J. Biochem. 2009;146(4)541–547
doi:10.1093/jb/mvp104

Membrane Localization of Protein-Tyrosine Phosphatase 1B is Essential for its Activation of Sterol Regulatory Element-Binding Protein-1 Gene Expression and Consequent Hypertriglyceridaemia

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Received March 30, 2009; accepted June 18, 2009; published online July 15, 2009

Protein-tyrosine phosphatase 1B (PTP1B) is a major regulator of insulin sensitivity. We have described a novel action of PTP1B in the induction of sterol regulatory element-binding protein-1 (SREBP-1) gene expression through activation of protein phosphatase 2A (PP2A). PTP1B is anchored to the endoplasmic reticulum membrane via its C-terminal tail. We have previously reported that membrane localization of PTP1B is essential for PP2A activation, which is crucial for enhancing SREBP-1 gene expression in *in vitro* experiments. In this study, we further investigated the physiological importance of membrane localization of PTP1B *in vivo*. We found that transient liver-specific overexpression of wild-type PTP1B (PTP1B-WT) using adenovirus-mediated gene transfer was associated with hypertriglyceridaemia and enhanced hepatic SREBP-1 gene expression in mice. However, overexpression of the C-terminal truncated PTP1B (PTP1BΔCT) failed to increase hepatic SREBP-1 expression or serum triglyceride levels, despite causing insulin resistance. Our results indicate that activation of PTP1B in the liver could induce hypertriglyceridaemia and that anchoring of PTP1B to the membrane is crucial for its action.

Key words: endoplasmic reticulum, protein phosphatase 2A, protein-tyrosine phosphatase 1B, sterol regulatory element-binding protein-1.

Abbreviations: ER, endoplasmic reticulum; G6Pase, glucose 6-phosphatase; PEPCK, phosphoenolpyruvate carboxykinase; *p*-NPP, *para*-nitrophenyl phosphate; PP2A, protein phosphatase 2A; PTP1B, protein-tyrosine phosphatase 1B; PTP1BΔCT, C-terminal-truncated PTP1B; PTP1BΔCT-CAAX, membrane-targeted PTP1BΔCT; SREBP, sterol regulatory element-binding protein; WT, wild-type.

Protein-tyrosine phosphatase 1B (PTP1B) is a major regulator of insulin sensitivity, and deletion of its gene leads to augmented insulin sensitivity and resistance to obesity induced by a high-fat diet (*1*, *2*). Studies from our laboratories and those of others, have shown that hyperinsulinaemia and increased carbohydrate intake stimulate PTP1B gene expression, which can cause insulin resistance (*3*–*5*), and that overexpression of PTP1B in liver, muscle and adipose tissue leads to insulin resistance (*1*, *6*–*9*). Therefore, PTP1B is considered a suitable therapeutic target for reducing insulin resistance (*10*).

In addition to acting as a negative regulator of insulin signalling, we previously reported a new aspect of PTP1B action; stimulation of sterol regulatory element binding protein (SREBP-1c) gene expression (*11*, *12*), a key transcription factor involved in the stimulation of lipogenesis in the liver (*11*, *12*). We also showed that high-fructose feeding caused insulin resistance with increased hepatic SREBP-1 mRNA content and PTP1B abundance in the liver (*3*, *13*). PTP1B directly enhances SREBP-1 gene expression by up-regulating Sp1 transcriptional activity through an increase in protein phosphatase 2A (PP2A) activity (*3*). Overexpression of wild-type PTP1B (PTP1B-WT), which associates with PP2A, decreases the level of PP2A C-subunit phosphorylation, stimulating its phosphatase activity (*3*, *14*). The role of the 'PTP1B-PP2A axis' in the stimulation of SREBP-1 gene expression was further supported by experiments using C-terminal truncated PTP1B (PTP1BΔCT), which does not localize to the membrane (*14*). PTP1B is anchored to the endoplasmic reticulum (ER) via its C-terminal tail (*15*). We found that changes in intracellular localization of PTP1B, induced by truncation of its C-terminal tail, does not alter its inhibitory effects on insulin signalling in 3T3-L1 adipocytes and Fao hepatoma cells (*14*, *16*). In contrast, overexpression of PTP1BΔCT does not induce SREBP-1 gene expression in Fao cells and mouse

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cultured hepatocytes (*14*). Consistent with this finding, PTP1BΔCT failed to bind PP2A, resulting in impaired PP2A activation in these cells (*14*). These results further support our hypothesis that PP2A activation by PTP1B is crucial for enhancing SREBP-1 gene expression. Simultaneously, these results revealed that membrane localization of PTP1B is essential for PP2A activation and enhancing SREBP-1 gene expression, but is not required for inhibition of insulin signalling.

