Jpn. J. Cancer Res. 81, 161–168, February 1990

Particulate-associated Protein Phosphatases of Rat Hepatomas as Compared with the Enzymes of Rat Liver

Ryuzaburo Shineha,[1,2] Kunimi Kikuchi,[3] Shinri Tamura,[4] Akira Hiraga,[1] Yoichi Suzuki[5] and Shigeru Tsuiki[1,6]

[1]*Biochemistry Laboratory, Research Institute for Tuberculosis and Cancer, Tohoku University, Seiryo-machi, Aoba-ku, Sendai 980, Departments of* [2]*Surgery and* [5]*Pediatrics, Tohoku University School of Medicine, Seiryo-machi, Aoba-ku, Sendai 980,* [3]*Section of Biochemistry, Institute of Immunological Science, Hokkaido University, Kita-15-jo Nishi-7-chome, Kita-ku, Sapporo 060 and* [4]*Department of Biochemistry, Hirosaki University School of Medicine, Zaifu-cho, Hirosaki 036*

In the course of investigating the neoplastic alterations of protein phosphatases, the particulate fractions of rat liver and AH-13, a strain of rat ascites hepatoma, were chromatographed on DEAE-cellulose and assayed for protein phosphatase using glycogen synthase D and phosphorylase *a* as substrates. The synthase phosphatase activity of rapidly growing AH-13 was due almost entirely to a divalent cation-inhibited protein phosphatase, tentatively designated phosphatase N, the level of which was elevated remarkably in the hepatoma as compared with liver. Other hepatomas including primary hepatoma induced with 3′-methyl-4-dimethylaminoazobenzene also exhibited high levels of this phosphatase. Phosphatase N exhibited M_r=49,000 (gel filtration) and has been partially purified with little alteration in properties. Partially purified phosphatase N was inhibited by divalent cations, rabbit skeletal muscle polypeptide inhibitor-2 and heparin, and released the catalytic subunit of type-1 protein phosphatase upon tryptic digestion. It is therefore apparent that phosphatase N is a type-1 protein phosphatase. There is some evidence to suggest that the high levels of phosphatase N in neoplastic cells are due primarily to enhanced synthesis of its non-catalytic (regulatory) subunit.

Key words: Protein phosphatase — Synthase phosphatase — Phosphorylase phosphatase — Rat liver — Rat hepatoma

We have previously reported that hepatocarcinogenesis profoundly affects the protein phosphatase activity of rat liver cytosol.[1)] Although the phosphatase activity is also distributed heavily in the particulate fraction,[2)] our study was not extended to that fraction at that time, since little was then known about rat liver particulate protein phosphatase.

The protein phosphatase of rat liver particulate fraction was studied recently by the groups of Cohen[3–5)] and of Stalmans[6–8)]; both groups agree that the enzyme has a molecular form in which the catalytic subunit of type-1 protein phosphatase[9,10)] is complexed to another protein component(s) that may determine the intracellular location and/or substrate specificity of the enzyme. Purification of such a multi-subunit type-1 protein phosphatase from liver particulate fractions, however, has been hampered by its tendency to dissociate and its susceptibility to proteolytic degradation. It appears that rat liver particulate fraction contains more than a single species of type-1 protein phosphatase.[2,5,7)]

Chromatographic studies carried out in this laboratory several years ago, on the other hand, have revealed that a divalent cation-inhibited protein phosphatase named P-1, together with P-2, represents the major phosphorylase phosphatase activity of rat liver particulate fraction.[2)] Under the same assay conditions, a peak of (glycogen) synthase phosphatase activity coeluted with phosphatase P-1. This activity, however, was so small that we could hardly determine whether the peak represents another type of particulate protein phosphatase or is simply the synthase phosphatase activity of phosphatase P-1.[2)] We have more recently found that a peak of synthase phosphatase activity apparently identical to the above synthase phosphatase peak in elution position and inhibition by divalent cations is increased to a marked degree in the particulate fraction of rat hepatomas without a concomitant increase in phosphatase P-1. The present paper deals with the results of the experiments designed to extend this finding.

MATERIALS AND METHODS

Hepatomas and tissues Yoshida ascites hepatoma AH-13 was inoculated intraperitoneally into male adult Donryu rats. The tumor cells were then harvested 4 days later, unless otherwise specified. Two other transplantable

[6] To whom reprint requests should be addressed.

Abbreviations used: MeDAB, 3′-methyl-4-dimethylaminoazobenzene; TLCK, *N*-tosyl L-lysyl chloromethylketone; TPCK, *N*-tosyl L-phenylalanyl chloromethylketone; PMSF, phenylmethanesulfonyl fluoride.

hepatomas, AH-130 and AH-109A, and one primary hepatoma were also used. AH-130 was prepared in the same way as AH-13, but AH-109A was inoculated subcutaneously and harvested 12 days later. The primary hepatoma was induced in male adult Wistar rats by feeding them with MeDAB.[11] Male adult Donryu rats, starved for 2 days and then refed for 1 day, served as the source of control liver.

