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Cell Cycle Dependent Gene Expressions and Activities of Protein Phosphatases PP1 and PP2A in Mouse NIH3T3 Fibroblasts

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SUMMARY: We determined the mRNA levels and the activities in nuclear and non-nuclear fractions of protein phosphatase type 1 (PP1) and type 2A (PP2A) through the cell cycle in synchronized mouse NIH3T3 fibroblasts. The mRNA level for PP1α was gradually elevated in late G1 phase, began to decrease in M phase, and reached the control level with entering into the next G1 phase. The mRNA level for PP2A was rapidly increased in early G1 phase, kept at the high level, and decreased after S phase. In nuclear fractions of cells, spontaneous activities of both PP1 and PP2A were gradually increased until M phase and rapidly decreased with entering the next G1 phase, while in non-nuclear fraction such dramatic alterations were not observed. Potential activities of PP1 in both fractions revealed by Co^{2+}-trypsin treatment showed an oscillaion patterns similar to those of the spontaneous activities. These results strongly suggest that cell cycle dependent gene expressions and activities of PP1 and PP2A play roles in DNA synthesis and mitosis during the cell cycle.

In the past several years there has been increasing evidence indicating specific phosphorylation and dephosphorylation of nuclear proteins during cell growth. Although it is well-known that the phosphorylation plays crucial roles in signal transduction for cell proliferation, differentiation and carcinogenesis, little has been focused on the implication of protein phosphatases for the mitotic events.

Four principal serine/threonine specific protein phosphatases termed PP1, PP2A, PP2B and PP2C and several protein tyrosin

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phosphatases have been identified (1, 2). Recently it has been reported that the protein phosphatases play important roles in the cell division cycle. CDC25, a tyrosin phosphatase, dephosphorylates and activates $p34^{cdc2}$ kinase in fission yeast (3) and human (4). Histone H1 phosphorylated by $p34^{cdc2}$ is dephosphorylated by PP2A1 (5). Inhibition of mitosis by okadaic acid suggests the possibility of involvement of PP2A in the transition from metaphase to anaphase (6). Okadaic acid also induces cell cycle arrest at either G1/S or G2/M depending on dose in myeloid leukemic cells (7). It was also reported that okadaic acid induces mitotic arrest and enhances nuclear protein phosphorylation in human leukemia K562 cells (8).

We have recently reported remarkable elevations in PP1 and PP2A mRNA levels at preneoplastic stages during hepatocarcinogenesis and in regenerating livers (9). We also found the specific overexpression of PP1α mRNA in a transplantable hepatoma cell line, AH13(10). Very recently, we reported that the potential activity of nuclear PP1 was increased 2.4-fold at 12 h after partial hepatectomy compared to the control level (11).

To clarify further the mechanism and the significance of these phenomena in regulation of cell proliferation, we have examined the mRNA levels and activities of PP1 and PP2A in nuclear and non-nuclear fractions during the cell cycle using synchronized mouse NIH3T3 fibroblasts.

MATERIALS AND METHODS

Cell culture: Mouse NIH3T3 fibroblasts were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% calf serum. In experiments on serum stimulation of cell growth, cells were initially grown to confluency and then placed in serum-free DMEM for 36 h. The cells were then stimulated by replacing the serum-free DMEM with fresh DMEM containing 10% calf serum, harvested at appropriate times and counted. The cells were subjected to flow cytometry, Northern blot analysis and phosphatase activity assay.

Cell cycle analysis: The harvested cells were washed with phosphate-buffered saline (PBS), fixed with PBS-ethanol mixture (1:1 v/v), then rewashed with PBS. After centrifugation, the cells were resuspended in PBS containing 50 μg/ml propidium iodide, 0.5 mg/ml RNase and 0.1% NaN3, and incubated at room temperature for 15 min. After the incubation, the suspension was mixed with 1 vol. of PBS and passed through a nylon mesh. G1, S and G2+M phases of the cell cycle were analyzed by FACScan flow cytometer (Becton Dickinson).

cDNA probes: The 1.4 kb full-length cDNA of rat PP1α and the 410 bp BstEII-BglII fragment of rat PP2A cDNA (12) were labeled with [α-^{32}P]dCTP by using Multiprime DNA Labeling Kit (Amersham International plc, England).

