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Original Research

Mitosis-specific phosphorylation of PML at T409 regulates spindle checkpoint

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Abstract: During mitosis, Promyelocytic leukemia nuclear bodies (PML NBs) change dramatically in morphology and composition, but little is known about function of PML in mitosis. Here, we show that PML is phosphorylated at T409 (PML p409) in a mitosis-specific manner. More importantly, PML p409 contributes to maintain the duration of pro-metaphase and regulates spindle checkpoint. Deficient PML p409 caused a shortening of pro-metaphase and challenged the nocodazole-triggered mitotic arrest. T409A mutation led to a higher frequency of misaligned chromosomes on metaphase plate, and subsequently death in late mitosis. In addition, inhibition of PML p409 repressed growth of tumor cells, suggesting that PML p409 is a potential target for cancer therapy. Collectively, our study demonstrated an important phosphorylated site of PML, which contributed to explore the role of PML in mitosis.

Key words: PML, phosphorylation, mitosis, spindle checkpoint.

Introduction

The promyelocytic leukemia protein (PML) was initially linked to the pathogenesis of acute promyelocytic leukemia because PML is almost invariably expressed as an oncogenic fusion protein (PML-RARα) in APL as a result of the t (15;17) translocation (1,2). PML function largely depends on its ability to form nuclear bodies (PML NBs). PML NBs are discrete nuclear structures that are thought to be organizing centers for protein assembly, allowing for more efficient regulation of cellular outcomes (3-6). Recent studies have suggested a role for PML in multiple cellular processes, including reproduction, apoptosis, differentiation, senescence, and viral defense (7-9).

Mitotic cells undergo dramatic reorganization and disassembly of the nucleus, including nuclear envelope breakdown, chromatin condensation, and disruption of subnuclear compartments such as the nucleolus and PML NBs (10-12). Morphology, organization, structure, and movements of PML NBs change dramatically during mitotic progression (5). Immunofluorescence analysis indicated that the 'core' components of PML NBs, such as SP100 and DAXX, exit the bodies, whereas PML protein is accumulated in mitotic PML NBs termed MAPPs (mitotic accumulations of PML proteins) (13). The MAPPs are fewer and larger than interphase PML NBs, which were thought to enhance reformation of interphase PML NBs during early G1. Time-lapse imaging of live cells demonstrated that PML NBs became mobile in mitosis, but there was limited exchange of PML in and out of MAPPs based on fluorescence recovery after photobleaching (FRAP) (14). On the other hand, biochemical studies have indicated that subtle modifications of PML, whereby it loses its SUMOylation and becomes phosphorylated, occur rapidly (15-17). However, it remains largely unknown how and why PML NBs structures are disassembled and reassembled in mitosis.

In the present study, we identified a mitosis-specific phosphorylation of PML at T409 (PML p409), a modification that was required for maintenance of pro-metaphase time and was associated with the spindle assembly checkpoint (SAC). Inhibition of PML p409 challenged nocodazole-triggered mitotic arrest, and led to chromosome mis-alignment on the metaphase plate and subsequently increased apoptosis in late mitosis. Hence, this work reveals the role of PML in the SAC and provides new insights into the function of PML NBs.

Materials and Methods

Cell lines and cell synchronization

The cell lines MCF-7 and HeLa were grown in DMEM medium (31600-034, GIBCO, USA) containing 10% FBS and penicillin/streptomycin. Human bone marrow stromal cells (BMSCs) were a generous gift from Doctor Duan Haifeng. All the results in the paper came from the experiments using MCF-7 cells, and most of them were confirmed by HeLa and BMSCs. For synchronization in G1, S and M phase, cells were treated with mimosine (0.4 mmol/L), thymidine (2 mmol/L), nocodazole (50 ng/ml), and taxol (50μmol/L) for at least 16 hours. Double-thymidine block and nocodazole arrest were done as described in the references (18). For mitotic cells, nocodazole-arrested cells were shaked off and enriched by centrifugation. The enriched cells were released in prewarmed fresh medium and harvested at different times. These cells were also attached onto glass slides for immunofluorescence using cytospin (Shandon, USA) according to manufacture's instruction.

