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Gene Expressions of Protein Tyrosine Phosphatases in Regenerating Rat Liver and Rat Ascites Hepatoma Cells

Takaya Kitamura, Koji Nakamura, Yusuke Mizuno and Kunimi Kikuchi[1]

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

mRNA levels for ten protein tyrosine phosphatases (PTPs), PTP-S, PTPH1, PTP-1, GLEPP1, LRP, PTP1D, PTPG1, PTPγ, PTPδ, and LAR, were determined during regeneration of rat liver, and mRNA levels for 5 PTPs, PTP-S, PTP-1, PTPγ, PTPδ, and LRP, were determined in three lines of rat ascites hepatoma cells. In regenerating rat liver, the expression patterns of PTP genes after partial hepatectomy could be classified into four groups. In group 1 (PTP-S and PTPH1), the mRNA levels increased rapidly, reached a maximum 7 h after partial hepatectomy, remained at a plateau for 1–2 days and then decreased gradually. In group 2 (PTP-1, GLEPP1, and LRP), the mRNA levels showed two peaks on days 1 and 5, and then decreased gradually. In group 3 (PTP1D and PTPG1), the mRNA levels increased rapidly, reached a maximum at 7 h, remained high for several days, and then did not decrease but rather increased after day 7. In group 4 (PTPγ, PTPδ, and LAR), the mRNA levels remained constant for the first 5 days and increased over the control levels after day 7. In rat ascites hepatomas, gene expression of non-receptor-like PTPs (PTP-S and PTP-1) showed various neoplastic alterations, whereas mRNAs of receptor-like PTPs (PTPγ, PTPδ, and LRP) were lost or drastically decreased.

Key words: Protein tyrosine phosphatase — Gene expression — Regenerating rat liver — Partial hepatectomy — Ascites hepatoma

Phosphorylation and dephosphorylation of target proteins at tyrosine residues are recognized as one of the key mechanisms in regulating many cellular functions such as cell proliferation and differentiation.[1] Although considerable information has been accumulated on PTKs[2], little is known about the physiological functions of PTPs.[2,3] PTPs can be structurally divided into two major classes. One is nonreceptor-like PTPs of low molecular weight, which possess a single catalytic domain flanked at either the amino or carboxyl terminus by divergent regulatory domains. These domains include a membrane-association domain (PTP-1[4]/PTP1B[5], P19-PTP[6]/PTPG1[7]/PTP-PEST[8]), a nuclear localization domain (PTP-S[9]/T-cell PTP[10]/MPTP[11]), an SH2 domain (PTP1C[12]/SH-PTP1[13]/HCP[14]/SHP[15], PTP2C[16]/SH-PTP2[17]/PTP1D[18]), and a cytoskeletal domain (PTPH1[19]). The other class consists of receptor-like PTPs, which generally possess two tandem repeated catalytic domains, a single transmembrane region, and a variety of extracellular domains characteristic of either a receptor or a cell adhesion molecule, except PTPβ[20] and GLEPP1,[21] which contain only a single catalytic domain. Ligands that bind the extracellular domains of these receptor-like PTPs have not yet been identified. The divergent extracellular domains of these PTPs include a small glycosylated segment (LRP[22]/PTPα,[20] PTPε[20]), tandem repeats of immunoglobulin-like and fibronectin type III domains similar to N-CAM (PTPδ,[20] PTPμ,[20] LAR[23]), and a long stretch of amino acid sequence with a carbonic anhydrase-like domain (PTPγ[20]).

Some of the PTPs may be tumor suppressors because of their potential ability to reverse PTK actions.[24] However, recent studies have revealed multifunctional actions of PTPs in addition to the suppressive role in proliferation.[25] The rapid restoration of liver mass subsequent to partial hepatectomy offers a good system for studies on growth-related events in physiologically controlled situations *in vivo*. Also, analysis of neoplastic alterations in PTP gene expressions will provide important information to elucidate the regulatory roles of PTPs in cell proliferation. Thus, we have examined here gene expressions of PTPs throughout the course of regeneration after partial hepatectomy, as well as in three rat ascites hepatomas, and compared them to the controls. mRNA levels in sham-operated livers were also determined. The results are discussed with reference to regeneration of liver.

MATERIALS AND METHODS

Animals and surgery Male Wistar rats (7–11 weeks old) were obtained from Japan SLC, Inc. The animals were

[1] To whom correspondence should be addressed.

