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Gene Expressions and Activities of Protein Phosphatases PP1 and PP2A in Rat Liver Regeneration After Partial Hepatectomy

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SUMMARY: We have examined the levels of gene expressions and activities of protein phosphatases, PP1 and PP2A, in rat regenerating livers. PP1α mRNA started to increase from 6 h after partial hepatectomy (PH) and showed two peaks at 12 and 48 h. PP2A mRNA level showed two peaks at 6 and 10~12 h. Protein phosphatase activities were determined both in non-nuclear fraction and in nuclei. While spontaneous PP1 activity in non-nuclear fraction was nearly constant, potential PP1 activity revealed by Co^{2+}-trypsin treatment showed a small peak between 7 and 12 h. In nuclei, both spontaneous and potential PP1 activity began to increase from 4~7 h after PH, reached a maximum (about 2.5-fold over control levels) at 12 h, the time which corresponds to the G1 to S transition in the cell cycle, and then declined back to control levels by 7 days. PP2A activity in non-nuclear fraction was nearly constant in both spontaneous and potential forms. PP2A activity in both forms in nuclei was very low throughout. These results suggest the possibility that PP1 in nuclei plays some role in the G1 to S transition in the cell cycle of hepatocyte proliferation.

For the recent decade numerous studies on signal transduction have demonstrated that the regulation of a variety of cellular process including cell growth and oncogenesis involve multiple protein phosphorylation cascades (1). Recently, however, the physiological roles of protein phosphatase, which functionally counteracts protein kinase, on cellular events have also been extensively studied and many important aspects of this enzyme

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Abbreviation: PH, partial hepatectomy.

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related to cell growth have been revealed (2,3). For example, in yeast mutations of PP1 gene induce abnormal cell division (4). PP2A dephosphorylates histone H1 phosphorylated by $p34^{cdc2}$ (5). Okadaic acid, a potent inhibitor of PP1 and PP2A, has strong promotor action in skin carcinogenesis (6).

We previously purified protein phosphatases and reported the properties (7-9). We also recently reported the increase of PP1α, PP2A and PP2C mRNAs in chemical hepatocarcinogenesis and specific overexpression of PP1α mRNA in a rat ascites hepatoma cell line, AH13 (10).

To elucidate the physiological significance of these enzymes, we analyzed their gene expressions and enzyme activities in liver regeneration which is known as a useful system to examine the mechanism of hepatocyte proliferation.

MATERIALS AND METHODS

ANIMALS: Inbred adult male WKAH/HKm rats(180~200 g) purchased from Japan SLC. Inc. were housed in a temperature-controlled room with 12 h alternating light and dark cycles and acclimated to this environment prior to the experiments. The rats were partially hepatectomized (about 70% resection) by the method of Higgins and Anderson (11). The time of the surgery was varied so that all the rats would be killed between 9 and 11 a.m. Food was supplied *ad libitum*, but was withdrawn 15~18 h before killing.

NORTHERN BLOT ANALYSIS: Total RNA (20μg) isolated from rat livers by the AGPC method (12) was electrophoresed on 1.2% agarose gel containing 6% formaldehyde and transferred to a nitrocellulose membrane. Probes were prepared as follows: The PstI-SmaI fragment (600 bp) of rat PP1α cDNA (13), the BstII-BglII fragment (410 bp) of rat PP2Aα cDNA (14) and the PstI fragment (1030 bp) of rat albumin cDNA (kindly provided by Y.Fujii-Kuriyama, Tohoku University, through M. Sakai, Hokkaido University) were labelled with [α-^{32}P]dCTP using the Multiprime DNA Labeling Kit (Amersham, England). Hybridization was performed as described previously (10). The filters were reused after washing in 0.1 x Denhardt's solution containing 2 mM EDTA and 5 mM Tris-HCl, pH 8.0, at 70°C for 2 h. We used the rat albumin cDNA as a control (Fig. 1C), because the level of albumin mRNA do not vary during at least the first 24 h after PH (15).