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Antibodies used for western blots and immunofluorescence

The antibodies used in this paper were as follows: mouse PML antibody (PG-M3, Santa Cruz, USA), rabbit PML antibody (H-238, Santa Cruz), rabbit PML antibody (A301-167A, Bethyl, USA), goat β-Actin (I149, Santa Cruz), mouse Myc antibody (M20002, Clontech), mouse GFP antibody (M048-3, MBL, Japan), and mouse α-tubulin-FITC antibody (F2168, Sigma, USA). PML p409-specific antibody was produced by MBL Company (Japan). Briefly, phosphorylated CPEAASTpPRDP peptide was used to immunize rabbits for 2 months. Specific antibody was purified from serum by affinity chromatography with phosphorylated and unphosphorylated peptides. Western blotting and immunofluorescence analysis was performed as previously described (19).

Plasmids and dsRNAi

GFP- or Myc-tagged PML (PML IV, GI: 34813 in the Genbank) vectors were constructed as described in previous paper (19,20). PML mutants (T409A and T409D) were produced by PCR using long mutation primers. Small dsRNAi (No.1 5′-AAGAGUCGGCCGACUUCUGGU-3') and (No.2 5′-GCAUCUACUGCCGAGGAUGUU-3') was synthesized by GenePharma (Shanghai, China). Plasmid and dsRNAi transfection was performed using Fugene 6 (Roche,USA) and Interferin (Polyplus-transfection SA, France), respectively, according to the manufacturers' instructions.

Mitotic index quantification

Cells cultured in 6-well plates were transfected with blank vector, wild type PML fused protein or PML mutants. After 48 hours, transfected cells were fixed with 4% paraformaldehyde and stained with 4,6-diamidino-2-phenylindole (DAPI) and α-tubulin. All steps in this experiment avoid loss of mitotic cells. Cells in different mitotic stages were selected by the morphology of the spindle microtubules, chromosomes, and nuclear envelope. DNA content analysis and Annexin V staining by flow cytometry were done as previously described (19).

Results

PML p409 was identified by mass spectrometry and a phospho-epitope antibody

To identify novel PML phosphorylation sites, immunoprecipitations were performed using Myc antibody in MCF-7 cells over-expressing Myc-PML. Immunoprecipitated Myc-PML fusion protein was subjected to SDS-PAGE separation and analyzed by mass spectrometry (data are not shown). Using this approach, PML threonine 409 was identified as a phosphorylation site. (Table 1). The 409 threonine of PML protein is in the fourth exon of the PML gene and is the consensus amino acid of all known human PML isoforms (20). PML p409 had been found in several isoforms of human PML in Phospho Site Plus database (www.phosphosite.org). Interestingly, the corresponding mouse PML threonine is also detected phosphorylated by mass spectrometry, suggesting that PML p409 may be functionally conserved (Fig. 1).

PML p409-specific antibody was purified from immunized rabbit serum and was verified by western blots. Firstly, only wild-type GFP-PML fused protein, but not unphosphorylated -mutant (T409A) could be detected in western blots by this antibody (Fig. 2A). Consistently, IFC shows PML p409-specific antibody could co-localized with transient over-expression of PML wild type other than T409 mutant (Fig. 2B). When endogenous PML was knocked down by siRNAs, the major bands recognized by the phospho-epitope antibody were also reduced (Fig. 2C). Next, bands recognized by our antibody disappeared when the mitotic lysate were treated with lambda phosphatase (λ-PPase) (Fig. 2D). Collectively, these data indicated that the phospho-epitope antibody can specifically recognize PML p409.

Table 1. PML IV protein phosphorylation site were identified by Mass spectrometry.