Preparation of the particulate fraction All preparative operations described below were carried out at 0–4°C. AH-13 cells were rinsed, suspended in 4 vol of buffer A (0.4 *M* sucrose/50 m*M* glycylglycine, pH 7.4/5 m*M* EDTA) and homogenized by ultrasonication at 10 kHz for 1 min. Livers were rinsed, suspended in buffer A as above and homogenized with a glass/Teflon homogenizer for 2 min. The liver homogenates were then ultrasonicated at 10 kHz for 1 min so that any specific effect of ultrasonication on the tumor cells other than homogenization could be canceled out. In some experiments, buffer A contained a cocktail of protease inhibitors (0.1 m*M* TLCK/0.1 m*M* TPCK/0.1 m*M* PMSF/0.5 m*M* benzamidine/2 m*M* EGTA), but this scarcely affected the experimental results. The homogenates were centrifuged at 5,000*g* for 15 min, and the supernatant was further centrifuged at 105,000*g* for 1 h. The high-speed pellet was suspended in a minimum volume of buffer B (10 m*M* Tris-HCl, pH 7.4/5 m*M* mercaptoethanol/2% glycerol), homogenized with a glass/Teflon homogenizer for 2 min and used as the particulate fraction.

Preparation of KCl-extracts AH-13 and livers were homogenized as described above except that buffer C (0.5 *M* KCl/10 m*M* Tris-HCl, pH 7.4/5 m*M* mercaptoethanol/5 m*M* $MnCl_2$/20% glycerol) replaced buffer A. After successive centrifugation at 5,000*g* (15 min) and 105,000*g* (1 h), the final supernatant was mixed with 2 vol of saturated ammonium sulfate solution, and the precipitate formed was collected by centrifugation. Dissolution of the precipitate in a minimum volume of buffer B followed by gel filtration on Sephadex G-25 gave a solution hereafter referred to as "KCl-extract." Essentially the same procedure was used to prepare KCl-extracts from AH-130, AH-109A, MeDAB-primary hepatoma and regenerating liver.

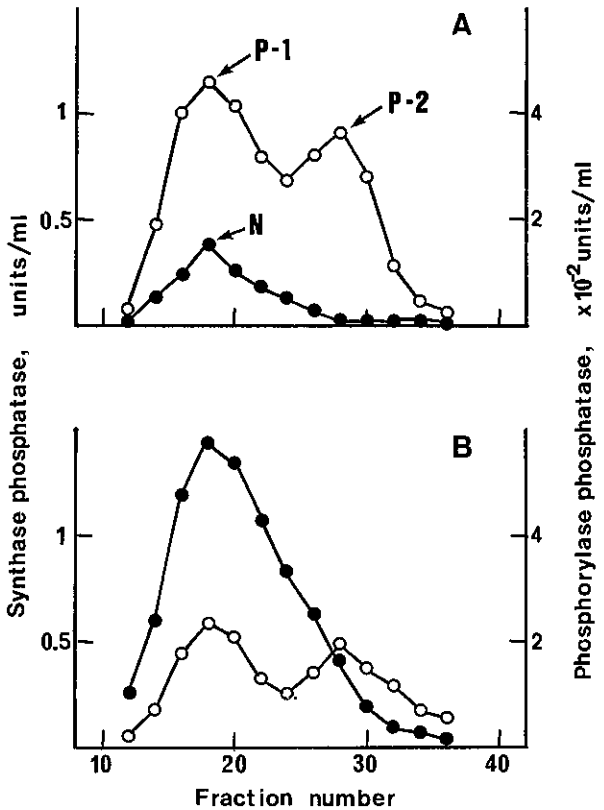
Partial purification of phosphatase N The KCl-extract prepared from 27 ml of packed AH-13 cells was applied to a DEAE-cellulose column (2.5×10 cm) previously equilibrated with buffer B. The column was washed with 200 ml of buffer B and developed with a linear 0–0.5 *M* NaCl gradient in 400 ml of buffer B. After dialysis against buffer B using a dialyzing apparatus described previously,[12] the eluate was collected in 10 ml fractions and assayed for synthase phosphatase activity under the conditions described below. The active fractions were pooled and applied to a second column of DEAE-cellulose (2.5×10 cm) previously equilibrated with buffer B/0.05 *M* NaCl. The column was washed with the equilibrating buffer and developed with a linear 0.05–0.4 *M* NaCl gradient in 400 ml of buffer B. After dialysis against buffer B, 10 ml fractions were collected and assayed as above. The active fractions were pooled and applied to an aminohexyl-Sepharose-4B column (1.5×5 cm) previously equilibrated with buffer B/0.15 *M* NaCl. The column was washed with the equilibrating buffer, and phosphatase N was eluted with a linear 0.15–0.5 *M* NaCl gradient in 200 ml of buffer B. The eluate was dialyzed against buffer B, collected in 5 ml fractions and assayed. In one experiment, all the purification buffers including buffer C contained protease inhibitors (see above). Little change, however, was noticed in the elution profile or yield of phosphatase N.