[2] Abbreviations used in this paper: PTK, protein tyrosine kinase; PTP, protein tyrosine phosphatase; LAR, leukocyte common antigen related protein; LRP, leukocyte common antigen related phosphatase; RT-PCR, reverse transcriptase-polymerase chain reaction; N-CAM, neural cell adhesion molecule.

kept in a temperature-controlled room with 12-h alternating light and dark cycles. Partial hepatectomy was performed under diethylether anesthesia by the method of Higgins and Anderson.[26] The time of surgery was varied so that all the animals would be killed between 9 and 11 a.m. Sham-operated rats were subjected to ether anesthesia and laparotomy but without partial hepatectomy. Control rats were not subjected to ether anesthesia or surgery. Food was supplied *ad libitum*, but was withdrawn 12–15 h before killing.

Male Donryu rats weighing 150–200 g (5 weeks old) and ascites hepatoma cells were obtained from Nippon Rat Co., Urawa and the Cancer Cell Repository, Tohoku University, Sendai, respectively. The ascites hepatomas were inoculated intraperitoneally into rats. The rats were killed for harvesting of the rapidly growing hepatoma cells at 6–14 days after inoculation, depending on the cell growth rate. The hepatoma cells were washed with physiological saline 3 times at 4°C to remove erythrocytes.

RT-PCR and DNA sequencing Total RNA was prepared from rat liver 7 days after partial hepatectomy by the guanidium thiocyanate procedure,[27] and was converted to single-stranded cDNA by reverse transcriptase with oligo(dT) primer. The synthesized cDNA was used in the PCR as a template with two sets of oligonucleotide primers. Set 1 consisted of the sense primer corresponding to the amino acid sequence KC(A/D)QYWP (5′-GCGGAATTCAAGTG(C/T)G(A/C)ICA(A/G)TA-(C/T)TGGCC-3′) with an *Eco*RI site and the antisense primer to the amino acid sequence HCSAG(I/V)GR (5′-CGCGGATCCGICCIA(C/T)ICCIGCI(C/G)(A/T)(A/G)CA(A/G)TG-3′) with a *Bam*HI site. Set 2 consisted of the sense primer corresponding to the amino acid sequence WRMVW (5′-CTGAATTCTGGAGGA-TGGTGTGG-3′) with an *Eco*RI site and the antisense primer corresponding to the amino acid sequence HCSAGVG (5′-ATGGATCCCGCCCAACACCAGC-ACTGCAGTG-3′) with a *Bam*HI site, designed from conserved regions of the PTP domain.[13] The PCR was carried out in 30 temperature cycles consisting of 94°C, 55°C, and 72°C for periods of 1, 2, and 3 min, respectively, in a Perkin-Elmer-Cetus thermal cycler. The PCR products were isolated and cloned into pCRII vector (Invitrogen, The Netherlands). The nucleotide sequences were determined by the chain termination method[28] using a dye termination cycle sequencing kit (Applied Biosystems, USA) and a 373A DNA sequencer (Applied Biosystems).

Isolation of RNA Total RNA was extracted from regenerating livers at various times after partial hepatectomy, livers after sham operation, and ascites hepatoma cells. Poly(A)$^+$ RNA was isolated by oligo(dT)-cellulose column chromatography from regenerating livers as described previously,[29] except for the use of Oligotex-dT30 (Takara Shuzo, Kyoto) for isolation from ascites hepatoma cells.

Northern blot analysis Equal amounts of poly(A)$^+$ RNA (5 or 10 μg) were electrophoresed on 1% agarose gel containing 17% formaldehyde, transferred to nitrocellulose membranes (Schleicher and Schuell, Germany) and fixed by baking at 80°C for 2 h under vacuum. The membranes were hybridized in 50% formamide, 1×Denhardt's solution, 5×SSC, 50 m*M* NaH_2PO_4, pH 6.5, 1% glycine, and 100 μg/ml boiled salmon sperm DNA at 42°C overnight with ^{32}P-labeled cDNA probes. These probes were labeled by the random prime method using a Mega Prime Labeling kit (Amersham, England). The blots were then washed twice with 2×SSC containing 0.1% SDS at room temperature for 5 min, followed by 0.1×SSC containing 0.1% SDS at 50°C for 15 min and were exposed to XRP-5 X-ray film (Eastman Kodak Company, USA) at −80°C with intensifying screens. The intensity of bands was quantified with a densitometer (Molecular Dynamics, Tokyo). The membranes were reused several times for other probes after washing twice at 70°C for 1 h in 2.5 m*M* tris-HCl, pH 8.0, 0.1 m*M* EDTA, 0.05% sodium pyrophosphate and 0.05× Denhardt's solution. The mRNAs of PTPs were identified on the basis of criteria of high specificity and length (kb) of the bands. Under these conditions, the mRNAs of rat PTP-S,[10] PTP1D,[18] PTPG1,[7] GLEPP1, and LRP[22] were detected as single bands of 1.8, 2.7, 4.4, 3.3 and 2.7 kb, respectively, and those of PTPH1,[19] PTP-1,[4] PTPγ,[30] PTPδ,[31] and LAR[32,33] as pairs of bands of 3.4 and 4.9, 1.9 and 3.5, 4.9 and 7.6, 5.1 and 7.3, and 4.4 and 5.5 kb, respectively. The values were all similar to those previously reported, except LAR.