Phosphosite	Peptide
1.Ser 117	R.RLSVYR.Q + Phospho (ST)
2.Ser 36	R.QPSPSPSPTER.A + Phospho (ST)
3.Ser 36+Ser38	R.QPSPSPSPTER.A + 2 Phospho (ST)
4.Ser 48	R.APASEEEFQFLR.C + Phospho (ST)
5.Ser 527	K.AVSPPHLDGPPSPR.S + Phospho (ST)
6.Ser 518+Ser527	K.AVSPPHLDGPPSPR.S + 2 Phospho (ST)
7.Ser 504	R.LARSSPEQPRPSTSK.A + Phospho (ST)
8.Ser 565	R.VVVISSSEDSDAENSSSR.E + Phospho (ST)
9.Ser 562+Ser565	R.VVVISSSEDSDAENSSSR.E + 2 Phospho (ST)
10.Ser 583	R.ELDDSSSESSDLQLEGPSTLR.V + Phospho (ST)
11.Ser 403	K.ASPEAASTPRDPIDVDLPEEAER.V + Phospho (ST)
12.Thr 409	K.KASPEAASTPRDPIDVDLPEEAER.V + Phospho (ST)
13.Ser 530	R.SPVIGSEVFLPNSNHVASGAGEAEER.V + Phospho (ST)

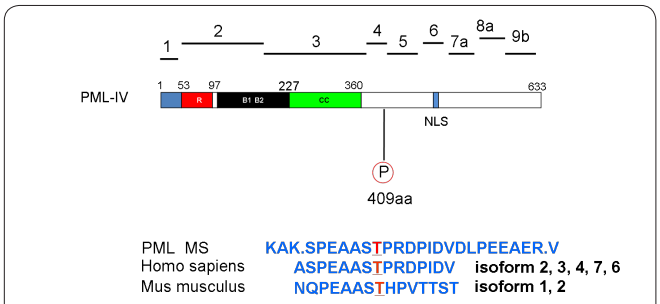

Figure 1. Schematic representation of PML modular organization and its major domain. PML threonine 409 is in the fourth exon of the PML gene and is a consensus amino acid among all known human PML isoforms, even though it is not in the well-known RBCC domain. According to the data form high throughput mass spectrometry human PML isoforms 2, 3, 4, 6 and 7 all had phosphorylated at T409. Mouse PML gene has two isoforms, and both of them can be phosphorylated at similar threonine. Color boxes in the figure represent important domains or elements of PML protein. R (red), RING-finger domain. B1 B2 (black), Box1 and Box2 domain. CC (green), Coiled-coil domain. NLS(blue), nuclear location signal.

PML p409 was mitosis-specific expression

Serendipitously, we found in immunol staining that the level of PML p409 was highest in mitotic cells (supplementary Figure 1). To confirm if the PML p409 is associated with cell cycle, MCF-7 cells were arrested in G1, S and M phase by addition of various drugs. The fraction of PML with phosphorylated T409 was significantly higher in mitosis-arrested cells (Fig. 3A). Next, the kinetics of PML phosphorylation was examined during cell cycle. PML p409 peaked at 14-16 h after the release from the thymidine, when most cells entered mitosis (Fig. 3B). Therefore, PML p409 was mitosis-specific expression.

Immunofluorescence analysis showed that location

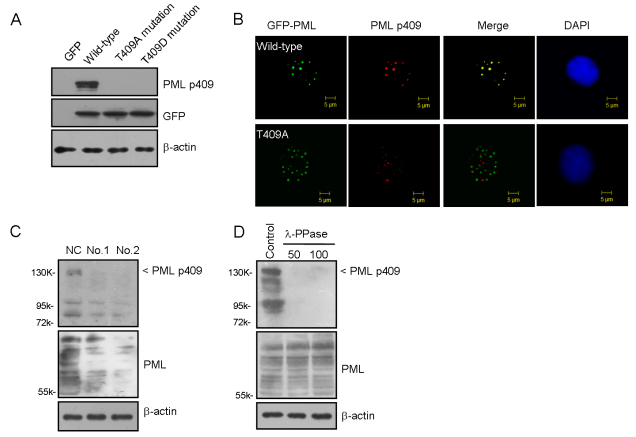

Figure 2. PML p409 antibody is specific. **A**. Lysates of MCF-7 cells transfected with GFP blank vector, wild-type GFP-PML (WT) or GFP-PML mutants (T409A and T409D) were resolved on an SDS-PAGE gel and blotted using the indicated antibodies. **B**. Colocalization of PML p409 with PML wild type other than mutant Cells were transcient transfected GFP-PML and GFP-PML-T409A, fixed and counterstained with PML p409(red) and anti-GFP(green). Colocalization foci appears yellow in the merge image. Scale bar,5μm. **C**. Lysates of MCF-7 cells transfected with negative control siRNA (NC) or PML siRNA (No.1 and No.2) were blotted with the indicated antibodies. **D**. Lysates of nocodazole-arrested MCF-7 cells were incubated withλ-PPase, and were blotted with the indicated antibodies.