Substrates and assays Glycogen synthase D was prepared from rat liver as described previously.[13] Crystalline rabbit skeletal muscle phosphorylase *a* was purchased from Boehringer (Mannheim). Synthase phosphatase and phosphorylase phosphatase activities were assayed by the formation of synthase I from synthase D and the conversion of phosphorylase *a* to phosphorylase *b*, respectively. The assay conditions were as described previously[12, 14] except that no divalent cations were included in the assay mixture unless otherwise specified. The units of synthase phosphatase and phosphorylase phosphatase activities were defined as described previously.[14] Dephosphorylation of ^{32}P-labeled phosphohistone was measured as described previously[15] except that divalent cations were absent. Caseinolytic activity was tested with ^{14}C-methylated casein[16] as the substrate under conditions comparable to those for phosphatase N assay. Protein was determined by the method of Bradford.[17]

Other materials Polypeptide inhibitor-2, a specific inhibitor of type-1 protein phosphatase,[18] was prepared from rabbit skeletal muscle by the method of Yang *et al.*[19] The step-1 preparation was used. DEAE-cellulose was purchased from Whatman (Kent), Sephadex G-200 and aminohexyl-Sepharose 4B from Pharmacia (Uppsala) and Novo heparin from Kodama (Kyoto).

RESULTS

Synthase phosphatase activity of AH-13 particulate fraction We have previously demonstrated that there are two types of synthase phosphatase activity in rat liver particulate fraction.[2] One of these requires Mn^{2+} and has been designated phosphatase M.[2] The other activity, then designated N, is minor (Fig. 1A); it coelutes from DEAE-cellulose with P-1, a major phosphorylase phosphatase activity in the particulate fraction (see Fig. 1A), and is inhibited by divalent cations as is P-1. It was



then considered that the activity N could be the synthase phosphatase activity of phosphatase P-1.

When the particulate fraction of AH-13 cells, harvested at 4 days after inoculation, was chromatographed on DEAE-cellulose, however, the synthase phosphatase activity N was found to be more than 3-fold higher as compared with the particulate fraction of rat liver (Fig. 1B). The phosphorylase phosphatase activity P-1, on the other hand, was rather decreased (Fig. 1B). These results indicate that phosphatase N is a protein phosphatase that is distinct from phosphatase P-1.

Synthase phosphatase activity of KCl-extracts The subsequent studies have revealed that the homogenization in buffer A releases a substantial portion of phosphatase N into the cytosolic fraction. Since phosphatase N still bound to the particulate fraction was readily released with 0.5 *M* KCl, the quantitation of the phosphatase was performed by extracting cells or tissues directly with

Fig. 1. Chromatography on DEAE-cellulose of the particulate-bound protein phosphatases of liver (A) and AH-13 (B). The particulate fraction prepared from 15 g of tissue was applied to a DEAE-cellulose column (2.5×10 cm) equilibrated with buffer B and developed with a linear 0–0.5 *M* NaCl gradient in 400 ml of buffer B. The eluate was dialyzed against buffer B (see text) and collected in 10 ml fractions. The fractions were then assayed for synthase phosphatase (●) and phosphorylase phosphatase activities (○).

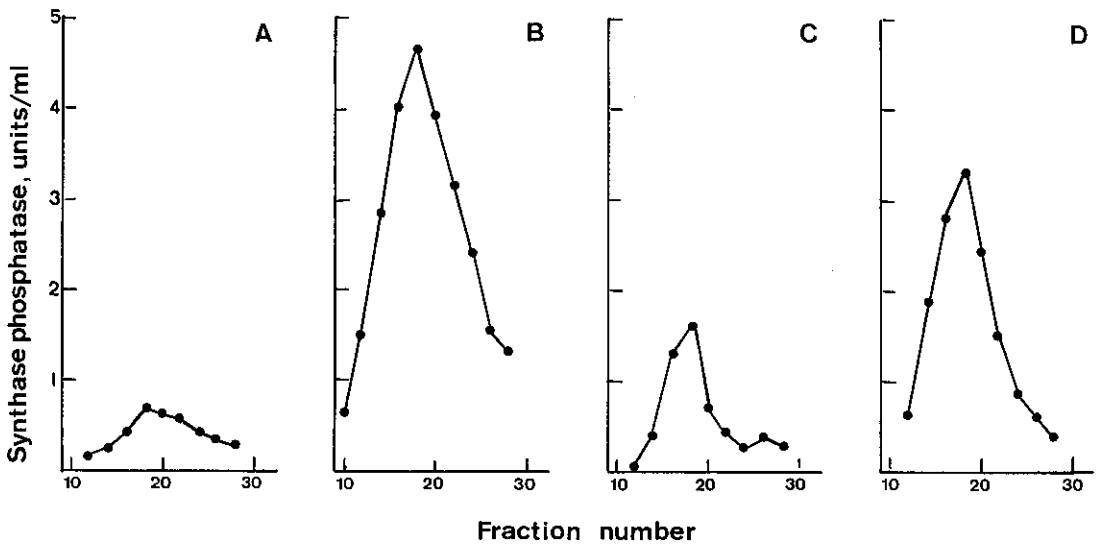


Fig. 2. Phosphatase N as revealed by chromatography of the KCl extracts on DEAE-cellulose. The KCl extracts from 10 g of liver (A), AH-13 cells at 4 (B) and 5 days after inoculation (C) and hepatoma induced by MeDAB (D) were chromatographed on DEAE-cellulose as described in the legend to Fig. 1 except that a column of 2.5×8 cm was developed with 200 ml of buffer B and that 5 ml fractions were collected. The fractions were assayed for synthase phosphatase activity.

