

Original article:

## Expression of the protein phosphatases PP1 $\alpha$ and PP1 $\gamma$ 1 in rat ascites hepatoma cell lines and in rat hepatocyte cell line

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### ABSTRACT

Expressions of protein phosphatases 1 $\alpha$  (PP1 $\alpha$ ), and 1 $\gamma$ 1 (PP1 $\gamma$ 1) was determined in cultured rat ascites hepatoma cell lines (AH-13, AH-109A) and in the normal rat hepatocyte cell line RLN-B2 by Northern blot analysis. High levels of PP1 $\alpha$  mRNA expression were observed only in the hepatoma cells. In RLN-B2 cells only PP1 $\gamma$ 1 mRNA was increased compared to normal rat liver. In all cell types, mRNA levels of PP1 $\gamma$ 1 decreased as a function of harvest time. The present data show that PP1 $\alpha$  mRNA is expressed at high levels in hepatoma cells in vivo, but rapidly decreased under critical nutritional conditions.

**Keywords:** Protein phosphatase, PP1 $\alpha$ , PP1 $\gamma$ 1, ascites hepatoma, cell culture

### INTRODUCTION

It is now well established that protein phosphorylation/dephosphorylation is a major regulatory mechanism for various cellular functions. Increased expression of PP1 $\alpha$ , PP2A $\alpha$ , and PP2C $\alpha$  has been observed during chemical hepatocarcinogenesis (Kitamura et al., 1992), in primary hepatomas (Kitamura et al., 1992; Kitamura et al., 1994), in rat ascites hepatoma cell lines (Kitamura et al., 1994; Saadat et al., 1994; Saadat et al., 1995a), and in regenerating rat liver after partial hepatectomy (Kakinoki et al., 1992). The level of PP1 $\gamma$ 1 mRNA and also the amount of catalytic subunit of PP1 $\gamma$ 1 increased in some of the examined rat ascites hepatoma cell lines (Saadat et al., 1994; Saadat et al., 1995a, b; Takizawa et al., 1994).

In all of these studies the alterations in ascites hepatoma cell lines were compared with rat liver. Also it was reported that about 7% of the cells in the ascites are likely to be cells derived from the peritoneal lining or phagocytes, which have entered the peritoneal cavity from the blood (Mukai et al., 1987). Although the in vivo growth behavior of ascites hepatomas was well described (Patt and Straube, 1956; Hatayama and Sato, 1980; Tessitor et al., 1987), there are some points that would become clear by controlling several factors using in vitro culture conditions. Therefore, in an attempt to further understand role(s) of PP1 $\alpha$  and PP1 $\gamma$ 1 in hepatomas, the mRNA levels of PP1 $\alpha$  and PP1 $\gamma$ 1 in ascites hepatoma cells were examined under in vitro conditions.

## MATERIALS AND METHODS

### *Cell culture*

Two Yoshida ascites hepatoma cell lines, AH-13 and AH-109A, were harvested 4-5 days after transplantation, washed with serum free medium to remove the ascites fluid, and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) heat inactivated calf-serum, at a density of  $3 \times 10^5$  cells/ml, in 35-mm plastic culture dishes, at 37°C in 5% CO<sub>2</sub>. It should be noted that about 7% of the cells in the ascites are likely to be cells derived from the peritoneal lining or phagocytes, which have entered the peritoneal cavity from the blood (Mukai et al., 1987). In order to remove unwanted cells and also to receive physiological adaptation of hepatoma cells to the medium, these cells were cultured for 2 weeks. By this procedure, host cells were eliminated and virtually not detected in the cell suspension after 2 weeks of culture. During this period the medium was changed every 2 days.