In this study, to further explore the physiological role of PTP1B membrane localization, we performed *in vivo* transfection experiments. Transient liver-specific overexpression of PTP1B-WT protein by adenovirus infection led to hypertriglyceridaemia with enhanced SREBP-1 gene expression in mice. However, overexpression of PTP1BΔCT failed to increase SREBP-1 mRNA expression or serum triglyceride levels, despite causing insulin resistance. Our *in vivo* study demonstrated that abnormal activation of PTP1B in the liver does not cause only insulin resistance but also hypertriglyceridaemia, and confirmed that anchoring of PTP1B on the membrane is crucial for its enhancement of SREBP-1c gene expression and leading to hypertriglyceridaemia.

MATERIALS AND METHODS

Materials—Human insulin was kindly provided by Eli Lilly, Inc. (Indianapolis, IN, USA). Monoclonal PTP1B antibody was purchased from Oncogene Research Products (San Diego, CA, USA). Polyclonal PTP1B antibody and polyclonal anti-PP2A-C (C subunit of PP2A) antibody were obtained from Upstate Biotechnology, Inc. (Lake Placid, NY, USA). Anti-β-actin and anti-PP2A antibodies and Protein A/G agarose were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Horseradish peroxidase-linked anti-rabbit and -mouse antibodies were from Amersham Biosciences Corp. (Piscataway, NJ, USA). All radioisotopes were obtained from DuPont-NEN (Boston, MA, USA). XAR-5 film was obtained from Eastman-Kodak Co. (Rochester, NY, USA). All other reagents and chemicals were purchased from Sigma (St Louis, MO, USA).

Preparation of Recombinant Adenovirus—The recombinant adenovirus encoding rat PTP1B-WT was prepared as described previously (*8*, *14*, *17*). A 35-amino-acid segment was deleted from the C-terminal (ΔCT) of PTP1B cDNA by polymerase chain reaction (PCR), as described previously (*14*). Lac-Z-encoding adenovirus was used for the control.

In vivo *Transfection Experiments*—Six-week-old male CBA/JNCrj mice (Charles River Japan Inc., Kanagawa, Japan) were each injected, via the tail vein, with adenoviral vector (LacZ, PTP1B-WT and PTP1BΔCT), at a dose of 5×10^8 plaque-forming units (dissolved in 150 μl of saline), according to the method of Miyake *et al*. (*18*). Experiments were performed under overnight fasting conditions, 6 days after adenovirus injection. Plasma glucose, triglyceride and insulin levels were measured, after overnight fasting, as described previously (*3*). They were also measured 4 h postprandial, as described previously (*19*). The liver tissues were removed, immediately frozen in liquid nitrogen and stored at −80°C until use. All experiments were approved by the Shiga University of Medical Science Animal Care Committee and were in accordance with the university guidelines for the use and care of laboratory animals.

Preparation of Whole Cell Lysates and Immunoprecipitation—Liver tissues were lysed using a previously described method (*3*). For immunoprecipitation, cell lysates were incubated with primary antibody for 5 h at 4°C and with protein A/G-agarose for an additional 2 h. The immunoprecipitates were washed, resuspended in Laemmli sample buffer containing 100-mM DTT, and heated for 5 min at 100°C.

Immunoblotting—Whole-cell lysates and antibody immunoprecipitates were resolved by SDS–PAGE, transferred onto polyvinylidene difluoride membranes and probed with the indicated antibodies. Blots were then incubated with horseradish peroxidase-linked secondary antibody, followed by chemiluminescence detection. The corresponding bands were scanned and quantified with Scion Image and normalized to β-actin.

Northern Blot Analysis—Northern blot analysis was performed, as previously described (*13*). The probes for phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) were kindly provided by Dr D.K. Granner (Vanderbilt University, Nashville, TN) and Dr H. Nakajima (Osaka Medical Center for Cancer and Cardiovascular Disease, Osaka, Japan), respectively (*20*, *21*). The cDNA probe for SREBP-1 was generated by reverse transcriptase PCR amplification, from total RNA isolated from the rat liver, as previously reported (*13*). The corresponding bands were scanned and quantified with Scion Image and normalized to the signal generated with a probe for 18S ribosomal RNA.

