Jpn. J. Cancer Res. 85, 274–278, March 1994

Selective Increases in Isoform PP1α of Type-1 Protein Phosphatase in Ascites Hepatoma Cells

Norio Takizawa, Yusuke Mizuno, Mostafa Saadat and Kunimi Kikuchi[1]

Section of Biochemistry, Institute of Immunological Science, Hokkaido University, Kita-15, Nishi-7, Kita-ku, Sapporo, Hokkaido 060

The amounts of four isoforms of the catalytic subunit of type-1 protein phosphatases, PP1α, PP1γ1, PP1γ2, and PP1δ, have been determined in extracts of rat ascites hepatomas, AH131A, AH13, AH13NMOR, AH143A, and Yoshida sarcoma, and compared to those of rat liver by Western blot analysis. The amount of PP1α was increased over three times in all five hepatomas. The amount of PP1γ1 was increased over two times in AH13, AH13NMOR, and AH143A. The amount of PP1δ was selectively increased about 4 times in AH131A and AH143A. The PP1γ2 protein was undetectable in both liver and hepatomas. There was good parallelism between the general increase in only PP1α protein in the hepatomas and the previous data demonstrating the general increase in PP1α mRNA in numerous ascites hepatomas. These results suggest that PP1α plays important roles in the expression of malignant phenotype, that its amount is under strict control at the transcription level, and that PP1γ1 and PP1δ play different roles in the expression of some phenotype(s) of the ascites hepatomas.

Key words: Protein phosphatase — Type-1 protein phosphatase — PP1 isoforms — Serine/threonine protein phosphatase — Ascites hepatoma

Protein dephosphorylation is one of the major regulatory mechanisms of cell proliferation.[1–3] So far, four principal types of serine/threonine protein phosphatases, termed PP1, PP2A, PP2B, and PP2C, have been identified.[1–3] PP1 has been reported to have at least four isoforms, termed PP1α, PP1γ1, PP1γ2, and PP1δ.[4–7] Recently, we reported selective high expression of PP1α mRNA in rat poorly differentiated ascites hepatomas.[7–10] We also demonstrated elevations of PP1α mRNA and nuclear PP1 activity in rat liver at 10–12 h after partial hepatectomy.[11] To investigate the significance of the overexpression of PP1 genes in ascites hepatomas, we first established a method for quantitative determination of each catalytic subunit of the four PP1 isoforms by Western blot analysis, and then compared the amounts between various hepatomas and liver. Very recently, Shima *et al.* reported the amounts of PP1 isoforms in various rat tissues.[12] The relationship of the present results to the previous findings is discussed.

MATERIALS AND METHODS

Tissues and hepatoma cells Male Donryu rats weighing 150–200 g and fed *ad libitum* were used. Yoshida ascites hepatoma cells including AH13, AH13NMOR,[2] AH131A, AH143A, and Yoshida sarcoma, were obtained from the Cancer Cell Repository, Tohoku University. The cells were harvested 6–14 days after inoculation into rats, washed with physiological saline and stored at −80°C until use.

Antisera Peptides corresponding to the carboxyl-terminal region of PP1 isoforms with an additional cysteine residue, CRPITPPRNSAKAKK for PP1α, CPPRGMITKQAKK for PP1γ1, CQKASNYRNNT-VLYE for PP1γ2, and CSEKKAKYQYGGLNSG for PP1δ, after being purified by reversed-phase liquid chromatography, were coupled to keyhole limpet hemocyanin using sulfosuccinimidyl 4-(N-maleimidomethyl)-cyclohexane-1-carboxylate according to the manufacturer's instructions (Pierce, Rockford, IL). The peptide conjugates were dialyzed against phosphate-buffered saline to remove excess coupling agent. Portions (0.2 mg) were emulsified with complete Freund's adjuvant and injected into New Zealand white rabbits. Antisera with high titer were obtained after several immunizations at 4- to 5-week intervals with the conjugates emulsified with incomplete Freund's adjuvant.