Cell numbers were counted by trypan blue exclusion. In cell culture, the growth rate was slow down during the first 2-3 h, became rapid up to 48 h, and if the medium was not changed, the growth rate decreased again (up to 72 h), and then became negative (up to 96 h). The number of dead cells increased rapidly between 72 to 96 h. The growth curves of cultured hepatoma cells without medium change are composed of four phases: the latent, exponential, stationary (or quasi-stationary), and dead phases. More than 30 years ago, the same pattern was well described by Watanabe and Okada for cultured mouse L5178Y leukemic cell line (Watanabe and Okada, 1967a, b). It is suggested that the nutritional limitation is the main cause of cell death under these conditions. It was reported that AH-13 grows in rat ascites more rapidly than AH-109A (Yoshida, 1971). Under

the cultural conditions, there was no difference between them during the first 24 h, but after that, AH-13 grew more rapidly than AH-109A. The ability of island formation in ascites is different between hepatomas. So far AH-13 and AH-109A were classified as free and island types, respectively (Watanabe and Okada, 1967b). In AH-13, free cells were always kept at high level, while in AH-109A, free cells rapidly decreased and large islands had a maximum appeared.

Therefore, hepatoma cells appeared to be exposed to various microenvironments which malnutrition was more severe around large islands compared to free cells and even small islands. It is self-evident that under such circumstances malnutrition is another cause of the slow growth rate of AH-109A compared to AH-13 in both of in vivo and in vitro cultures.

The RLN-B2 cell line which originated from liver tissue of a male 7-day-old Donryu rat (Masuji et al., 1974) was cultured in DMEM supplemented with 20% (v/v) heat inactivated calf-serum, at a density of  $2.5 \times 10^5$  cells/ml, in 35-mm plastic culture dishes, at 37°C in 5% CO<sub>2</sub>.

### *RNA extraction and Northern Blotting*

Total RNA was extracted and purified from cells using a single step method of acid guanidinium thiocyanate-phenol-chloroform extraction (Chmczynski and Sacchi, 1982). The amount of RNA was determined from the absorbance at 260 nm using a Beckman DU-50 spectrophotometer. Total RNAs (20 µg/lane) were electrophoresed on a 1.2% agarose gel containing 6% formaldehyde. Northern blotting was carried out as described previously (Saadat et al., 1994). The following cDNA probes were used: the full-length of rat PP1α cDNA (Kitamura et al., 1991), the *Apa-Hind III* fragment (800 bp) of rat PP1γ1 cDNA (Sasaki et al., 1990), the *BstII-BglII*

fragment (410 bp) of rat PP2A $\alpha$  cDNA (Kitagawa et al., 1988), and the *PstI-Sall* fragment (550 bp) of rat PP2C $\alpha$  cDNA (Tamura et al., 1989). The autoradiographs of the blots were analyzed with a Personal Densitometer (Molecular Dynamics, Japan, Tokyo) to determine the relative amounts of mRNAs of PP1 $\alpha$ , PP1 $\gamma$ 1, PP2A $\alpha$ , and PP2C $\alpha$ .