0.5 *M* KCl so that loss of the enzyme into the cytosolic fraction could be avoided. It should be noted that although phosphatases M[2] and IA[13] are also supposed to exist in the extract, they are inactive since no divalent cation is present in the assay mixture. In the experiments shown in Fig. 2 (A and B), the KCl-extracts of liver and AH-13 were chromatographed on DEAE-cellulose. The level of phosphatase N was more than 5-fold higher in the hepatoma cells than in liver. It is also remarkable that phosphatase N decreased abruptly at 5 days after inoculation (Fig. 2C). This sudden decrease in phosphatase N is probably due to the known transition in ascites tumor cells from an initial rapid cell multiplication to a period of decelerated growth occurring 4–5 days after inoculation.[20,21] An increase in phosphatase N has been found not only in AH-13 cells but also in all the rat hepatomas so far examined, either ascitic (AH-130) or solid (AH-109A) and either transplantable (AHs) or primary. The results obtained for MeDAB-induced primary hepatoma are shown in Fig. 2D.

Molecular size of phosphatase N In order to determine the molecular size of AH-13 phosphatase N, the enzyme recovered from DEAE-cellulose (Fig. 2B) was immediately applied to a Sephadex G-200 column. As shown in Fig. 3, the bulk of synthase phosphatase activity eluted as a sharp peak with an elution volume corresponding to M_r=49,000 although there were a few additional peaks, whose molecular size was greater than that of the major activity. It is clear that phosphatase N is a protein with a molecular weight of about 49,000.

Partial purification of phosphatase N Phosphatase N was purified from the KCl-extracts of AH-13 by sequential chromatographies on (i) DEAE-cellulose, (ii) the second DEAE-cellulose and (iii) aminohexyl-Sepharose-4B. At every step, the phosphatase eluted as a single peak, although the shape became broader with the progress of purification (Fig. 4). After these steps, the purification was 11-fold with a recovery of 67%. The purification was also followed by measuring the phosphorylase phosphatase activity. After the first, second and third steps of

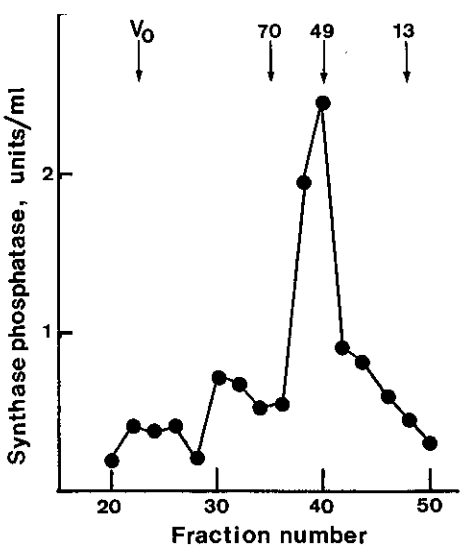


Fig. 3. Gel filtration on Sephadex G-200 of phosphatase N. Fractions 20 and 21 in Fig. 2B were pooled, concentrated to a few ml by using a concentration column (DEAE-cellulose), applied to a Sephadex G-200 column (2.5×40 cm) equilibrated with buffer C and eluted with the same buffer at a flow rate of 10 ml/h. After dialysis against buffer C minus 0.5 *M* KCl, fractions of 4 ml were collected and assayed for synthase phosphatase activity. The arrows indicate the elution positions of blue dextran (V_0), bovine serum albumin (70k), horseradish peroxidase (49k) and cytochrome *c* (13k).

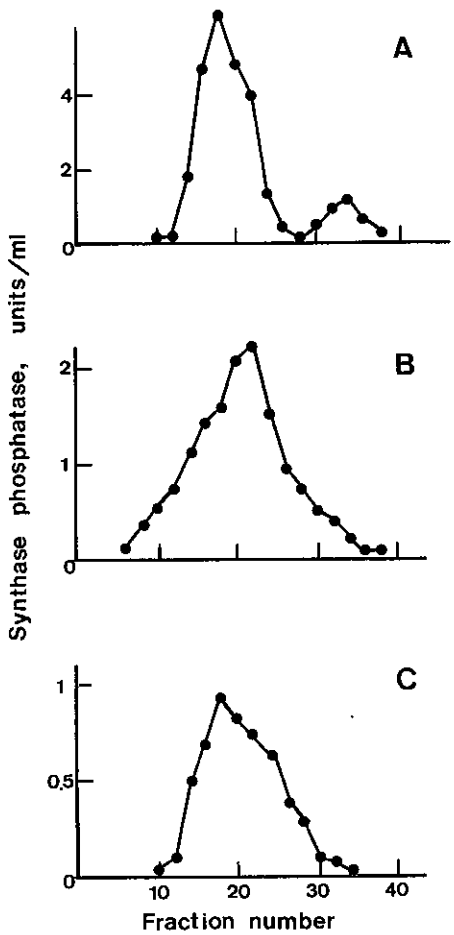


Fig. 4. Partial purification of phosphatase N from AH-13 cells by sequential chromatographies on DEAE-cellulose (A), the second DEAE-cellulose (B) and aminohexyl-Sepharose-4B (C). The procedures were described in detail in the text.