Extraction AH cells or tissues were homogenized on ice with a Sonifer 450 (Branson Ultrasonics Corp., Danbury, CT) in three volumes of cold buffer containing 50 m*M* Tris-HCl, pH 7.0, 0.25 *M* sucrose, 2 m*M* EDTA, 2 m*M* EGTA, 4 μg/ml leupeptin, 0.1 m*M* benzamidine, 0.1 m*M* phenylmethylsulfonyl fluoride, 0.5 m*M* tosylphenylalanine chloromethyl ketone, 0.1% 2-mercaptoethanol, and 0.5% Nonidet P-40. The homogenates were centrifuged at 8,000*g* for 20 min at 4°C and the resulting supernatants were used as crude extracts.

[1] To whom correspondence should be addressed.

[2] Abbreviations used in this paper: NMOR, nitrogen mustard N-oxide-resistant; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Western blot analysis The extracts were electrophoresed on a 12% polyacrylamide gel by the method of Laemmli[13] and transferred electrophoretically to a nitrocellulose membrane. The blot was blocked with skim milk, incubated for 1 h with anti-PP1 antisera diluted 100- to 500-fold, washed with phosphate-buffered saline containing 0.1% Tween, and then incubated with 1 μg/ml horseradish peroxidase-labeled donkey anti-rabbit IgG antibody (Chemicon, Temecula, CA) for 1 h. Detection of the immunoreactive bands was performed with an ECL Western blotting detection kit (Amersham International plc, England). The intensity was evaluated with a scanning densitometer (Molecular Dynamics, Sunnyvale, CA). The molecular mass was estimated from the mobility of the band relative to biotinylated standard proteins; phosphorylase (99 kDa), bovine serum albumin (68 kDa), ovalbumin (46 kDa) and carbonic anhydrase (32 kDa).[14]

RESULTS

Western blot analysis of PP1α, PP1γ1, PP1γ2, and PP1δ Western blot analyses were carried out for extracts from various rat tissues including testis and liver by using antisera raised against the carboxyl-terminal peptides of PP1α, PP1γ1, PP1γ2, and PP1δ. Because the tissue distributions of the four isoforms of PP1 were similar to those reported by Shima *et al.*,[12, 15] only two representative patterns of testis and liver extracts are shown in Fig. 1. In accordance with their reports, only testis among the tissues examined showed immunoreactive bands for all of the four PP1 isoforms, at 38.5, 37.0, 39.5, and 40.0 kDa for PP1γ1, PP1δ, PP1α, and PP1γ2, respectively, whereas the other tissue extracts showed no immunoreactive band for PP1γ2. Each of the antisera detected only one main band of slightly different mobility in the range of 36–40 kDa, the region to which the PP1 isoforms are expected to migrate.[12, 16] Therefore, these bands appeared to represent the respective PP1 isoforms.

To confirm the specificity of each antiserum, immunoreactions were carried out in the presence of the peptides used for immunization. As shown in Fig. 2, each immunoreaction was selectively inhibited only by the peptide used for the immunization, and no cross-reaction was seen among the four isoforms. These results indicate that the antisera used are specific enough to determine each isoform of PP1 under the experimental conditions employed.

PP1α, PP1γ1, PP1γ2, and PP1δ in ascites hepatomas Amounts of the four isoforms of PP1 catalytic subunit in ascites hepatomas were compared to those of control liver by Western blot analysis (Fig. 3). The amount of

