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Protein-Tyrosine Phosphatase 1B Associates with Insulin Receptor and Negatively Regulates Insulin Signaling without Receptor Internalization

Kun Shi[1], Katsuya Egawa[1], Hiroshi Maegawa[1,*], Takaaki Nakamura[2], Satoshi Ugi[1], Yoshihiko Nishio[1] and Atsunori Kashiwagi[2]

[1]Division of Endocrinology and Metabolism, Department of Medicine, and [2]Department of Anatomy, Shiga University of Medical Science, Otsu 520-2192

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Phosphorylated platelet-derived growth factor (PDGF) receptor becomes internalized and then is dephosphorylated by protein-tyrosine phosphatase (PTP) 1B at the endoplasmic reticulum (ER). However, it remains unclear where PTP1B dephosphorylates insulin receptor and inhibits its activity. To clarify how and where PTP1B could interact with insulin receptor, we overexpressed a phosphatase-inactive mutant, PTP1BC/S, in 3T3-L1 adipocytes. Although PDGF receptor was maximally associated with PTP1BC/S at 30 min after PDGF stimulation, the maximal association of insulin receptor with PTP1BC/S was attained at 5 min after insulin stimulation. Furthermore, dansylcadaverine, a blocker of receptor internalization, inhibited this PDGF-induced association of PTP1BC/S with its receptor. However, dansylcadaverine did not affect the insulin-stimulated association of PTP1BC/S with insulin receptor, as well as dephosphorylation of insulin receptor by PTP1B. These results indicate that PTP1B might interact with insulin receptor and deactivate it without internalization. Finally, we overexpressed the wild-type and cytosolic-form of PTP1B to determine the role of ER-anchoring of PTP1B, and found that both inhibited insulin signaling equally. Thus, our data indicate that localization of PTP1B at the ER is not needed for insulin receptor dephosphorylation by PTP1B.

Key words: insulin signaling, intracellular localization, protein-tyrosine phosphatase 1B, receptor internalization, 3T3-L1 adipocytes.

Abbreviations: DC, dansylcadaverine; ER, endoplasmic reticulum; FCS, fetal calf serum; IRS, insulin receptor substrate; MOI, multiplicity of infection; PDGF, platelet-derived growth factor; PMSF, phenylmethylsulfonyl fluoride; pNPP, *p*-nitrophenyl phosphate; PTP, protein-tyrosine phosphatase.

Several lines of evidence have demonstrated that protein-tyrosine phosphatase (PTP) 1B negatively regulates insulin signaling and plays an important role in the acceleration of insulin resistance (*1–3*). We reported that exposure of rat 1 fibroblasts expressing human insulin receptors to high-glucose conditions impaired the insulin-stimulated tyrosine phosphorylation of both insulin receptor and insulin receptor substrate (IRS)-1 due to the increased PTP1B expression and activity (*4*). Furthermore, overexpression of PTP1B inhibited insulin signaling, such as tyrosine phosphorylation of insulin receptor and IRS-1, activation of phosphatidylinositol (PI) 3-kinase, and phosphorylation of Akt and mitogen-activated protein kinase, and insulin's metabolic effects such as glycogen synthesis in L6 myocytes and Fao hepatoma cells (*5*), as well as 3T3-L1 adipocytes (*6*, *7*).

Since the initial events of insulin signaling, *i.e.*, the binding of insulin to its specific receptor and activation of its receptor tyrosine kinase, occur at the plasma membrane, the localization of signaling molecules at the plasma membrane may be critical for regulation of insulin signaling. We previously reported that membrane targeting of PI 3-kinase (p110CAAX) was sufficient to enhance its downstream effects, even though overexpression of wild-type PI 3-kinase was not (*8*). Moreover, we recently found that overexpression of a membrane-targeted phosphoinositide-dependent protein kinase (PDK)-1 triggered phosphorylation of Akt and GSK-3, as did that of p110CAAX (*9*). These findings support the importance of the intracellular localization of signaling molecules in insulin signal transduction.