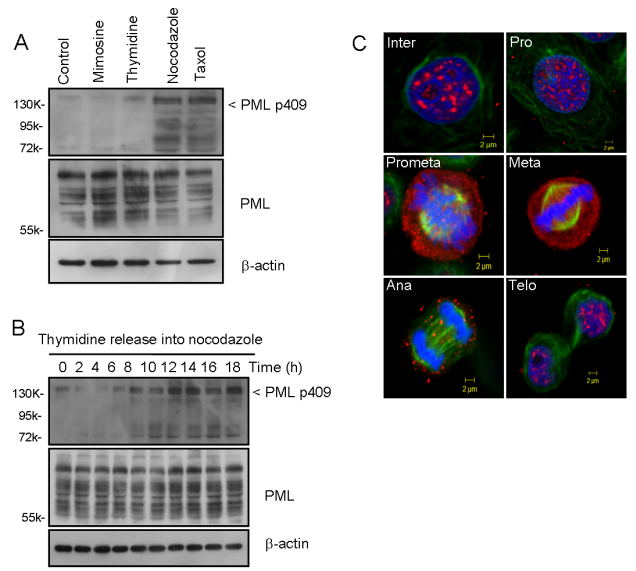

Figure 3. Phosphorylation of PML at T409 is mitosis-specific. **A**. After treatment with mimosine, thymidine, nocodazole and taxol, MCF-7 cells were arrested in late G1 (75%), S (32%) and G2/M (60% and 66%), respectively. Extracts from these cells were dissolved in SDS buffer, separated on SDS-PAGE and blotted with the indicated antibodies. **B**. MCF-7 cells were synchronized by a double thymidine treatment and then released into nocodazole. Cell extracts were prepared at the times indicated after thymidine release. Immunoblots analysis was carried out using the indicated antibodies. **C**. Localization of PML p409 was distinct and dynamics. Immunofluorescence analysis was performed with PML p409 (red) antibody and α-tubulin (green) antibody, and nuclei were counterstained with DAPI (blue) in MCF-7 cells. Cells in different mitotic stages including interphase (Inter), prophase (Pro), pro-metaphase (Pro-meta), metaphase (Meta), anaphase (Ana), and telophase (Telo), were selected according to chromosome and microtubule morphology. Scale bar, 2μm.

of PML p409 is different from that of general PML protein throughout cell cycle. PML p409 gathered as irregular shape and distributed among dense chromatin in interphase cells. During mitosis, PML p409 re-localized in many smaller clusters in prophase and gradually diffused throughout the entire cell during pro-metaphase or metaphase. In anaphase, PML p409 reassembled into small particles around the chromosomes. Finally, PML p409 returned to the irregular shape and location in telophase (Fig. 3C). Similarly, expression pattern of PML p409 in MCF-7 was same as in that of HeLa and BMSCs. In addition, location of general PML during mitotic progression was also detected and the results were same as described in the previous papers (17,18) (data are not shown). Taken together, our data indicated that PML p409 was distinctly localized during mitotic progression, suggesting that function of PML p409 may be associated with mitosis.

PML p409 regulated spindle assembly checkpoint

To explore the role of PML p409 in mitosis, we monitored the mitotic progression by counting of cells in different mitotic stages. T409A mutation significantly decreased the percentage of mitotic cells in pro-metaphase, compared with empty vector, wild-type PML or T409D mutation (Fig. 4A). In mitosis, the SAC control duration of pro-metaphase until all chromosomes are accurately attached by spindle microtubule (21,22).