RESULTS

Isolation and sequencing of cDNAs for rat PTPs By PCR amplification with primers corresponding to the conserved catalytic domains of PTPs, 52 PCR products were isolated from regenerating rat liver, normal rat liver and testis, and sequenced. Fig. 1 summarizes the amino acid sequences deduced from the cDNA sequences of the isolated clones. From the high homologies of the sequences between the isolated clones and the known PTPs, PTP3 (95.5%, 96.9%), PTP7 (86.7%, 97.8%), PTP8 (94.3%, 93.9%), PTP11 (84.0%, 92.9%), PTP16 (88.3%,91.6%), PTP17 (86.9%, 88.8%), PTP36 (82.0%, 88.5%), PTP54 (82.5%, 87.4%), PTP59 (92.3%, 96.8%), PTPB1 (98.6%, 99.2%), and TE3 (99.2%, 98.4%) are concluded to correspond to LRP,[22] PTPδ,[20] PTP-S,[9] PTP1D,[18] PTPγ,[20] PTP1C,[12] GLEPP 1,[21] PTPH1,[19] PTPG1,[7] LAR,[23] and PTP-1,[4] respectively, of rat (the figures in the parentheses correspond to nucleotide and amino acid identities, respectively).

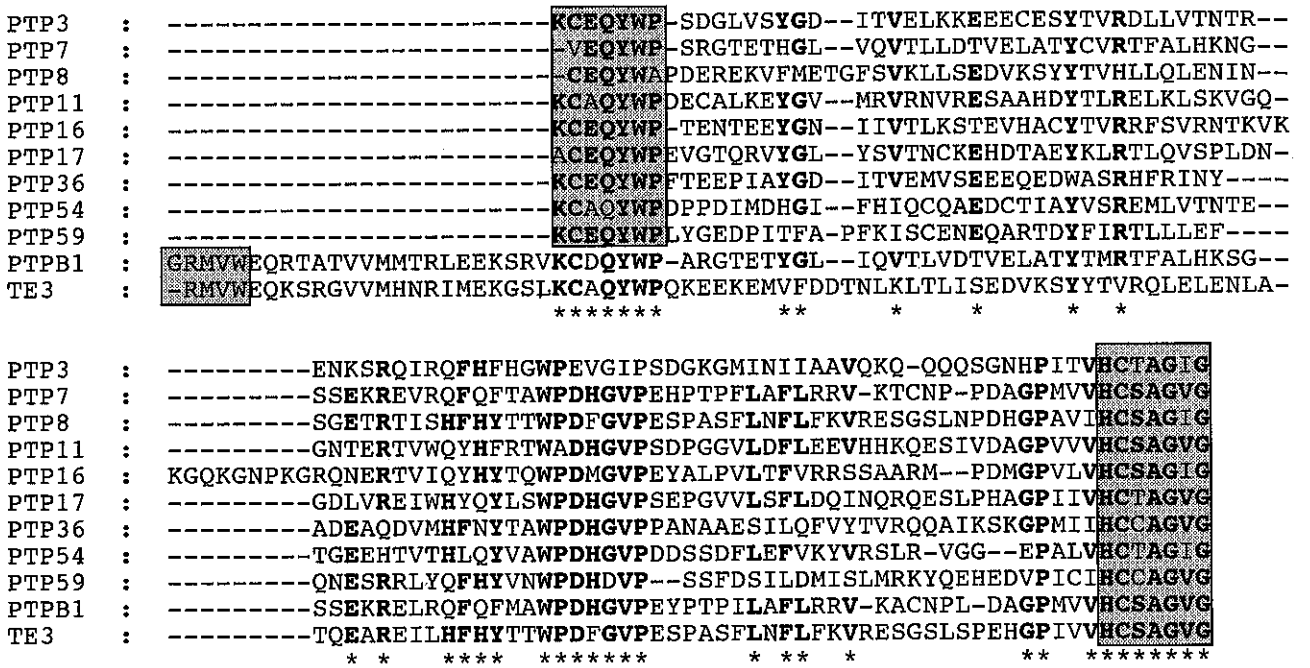

Fig. 1. Alignment of amino acid sequences deduced from the cloned PTPs obtained by RT-PCR. PTP3, 7, 8, 11, 16, 17, 36, 54, and 59 were isolated from regenerating rat liver. PTPB1 and TE3 were isolated from normal rat liver and testis, respectively. The positions where six or more sequences share an identical amino acid are indicated by asterisks and printed in bold. The amino acid sequences corresponding to the nucleotide sequences of the primers used in PCR are indicated by shaded boxes. The single-letter code is used.

mRNA levels of PTPs in regenerating rat livers mRNA levels of PTPs in regenerating rat livers were determined by using the cDNA clones as probes. As shown in Fig. 2A-1, the mRNA level of PTP-S rapidly increased approximately 4-fold by 7 h after partial hepatectomy compared to the control, then decreased gradually. In sham-operated rats without partial hepatectomy, a similar expression pattern was observed (data not shown), but the increase of the mRNA level of partially hepatectomized rats was much larger than that in sham-operated