PREPARATION OF NON-NUCLEAR FRACTION AND NUCLEI: Nuclei were isolated essentially by the method of Blobel and Potter (16) with a slight modification as described by Kuret et al.(17). Animals were decapitated, and livers were removed quickly and chilled immediately in several volumes of ice cold 0.25 M sucrose in solution A (50 mM Tris-HCl, pH 7.5, 25 mM KCl, 0.01 mM leupeptin, and 0.1% 2-mercaptoethanol) and homogenized in the same buffer. The homogenate was filtered through cheese cloth and was mixed with 2 vols of 2.3 M sucrose in solution A. The mixture was underlaid by one volume of 2.3 M sucrose in solution A and then centrifuged at 124000 x *g* for 30 min using a swinging bucket rotor at 4°C. The resulting supernatant was termed non-nuclear fraction. The nuclear

pellets were resuspended in solution B (50 mM Tris-HCl, pH 7.5, 25 mM KCl, 5 mM $MgCl_2$, 0.1% 2-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride, and 1 mM benzamidine) containing 0.25 M sucrose and were made 0.5% (w/v) in Triton X-100, rehomogenized and recentrifuged for 5 min at 800 x *g*. The white nuclear pellets were resuspended in solution B containing 10 mM EDTA (2.5ml/g liver). To the suspension, 0.33 vols of 50 mM Tris-HCl, pH 7.5, 0.1% 2-mercaptoethanol, 2 M NaCl were added, then stirred for 30 min. The resulting suspension was termed nuclear lysate.

PROTEIN PHOSPHATASE ASSAY: Activities of PP1 and PP2A were measured by the method of Cohen et al.(18) with a slight modification as described previously (19). Briefly, ^{32}P-labelled rabbit skeletal muscle phosphorylase a (10^6 cpm/nmol) was used as a substrate. After pre-incubation, assays were carried out for 10 min and terminated by adding 20 mM silicotungstic acid in 0.01 M H_2SO_4. PP1 is the activity that is sensitive to inhibitor-2 and PP2A is the activity that is unaffected by inhibitor-2. No PP2B or PP2C activity is detected under these conditions, as demonstrated previously (19). PP1 and PP2A activities in nuclei were defined as the activities of the resuspended nuclear pellets just before lysis subtracted from the activities of nuclear lysates. PP1 activities of resuspended nuclear pellets before lysis of nuclei were usually about 10% of the activities in nuclear lysates, whereas PP2A activities before and after lysis of nuclei were hardly detected (data not shown). Treatment of non-nuclear fraction with Co^{2+}-trypsin and 2-mercaptoethanol was carried out as described elsewhere (19,20), except that the concentration of TPCK-trypsin was 15 μg/ml, and that of 2-mercaptoethanol about 0.75 M. PP1 and PP2A existed as holoenzymes are dissociated to the catalytic subunits by the treatments with Co^{2+}-trypsin and with 2-mercaptoethanol, respectively (21,22). In this paper, the enzyme activity before the treatment was termed spontaneous activity and the one after the treatment was termed potential activity. One unit of activity (U) was defined as the amount of enzyme which catalyzes the release of 1 μmol of phosphate per min.

PROTEIN DETERMINATION: Protein concentration was measured by the procedure of Bradford(23), using bovine serum albumin as the standard.

RESULTS

EXPRESSION OF PP1α mRNA: PP1α mRNA detected during liver regeneration was about 1.7 kb species (Fig. 1A). The level of PP1α mRNA began to increase gradually from 6 h after PH, reached a maximum at 12 h and then declined slightly. There was the second peak at 48 h and the level was subsequently kept at relatively high until day 7.

EXPRESSION OF PP2A mRNA: As previously reported (14), two mRNA bands were detected, namely the major one of 2.0 kb and the minor one of 2.7 kb. The two bands showed similar patterns of change in quantity during liver regeneration (Fig. 1B). Two peaks of the mRNA level were observed: the first peak at 6 h , the second peak