RNase Protection Assay—The amount of SREBP-1c mRNA was assessed by RNase protection assay, as previously described (*3*, *22*). Aliquots of total RNA (10 μg), from each sample, were incubated with the SREBP-1 cRNA probe or a cRNA probe for β-actin mRNA. After digestion with RNase A/T1, protected fragments corresponding to SREBP-1a and SREBP-1c were quantified with Scion Image and normalized to β-actin.

Measurement of Phosphatase Activity—PTP1B and PP2A phosphatase activities were measured using *para*-nitrophenyl phosphate (*p*-NPP) as a substrate (*9*, *23*, *24*). Liver tissue was lysed and incubated with anti-PTP1B polyclonal antibody or anti-PP2A-C polyclonal antibody and Protein A/G Plus-agarose, at 4°C for 3 h, under constant rotation. Immunoprecipitants were washed three times and incubated with 50 μl of buffer containing 10-mM *p*-NPP, for 1 h at 37°C. The amount of *para*-nitrophenol produced was determined by measuring absorbance at 405 nm.

Statistical Analysis—Data are expressed as mean ± SEM of four separate experiments, unless otherwise stated. Tukey–Kramer HSD test was used to determine any significant differences among three or more groups. A P-value <0.05 was considered significant.

RESULTS AND DISCUSSION

Overexpression of PTP1B-WT and PTP1BΔCT in Mouse Liver—To investigate the physiological role of

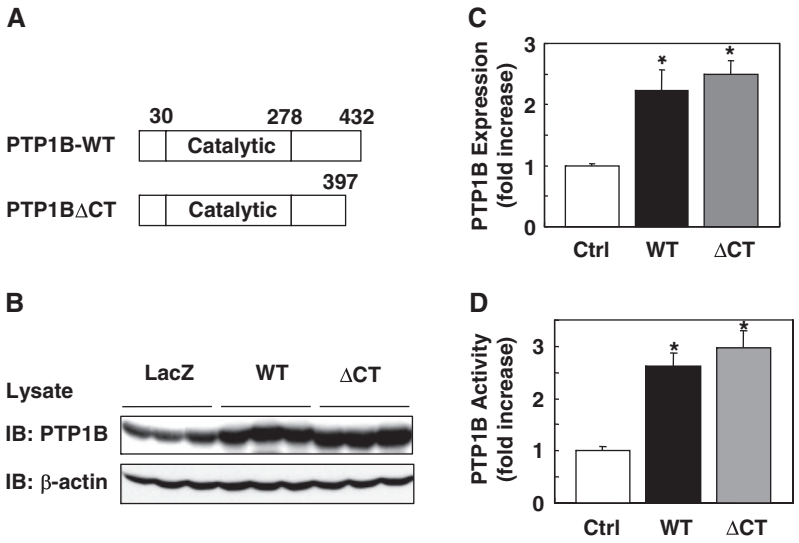

Fig. 1. **Liver-specific overexpression of PTP1B-WT and PTP1BΔCT.** Mice were injected with adenovirus encoding LacZ, PTP1B-WT (WT) or PTP1BΔCT (ΔCT). (A) Structure of PTP1B-WT and PTP1BΔCT. (B) Protein expression of PTP1B in the liver was analysed by western blotting using anti-PTP1B antibody. Loading difference was checked by β-actin immunoblotting. (C) Quantitative analysis of the results shown in (B) generated using a desk scanner. Data are mean ± SEM of 15–20 mice. $^{*}P < 0.01$ versus control mice. (D) PTP1B phosphatase activity in the liver measured as described in MATERIALS AND METHODS section. Data are mean ± SEM of 6–7 mice. $^{*}P < 0.01$ versus control mice. IB, immunoblot.

Table 1. **Body-weight change in mice before and after viral injection.**

Virus	Day 0 (g)	Day 6 (g)	Change (%)
LacZ	21.7 ± 0.7	23.5 ± 0.8	8.1 ± 0.5
PTP1B-WT	21.6 ± 0.6	23.1 ± 0.7	7.0 ± 0.5
PTP1BΔCT	21.9 ± 0.7	23.8 ± 0.7	8.8 ± 0.4

Data are mean ± SD (n = 15–20). PTP1BΔCT, C-terminal-truncated PTP1B.