rats. PTPH1 showed a similar expression pattern to PTP-S (Fig. 2A-2). In sham-operated rats, the expression pattern of PTPH1 was very similar to that in partially hepatectomized rats (data not shown). Therefore, the rapid increase in mRNA levels of PTPH1 in regenerating liver seems to be mostly due to surgical stress. PTP-1 showed two transcripts of 1.9 kb and 3.5 kb, as previously reported (Fig. 2A-3).[4] The 3.5 kb transcript showed a rapid increase at 7 h, and then decreased slightly. It increased again and reached a maximum on day 5, and then decreased gradually. On the other hand, the mRNA level of 1.9 kb increased slowly and remained high on days 2–5, then rapidly decreased to the control level. In sham-operated rats, the mRNA levels of 1.9 kb and 3.5 kb peaked at 7 h (Fig. 2B-1). The possibility that circadian rhythm influences the gene expression is excluded, because all the sham-operations were conducted so that the liver would be removed at about 9 a.m., under which conditions the mRNA levels were remarkably increased by 7 h after the sham-operation, but were at control levels 10 days after it. From these results, it was strongly suggested that the rapid increase at 7 h in mRNA of PTP-1 is due to surgical stress. The mRNA levels of GLEPP1 (Fig. 2A-4) and LRP (data not shown) showed similar patterns to that of 3.5 kb PTP-1 mRNA. The mRNA levels of PTP1D and PTPG1 increased rapidly, reached a maximum at 7–14 h, and maintained high levels at least until day 14 (Fig. 2A-5, 6). Sham-operated rats also showed rapid increases by 7 h (data not shown). The mRNA levels of PTPγ, PTPδ, and LAR showed almost no change for the first 5 days, but increased over the control levels after day 7 (Fig. 2A-7, 8, 9). In sham-operated rats, the mRNA level of PTPδ was rather decreased on day 10 (Fig. 2B-2).

From these results, the expression patterns of PTP genes in regenerating rat livers after partial hepatectomy were classified into 4 groups. Group 1 contains PTP-S and PTPH1; their gene expressions were increased tran-