## RESULTS

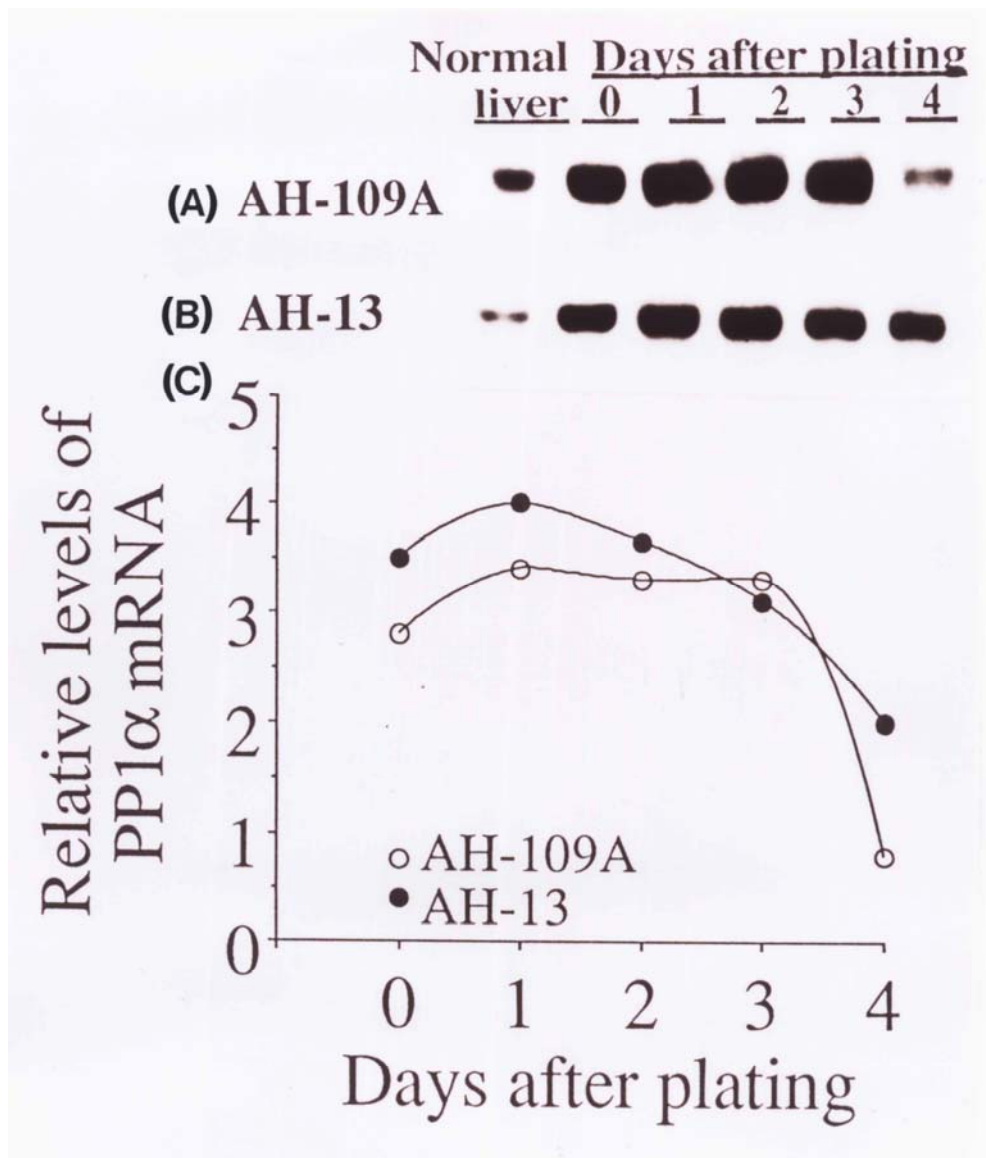
Figure 1 shows that the PP1 $\alpha$  mRNA was higher (about 3 to 4 folds) in both cultured hepatomas than those of the normal liver up to day 3. mRNA levels of PP1 $\alpha$  were always higher in AH-13 than AH-109A. Relative level of PP1 $\alpha$  in AH-109A dramatically decreased to 0.5 on day 4, whereas its level also decreased in AH-13 but it was still higher than the normal level. In contrast to PP1 $\alpha$  mRNA levels, PP1 $\gamma$ 1 was higher in AH-109A compared to AH-13 (Fig. 2). Although in rapidly growing stages, AH-13 compared to AH-109A contained lower amount of PP1 $\gamma$ 1 mRNA, but its level became higher than those of AH-109A on day 4. It should be noted that PP1 $\gamma$ 1 mRNA level of AH-109A decreased faster than that of AH-13 after day 1. The fluctuations were greater in AH-109A than AH-13 and also it was greater for PP1 $\gamma$ 1 than those for PP1 $\alpha$ .

In order to exclude a possibility of RNA degradation in living hepatomas at quasi-

