to be activated by Cdk2 in the nucleolus of proliferating cells

The nucleolus is an active and dynamic nuclear domain and plays a major role in compartmentalization of nuclear function. The cdks have been shown to govern formation and maintenance of the nucleolus (24). The discoveries of PARP10 and its phosphorylation by cdk2 in the nucleolus have advanced our understanding of cdks' roles in nucleolar function and/or formation. Our observations in the present report

indicate that the PARP10 is present in the cytoplasm as well as the nucleus with the exception of T101 phosphorylated species associated with the nucleolus of proliferating cells exclusively. This indicates that T101 phosphorylation by cdk2 may be important for Pol I-associated functions in the proliferating cells. What makes the difference of ribosome biogenesis or Pol I-dependent process between differentiated/quiecent cells and proliferating/growing cells? Apparently, phosphorylated PARP10 plays very important role in determining the Pol I-dependent process (e.g., rRNA transcription or rDNA replication) for cells going into cell cycle. Our results of PARP10 knockdown suggest that the level of PARP10 is important for the survival of proliferating cells. The cytoplasmic PARP10 does not seem to associate with microtubules or cytoskeletons. The Cdks have been shown to govern formation and maintenance of the nucleolus. Cytoplasmic function of PARP10 is likely to be independent of cdks. The level of cytoplasmic PARP10 does not seem to change when cells get differentiated. This is in strikingly contrast to the appearance of cdk2-dependent phosphorylation of PARP10 in the nucleolus of proliferating cells. Apart being phosphorylated in the nucleolus of proliferating cells, the level of PARP10 may have functions in the homeostasis of terminally differentiated cells. This will be the target of future studies. Functions of PARP10 in the cytoplasm, nucleoplasm and nucleolus may all contribute to the smooth cell cycle progression. Although cdks

could govern formation and maintenance of the nucleolus, their targets remain poorly defined. Some well documanted examples such as the increase of rDNA transcription during G1 progression appears to depend on phosphorylation of UBF by G1-specific CDK (36). On the other hand, rDNA transcription is repressed at mitosis by cdk1 phosphorylation of components of the rDNA transcription machinery (37, 24). Our finding that cdk2 phosphorylated PARP10 colocalizes with Pol I in the nucleolus suggests that it may play crucial role in the transcription of rDNA or the maintaining the dynamics of rDNA.

The phosphorylated PARP10 is very susceptible to phosphatase activity as demonstrated by the fact that phosphorylated PARP10 can only be detected by Western blot when the cells were lysed directly with hot SDS sample buffer. It can no longer be detected when the whole cell extracts were prepared. This suggests that the phosphorylation and dephosphorylation of PARP10 may be tightly regulated in a temporal- and spatial-specific manner. Our present results have laid foundation for future studies of the functions of PARP10.

References

1 Shall, S., and G. de Murcia. (2000) Mut. Res. 460:1-15.

2 Smith, S., I. Giriat, A. Schmitt, and T. de Lange. (1998) Science 282:1484-1487.

3 Shall, S. (2002) BioEssays 24:197-201.

4 Ame, J.C., V. Rolli, V. Schreiber, C. Niedergang, F. Apiou, P. Decker, S. Muller, T.

5 Babiychuk, E., P.B. Cottrill, S. Storozhenko, M. Fuangthong, Y. Chen, M.K.

6 Berghammer, H., M. Ebner, R. Marksteiner, and B. Auer. (1999) FEBS Lett. 449:259-263.

7 Johansson, M. (1999) Genomics 57:442-445.

8 Kickhoefer, V.A., A.G. Stephen, L. Harrington, M.O. Robinson, and L.H. Rome. (1999) J. Cell Biol. 146:917-928.

9 Smith, S., and de Lange, T. (1999). Cell cycle dependent localization of the telomeric PARP, tankyrase, to nuclear pore complexes and centrosomes. J. Cell Sci. 112:3649-3656.

10 Lyons, R.J., R. Deane, D.K. Lynch, Z.S. Ye, G.M. Sanderson, H.J. Eyre, G.R Sutherland, and R.J. Daly. (2001) J. Biol. Chem. 276:17172-17180.