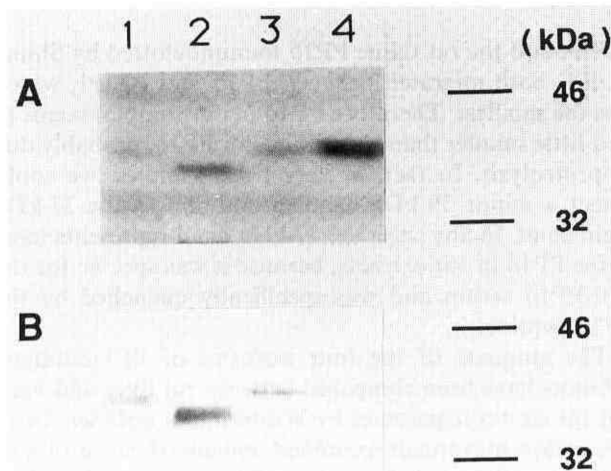

Fig. 1. Western blot analysis of PP1α, PP1γ1, PP1γ2, and PP1δ. Extracts (2 μl/4 mm-lane) of rat testis (A) and liver (B) were subjected to SDS-PAGE followed by transfer to a nitrocellulose membrane. The blots were incubated with antisera against PP1γ1 (lane 1), PP1δ (lane 2), PP1α (lane 3), and PP1γ2 (lane 4). Immunoreactive bands were visualized as described under "Materials and Methods." The positions of the molecular-mass markers ovalbumin (46 kDa) and carbonic anhydrase (32 kDa) are indicated.

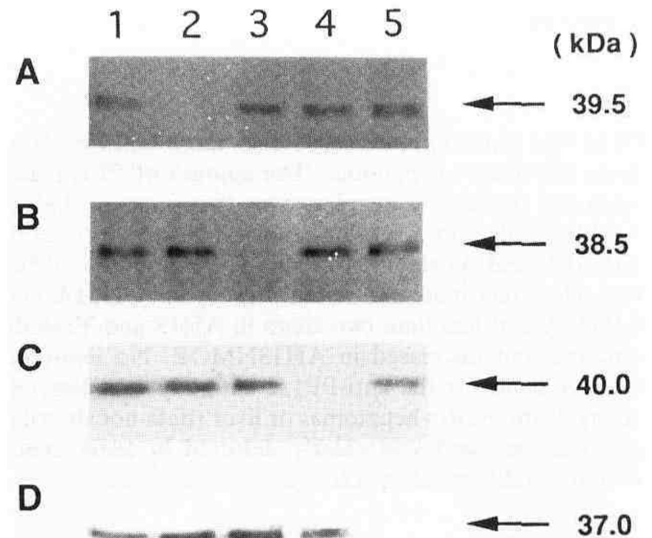

Fig. 2. Competition with peptides. Extracts (2 μl/4 mm-lane) of rat brain (A and B), testis (C), and liver (D) were analyzed by Western blotting. These tissues were used because they provided clear bands for each isoform. The blots were incubated with antisera against PP1α (A), PP1γ1 (B), PP1γ2 (C), and PP1δ (D) in the presence of 10 μM peptides. Lanes: 1, no peptide; 2, peptide of PP1α; 3, peptide of PP1γ1; 4, peptide of PP1γ2; 5, peptide of PP1δ.