In the case of PTP, its intracellular localization and compartmentalization may also play important roles in substrate specificity determination (*10*). Receptor-type PTPs, such as PTPα and leukocyte common antigen-related phosphatase (LAR), are localized predominantly in the plasma membrane and to a lesser extent in heavy microsomes. This distribution is similar to that of insulin receptor. PTP1B and IRS-1 are present in light microsomes and cytosol, whereas SHP2 is exclusively cytosolic. In accordance with its intracellular localization, PTP1B dephosphorylates insulin receptor, and dephosphorylated IRS-1 most potently (*11*). It has been reported that PTP1B becomes anchored to the endoplasmic reticulum (ER) via the 35 COOH-terminal amino acids and is activated after release into the cytosol through truncation of

*To whom correspondence should be addressed. Tel: +81-77-548-2222, Fax: +81-77-543-3858, E-mail: maegawa@belle.shiga-med.ac.jp

its COOH-terminus (*12*, *13*). However, Haj *et al.* recently indicated that PTP1B-catalyzed dephosphorylation requires endocytosis of the receptors for epidermal growth factor and platelet-derived growth factor (PDGF) at specific sites on the surface of the ER (*14*). Thus, it remains unclear how and where insulin receptor interacts with the PTP1B molecule and tyrosine-phosphorylation of the receptor is abolished by PTP1B. Therefore, in the present study, we investigated whether or not the internalization of insulin receptor at the ER is prerequisite for its dephosphorylation by PTP1B, which is found in PDGF receptor deactivation.

For the above investigation, we employed the adenovirus-mediated gene transfer technique and an inhibitor of receptor internalization. In the present study, we found that receptor internalization was not nessesary for dephosphorylation of insulin receptor by PTP1B, and that ER-anchoring of PTP1B was not essential for the inhibitory effect on insulin signaling of PTP1B.

MATERIALS AND METHODS

Materials—Human insulin was provided by Eli Lilly, Inc. (Indianapolis, IL, USA). Anti-PTP1B polyclonal, anti-IRS-1, anti-p85 N-SH2, and anti-Shc antibodies were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY, USA). Anti-phosphospecific-Akt and -p70S6 kinase antibodies were obtained from Cell Signaling Technology, Inc. (Beverly, MA, USA). Anti-Akt1, anti-p70S6 kinase, and horseradish peroxidase-linked anti-rabbit and anti-mouse antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-phospho-tyrosine antibodies were obtained from Transduction Laboratories (Lexington, KY, USA). Monoclonal PTP1B antibodies were from Oncogene Research Products (San Diego, CA, USA). Dulbecco's modified Eagle's (DME) medium and fetal calf serum (FCS) were purchased from Life Technologies (Rockville, MD, USA). Porcine [^{125}I]Tyr-A_{14}-insulin was purchased from PerkinElmer Life Science (Boston, MA, USA), and other radioisotopes were from Du-Pont-NEN (Boston, MA, USA). XAR-5 film was obtained from Eastman-Kodak (Rochester, NY, USA). All other reagents and chemicals were purchased from Sigma (St. Louis, MO, USA).

Cell Culture—3T3-L1 preadipocytes, which were provided by Dr. J. M. Olefsky (University of California, San Diego, La Jolla, CA, USA), were grown and maintained in DME high-glucose medium containing 50 U/ml penicillin, 50 μg/ml streptomycin, and 10% FCS in a 10% CO_2 environment. The cells were allowed to grow until 2 d postconfluency, and then caused to differentiate by the addition of the same medium containing isobutylmethylxanthine (500 μM), dexamethasone (25 μM), and insulin (4 μg/ml) for 3 d, and then the addition of the medium containing insulin for 3 d. The medium was then changed every 3 d until the cells were fully differentiated, typically after 14 d. Prior to the experimentation, the adipocytes were trypsinized and reseeded onto appropriate culture dishes.