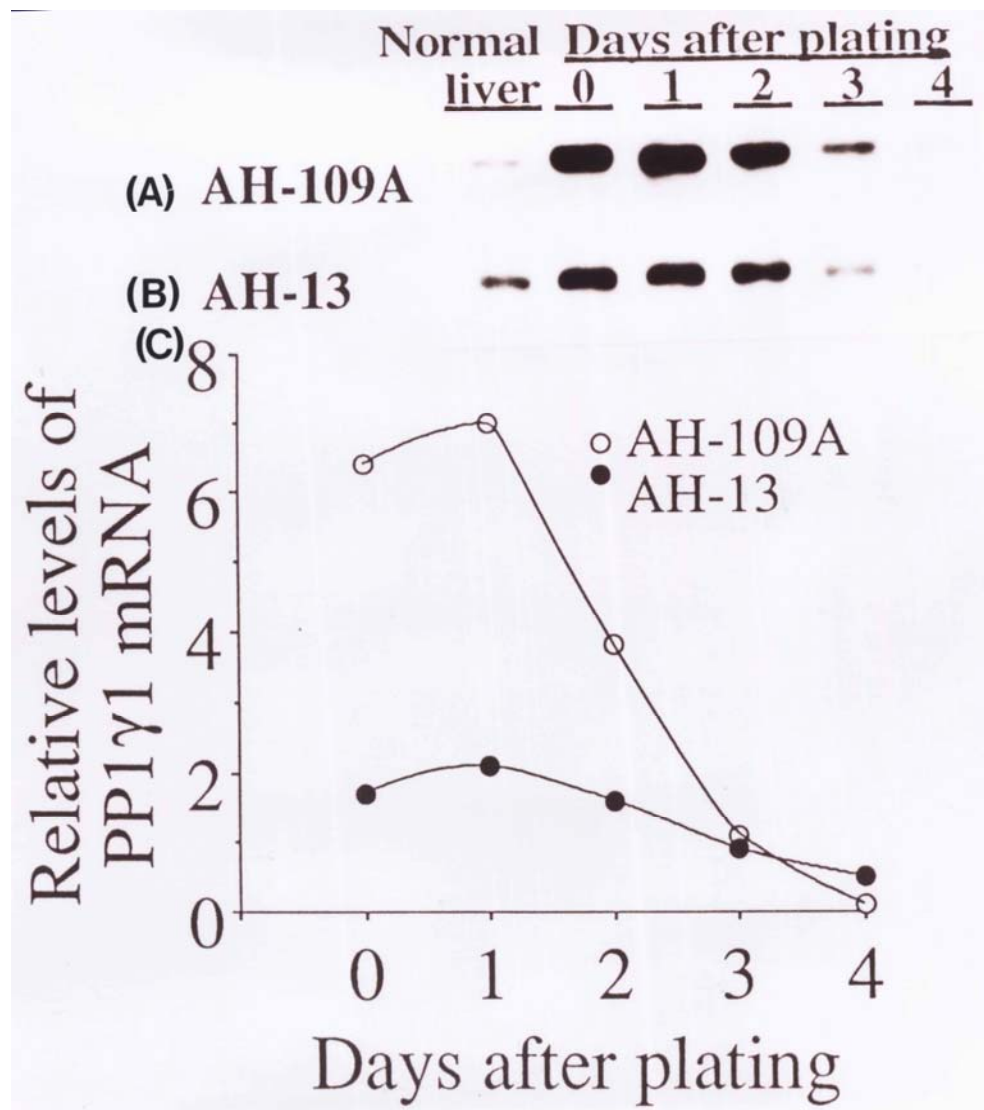
stationary/dead phases, analysis of PP2C $\alpha$  mRNA levels was performed. PP2C $\alpha$  mRNA levels were decreased in hepatomas compared to normal liver but not correlated with harvest time (data not shown).

Cell density increases as a function of harvest time. To determine the effect(s) of cell density on the alterations of protein phosphatase mRNA levels, AH-109A was cultured at 4 different cell densities ( $3 \times 10^5$ ,  $6 \times 10^5$ ,  $1 \times 10^6$ , and  $3 \times 10^6$  cells/ml) and harvested 12 h after plating. No relationship between mRNA levels of PP1 $\alpha$  and PP1 $\gamma$ 1 and cell densities was observed (data not shown).