Northern blot analysis: Total cellular RNA (20 μg) prepared by the procedure of Chomczynski et al. (13) was separated by

electrophoresis on 1% agarose gel containing 6% formaldehyde and transferred to a nitrocellulose membrane (Schleicher & Schuell, Germany). After being baked at 80°C for 2 h under vacuum, the membranes were prehybridized in 5 x SSC, 1 x Denhardt's solution, 50 mM NaH_2PO_4, pH 6.5, 1% glycine, 100 μg/ml denatured E.coli DNA, 50% formamide for 1 h at 42°C. Hybridization was performed in the same solution containing the radioactive cDNA probes at 42°C overnight. The membranes were washed twice in 2 x SSC and 0.1% SDS at room temperature for 5 min, followed by washing twice in 0.1 x SSC and 0.1% SDS at 50°C for 15 min. The membranes were exposed to XRP-5 X-ray film (Eastman Kodak Company, NY) at -80°C with intensifying screens. The membranes were reused after the old probes had been washed out by incubating the membranes in 2.5 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, 0.05% sodium pyrophosphate and 0.05 x Denhardt's solution at 70°C for 1 h.

Preparation of nuclear and non-nuclear fractions: The harvested cells were adjusted at 10^7/ml and lysed with NP-40 lysis buffer (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 0.5% (v/v) NP-40) at 10^7 cells/ml and centrifuged at 500 x g for 5 min at 4°C (14). The supernatant was termed non-nuclear fraction. The pellet after being washed with the same buffer was termed nuclear fraction, which was then lysed at 2 x 10^7 cells/ml by the method of Kret et al. (15) and used for enzyme assays.

Protein phosphatase assay: Activities of PP1 and PP2A in the nuclear and non-nuclear fractions were measured by the method of Cohen et al. (16) with a slight modification (17). PP2A activity was determined in the presence of inhibitor-2 to inhibit PP1 activity. PP1 activity was calculated by deducting the phosphatase activity measured in the presence of inhibitor-2 from the one in its absence. PP1 activity was also measured after Co^{2+}-trypsin treatment as described previously (17, 18). PP1 activities before and after the treatment were termed spontaneous and potential activities, respectively. One unit of activity (U) was defined as the amount of enzyme which catalyzes the release of 1 μmol of phosphate per min. Activities of PP1 and PP2A were presented as milli unit per 10^7 cells.

RESULTS

Expression of PP1 and PP2A in mouse NIH3T3 fibroblasts during the cell cycle: Cell cycle dependent expressions of PP1 and PP2A after stimulation of cell growth by serum addition were investigated.

Figure 1 shows the results for the stages of the cell cycle from G0/G1 to mid-S phase. Figure 1A shows the number of cells at G1, S and G2+M phases. Figure 1B and 1C show alterations in the mRNA levels of PP1α and PP2A, respectively, for 18 hours after serum re-addition. The mRNA level for PP1α began to increase from 2 h after the serum stimulation and reached a maximum at 8-10 h, corresponding to late G1 phase. The mRNA level for PP2A was increased and reached a maximum 2 h after the stimulation, and remained at the high level until late S phase.