Primary cultured rat hepatocytes were isolated from non-fasting 200-g male Sprague-Dawley rats by the collagenase method described previously (*15*). The animals were anesthetized, and then their livers were perfused *in situ* via the portal vein at 25 ml/min with Krebs-Ringer buffer (KRB), and then with KRB containing collagenase (Sigma) for 10 min at the same flow rate. The dissociated cells were dispersed by shaking, followed by filtration at 4°C through gauze into an equal volume of ice-cold DME medium containing 10% FCS, 100 U/ml penicillin, and 100 mg/ml streptomycin. The cells were then precipitated and washed twice at 4°C with the same medium. The cells in suspension (8 × 106) were plated onto tissue culture treated glass slides (Becton Dickinson Labware, Franklin Lakes, NJ, USA) in William's E Medium (Sigma) supplemented with 10% FCS, 6 ng/ml insulin, 100 nM triiodothyronine, 100 nM dexamethasone, 100 U/ml penicillin, and 100 mg/ml streptomycin. After incubation at 37°C under 10% CO_2 for 2 h, the cells were washed twice with PBS, and then incubated with DME medium supplemented with 6 ng/ml insulin, 100 nM triiodothyronine, 100 nM dexamethasone, 100 U/ml penicillin, and 100 mg/ml streptomycin.

Ad-E1A–transformed human embryonic kidney cell line 293 was cultured in DME high-glucose medium containing 50 U/ml penicillin, 50 μg/ml streptomycin and 10% FCS in a 5% CO_2 environment.

Preparation of Recombinant Adenoviruses—The recombinant adenoviruses containing wild-type PTP1B (PTP1BWT) or cysteine^{215}/serine^{215} mutant PTP1B (PTP1BC/S) and their cDNAs were kindly provided by Dr. M. Bryer-Ash (University of California, Los Angeles, Los Angeles, CA) (*16*). A 35–amino-acid deletion from the COOH-terminal of PTP1B (PTP1BΔCT) cDNA was generated by PCR reaction using the following pair of primers: 5′-gtacggtgggaggtctatat-3′ and 5′-ggggtcgacctattcctc-ctcggttggggacagctcttcctc-3′, and then the resulting fragment was subcloned into the pACCMVpLpASR (+) plasmid (*17*). After confirmation of the sequence, the resulting recombinant plasmid was cotransfected into 293 packaging cells with the pJM17 plasmid (*18*), which carries the Ad5 genomic DNA, and propagated as described previously (*19*). Mature recombinant Ad5 encoding PTP1BΔCT was thus generated through *in vivo* homologous recombination between these two plasmids as described previously (*5*, *8*).

Cell Treatment—3T3-L1 adipocytes were infected at a multiplicity of infection (MOI) of 10–50 plaque forming units (pfu)/cell for 16 h with a stock of either a control recombinant adenovirus (Ad5-ctrl) containing the cytomegalovirus promoter, pUC 18 polylinker, and a fragment of the SV40 genome, or the recombinant adenovirus containing PTP1BC/S (Ad5-PTP1BC/S), PTP1BWT (Ad5-PTP1BWT), or PTP1BΔCT (Ad5-PTP1BΔCT). Transfected cells were incubated for 56 h at 37°C under 10% CO_2 in DME high-glucose medium containing 2% heat-inactivated serum, followed by incubation in the starvation medium required for the assay. Primary cultured rat hepatocytes were infected at 10 MOI with various recombinant adenoviruses as described above for 2 h and then incubated for 24 h at 37°C under 10% CO_2. The efficiency of the adenovirus-mediated gene transfer was approximately 90%, as measured by immunocytochemistry. The survival of cells was unaffected by incubation of the cells with the different adenovirus constructs since the total protein level remained the same in infected and uninfected cells.