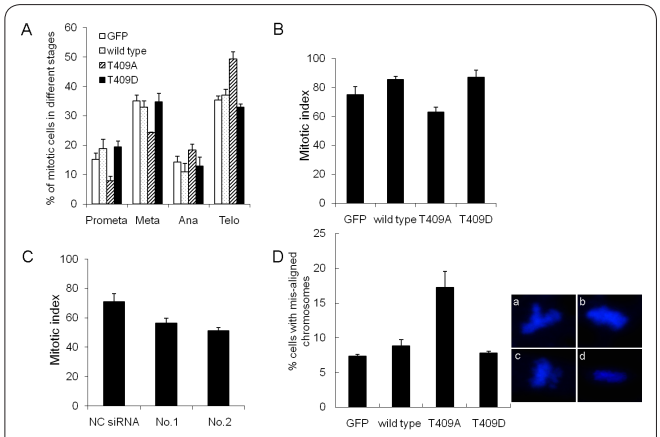

Figure 4. PML p409 participate in spindle checkpoint. Regulation. **A**. MCF-7 cells were transfected with GFP empty vector, wild type GFP-PML or its mutants (T409A and T409D). After 48 hours, cells were fixed with 4% paraformaldehyde and stained with DAPI. GFP-positive cells in different mitotic stages (Pro-meta, pro-metaphase; Meta, metaphase; Ana, anaphase; Telo, telophase) were calculated. Error bars, s.e.m. Over 4000 mitotic cells were counted in three independent experiments. **B**. MCF-7 cells were transfected as described in Fig.2. After 24 hours, nocodazole was added. Next, cells were fixed and stained 12 hours later. Mitotic index were determined in GFP-positive cells. Error bars, s.e.m. Over 1,000 cells were counted in three independent experiments. **C**. MCF-7 cells were transfected with negative control siRNA (NC) or PML siRNAs (No.1 and No.2). After 48 hours, nocodazole was added. Next, cells were fixed and stained 12 hours later. Mitotic index were determined. Error bars, s.e.m. Over 1,000 cells were counted in three independent experiments. **D**. MCF-7 cells were transfected as described in Fig.5. Mitotic cells with abnormal chromosome congression were quantified. Examples of misaligned chromosomes (a, b, and c) and normal chromosomes (d) were in right panel. Error bars, s.e.m. Over 1,000 mitotic cells were counted in three independent experiments.

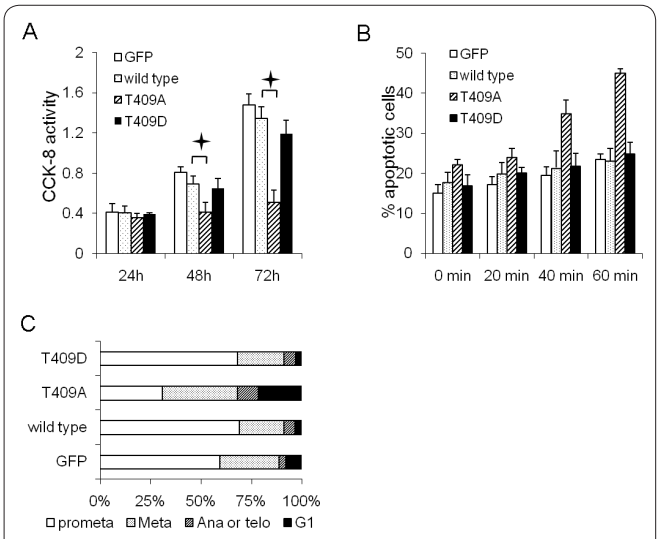

Figure 5. Inhibition of PML p409 suppresses growth of tumor cells. **A**. Number of viable cells were detected by Cell Counting Kit-8 (CCK-8) at the indicated time after transfection with GFP, wild-type PML or PML mutants (T409A or T409D). Data are shown as mean±s.d. Statistical analysis were carried out by student't-test. Stars mean $p<0.01$. **B**. MCF-7 cells were treated with nocodazole. After 16 hours, mitotic cells were shaked off, enriched by centrifugation and released into fresh medium. Apoptotic cells was calculated by Annexin V binding and propidium iodide staining at indicated time after release from nocodazole treatment. Data are shown as mean±s.d. **C**. Mitotic cells were enriched and released as in (B). Cells in prometaphase (prometa), metaphase (meta), anaphase (ana) and interphase (inter) were calculated. Over 2,000 cells were counted in three independent experiments.