PTP1B localization *in vivo*, CBA/JNCrj mice were injected with recombinant adenoviruses encoding LacZ, PTP1B-WT, or PTP1BΔCT, which does not localize to the membrane (*14*) (Fig. 1A), via the tail vein. At 6 days post-adenovirus injection, the expressions of PTP1B proteins in the liver were 2- to 3-fold higher than endogenous protein (Fig. 1B and C). PTP1B-WT and PTP1BΔCT were expressed at similar levels. Phosphatase activities of PTP1B were comparable to their protein expression levels (Fig. 1D).

There was no significant difference in weight between mice in any of the virally injected groups (Table 1). Furthermore, weight gain after viral injection did not differ among all groups, suggesting food intake was not significantly changed.

Overexpression of both PTP1B-WT and PTP1BΔCT in Liver Leads to Insulin Resistance in Mouse—As shown in Fig. 2A and B, hepatic overexpression of PTP1B-WT and PTP1BΔCT, in the virally injected mice, was associated with significant elevation of fasting plasma glucose and insulin levels, compared with the control mice. Furthermore, although plasma glucose levels in postprandial state were similar among the three groups, plasma insulin levels were higher in PTP1B (WT and ΔCT) infected mice (Fig. 2B). These results suggest that PTP1B leads to insulin resistance *in vivo* and support our previous reports that PTP1B-WT and PTP1BΔCT equally inhibit insulin signalling in 3T3-L1 adipocytes and Fao cells (*14*, *16*). Since we did not observe any change in body weight, food intake is unlikely to account for altered glucose homeostasis.

Several observations suggest that the liver is a major site of the peripheral action of PTP1B in regulating glucose homeostasis. Hyper-insulinaemic euglycaemic clamp studies revealed a marked enhancement of insulin-stimulated glucose uptake into skeletal muscle but also a trend toward greater suppression of hepatic glucose output in mice lacking PTP1B (*2*). Furthermore, liver-specific re-expression of PTP1B in these mice led to marked attenuation of their enhanced insulin sensitivity (*1*).

In support of these findings, we also observed significant increases in mRNA levels of enzymes involved in gluconeogenesis, such as PEPCK and G6Pase, in PTP1B-WT- and PTP1BΔCT-overexpressing livers of fasted mice (Fig. 3). The expression of these gluconeogenic genes is controlled mainly by insulin and glucagon (*25*) and has shown that inhibition of insulin signalling in the liver led to enhancement of expression of these genes and an increase in gluconeogenesis, resulting in fasting hyperglycaemia (*18*, *26*, *27*).

Taken together, these results clearly prove that hepatic PTP1B could lead to insulin resistance *in vivo* and that membrane localization of PTP1B is not required for its effect on insulin resistance.

PTP1B-WT but not PTP1BΔCT Leads to Hypertriglyceridaemia in Mouse—We previously reported that overexpression of PTP1B directly enhances SREBP-1 gene expression through PP2A activation, revealing a new aspect of PTP1B action (*3*). Furthermore, we also reported that membrane localization of