Insulin Binding Assay—Differentiated 3T3-L1 adipocytes in 6-well dishes were pretreated with or without 100 μM dansylcadaverine (DC) for 30 min. The cells were then incubated with 0.02 nM [^{125}I]Tyr-A_{14}-insulin with or without 5 μM cold insulin at 4°C overnight. The binding assay was performed as described previously with some modification (*20*).

Subcellular Fractionation—An abbreviated differential centrifugation procedure was used to obtain cytosol, intracellular membrane and plasma membrane fractions from 3T3-L1 adipocytes expressing PTP1BWT or PTP1BΔCT as described previously with some modification (*21*). Cells were homogenized in HES buffer (20 mM Hepes, pH 7.4, 1mM EDTA, 250 mM sucrose) supplemented with 0.1 mM sodium orthovanadate and 1mM phenylmethylsulfonyl fluoride (PMSF). The homogenate was centrifuged at 19,000 × *g* for 20 min, and the resulting supernatant was centrifuged at 180,000 × *g* for 75 min at 4°C, which yielded the cytosol fraction as a supernatant. The pellet was solubilized in ice-cold HES buffer containing 1% Triton X-100 as the intracellular membrane fraction. The pellet obtained on the initial spin was layered onto 1.12 M sucrose in HES buffer, followed by centrifugation at 100,000 × *g* for 60 min. This yielded a white fluffy band at the interface (plasma membrane fraction). The plasma membrane fraction was resuspended in HES and then pelleted at 40,000 × *g* for 20 min. All fractions were resuspended in HES to a final protein concentration of 1–2 mg/ml and stored at –20°C.

Immunohistochemical Analysis—Primary cultured rat hepatocytes were stained as described previously (*22*). In brief, the cells were fixed for 2 h with 4% paraformaldehyde, and then washed for 4 days with 0.1 M PBS containing 0.1% Triton X (PBST), followed by incubation with a monoclonal antibody against PTP1B diluted to 1:5,000 in PBST. The cells were then incubated with species-specific secondary antibodies conjugated to green fluorescence protein. The positive reaction was observed under a fluorescence microscope (Olympus IX70; Olympus Optical Co., Ltd., Tokyo, Japan), and images were taken with a CCD camera (Cool SNAP/HQ; Nippon Roper Co., Chiba, Japan).

Measurement of PTP Activity—PTP activity in the cell extracts was determined by measuring the hydrolysis of p-nitrophenyl phosphate (pNPP) as described previously (*6*). In brief, cells were infected with the various Ad5-PTP1B constructs as described above. After incubation for 3 days, cell extracts were prepared in solubilizing buffer (20 mM Tris-EDTA, pH 7.5, containing 140 mM NaCl, 1 mM EDTA, 1% [v/v] Nonidet P-40, 1 mM PMSF, 1 μg/ml aprotinin). Aliquots of the cell extracts (100 μg of total protein in 200 μl of buffer) were incubated with 1 μg monoclonal anti-PTP1B antibodies at 4°C for 1 h with constant rotation, and then with Protein A/G Plus-agarose (Santa Cruz Biotechnology) for an additional 2 h. The immunoprecipitate was collected by centrifugation at 14,000 × *g* for 5 min. After hydrolysis of pNPP at 37°C for 60 min in a reaction buffer (20 mM Tris, pH 7.5, 10 mM dithiothreitol) containing 10 mM pNPP, the reaction was quenched by the addition of 100 μl of 1 M NaOH, and then the amount of product (p-nitrophenyl) was measured by determining the absorbance at 405 nm with a spectrophotometer. The nonenzymatic hydrolysis of pNPP was corrected by measuring the increase in absorbance at 405 nm observed in the absence of enzyme.