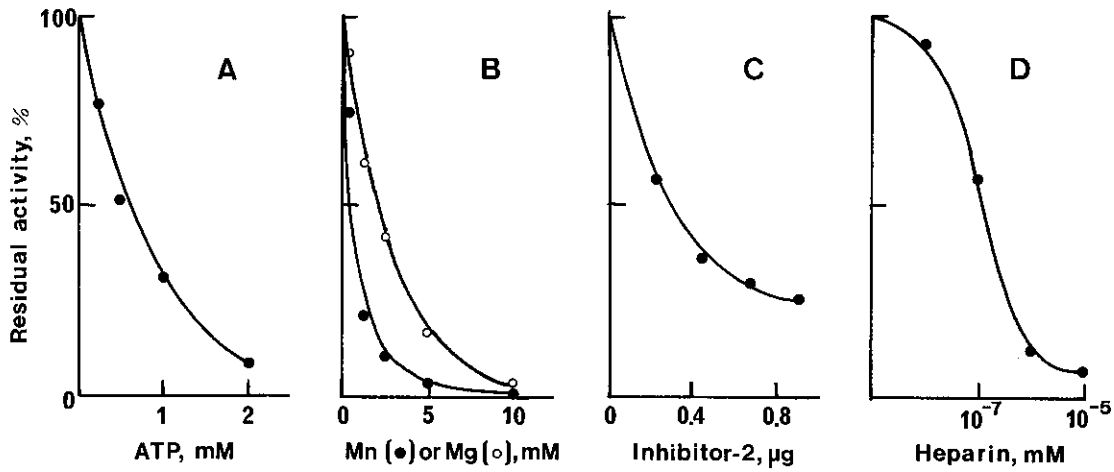


Fig. 5. Effects of increasing concentrations of ATP (A), $MnCl_2$ and $MgCl_2$ (B), and heparin (D) and increasing amounts of polypeptide inhibitor-2 (C) on the synthase phosphatase activity of partially purified phosphatase N. The assay mixture contained the indicated concentrations of these inhibitors.

chromatography, the values of the phosphorylase phosphatase (units)/synthase phosphatase (units) activity ratio were calculated to be 96, 92 and 133, respectively. The increase found after the third step may be due to a modification of the enzyme. It is of interest that proteolysis usually decreases the synthase phosphatase activity of the particulate-associated protein phosphatase but increases its phosphorylase phosphatase activity.[3,7] Inclusion of protease inhibitors in purification buffers as well as buffer C, however, had little influence on the purification data. The purified phosphatase N exhibited an unchanged molecular weight of 49,000 by gel filtration on Sephadex G-200. The enzyme was capable of releasing [^{32}P]phosphate from ^{32}P-labeled phosphohistone, but it exhibited no caseinolytic activity under assay conditions comparable to those used for synthase phosphatase activity. The following experiments were conducted using this purified preparation of phosphatase N.

Properties of phosphatase N Phosphatase N was inhibited by 20% by 0.2 m*M* ATP (Fig. 5A), which, however, inhibited cytosolic protein phosphatases IB and II (type-2A according to Ingebritsen and Cohen[22]) by more than 80%.[12,23] Phosphatase N was also distinguished from the cytosolic phosphatases by its sensitivity to inhibition by Mn^{2+} and Mg^{2+} (Fig. 5B), polypeptide inhibitor-2 (Fig. 5C) and heparin (Fig. 5D). The relatively low sensitivity to ATP inhibition[9] and effective inhibition by divalent cations,[24] inhibitor-2[18,22] and heparin[25] have been considered to be characteristic features of protein phosphatases classified as type-1 by Ingebristsen and Cohen.[22] The results reported in Fig. 5 therefore provide a strong indication that phosphatase N is a type-1 protein phosphatase.

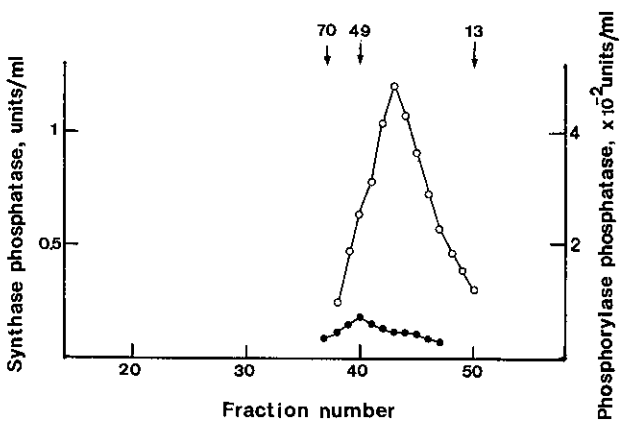


Fig. 6. Gel filtration on Sephadex G-200 of trypsinized phosphatase N. Fraction 20 in Fig. 4C was digested with 0.25 mg/ml of trypsin for 30 min at 30°C, and after removal of trypsin by using an aminohexyl-Sepharose-4B column, the digest was chromatographed on Sephadex G-200. The conditions were the same as described in the legend to Fig. 3. The eluted fractions were assayed for synthase phosphatase (●) and phosphorylase phosphatase activities (○).