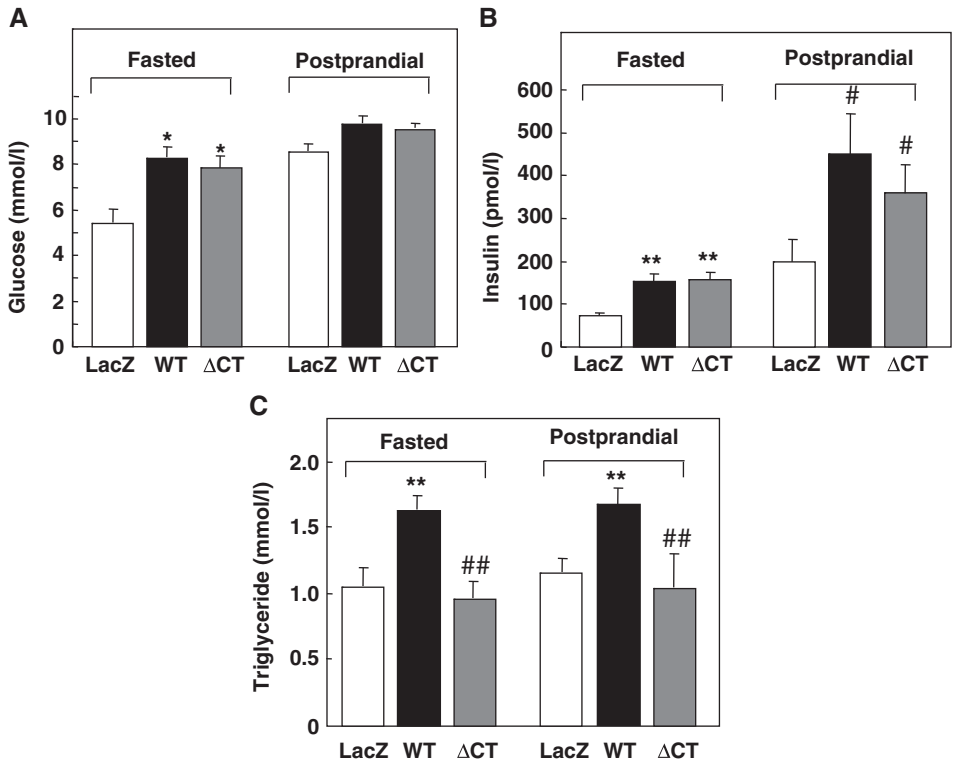

Fig. 2. **Effects of liver-specific overexpression of PTP1B-WT and PTP1BΔCT on metabolic parameters in mice.** (A) Plasma concentrations of glucose (A), insulin (B) and triglycerides (C) of mice in fasted and postprandial states. Data are mean ± SEM of 15–20 mice. $^{*}P < 0.05$, $^{**}P < 0.01$ versus control mice, $^{\#}P < 0.05$ versus control mice in postprandial state and $^{\#\#}P < 0.01$ versus mice injected with PTP1B-WT.

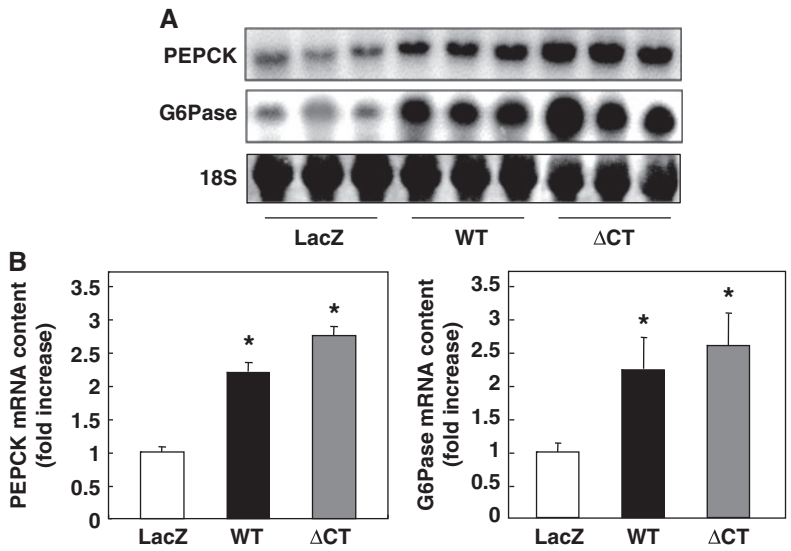

Fig. 3. **Both PTP1B-WT and PTP1BΔCT enhance the expression levels of gluconeogenic genes.** (A) Six days after injection, total RNA was isolated from the liver. The mRNA contents of PEPCK and G6Pase in the fasted liver were assessed by northern blotting. (B) Quantitative analysis of the results shown in (A) generated using a desk scanner. Data are mean ± SEM of 6–7 mice. $^{*}P < 0.01$ versus control mice.

PTP1B is necessary for this action to occur (*14*). To determine whether our observations in cultured cells could be observed *in vivo*, we measured plasma triglyceride levels in mice. As shown in Fig. 2C, hepatic overexpression of PTP1B-WT in mice significantly increased plasma triglyceride levels under both fasting and postprandial conditions, whereas overexpression of PTP1BΔCT did not increase plasma triglyceride levels.