Therefore, PML p409 may be implicated in the regulation of SAC in mitosis. Next, we test if T409A mutation could challenge spindle poison-triggered mitotic arrest. Mitotic index was significantly reduced in PML T409A mutant- expressing cells in the presence of nocodazole (Fig. 4B). Similarly, PML knockdown also compromised the ability of the cells to arrest in mitosis (Fig.4C). As the defects of spindle checkpoint can lead to chromosome misalignment or mis-segregation during mitosis, we assessed metaphase chromosome morphology in mitotic cells. It was shown that T409A mutation produced more misaligned chromosomes on the metaphase plate (Fig. 4D). Collectively, these data showed that PML p409 led to prolonged pro-metaphase and regulated the spindle checkpoint in mitosis.

Inhibition of PML p409 suppressed growth of tumor cells and induced mitotic apoptosis

Previous studies reported that PML was absent in many solid and liquid tumors and was functional in tumor suppression (5). We evaluated the effect of PML p409 on the growth of MCF-7 cells by CCK-8 staining. T409A mutation repressed growth of MCF-7 cells more severely than wild type PML or T409D mutation (Fig. 5A). It is generally believed that depletion or inactivation of SAC proteins lead to severe mitotic defects and subsequently death in late mitosis (22). So, we investigate if effect of T409A mutation depends on its function in the SAC. Apoptosis of cells released from nocodazole-treatment at the indicated times was detected, while mitotic stages of these cells were determined. Apoptosis triggered by T409A mutation occurred apparently at 40 and 60 minutes after release (Fig. 5B), when these cells were just exiting from metaphase and most were in anaphase or telophase (Fig. 5C). Hence, it is concluded that T409A mutation led to more cell death in late mitosis because of the mitotic defects caused by deficient PML p409.

Discussion

It had been reported by Everett et al in 1998 that there was an unstable, mitosis-specific PML isoform of about 140 kDa in immunoblot analysis (17). In addition, this PML isoform was phosphorylated, because phosphatase inhibitors resulted in the appearance of this isoform. However, the function of the isoform could not be further explored, as the mitosis-specific phosphorylated site was unknown. In the current study, phosphorylation of PML at T409 was unexpectedly found to be mitosis-specific. Coincidently, PML p409 specific antibody also recognized one band at about 140 kDa in western blots (Fig. 3A). Collectively, phosphorylation of PML at T409 physically happens during mitosis, and it may be a key clue for exploring the role of PML in mitosis.

Mitosis is one of the most important events in cell cycle, and errors in the choreography of this process can lead to aneuploidy or genetic instability, fostering cell death or disease (21, 22). The SAC is by far the most robust mechanism for ensuring the fidelity of chromosome segregation by monitoring chromosome attachment and delaying mitotic progression to allow extra time to correct defects (22,23). Our data indicate that PML p409 was involved in the regulation of SAC: deficient PML p409 accelerated pro-metaphase and overrode nocodazole-arrested mitosis. Furthermore, inhibition of PML p409 resulted in more misaligned chromosomes in metaphase, and subsequent mitotic death in late mitosis. In general, most proteins associated with the SAC are temporally localized to the kinetochore, but PML p409 was diffuse in almost all cells. Therefore, we propose that phosphorylated PML may not work as a direct regulator of the SAC, but may regulate localization or modification of some key SAC factors (8,22). Clearly, PML function in the SAC needs further study.

In 2010, Yang et al showed that a small-molecule inhibitor of BMK1/ERK5 suppressed tumor growth through inhibition of PML p409 (24). It is amazing how PML p409 became a target for cancer-therapy, as PML is considered as a tumor suppressor gene (5,25). However, our data produced another possibility that inhibition of PML p409 could lead to SAC defect and induce apoptosis of mitotic cells. It was shown that cancer cell lines undergo apoptosis when they are depleted of SAC components. This leads a strategy for cancer-therapy that SAC inactivation might become a desirable way to kill rapidly dividing cancer cells. So, our results provide possible reason that PML p409 may became a potential therapeutic target in PML-expressing tumors.

Acknowledgments

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