Release of the catalytic subunit of type-1 protein phosphatases from phosphatase N In skeletal muscle, type-1 protein phosphatases are composed of a catalytic subunit characteristic of the type-1 phosphatases (M_r=37,000–38,000)[26,27] and another protein component(s) that may

serve to specify the intracellular location and/or substrate preference of the enzymes.[28-30] Digestion of the holoenzymes with proteases results in release of the catalytic subunit, which, however, exhibits a decreased molecular weight (32,000–33,000) due to a limited proteolysis.[24, 31] Furthermore, this type of dissociation in the liver enzymes caused by trypsin has been shown to bring about a marked increase in phosphorylase phosphatase activity and an almost complete loss of synthase phosphatase activity.[3, 7, 32] To further elucidate the nature of phosphatase N, therefore, the enzyme was digested with trypsin. When the digest was chromatographed on Sephadex G-200, phosphorylase phosphatase activity eluted as a sharp single peak with M_r=32,000 while synthase phosphatase activity could hardly be detected (Fig. 6). The enzyme eluted from the column was potently inhibited by inhibitor-2 (data not shown). These results establish that phosphatase N is a type-1 protein phosphatase.

DISCUSSION

We have previously reported that rat liver particulate fraction contains a major phosphorylase phosphatase activity termed P-1, which coelutes from DEAE-cellulose with a minor activity of synthase phosphatase[2] (see Fig. 1A). In the present work, we have demonstrated that this synthase phosphatase activity, now termed N, is increased to a marked degree in hepatoma cells while P-1 is rather decreased (Fig. 1B). The phosphorylase phosphatase/synthase phosphatase activity ratio of hepatic P-1 fractions was more than 1000 (Fig. 1A) while that of hepatoma fractions containing N was less than 150 (Fig. 1B). It therefore appears that peak N represents a protein phosphatase, phosphatase N, that is distinct from phosphatase P-1 responsible for the activity P-1. Upon hepatocarcinogenesis, the two enzymes appear to be affected in opposite directions.

This conclusion raises the question of how phosphatase N of rat hepatoma is related to phosphatase P-1 of rat liver. The previous work has shown that in phosphatase P-1, the type-1 catalytic subunit is complexed to another protein component(s).[2] Phosphatase N is also an oligomeric (most probably dimeric) enzyme containing the type-1 catalytic subunit, as judged from its higher molecular weight than the catalytic subunit and its conversion to the catalytic subunit only by tryptic digestion (Fig. 6). Furthermore, both enzymes are associated with the particulate fraction with a linkage cleavable with salt and are potently inhibited by divalent cations. It has repeatedly been pointed out that the non-catalytic (regulatory) subunit(s) of type-1 protein phosphatase is extremely sensitive to proteolysis and that the proteolysis results in an increase in phosphorylase phosphatase activity but a decrease in synthase phosphatase activity.[3, 7, 32] The hypothesis that phosphatase N is simply an intact or less impaired form of phosphatase P-1, however, is unlikely since the molecular weight of phosphatase P-1 is somewhat higher (50,000 by gel filtration[2]) than that of phosphatase N.

The most reasonable explanation appears to be that phosphatase N contains a regulatory subunit (subunit *n*) distinct from that of phosphatase P-1 (subunit *p-1*): it has been claimed that the distinctive properties of different type-1 protein phosphatases should be conferred by regulatory subunits.[5, 7, 33] If this assumption is correct, then the high levels of phosphatase N in AH-13 cells should be a consequence of enhanced synthesis of subunit *n*; the synthesis of the type-1 catalytic subunit may not be enhanced in the hepatoma cells since in these cells, the high levels of phosphatase N appears to be attained at the expense of phosphatase P-1 (see Fig. 1). Studies are now in progress to compare the level of expression of the type-1 catalytic subunit mRNA between rat liver and hepatomas.