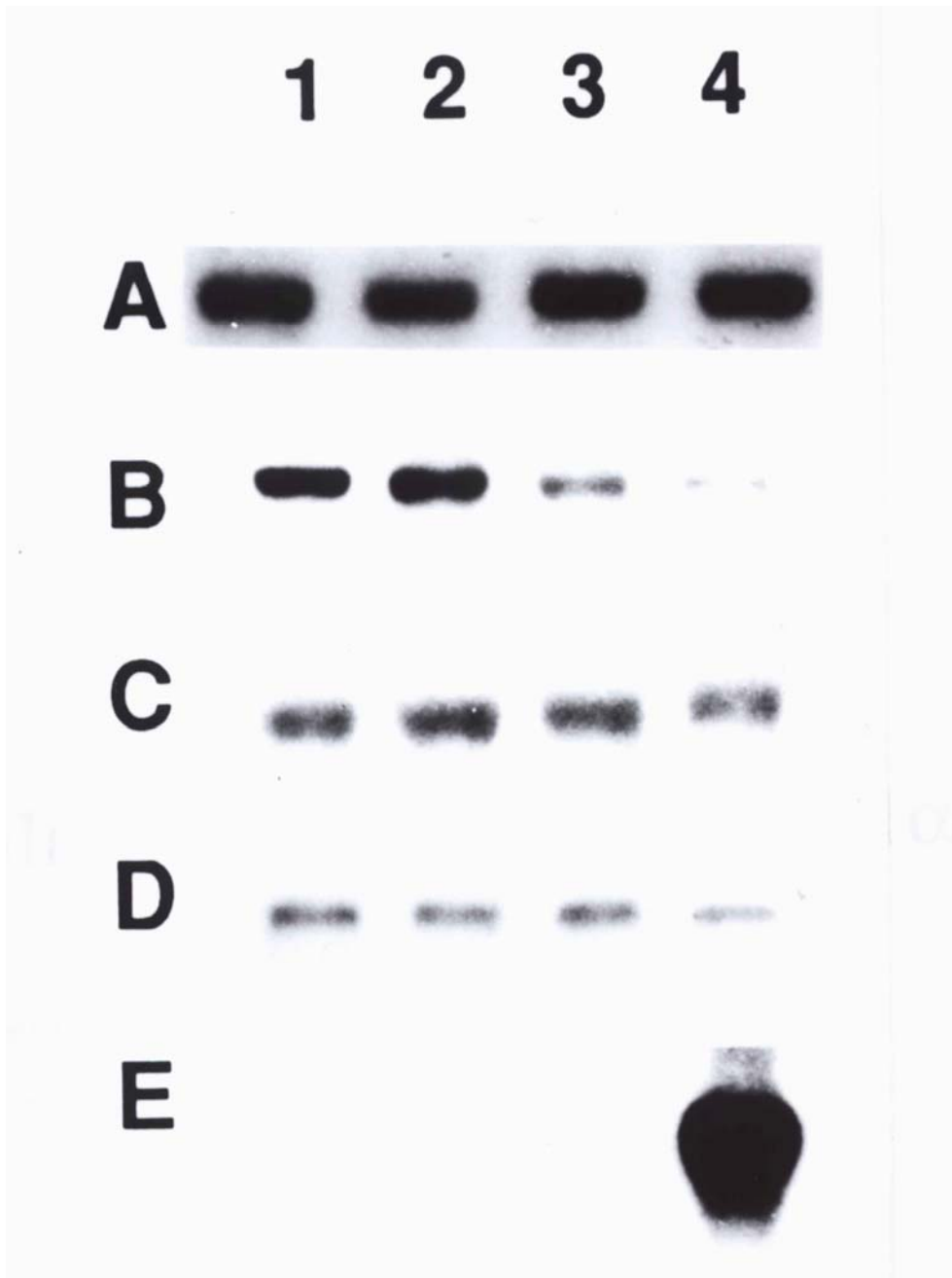
In culture of RLN-B2 cell line (normal rat hepatocyte cell line) the number of cells, after a lag phase, increased rapidly, then decreased, followed by a confluent stage without increasing the number of dead cells. Figure 3 shows that the mRNA levels of PP1 $\alpha$ , PP2A $\alpha$ , and PP2C $\alpha$  in RLN-B2 cells were not increased compared to normal rat liver and there was no relationship between cell growth rates and these mRNA levels. Only the PP1 $\gamma$ 1 mRNA level was increased remarkably in rapidly growing RLN-B2 cells compared to normal liver and decreased significantly as a function of harvest time. Albumin gene, a marker of differentiate functions, was not expressed in this cell line.



**Figure 1:** Northern blot analysis of PP1 $\alpha$  in cultured ascites hepatoma cells. Hepatoma cells were harvested on days 0-4 after plating. In order to analyze the PP1 $\alpha$  mRNA levels in AH-109A (A) and AH-13 (B) at different growth stages, the bands were scanned, values were normalized against those of normal liver, and the calculated relative amounts were plotted in panel C.