To investigate the reason for this difference, we measured mRNA contents of SREBP-1a and SREBP-1c in the

liver in the fasting state by RNase protection assay. Since PTP1B can enhance SREBP-1 gene expression in the absence of insulin in rat hepatocytes and Fao cells (*3*, *14*), and by several other factors in the postprandial state, experiments were performed under overnight fasting conditions. As previously reported (*28–30*), the amount of SREBP-1c mRNA was higher than that of SREBP-1a in mouse liver (Fig. 4A). Overexpression of PTP1B-WT, but not PTP1BΔCT, increased both SREBP-1a and SREBP-1c mRNA (Fig. 4A), which is consistent with our previous *in vitro* study (*14*). The stimulatory effect of PTP1B was remarkable on the SREBP-1c mRNA expression.

These results prove our hypothesis that the C-terminal region of PTP1B is critical for SREBP-1 gene expression, and the associated hypertriglyceridaemia.

PTP1BΔCT Fails to Associate with PP2A and Activate its Activity in Mouse Liver—To further analyse the molecular mechanism of the difference between PTP1B-WT and PTB1BΔCT in stimulating SREBP-1c expression, we examined the association of exogenously expressed PTP1B-WT and PTP1BΔCT with endogenous PP2A in mouse liver. We used a monoclonal PTP1B antibody as it recognizes rat but not mouse PTP1B (Supplementary Figure 1). Thus, we could evaluate the association of PP2A with only exogenously expressed PTP1B, by using an adenovirus encoding rat PTP1B. Mouse liver lysates infected with PTP1B-WT and PTP1BΔCT were immunoprecipitated with anti-PP2A antibody. The immunoprecipitates were then analysed by western blotting with monoclonal anti-PTP1B antibody. Significant association of PTP1B-WT with PP2A was observed in the reciprocal immunoprecipitation study (Fig. 5A). In contrast to this, PTP1BΔCT could not associate with PP2A (Fig. 5A). Furthermore, overexpression of PTP1B-WT but not PTP1BΔCT enhanced PP2A activity in mice liver (Fig. 5B). These results are consistent with the findings of our *in vitro* studies (*14*), where we have reported that PTP1B can stimulate SREBP-1 gene expression through binding and enhancement of PP2A activity, whereas PTP1BΔCT cannot bind and activate PP2A, resulting in impaired SREBP-1 gene expression (*14*). These results suggest that the ability of

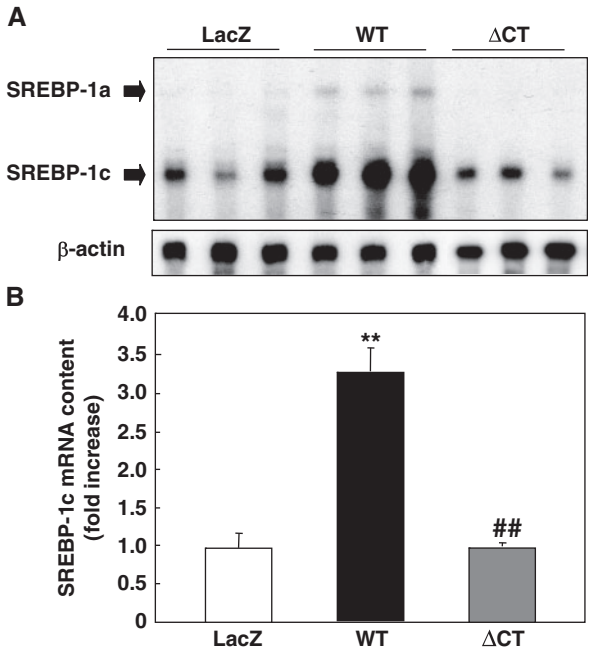

Fig. 4. **PTP1B-WT but not PTP1BΔCT enhances the expression of SREBP-1c.** (A) mRNA contents of SREBP-1a and SREBP-1c in the fasted liver were assessed by RNase protection assay. (B) Quantitative analysis of the results of SREBP-1c shown in (A) generated using a desk scanner. Data are mean ± SEM of 6–7 mice. $^{**}P < 0.01$ versus control mice. $^{\#\#}P < 0.01$ versus mice injected with PTP1B-WT.