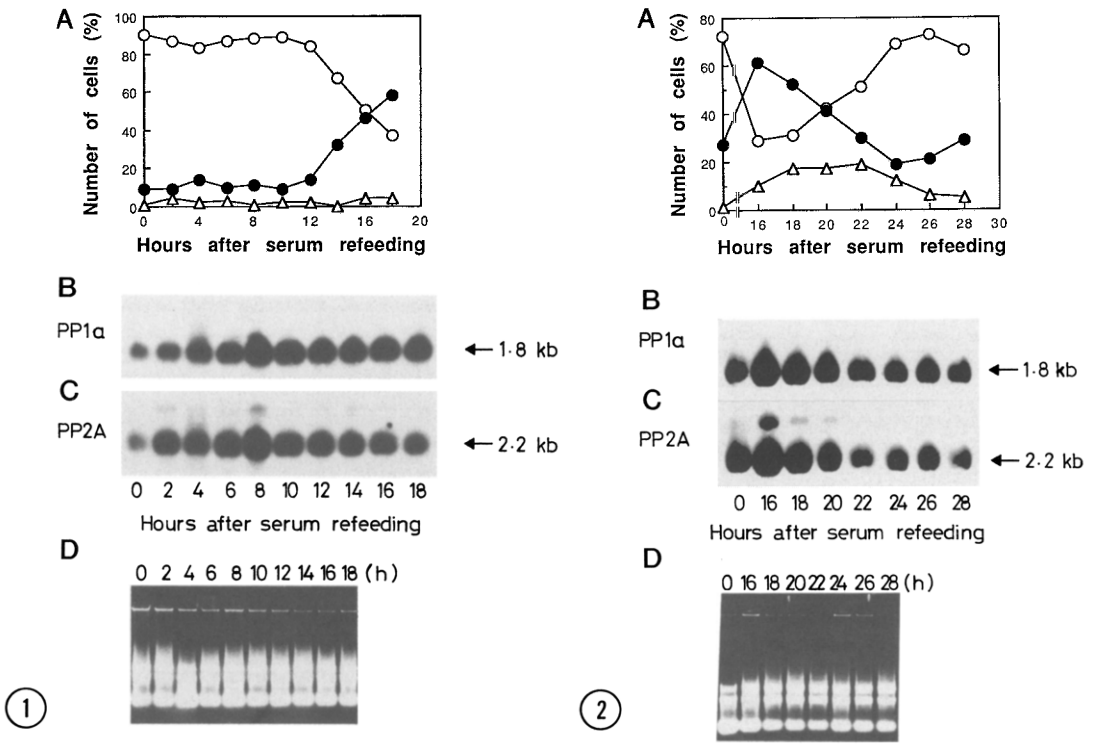

Figure 1. Northern blot analysis of PP1α and PP2A from G0/G1 to mid-S phase during the cell cycle. (A) The percentages of cells in G1 phase (open circle), S phase (closed circle) and G2+M phase (triangle) were determined by flow cytometry at appropriate times after refeeding serum as described in Materials and Methods. 20 μg of total RNA extracted from cells at various times after refeeding was electrophoresed, transferred to a nitrocellulose membrane and hybridized with cDNAs for PP1α (B) and PP2A (C) as described in Materials and Methods. The ethidium bromide-stained gel demonstrates equal loading (D).

Figure 2. Northern blot analysis of PP1α and PP2A from S to the next G1 phase during the cell cycle. (A) The percentages of cells in G1 phase (open circle), S phase (closed circle) and G2+M phase (triangle) were determined by flow cytometry. 20 μg of total RNA extracted from cells at 0 h and 16-28 h after refeeding was electrophoresed, transferred, and hybridized with cDNAs for PP1α (B) and PP2A (C). The ethidium bromide-stained gel demonstrates equal loading (D).

Figure 2 shows the results for the subsequent 16-28 h after the serum stimulation, corresponding to mid-S phase to the next early G1. The mRNA level for PP1α was kept high for the first several hours, then began to decline at late M phase and reached to the control level at the next early G1 phase (Fig. 2B). The change in the mRNA level for PP2A was similar to that of PP1α (Fig. 2C). Similar results were obtained from the experiments in which NIH3T3 cells were synchronized at S phase by addition of excess thymidine and subsequently released from the arrest by replacing the medium with a thymidine free one (data not shown).

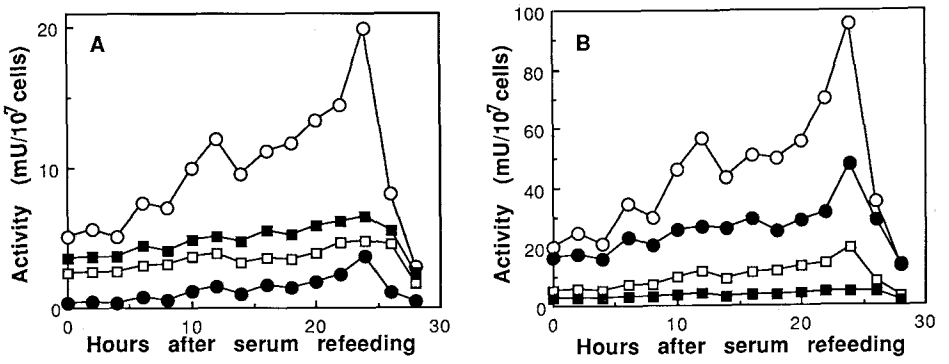

Figure 3. Protein phosphatase activities of PP1 and PP2A in nuclear and non-nuclear fractions during the cell cycle. (A) Spontaneous activities of PP1 (open symbol) and PP2A (closed symbol) in nuclear (circle) and non-nuclear fractions (square) were measured during the cell cycle as described in Materials and Methods. (B) Potential activities of PP1 revealed by Co^{2+}-trypsin treatment in nuclear (open circles) and non-nuclear fractions (closed circles) were measured as described in Materials and Methods. Spontaneous activities of PP1 in nuclear (open squares) and non-nuclear (closed squares) fractions were also presented for comparison. Each point represents average of duplicate determinations. These results were confirmed by determinations for another series of cell extractions at the same time points.