The level of phosphatase N is high in 4-day AH-13 cells but falls abruptly at 5 days (Fig. 2). Since the growth rate of ascites tumor cells *in vivo* decreases abruptly 4–5 days after inoculation,[20] it is possible that the abrupt decrease in phosphatase N may be due to this transition in growth rate. Whether or not this is in fact true may be determined if we could elucidate how the enzyme behaves time-dependently after partial hepatectomy, but this has not been done yet. Likewise, since no investigation has been made of fetal liver, we are uncertain if phosphatase N can be categorized as a oncofetal protein. To elucidate the physiological and/or pathological significance of phosphatase N, the determination of its exact subcellular location would be highly important. Alemany *et al.*[3] have shown that in the particulate fraction of rat liver, protein phosphatase is bound to glycogen particles and microsomes. Although substantial evidence has yet to come, we are inclined to consider that phosphatase N is bound to microsomes rather than glycogen for the following reasons: (i) phosphatase N is released from the particulate fraction with 0.5 *M* KCl while Schelling *et al.*[5] have stated that when synthase phosphatase was assayed, the activity bound to microsomes but not that bound to glycogen is released with salt; (ii) in our unpublished work, phosphatase P-1, also releasable with salt, has been shown to be located mainly in the microsomes; and (iii) AH-130 and MeDAB-induced hepatoma have been reported to contain little glycogen.[34, 35] The extremely low glycogen content of these hepatomas also suggests an apparent unrelatedness of phosphatase N to tissue glycogen synthesis even though AH-13 is known to accumulate a large amount of glycogen.[34]

ACKNOWLEDGMENTS

The authors are indebted to Dr. Testuro Nishihira for his advice. Thanks are also due to Mr. Kimio Konno and Ms. Akiko Kawasaki for their assistance. This work was supported in part by Grants-in-Aid for Cancer Research from the Ministry of Education, Science and Culture and the Ministry of Health and Welfare of Japan, by a grant from the Princess Takamatsu Cancer Research Fund and also by a Special Grant-in-Aid for Promotion of Education and Science in Hokkaido University provided by the Ministry for Education, Science and Culture of Japan.

(Received October 27, 1989/Accepted December 26, 1989)

REFERENCES

1) Kikuchi, K., Shineha, R., Hiraga, A., Tamura, S., Kikuchi, H. and Tsuiki, S. Cytosolic protein phosphatases of rat ascites hepatoma AH-13 as compared with those of rat liver: isolation and characterization of a novel protein phosphatase. *Gann,* **75,** 388–394 (1984).
2) Tsuiki, S., Kikuchi, K., Tamura, S., Hiraga, A. and Shineha, R. Glycogen synthase phosphatase and phosphorylase phosphatase of rat liver. *Adv. Prot. Phosphatase,* **1,** 193–214 (1985).
3) Alemany, S., Pelech, S., Brierley, C. H. and Cohen, P. The protein phosphatases involved in cellular regulation. Evidence that dephosphorylation of glycogen phosphorylase and glycogen synthase in the glycogen and microsomal fractions of rat liver are catalyzed by the same enzyme: protein phosphatase-1. *Eur. J. Biochem.,* **156,** 101–110 (1986).
4) Alemany, S. and Cohen, P. Phosphorylase *a* is an allosteric inhibitor of the glycogen and microsomal forms of rat hepatic protein phosphatase-1. *FEBS Lett.,* **198,** 194–202 (1986).
5) Schelling, D., Leader, D. P., Zammit, V. A. and Cohen, P. Distinct type-1 protein phosphatases are associated with hepatic glycogen and microsomes. *Biochim. Biophys. Acta,* **927,** 221–231 (1988).
6) Mvumbi, L. and Stalmans, W. High-affinity binding of glycogen-synthase phosphatase to glycogen particles in the liver. *Biochem. J.,* **246,** 367–374 (1987).
7) Bollen, M., Vandenheede, J. R., Goris, J. and Stalmans, W. Characterization of glycogen-synthase phosphatase and phosphorylase phosphatase in subcellular liver fractions. *Biochim. Biophys. Acta,* **969,** 66–77 (1988).
8) Bollen, M. and Stalmans, W. The modulator protein dissociates the catalytic subunit of hepatic protein phosphatase G from glycogen. *Biochem. J.,* **250,** 659–663 (1988).
9) Ingebritsen, T. S., Foulkes, J. G. and Cohen, P. The broad specificity protein phosphatase from mammalian liver. *FEBS Lett.,* **119,** 9014 (1980).
10) DePaoli-Roach, A. A. Synergistic phosphorylation and activation of ATP-Mg-dependent phosphoprotein phosphatase by F_A/GSK-3 and casein kinase II. *J. Biol. Chem.,* **259,** 12144–12152 (1984).
11) Miyagi, T. and Tsuiki, S. Studies on UDP-N-acetylglucosamine: α-mannoside β-N-acetylglucosaminyltransferase of rat liver and hepatomas. *Biochim. Biophys. Acta,* **661,** 148–157 (1981).
12) Tamura, S., Kikuchi, H., Kikuchi, K., Hiraga, A. and Tsuiki, S. Purification and subunit structure of a high-molecular-weight phosphoprotein phosphatase (phosphatase II) from rat liver. *Eur. J. Biochem.,* **104,** 347–355 (1980).
13) Hiraga, A., Kikuchi, K., Tamura, S. and Tsuiki, S. Purification and characterization of Mg^{2+}-dependent glycogen synthase phosphatase (phosphoprotein phosphatase IA) from rat liver. *Eur. J. Biochem.,* **119,** 503–510 (1981).
14) Kikuchi, K., Tamura, S., Hiraga, A. and Tsuiki, S. Glycogen synthase phosphatase of rat liver. Its separation from phosphorylase phosphatase on DE-52 columns. *Biochem. Biophys. Res. Commun.,* **75,** 29–37 (1977).
15) Tamura, S., Kikuchi, K., Hiraga, A., Kikuchi, H., Hosokawa, M. and Tsuiki, S. Characterization of multiple forms of histone phosphatase in rat liver. *Biochim. Biophys. Acta,* **524,** 349–356 (1978).
16) Rice, R. H. and Means, G. S. Radioactive labeling of proteins *in vitro. J. Biol. Chem.,* **246,** 831–832 (1971).
17) Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.,* **72,** 248–254 (1976).
18) Huang, F. L. and Glinsmann, W. H. Separation and characterization of two phosphorylase phosphatase inhibitors from rabbit skeletal muscle. *Eur. J. Biochem.,* **70,** 419–426 (1976).
19) Yang, S-D., Vandenheede, J. R. and Merlevede, W. A simplified procedure for the purification of the protein phosphatase modulator (inhibitor-2) from rabbit skeletal muscle. *FEBS Lett.,* **132,** 293–295 (1981).
20) Patt, H. M. and Straube, R. L. Measurement and nature of ascites tumor growth. *Ann. N.Y. Acad. Sci.,* **63,** 728–737 (1956).
21) Tsuiki, S., Sukeno, T., Saeki, R. and Takeda, H. Variations in carbohydrate-metabolizing activities of ascites tumor cells during growth *in vivo. Sci. Rep. Inst. Tohoku Univ.-C,* **13,** 223–228 (1967).
22) Ingebritsen, T. A. and Cohen, P. Protein phosphatases: properties and role in cellular regulation. *Science,* **221,** 331–338 (1983).
23) Tamura, S. and Tsuiki, S. Purification and subunit structure of rat-liver phosphoprotein phosphatase, whose molecular weight is 260 000 by gel filtration (phosphatase IB). *Eur. J. Biochem.,* **111,** 217–224 (1980).