11 Menissier-de Murcia, J and G. de Murcia. (1999) J. Biol. Chem.

274:17860-17868.

12 Augustin, A., C. Spenlehauer, H. Dumondl, J. Menissier-de Murcia, M. Piel, A.C.

13 Van Zon, A., M.H. Mossink, M. Schoester, A.B. Houtsmuller, G.L. Scheffer, R.L.

14 Liu, Y., B.E. Snow, V.A. Kickhoefer, N. Erdmann, W. Zhou, A. Wakeham, M.

15 Smith, S., and T. de Lange. (2000) Curr. Biol. 10:1299-1302.

16 Ma, Q., K.T. Baldwin, A.J. Renzelli, A. McDaniel, and L. Dong. (2001) Biochem. Biophys. Res. Commun. 289:499-506

17 Aguiar, R.C., Y. Yakushijin, S. Kharbanda, R. Salgia, J.A. Fletcher, and M.A. Shipp. (2000) Blood 96:4328-4334.

18 Katoh, M and M Katoj. (2003) Int. J Oncol 23:541-547.

19 Gao,G., X. Guo, and S.P. Goff. (2002) Science 297:1703-1706.

20 Hernandez-Verdun, D., P. Roussel, and J. Gebrane-Younes. (2002) J Cell Sci.115:2265-2270.

21 Carmo-Fonseca, M., L. Mendes-Soares, and I. Campos. (2000) Nat Cell Biol. 2:E107-112.

22 Moss, T., and V.Y. Stefanovsky. (2002) Cell 109:545-548.

23 Moss, T. (2004) Curr Opin Genet Dev. 14:210-217.

24 Sirri, V., D. Hernandez-Verdun, and P. Roussel. (2002) J Cell Biol. 156:969-981.

25 Blangy, A., H.A. Lane, P. d'Herin, M. Harper, M. Kress, and E.A. Nigg. (1995) Cell 83:1159-1169.

26 Sawin, K. E. and Mitchison, T. J. (1995) Proc. Natl. Acad. Sci. USA 92:4289-4293.

27 Matsuo, R., A. Murayama, Y. Saitoh, H. Sakaki, and K. Inokuchi. (2000) J. Neurochem. 74:2239-2249.

28 Kurt, R.A., R.M. Bernstein, S.F. Schluter, J. Park, J. Marchalonis, and E. Akporiaye. (1996) GenBank Database Accession No. U55178.

29 De Murcia, J.M., M. Ricoul, L. Tartier, C. Niedergang, A. Huber, F. Dantzer, V. Schreiber, J.C. Ame, A. Dierich, M. LeMeur, L. Sabatier, P. Chambon, and G. de Murcia. (2003) EMBO J. 22:2255-2263.

29 Dynek, J.N. and S. Smith. (2004) Science 304:97-100.

30 Krause, W.L. and J.T. Lis. (2003) Cell 113:677-683.

31 Kuimov, A.N., and S.M. Terekhov. (2003) Biochemistry (Mosc) 68:260-268.

32 Chi, N.W. and H.F. Lodish. (2000) J. Biol. Chem. 275:38437-38444.

33 Seimiya, H., and S. Smith. (2002) J. Biol. Chem. 277:14116-14126.

34 Sbodio, J.I., and N.-W. Chi. (2002) J. Biol. Chem:277:31887-331892.

35 Cook, B.D., J.N. Dynek, W. Chang, G. Shostak, and S. Smith. (2002) Mol. Cell Biol. 22:332-342.

36 Voit, R., M. Hoffmann, and I. Grummt. (1999) EMBO J. 18:1891-1899.

37 Heix, J., A. Vente, R. Voit, A. Budde, T.M. Michaelidis, and I. Grummt. (1998) EMBO J. 17:7373-7381.

38 Ame, J. C., C. Spenlehauer, and G. de Murcia. (2004) Bioessays 26:882-893.

Acknowledgements

This research was supported by grants from the National Science Council (NSC92-2320-B002-190 and NSC93-2320-B002-109) and the Institute of Biological Chemistry, Academia Sinica. We thank Ms Li-ping Tu and Rui Chao for technical assistance, Ming-Yue Lee for reading the manuscript and the Helix Research Institute of Japan for the cDNA clone FLJ14464.