Western Blotting—Ad5-ctrl-, Ad5-PTP1BC/S, Ad5-PTP1BWT-, or Ad5-PTP1BΔCT-infected cells were starved for 16 h in DME regular-glucose medium with 0.2% BSA. The cells were stimulated with or without 100 ng/ml insulin or 30 ng/ml PDGF for the indicated times at 37°C, and then lysed in solubilizing buffer comprising 20 mM Tris, 1 mM EDTA, 140 mM NaCl, 1% Nonidet P-40 (NP-40), 50 U/ml aprotinin, 1 mM Na_3VO_4, 1 mM PMSF and 50 mM NaF, pH 7.5, for 30 min at 4°C. The cell lysates were centrifuged to remove insoluble material. For Western blot analysis, whole cell lysates (20 μg of protein per lane) were denatured by boiling in Laemmli sample buffer containing 100 mM dithiothreitol and then resolved by SDS-PAGE. Proteins were transferred to nitrocellulose by electroblotting in Towbin buffer containing 20% methanol. For immunoblotting, membranes were blocked and probed with the specified antibodies. The blots were then incubated with horseradish peroxidase-linked secondary antibodies followed by chemiluminescence detection, according to the manufacturer's instructions (PerkinElmer Life Science Inc., Boston, MA).

2-Deoxyglucose Transport—Glucose transport was examined as described previously (*8*). Differentiated 3T3-L1 adipocytes were infected with various Ad5-PTP1B or Ad5-ctrl at 50 MOI as described above and then grown in medium containing heat-inactivated serum (2%) for 72 h. Serum- and glucose-deprived cells were incubated in DME medium in the absence (basal) or presence of the indicated concentrations of insulin for 1 h at 37°C. Glucose uptake was determined in duplicate or triplicate at each point after the addition of 10 μl of substrate (2-[^{3}H]deoxyglucose or L-[^{3}H]-glucose, 0.1 μCi, final concentration 0.01 mmol) to provide a concentration at which cell membrane transport was rate limiting. The value for L-glucose was subtracted to correct for the contributions of diffusion and trapping.

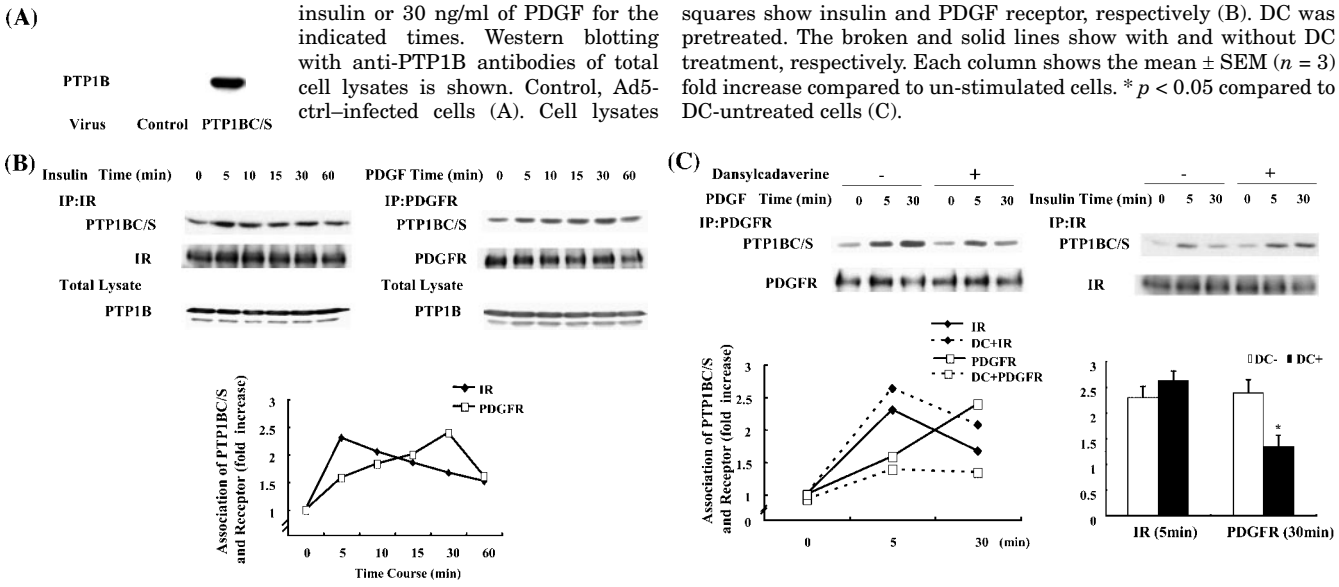
Glycogen Synthesis—Glycogen synthesis was measured as described previously (*23*). 3T3-L1 adipocytes were infected with various Ad5-PTP1B at 50 MOI for 16 h and then grown in medium containing 2% heat-inactivated serum for 56 h. The cells were serum starved for 16 h, and then the medium was replaced with DME medium containing 1% BSA. The cells were incubated with [^{14}C]-glucose (0.4 μCi/well) and 100 ng/ml insulin for 2 h in a CO_2 incubator, washed with ice-cold PBS 5 times, and then lysed with 2 N NaOH at 55°C for 45 min. Synthesized [^{14}C]-glycogen was precipitated with cold glycogen in 95% ethanol and washed, and then the radioactivity was measured.