24) Silberman, S. R., Spech, M., Nemani, R., Ganapathi, M. K., Dombradi, V., Paris, H. and Lee, E. Y. C. Isolation and characterization of rabbit skeletal muscle protein phosphatases C-I and C-II. *J. Biol. Chem.*, **259**, 2913–2922 (1984).
25) Gergely, P., Erdodi, F. and Bot, G. Heparin inhibits the activity of protein phosphatase-1. *FEBS Lett.*, **132**, 293–295 (1984).
26) Ballow, L. M., Brautigan, D. L. and Fischer, E. H. Subunit structure and activation of inactive phosphorylase phosphatase. *Biochemistry*, **22**, 3393–3399 (1983).
27) Tung, H. Y. L., Resink, T. J., Hemmings, B., Shenolikar, S. and Cohen, P. The catalytic subunits of protein phosphatase-1 and protein phosphatase-2A are distinct gene products. *Eur. J. Biochem.*, **138**, 635–641 (1984).
28) Yang, S-D., Vandenheede, J. R., Goris, J. and Merlevede, W. ATP·Mg-dependent protein phosphatase from rabbit skeletal muscle. I. Purification of the enzyme and its regulation by the interaction with an activating protein factor. *J. Biol. Chem.*, **255**, 11759–11767 (1980).
29) Hiraga, A., Kemp, B. E. and Cohen, P. Further studies on the structure of the glycogen-bound form of protein phosphatase-1 from rabbit skeletal muscle. *Eur. J. Biochem.*, **163**, 253–258 (1987).
30) Chisholm, A. A. K. and Cohen, P. Identification of a third form of protein phosphatase 1 in rabbit skeletal muscle that is associated with myosin. *Biochim. Biophys. Acta*, **968**, 392–400 (1988).
31) Gratecos, D., Deiwiler, T. C., Hurd, S. and Fischer, E. H. Rabbit skeletal muscle phosphorylase phosphatase. 1. Purification and chemical properties. *Biochemistry*, **16**, 4812–4817 (1977).
32) Mvumbi, L. Dopere, F. and Stalmans, W. The inhibitory effect of phosphorylase *a* on the activation of glycogen synthase depends on the type of synthase phosphatase. *Biochem. J.*, **212**, 407–416 (1983).
33) Cohen, P. T. W., Schelling, D. L., Cruz e Silva, O. B. Da, Barker, H. M. and Cohen, P. The major type-1 protein phosphatase catalytic subunits are the same gene products in rabbit skeletal muscle and rabbit liver. *Biochim. Biophys. Acta*, **1008**, 125–128 (1989).
34) Saheki, R., Sato, K. and Tsuiki, S. Glycogen synthesis and glycogen synthetase in rat ascites hepatomas of low and high glycogen content. *Biochim. Biophys. Acta*, **230**, 571–582 (1971).
35) Shatton, J. B., Donnelly, A. J. and Weinhouse, S. Metabolism of Neoplastic Tissues. XVI. Glucokinase activity and glycogen levels during hepatocarcinogenesis by azo dyes. *Cancer Res.*, **22**, 1372–1380 (1962).