Activities of PP1 and PP2A in nuclear and non-nuclear fractions: Whether the increases in the mRNA levels of PP1 and PP2A during the cell cycle are reflected in their enzyme activity levels was examined. The phosphatase activities are presented in Fig. 3 as milli unit per 10^7 cells, not per mg protein, because the protein concentrations in nuclear fractions varied during the cell cycle.

In nuclear fraction, activities of both PP1 and PP2A were increased from 6 h after serum stimulation, peaking at 24 h corresponding to mid-M phase, and then rapidly declined to the control levels (Fig. 3A). The activities at the peaks of PP1 and PP2A were approximately 3.9- and 11-fold, respectively, greater than those before the stimulation. On the other hand, in non-nuclear fraction, there were only slight increases in activities of PP1 and PP2A, 1.9- and 1.8-fold, respectively, compared to the control levels.

Potential activity of PP1 revealed by Co^{2+}-trypsin treatment: It has been reported that the treatment of the crude extracts with Co^{2+}-trypsin gives the highest activity of PP1 (17, 19), which reflects the total amount of PP1 catalytic subunit. The PP1 activities in both nuclear and non-nuclear fractions were activated 5- to 10-fold by the treatment, while the oscillation patterns were similar to those of their spontaneous activity before the treatment (Fig. 3B).

DISCUSSION

In this paper, we demonstrated that, in NIH3T3 fibroblasts, PP1 gene was highly expressed at S phase and that the activities of PP1 and PP2A reach a peak at mitosis.

PP1 activity in nuclei was greatly activated by Co^{2+}-trypsin treatment in the present work, which is contradictory to the previous report that PP1 in rat liver nuclei exists as a free catalytic subunit (15). We also confirmed that PP1 activity in rat liver nuclei was not activated upon Co^{2+}-trypsin treatment (11). Therefore, the present results suggest a possibility that PP1 in nuclei of mouse NIH3T3 fibroblasts exists as a complex with regulatory subunits, such as inhibitor-2. This possibility is consistent with the previous report that inhibitor-2 oscillates and peaks at S and M phases in both cytosol and nucleus of rat fibroblasts (20).

The activity of PP1 in nuclear fraction was increased 3.9-fold higher at M phase than the control level, whereas the increase in non-nuclear fraction was only 1.8-fold (Fig. 3A). There are at least two possible mechanisms to explain this fact. One is that PP1 protein at S to M phase translocates from cytosol into nucleus immediately after its translation. The fact that the increase in mRNA of PP1 preceded the increase in the activity might support this mechanism. Fernandez et al. have demonstrated using synchronized rat embryo fibroblasts that PP1 undergoes a marked relocation from cytosol into nucleus at the end of G2 phase (21). Whether previously synthesyzed PP1 or newly synthesized one relocates is not clear. Another possibility is a phosphorylation-dephosphorylation mechanism in cell cycle dependent regulation of nuclear PP1.

The mRNA for PP2A was continuously expressed from early G1 phase until M phase. This observation is compatible with the previous report presenting constant expression of PP2A in Western blots and the activity assay during the mitotic cell cycle using embryonic bovine tracheal cells (22). In the present work, however, the PP2A activity in nuclei was increased at M phase, whereas that in non-nuclear fraction was kept constant at a high level, about 10-fold that in nuclear fraction except at M phase (Fig. 3A). It might be possible that PP2A in cytosol redistributes into nucleus or that PP2A in nucleus is activated by some modifications, such as phosphorylation, at M phase. In any case, the increase in nuclear PP2A activity before mitosis is consistent with the fact that okadaic acid induces CDC2 kinase activation (23).

The present work together with others suggests that PP1 and PP2A participate in mitotic events, however, their roles in mitosis are not yet clear. To elucidate these points and get further insight into roles of the protein phosphatases in regulation of the cell cycle, analyses of these protein phosphatases at protein levels are now in progress.

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