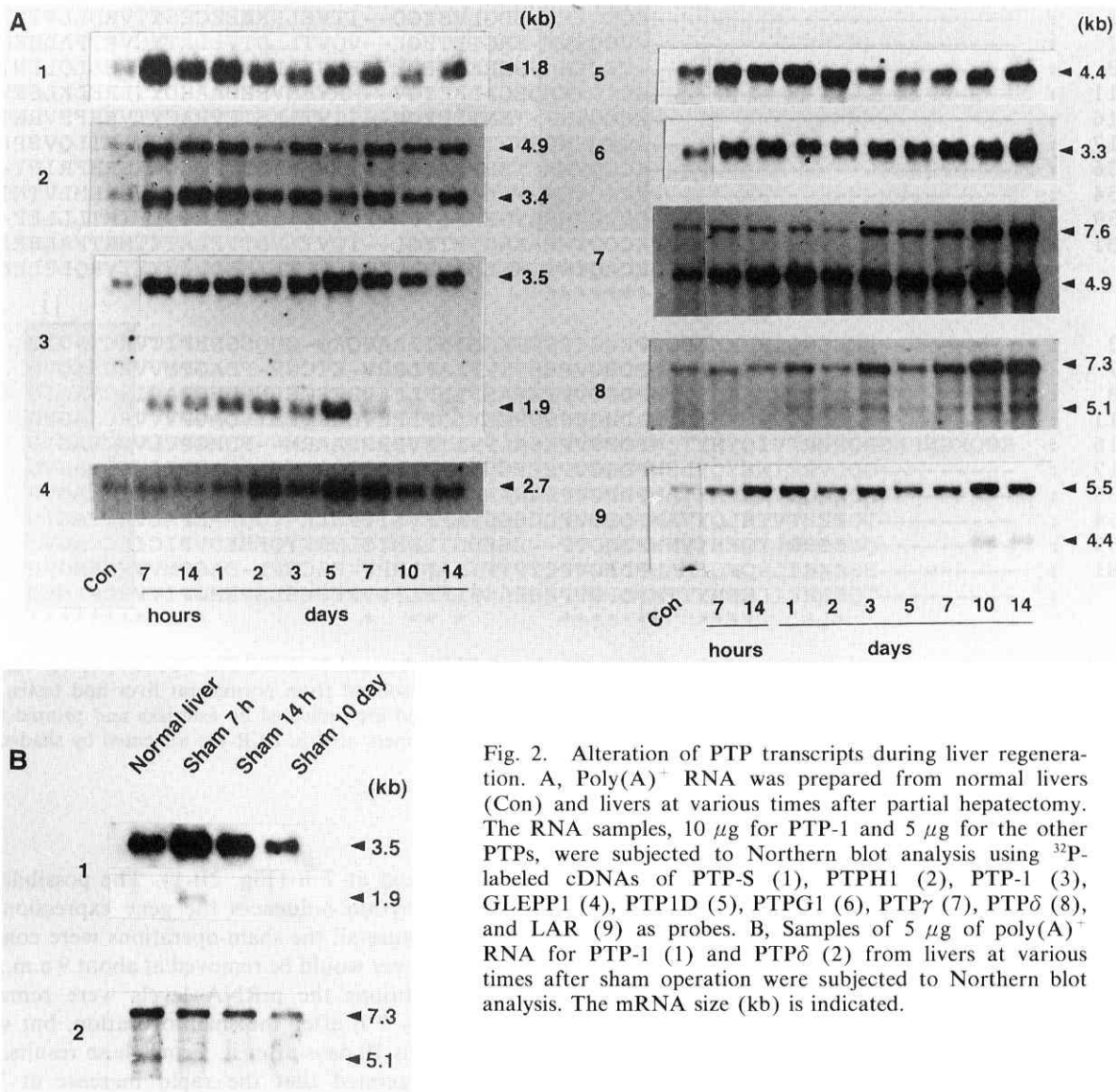

Fig. 2. Alteration of PTP transcripts during liver regeneration. A, Poly(A)$^+$ RNA was prepared from normal livers (Con) and livers at various times after partial hepatectomy. The RNA samples, 10 μg for PTP-1 and 5 μg for the other PTPs, were subjected to Northern blot analysis using ^{32}P-labeled cDNAs of PTP-S (1), PTPH1 (2), PTP-1 (3), GLEPP1 (4), PTP1D (5), PTPG1 (6), PTPγ (7), PTPδ (8), and LAR (9) as probes. B, Samples of 5 μg of poly(A)$^+$ RNA for PTP-1 (1) and PTPδ (2) from livers at various times after sham operation were subjected to Northern blot analysis. The mRNA size (kb) is indicated.

siently within 24 h after partial hepatectomy and then decreased gradually. Group 2 contains PTP-1, GLEPP1, and LRP (PTPα), the gene expressions of which increased and reached a maximum 5 days after partial hepatectomy (the time when the weight of regenerating liver recovered to that of control liver), then decreased gradually. Group 3 contains PTP1D and PTPG1, the gene expressions of which were similar to those in group 2 except that the expressions did not decrease but rather increased slightly after day 7. Group 4 contains PTPγ, PTPδ, and LAR, the mRNA levels of which were almost constant for the first 5 days, then increased over the control levels on day 7, after the liver weight had recovered to the control levels.