Statistics—The values are expressed as means ± SEM, unless otherwise stated. Scheffe's multiple comparison test was used to determine the significance of differences among more than three groups. $p < 0.05$ was considered significant.

RESULTS

Association of PTP1BC/S with Insulin or PDGF Receptor in 3T3-L1 Adipocytes—To determine how PTP1B could interact with insulin or PDGF receptor, we examined the time-course of receptor association with PTP1B. Because

Fig. 1. **Time course of association between PTP1BC/S and insulin receptor or PDGF receptor.** Differentiated 3T3-L1 adipocytes were infected with Ad5-PTP1BC/S at 50 MOI for 16 h. After 56-h incubation, the cells were starved for 16 h, pretreated with or without 100 μM dansylcadaverine (DC) for 30 min, and then stimulated with or without 100 ng/ml of insulin or 30 ng/ml of PDGF for the indicated times. Western blotting with anti-PTP1B antibodies of total cell lysates is shown. Control, Ad5-ctrl–infected cells (A). Cell lysates were immunoprecipitated (IP) with anti-insulin receptor (IR) antibodies or anti-PDGF receptor (PDGFR) antibodies. Immunocomplexes were analyzed by Western blotting with anti-PTP1B antibodies, and reblotted with insulin receptor or anti-PDGF receptor antibodies, respectively. The graph shows the fold increase in the association of PTP1BC/S and receptors. Closed diamonds and open squares show insulin and PDGF receptor, respectively (B). DC was pretreated. The broken and solid lines show with and without DC treatment, respectively. Each column shows the mean ± SEM (n = 3) fold increase compared to un-stimulated cells. * $p < 0.05$ compared to DC-untreated cells (C).



PTP1BWT is thought to dissociate from these receptors after dephosphorylation, we employed a phosphatase-defective cysteine^{215}/serine^{215} mutant of PTP1B (PTP1BC/S). PTP1BC/S was strongly expressed in 3T3-L1 adipocytes on Ad5-PTP1BC/S infection (Fig. 1A). As shown in Fig. 1B, the association between PDGF receptor and PTP1B became its maximum at 30 min after PDGF stimulation. In contrast, the insulin-stimulated association of PTP1B with insulin receptor became maximal within 5 min. Thus, PTP1B may interact with insulin receptor *via* a different mechanism from in the case of PDGF receptor.

Next, we examined whether insulin receptor internalization was also needed for insulin receptor to associate with PTP1B. Pretreatment with dansylcadaverine (100 μM), a specific inhibitor of receptor-internalization (*24*, *25*), did not interfere with the association of PTP1BC/S with insulin receptor, but it did interfere with that with PDGF receptor (Fig. 1C).