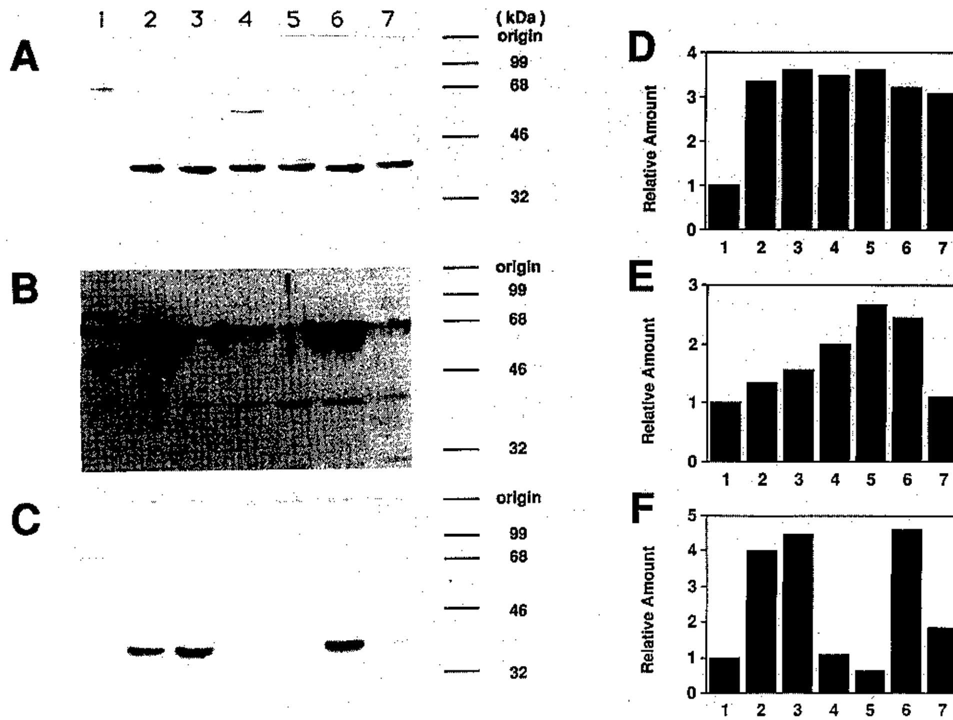

Fig. 3. PP1α, PP1γ1, and PP1δ in ascites hepatomas. Extracts containing 28 μg to protein (estimated by Lowry's method[23]) were loaded into the wells and subjected to SDS-PAGE. Lanes: 1, control liver; 2 and 3, AH131A; 4, AH13; 5, AH13NMOR; 6, AH143A; 7, Yoshida sarcoma. The blots were incubated with antisera against PP1α (A), PP1γ1 (B) and PP1δ (C). Densities of the immunoreactive bands relative to that of liver are plotted on D, E, and F for PP1α, PP1γ1, and PP1δ, respectively.

PP1α was uniformly increased over three times in all of these five ascites hepatomas. The amount of PP1γ1 was increased to varying degrees: over two times in AH13, AH13NMOR and AH143A, and less than two times in AH131A and Yoshida sarcoma. The amount of PP1δ was selectively increased about 4 times in AH131A and AH143A and less than two times in AH13 and Yoshida sarcoma, but decreased in AH13NMOR. No immunoreactive band for the anti-PP1γ2 antibody was detected in any of the ascites hepatomas or liver (data not shown), whereas the band was clearly detected in testis under similar conditions (Fig. 1A).

DISCUSSION

First we established a method to determine quantitatively the four isoforms of PP1 catalytic subunits, α, γ1, γ2, and δ, in rat tissue extracts using Western blot analysis. In the Western blotting patterns, we detected the four isoforms at slightly different migration distances. PP1δ appeared to be the smallest among the four isoforms on the basis of our data. However, it has been reported that the PP1δ expressed in *E. coli*[16] from rat cDNA and the rat tissue PP1δ immunoblotted by Shima *et al.*[12] both migrated between PP1α and PP1γ1, which was the smallest. Therefore PP1δ in our samples seems to be a little smaller than the full-length PP1δ, probably due to proteolysis. In fact, in very fresh samples, we could detect a minor 39-kDa band in addition to the 37-kDa main band. In any case, the 37-kDa band represents most of the PP1δ in our extracts, because it was specific for the anti-PP1δ serum and was specifically quenched by the PP1δ peptide.

The amounts of the four isoforms of PP1 catalytic subunits have been compared between rat liver and various rat ascites hepatomas by Western blot analysis. In all five ascites hepatomas examined, enhanced expression of PP1α was detected, being over three times higher than that of rat liver. In contrast, the PP1γ1 and PP1δ proteins were increased in only two or three among the five hepatomas. This difference in expression demonstrated that PP1α is under a control mechanism different from those for PP1γ1 and PP1δ, suggesting a difference in their physiological roles. In accordance with previous reports,[15] no immunoreactive band of PP1γ2 was detected in either the liver or the ascites hepatomas.