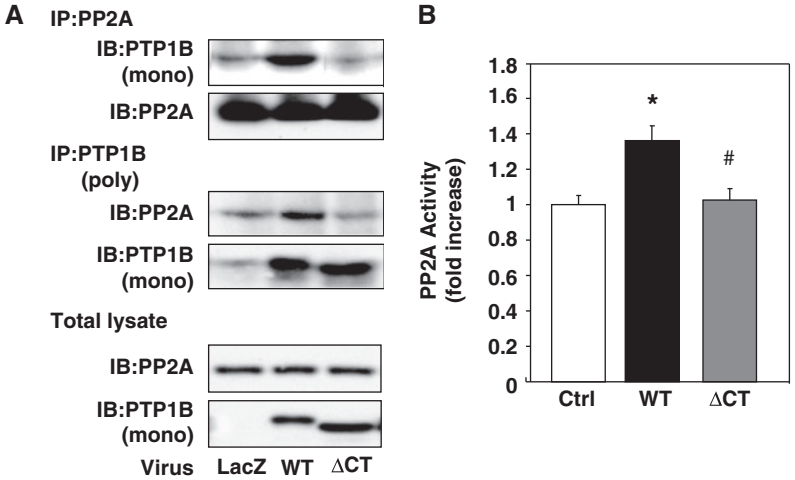

Fig. 5. **Associations of PTP1B-WT and PTP1BΔCT with PP2A in the liver.** (A) Associations of exogenously expressed PTP1B-WT (WT) and PTP1BΔCT (ΔCT). Mouse liver lysates were immunuprecipitated with anti-PP2A antibody, followed by Western blotting with anti-PTP1B monoclonal (mono) antibody and anti-PP2A antibody. Same lysates were immunoprecipitated with anti-PTP1B polyclonal (poly) antibody, followed by western blotting with anti-PP2A and anti-PTP1B monoclonal (mono) antibody. Representative results of four experiments are shown. (B) PP2A phosphatase activity in the liver was measured as described in MATERIALS AND METHODS section. Data are mean ± SEM of 6–7 mice. $^{*}P < 0.01$ versus control mice. $^{\#}P < 0.05$ versus mice injected with PTP1B-WT. IP, immunoprecipitate.

PTP1B to stimulate SREBP-1 gene expression is due to its association with PP2A. Although we did not assess the mechanisms of enhancement of SREBP-1gene expression in this study, we have shown that PTP1B enhances SREBP-1 gene expression by up-regulating Sp1 transcriptional activity via an increase in PP2A activity (*3*). The Sp1-binding sites are located at about −90 bp region of the SREBP-1a and SREBP-1c promoters and are thought to be important in the regulation of their gene expressions (*29*). Furthermore, we have shown that a defective mutation of the Sp1-binding sites in SREBP-1a and SREBP-1c promoters led to the cancellation of PTP1B effect on their promoter activities (*3*). Collectively, it is likely that activation of SP-1 transcription factor may be involved in this mechanism. Further study is needed to confirm this issue.

It is still unknown why PTP1BWT can associate with PP2A and PTP1BΔCT cannot. It is unlikely that the C-terminal region of PTP1B is responsible for the PP2A association, as the membrane-targeted PTP1BΔCT mutant (PTP1BCT-CAAX) can associate with PP2A and stimulate its activity despite the absence of the C-terminal region (*14*). The relationship between the subcellular localization of each PTP1B construct and PP2A may relate the ability of PTP1B to associate with PP2A. Further study is needed to clarify the physical role of the intracellular localization of PTP1B on its function.

In conclusion, our *in vivo* study demonstrated that abnormal activation of PTP1B in the liver not only causes insulin resistance but also hypertriglyceridaemia. We also confirmed that membrane anchoring of PTP1B is crucial for its enhancement of SREBP-1c gene expression, which leads to hypertriglyceridaemia. This study shows for the first time that hepatic PTP1B is involved in hypertriglyceridaemia. Based on these findings, we consider PTP1B a suitable therapeutic target for metabolic syndrome, which is characterized by insulin resistance and hypertriglyceridaemia.

SUPPLEMENTARY DATA

Supplementary data are available at *JB* online.

ACKNOWLEDGEMENTS

We thank Ms Yukie Nakai for the excellent technical assistance. We are grateful to Dr M. Bryer-Ash (UCLA), Dr D.K. Granner (Vanderbilt University) and Dr H. Nakajima (Osaka Medical Center for Cancer and Cardiovascular Disease) for providing adenovirus encoding PTP1B and cDNA probes.

FUNDING

Grant-in-aid from the Ministry of Education, Culture, Sports, Science and Technology, Japan (to S.U. and H.M.).

CONFLICT OF INTEREST

None declared.

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