**Figure 2:** Northern blot analysis of PP1γ1 in cultured ascites hepatoma cells. Hepatoma cells were harvested on days 0-4 after plating. In order to analyze the PP1γ1 mRNA levels in AH-109A (A) and AH-13 (B) at different growth stages, the bands were scanned, values were normalized against those of normal liver, and the calculated relative amounts were plotted in panel C.



**Figure 3:** mRNA levels of protein phosphatase in the RLN-B2 hepatocyte cell line. mRNA levels of PP1 $\alpha$  (A), PP1 $\gamma$ 1 (B), PP2A $\alpha$  (C), PP2C $\alpha$  (D), and albumin (E) in RLN-B2 cell harvested 1-3 days after plating (Lanes 1-3, respectively) were compared to those of normal liver (lane 4). The RLN-B2 was growing rapidly on days 1-2 and became confluent on day 3.

## DISCUSSION

Our present findings show that the PP1 $\gamma$ 1 levels decrease as a function of harvest time, and there is high similarity of PP1 $\gamma$ 1 changes between cultured hepatomas and RLN-B2 cells (Figs. 2 and 3). These results suggest that the PP1 $\gamma$ 1 expression levels are correlated with the cell growth rates. Our finding is consistent with the result of Cheng et al. study (Cheng et al., 2000). They reported that using antisense oligonucleotides in order to inhibit of PP1 $\gamma$ 1 gene expression, results in a dose-dependent inhibition of cellular proliferation, with growth arrest occurring after approximately 36-48 h, when PP1 $\gamma$ 1 mRNA expression was inhibited by more than 85% (Cheng et al., 2000).

Most interesting is the finding that only PP1 $\gamma$ 1 mRNA was increased in RLN-B2 cells compared to normal liver (Fig. 3). The finding that PP1 $\alpha$  mRNA level had no change in rapidly growing RLN-B2 hepatocytes supports the results of EGF stimulated hepatocytes (Kakinoki et al., 1994). It should be noted that the PP1 $\alpha$  mRNA level of RLN-B2 is not parallel with its high level in regenerating livers after partial hepatectomy (Kakinoki et al., 1992). It is also noteworthy that there are differences between regenerating liver and in vitro growing hepatocyte system in gene expressions of PP2A $\alpha$ , PP2C $\alpha$ , albumin and GST-P (Kitamura et al., 1994; Kakinoki et al., 1992) in addition to PP1 $\alpha$ . It might be suggested that the molecular mechanism(s) of increasing PP1 $\alpha$  mRNA levels is different between regeneration and hepatoma. The gene encoding PP1 $\alpha$  catalytic subunit was mapped at band q43 of rat chromosome 1 (Saadat et al., 1995c), near to H-ras oncogene (Gollahan and Aldaz, 1992). Further investigation is needed to test the possible influence of H-ras oncogene in

vicinity of the PP1 $\alpha$  gene during liver regeneration.

The present observations in cultured ascites hepatoma cells provide new evidence for high level of PP1 $\alpha$  mRNA in neoplastic cell growth. Previously it has been reported that the PP1 $\alpha$  mRNA level increased remarkably compared to normal rat liver during hepatocarcinogenesis (Kitamura et al., 1992) and all of ascites hepatomas (Kitamura et al., 1994; Saadat et al., 1994; Saadat et al., 1995a), but decreased unexpectedly in some primary hepatomas (Kitamura et al., 1992). The present data show that PP1 $\alpha$  mRNA level may decrease in hepatoma cells under critical nutritional conditions. Primary hepatomas like other solid tumors require plenty of blood supply, but actually the rate of neovascularization often fails to keep pace with tumor growth, therefore it is common for subpopulations of cells within solid tumors to experience nutritional poverty. Taken together, we concluded that PP1 $\alpha$  mRNA level was decreased in the primary hepatomas which were under very bad nutritional conditions. PP1 $\alpha$  mRNA levels suddenly decreased in living cells at dead phase. The reason why only PP1 $\alpha$  and PP1 $\gamma$ 1 decreased, whereas PP2C $\alpha$  and GST-P did not decrease at dead phase, is unclear at present. The different stability at mRNA level is a simple and also possible explanation for these observations.

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