We previously reported that the mRNA levels of PP1α, PP2A and PP2C are all increased during hepatocarcinogenesis, using the Solt-Farber model, but the levels are decreased to control levels in primary hepatomas.[7-9] We also found that the mRNA level of PP1α was selectively elevated in all ascites hepatomas examined, while those of PP1γ1, PP2Aα, and PP2Cα were slightly increased or decreased depending on the hepatoma.[9,10] These results strongly suggested important roles of PP1α in the expression of malignant phenotype in ascites hepatomas. The positive correlation between mRNA and protein content of PP1α demonstrates that PP1α in ascites hepatomas is under strict control of gene expression. Overexpressions of PP1 genes in ascites hepatomas seem to result in large pools of PP1 enzyme molecules. We previously reported an increase in PP1 activity in ascites hepatomas, although differential determination of activities of the isoforms is not yet possible.[17] In our previous experiments, however, high mRNA levels of protein phosphatases did not necessarily result in high enzymatic activities. For example, 6 h after partial hepatectomy, the mRNA levels of PP2A was increased more than 30 times, but the activity was almost unchanged.[11] Recently, we also reported that PP1 activity is transiently and selectively decreased upon the stimulation of CTLL-2 cells by interleukin-2, while the amount of PP1 remains constant.[18] Therefore, it should be emphasized that the PP1 protein levels in hepatoma cells are strictly regulated at the transcription level, because such a mechanism appears to be distinct from those underlying the observations described above. This mechanism seems to be characteristic of a rapidly proliferating state of cells.

The physiological functions of the four PP1 isoforms are of great interest but remain unknown at present. However, very recently, Zhang *et al.* reported the expression and characterization of PP1α, PP1γ1, PP1γ2, and PP1δ, and demonstrated that the four isoforms have similar enzymatic properties in terms of substrate specificity and sensitivity to okadaic acid and inhibitor-2.[16] Experiments with yeast mutations of protein phosphatase genes showed that the protein phosphatases are functionally replaceable between different isoforms within the same types, but not between different types.[19] Therefore, our present results may indicate an enhancement in the total function of PP1, including PP1α, PP1γ1, PP1γ2, and PP1δ, in ascites hepatomas.

We recently reported that in regenerating liver, PP1 activity in nuclei is transiently increased at 10–12 h after partial hepatectomy,[11] and we also reported that the mRNA level and activity of PP1 in NIH3T3 fibroblasts oscillate during the cell cycle.[20] These results altogether strongly suggest an important role of PP1 in regulation of the cell cycle. There is increasing evidence demonstrating that an increase in activity of protein kinase consisting of cyclin and cdk2 induces cell cycle progression from G1 to S through derepression by phosphorylating suppressor proteins such as Rb.[21,22] These reports seem to be contradictory to our present results. However, PP1 is a multifunctional enzyme and shows a broad subcellular distribution. Therefore our present data appear to suggest an involvement of some specific PP1 function in the expression of malignant phenotype. Further experiments are in progress.

ACKNOWLEDGMENTS

The authors thank the Cancer Cell Repository, The Research Institute for Tuberculosis and Cancer, Tohoku University, Sendai, for supplying Yoshida ascites hepatoma cells, and Dr. Kazumasa Ogasawara for synthesizing the peptides. Thanks are also due to Mrs. Eiko Yoshida for her skillful assistance. This work was supported in part by a Grant-in-Aid for Cancer Research, a Grant-in-Aid for General Scientific Research (B) and a Special Grant-in-Aid for Promotion of Education and Science, to Hokkaido University, all provided by the Ministry of Education, Science and Culture of Japan.

(Received September 30, 1993/Accepted December 17, 1993)

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