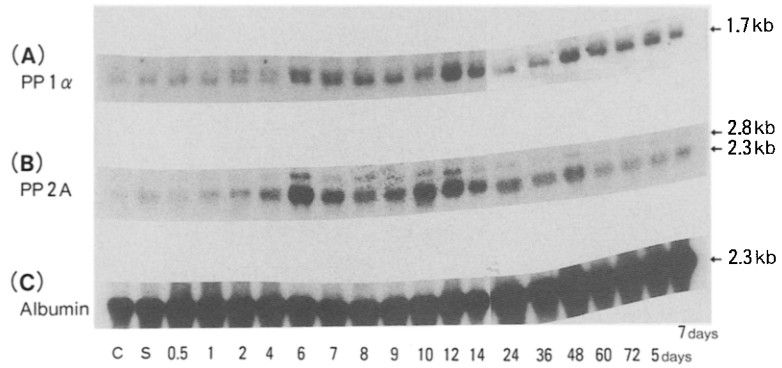

Fig. 1. Northern blot analysis of mRNA expressions of PP1α, PP2A and albumin in regenerating liver. 20 μg of total RNA prepared from livers at various times after PH was separated by electrophoresis, transferred to a nitrocellulose membrane and hybridized with ^{32}P-labelled probes for PP1α (A), PP2A (B) and albumin (C), as described in Materials and Methods. Numbers on right ordinate indicate the positions of transcripts for PP1α, PP2A and albumin, respectively. C, control; S, sham operation.

at 10~12 h after PH. Subsequently, the level declined back to about control level.

PP1 AND PP2A ACTIVITIES IN NON-NUCLEAR FRACTION: The spontaneous activity of PP1 remained nearly constant at about 1 mU/mg protein, whereas the potential activity was elevated approximately 1.5-fold at 7~12 h after PH as compared with the control level (Fig. 2). On the other hand, the spontaneous and potential activities of PP2A showed only slender increases until day 7 (Fig. 3).

PP1 AND PP2A ACTIVITIES IN NUCLEI: Since PP1 and PP2A activities in nuclei at each time point were unaffected by treatments with Co^{2+}-trypsin or 2-mercaptoethanol, respectively, only spontaneous activities are shown in Fig. 4. Spontaneous PP1 activity in nuclei began to increase from 4~7 h after PH, reached a maximum at 12 h, and then declined to about control level by day 7. The activities at 10, 12 and 24 h after PH were approximately 1.5-, 2.4- and 1.6-fold, respectively, over those of the control and the sham-operated livers. On the other hand, there was little activity of PP2A (either spontaneous or potential) in nuclei.

DISCUSSION

In the present work, we have demonstrated marked elevations of mRNA level and activity in nuclei of PP1 at 10~12 h after PH.

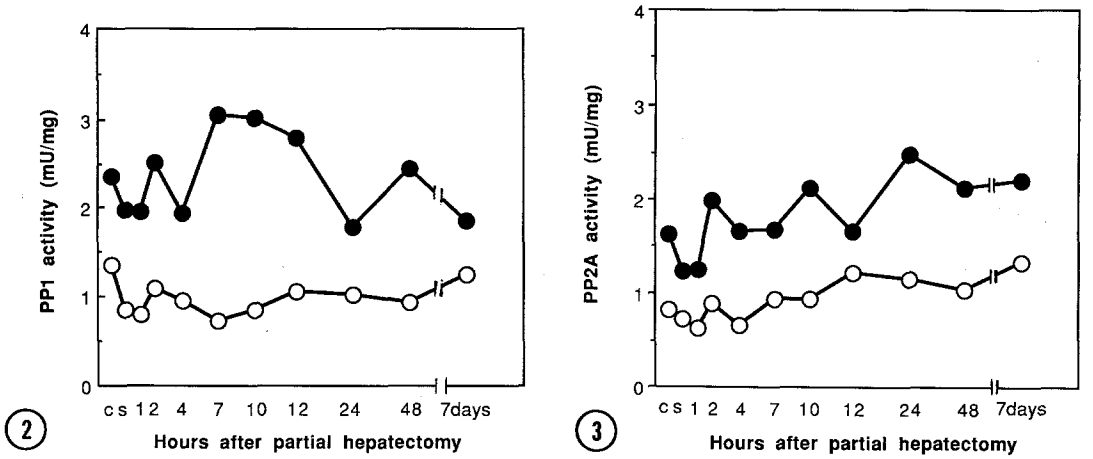

Fig. 2. PP1 activity in non-nuclear fraction. The spontaneous and potential activities of PP1 in non-nuclear fraction at various times after PH were measured. The spontaneous activity (open circle) represents the activity before treatment with Co^{2+}-trypsin and the potential activity (closed circle) represents the activity after the treatment, as described in Materials and Methods. Values of control (C), sham operation (S), 2 h, 24 h and 7 days represent averages of two rats.