Inhibition of Recepter-Internalization by Dansylcadaverine—Treatment with the present concentration of dansylcadaverine completely inhibited insulin-receptor internalization without affecting of the intracellular protein level of Akt, with a method described previously (*26*) (Fig. 2A). Preincubation with 100 μM dansylcadaverine did not affect the binding of insulin to its receptor (2.42 ± 0.32 pmol/500 μg protein to 2.37 ± 0.34 pmol/500 μg protein). Moreover, dansylcadaverine inhibited insulin-induced Shc phosphorylation, in accordance with the previous report that receptor internalization was necessary for Shc phosphorylation (*25*) (Fig. 2B). These results clearly demonstrate that dansylcadaverine actually inhibits receptor internalization in our system.

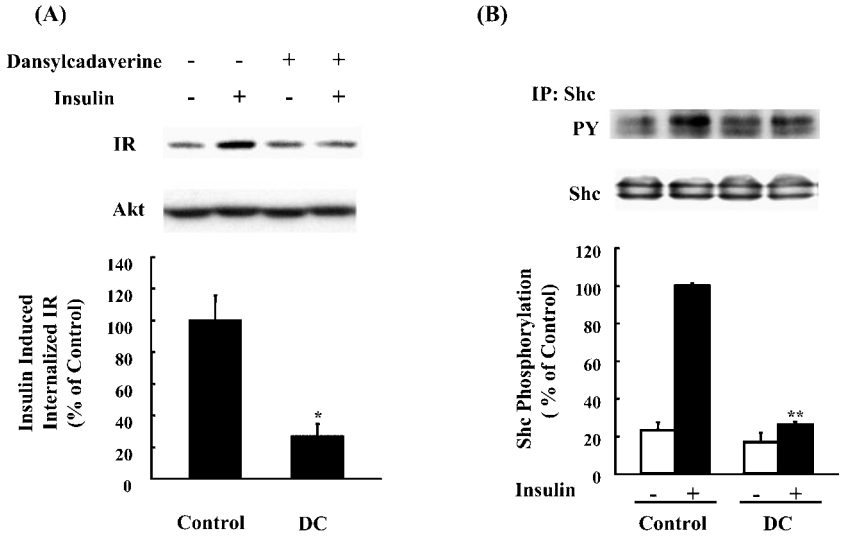


Fig. 2. **Dansylcadaverine inhibits receptor internalization.** Differentiated 3T3-L1 adipocytes were pretreated with or without (Control) 100 μM dansylcadaverine (DC) for 30 min, stimulated with or without 100 ng/ml of insulin for 30 min, and treated with 1 mg/ml trypsin for 30 min on ice, and then Western blotting was performed with anti-insulin receptor (IR) and Akt antibodies. Each column shows the mean ± SEM (n = 3) for the increased internalized insulin receptor. * $p < 0.05$ compared to DC-untreated cells (A). Differentiated 3T3-L1 adipocytes were pretreated with or without (Control) 100 μM DC for 30 min, stimulated with or without 100 ng/ml of insulin for 5 min, immunoprecipitated (IP) with anti-Shc antibodies, and then blotted with phospho-tyrosine (PY) antibodies. Each column shows the mean ± SEM (n = 3) percentage compared to insulin-stimulated DC-untreated cells. ** $p < 0.01$ compared to DC-untreated cells (B).