mRNA levels of PTPs in rat ascites hepatoma cells To elucidate neoplastic alterations of PTPs, gene expressions of PTPs (PTP-S, PTP-1, PTPγ, PTPδ, and LRP) were determined in three rat ascites hepatoma cell lines, AH13, AH143A, and AH311. Gene expressions of non-receptor-like PTPs (PTP-S and PTP-1) showed various changes in the three hepatomas, but did not show any common features characteristic of neoplastic alteration (Fig. 3-1, 2). The mRNA level of PTP-S was markedly decreased in AH13 and AH143A, but at similar levels in AH311, compared with the control. Levels of mRNA at 1.9 kb of PTP-1 were markedly increased in AH13 and those at 3.5 kb were increased in AH311, but decreased in AH143A, compared with control levels. On the other

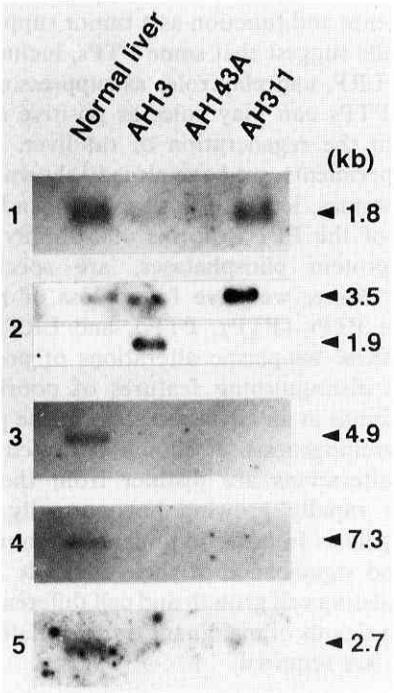

Fig. 3. Alteration of PTP transcripts in rat ascites hepatoma cells. Poly(A)$^+$ RNA was prepared with Oligotex-dT30 (Takara Shuzo, Kyoto) from liver and ascites hepatoma cells. Three μg samples of RNA were subjected to Northern blot analysis using ^{32}P-labeled cDNAs of PTP-S (1), PTP-1 (2), PTPγ (3), PTPδ (4), and LRP (5) as probes.

hand, very interestingly, mRNAs of receptor-like PTPs including PTPγ, PTPδ, and LRP were lost or at least drastically decreased in all three hepatoma strains examined (Fig. 3-3, 4, 5).

DISCUSSION

In the present study, we determined mRNA levels of PTPs in regenerating rat livers and in three rat ascites hepatoma cell lines. Alteration patterns of the mRNA levels during the regenerating process after partial hepatectomy could be classified into 4 groups. Group 1 increased rapidly at 7 h, remained at high levels for 1–2 days, and then declined gradually. Group 2 increased rapidly at 7 h, peaked on day 5, and then decreased gradually. Group 3 was similar to group 2 except that the expression levels did not decrease even after day 7. Group 4 maintained control levels until day 5 to 7, and then increased over the control levels after regeneration was completed.