Fig. 3. PP2A activity in non-nuclear fraction. The spontaneous and potential activities of PP2A in non-nuclear fraction at various times after PH were measured. The spontaneous (open circle) and potential (closed circle) activities represent the activities before and after treatment with 2-mercaptoethanol, respectively, as described in Materials and Methods. Values of control (C), sham operation (S), 2 h, 24 h and 7 days represent averages of two rats.

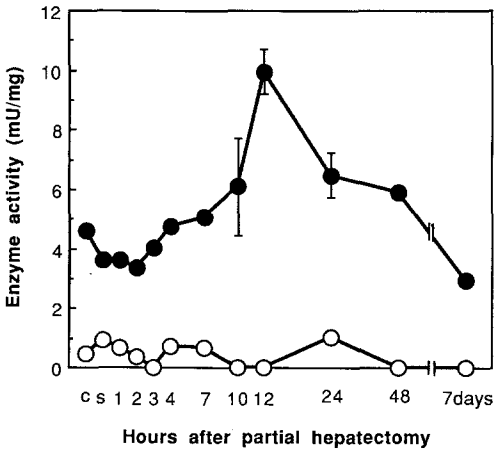

Fig. 4. PP1 and PP2A activities in nuclei. The spontaneous activities of PP1 (closed circle) and PP2A (open circle) in nuclei were determined at various times after PH. Values of 10, 12 and 24 h represent averages of three rats and error bars indicate one standard deviation. Values of control (C), sham operation (S), 2 h and 7 days represent averages of two rats.

The activity in nuclei was unaffected by treatment with Co^{2+}-trypsin, suggesting that PP1 might be present in the nuclei as a free catalytic subunit as previouly reported by Kuret et al.(17) and Jakes et al.(24). The physiological significance for the elevations of PP1 activity in nuclei and gene expression at 10~12 h after PH is still unknown.

The kinetics of the regeneration response in hepatocytes after two-thirds hepatectomy have been well described (25,26). DNA synthesis in these cells starts within 12~16 h and reaches a peak within 22~24 h. Accordingly, the time 10~12 h after PH represents pre-replicative phase which precedes DNA synthesis, that is , the G1 to S transition in the cell cycle. Our data, therefore, suggest the possibility that the activation of PP1 in nuclei is associated with the entry of hepatocytes into S phase from G1 phase during liver regeneration.

Brautigan et al.(27) reported that the level of inhibitor-2, a specific inhibitor of PP1, assayed by immunofluorescence staining, starts to increase in early S phase, peaking at mid-S phase in both cytoplasm and nucleus in rat fibrolasts and that inhibitor-2 might have a role in modulation, or suppression of $p34^{cdc2}$ kinase activity. Recently, there has been accumulating evidence that $p34^{cdc2}$ kinase is involved in the regulation of not only the G2 to M transition, but also the G1 to S transition (28,29) and is regulated by phosphorylation and dephosphorylation (30). Taking account of these facts, our present results indicate that PP1 in nuclei at the G1 to S transition might play some role in the regulation of $p34^{cdc2}$ kinase activity.

On the other hand, PP2A activity in either non-nuclear fraction or nuclei did not show any dynamic change, despite the characteristic changes in mRNA level which presented two peaks at 6 and 10~12 h after PH. These observations may be due, in part, to the usage of an inadequate substrate, phosphorylase a. In fact, there is evidence that nuclei contain an inactive form of PP2A, whose activity is expressed only in the presence of basic proteins such as histone H1 (21), and recently, Sola et al. (5) have demonstrated that PP2A dephosphorylates histone H1 which was phosphorylated by $p34^{cdc2}$.

In order to elucidate the functional significance of the induction of PP1 activity in nuclei and gene expression at 10~12hr after PH, we are attempting to perform further experiments.

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