Such various responses suggest that these PTP molecules are differentially involved in regulating multiple cellular functions. The physiological function of each PTP species remains uncertain at the present time, but PTPs in group 1 might be positively involved in triggering initiation of liver regeneration. For example, the rapid increase of PTP-S mRNA suggests that it belongs to the category of early response genes, such as *fos* and *jun*. PTP-S possesses a sequence homologous to c-*fos* and c-*jun*[9] and has binding ability to DNA.[34] It has been reported that gene expression of PTP-S (MPTP) is cell cycle-dependent and reaches the maximum at the G1 phase.[35] Therefore, it is possible that PTP-S is involved in positively regulating regeneration of rat liver after partial hepatectomy. PTPs in group 2 (PTP-1, GLEPP1, and LRP) might be involved in negatively regulating cell growth to suppress liver regeneration, because the mRNA levels peaked before, and decreased after the mass of the partially hepatectomized liver recovered to the normal level. The PTPs in group 3, expressed continuously at high levels, may be involved in constitutive functions during regeneration of hepatocytes. The PTPs in group 4 may play roles as suppressor(s) or be involved in highly differentiated cellular functions such as intercellular recognition and/or cell-matrix interaction, because the mRNA levels increased after the regeneration was almost completed. The lack of mRNAs of PTPγ, PTPδ, and LRP in ascites hepatoma cells strongly suggests that these genes may act as suppressor genes or be involved in highly differentiated functions. The PTPγ gene is located on human chromosome 3p21, a region frequently deleted in certain types of renal and lung carcinomas, suggesting that it is a candidate tumor suppressor gene.[24, 36] PTPγ, a member of a subfamily of receptor-like PTP, possesses a long extracellular domain containing a stretch of 266 amino acids with striking homology to carbonic anhydrase.[37] It is expressed in specific regions of the brain and in a variety of murine tissues including brain, lung, kidney, heart, skeletal muscle, liver, spleen, and testis.[37] It was also reported that the localization of PTPγ changes during brain development. PTPδ and LAR, members of another subfamily of receptor-like PTP, have sequence similarity to the neural cell adhesion molecule N-CAM. Changes in LAR expression were reported during nerve growth factor-induced PC12 pheochromocytoma cell differentiation and with contact-mediated inhibition of fibroblast growth.[32] It was also reported that LRP (PTPα), with a particularly short and heavily glycosylated extracellular domain, is involved in neural differentiation.[38] These facts suggest that the activities of these PTPs are regulated through cell-to-cell contact. Therefore, our present results with ascites hepatomas strongly suggest loss or dramatic decrease of cell-to-cell interaction, an important malignant phenotype. In some experiments, we have observed that mRNA levels of PTPγ, PTPδ, and LAR are slightly decreased in the early stages after partial hepatectomy. However, the decrease

is only slight, and loss of the expressions was never observed in regenerating liver. The difference in the gene expression between regenerating liver and ascites hepatoma strongly suggests that the lack of mRNAs of PTPγ, PTPδ, and LRP is not a simple reflection of rapid cell growth, but one of malignant alterations.

Very recently, Higashitsuji *et al.* demonstrated enhanced expression of multiple tyrosine phosphatases in the regenerating mouse liver by using total RNA.[39] In our preliminary experiments, mRNA bands of several PTPs, including PTPγ, PTPδ, and LAR, were undetectable in Northern blot analysis using total RNA, because of their extremely low concentrations. We also found that mRNA levels of some PTPs in sham-operated rats were markedly altered in the early stages after the operation. For these reasons, we carried out Northern blot analysis with the mRNA fraction instead of total RNA fraction, and sometimes in sham-operated rats as well as partially hepatectomized rats. Enhanced expressions of several PTPs were observed in regenerating rat liver, as reported in mouse by Higashitsuji *et al.*,[39] but their patterns after partial hepatectomy were characteristic of each species. For example, mRNA levels of mouse PTP-1 (PTP1B) reached a maximum on day 2, and returned to the control level on day 3, whereas in rat, it reached a maximum on day 5. The marked increase on day 2 in mouse LRP (PTPα) was not observed in the rat. It should be noted that mRNA levels of PTPγ, PTPδ, and LAR were increased in the rat on days 10–14.

Protein phosphatase consists of serine/threonine protein phosphatase (PP) and PTP. It has been well-established that oncogene products mostly possess protein tyrosine kinase activity,[40] and that protein tyrosine phosphorylation levels are increased in malignant transformation.[41] Therefore PTP can functionally counteract these oncogenes and function as a tumor suppressor. Our present results suggest that some PTPs, including PTPγ, PTPδ, and LRP, can play roles as suppressors, but also that other PTPs can play roles as positive or negative regulators in the regeneration of rat liver. Through a series of experiments, we have already shown that in rat ascites hepatomas, levels of both mRNA and protein of PP1α, one of the PP1 isoforms of a family of serine/threonine protein phosphatases, are specifically increased.[42–44] Here, we have found loss of mRNAs of receptor-like PTPs (PTPγ, PTPδ, and LRP). We conclude that these neoplastic alterations of protein phosphatase are distinguishing features of poorly differentiated hepatomas at an advanced stage in the progression of hepatocarcinogenesis. It should be noted that these neoplastic alterations are distinct from the responses observed in rapidly growing but normally controlled regenerating liver. In order to understand more precisely the roles and significance of these multiple PTP molecules in regulating cell growth and cell differentiation and in triggering signals of malignant transformation, further experiments are required.

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