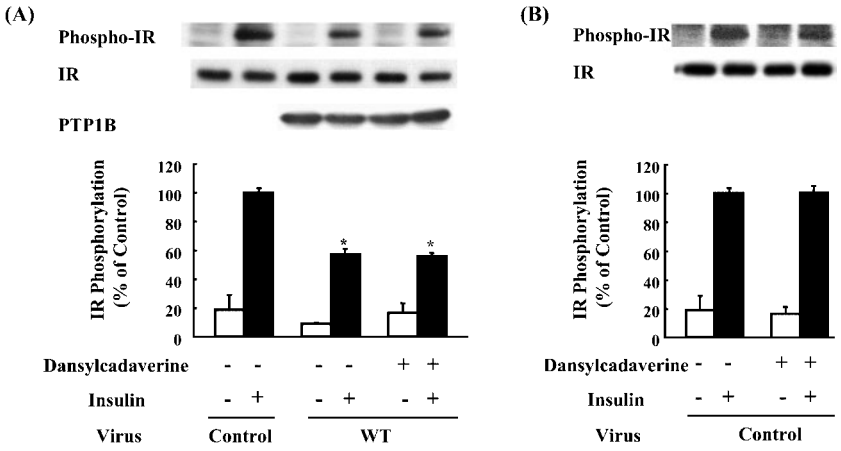


Fig. 3. **Dansylcadaverine does not affect decreased insulin receptor phosphorylation by PTP1B.** Ad5-PTP1BWT– (WT) or Ad5-ctrl–infected 3T3-L1 adipocytes (Control) were incubated with or without 100 μM dansylcadaverine for 30 min and then stimulated with 100 ng/ml of insulin for 5 min. The cells were lysed, and the analyzed by SDS-PAGE followed by Western blotting with anti-phospho-insulin receptor (Phospho-IR), anti-insulin receptor (IR), and anti-PTP1B (PTP1B) antibodies. Each column shows the mean ± SEM (n = 3) fold increase compared to insulin-stimulated control cells. $^{*}p < 0.05$ compared to insulin-stimulated control cells.

Effect of Dansylcadaverine on PTP1B-Induced Inhibition of Insulin Receptor Phosphorylation—To determine whether impaired receptor internalization could affect insulin-receptor dephosphorylation by PTP1B, we incubated cells with 100 μM dansylcadaverine. However, dansylcadaverine treatment did not interfere with the inhibitory effect of overexpressed PTP1BWT on insulin receptor phosphorylation (Fig. 3).

Expression of PTP1BWT and PTP1BΔCT in 3T3-L1 Adipocytes—To determine the role of ER-anchoring of PTP1B, PTP1BWT and PTP1B with a 35–amino-acid deletion at the COOH-terminus (PTP1BΔCT) were employed. To confirm their intracellular localization, 3T3-L1 adipocytes were infected with Ad5-PTP1BWT or Ad5-PTP1BΔCT at 50 MOI for 16 h, and then fractionated. PTP1BWT and PTP1BΔCT were expressed at similar levels in 3T3-L1 adipocytes, the increases being about 5–8 fold compared with in the case of endogenous PTP1B (*6*). As shown in Fig. 4A, PTP1BWT was mainly detected in the intracellular membrane fraction. In contrast, PTP1BΔCT was found almost exclusively in the cytosol fraction. After insulin stimulation, small amounts of PTP1BWT and PTP1BΔCT appeared in the plasma membrane fraction. Thus, portions of PTP1BWT and PTP1BΔCT moved to the plasma membrane after insulin stimulation. The immunohistochemical staining confirmed these observation (Fig. 4B).

Next, *in vitro* PTP activity was measured with pNPP as a substrate. As shown in Fig. 4C, overexpression of PTP1BWT and PTP1BΔCT stimulated PTP activity by

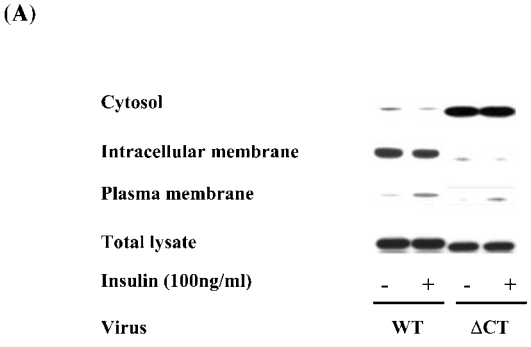
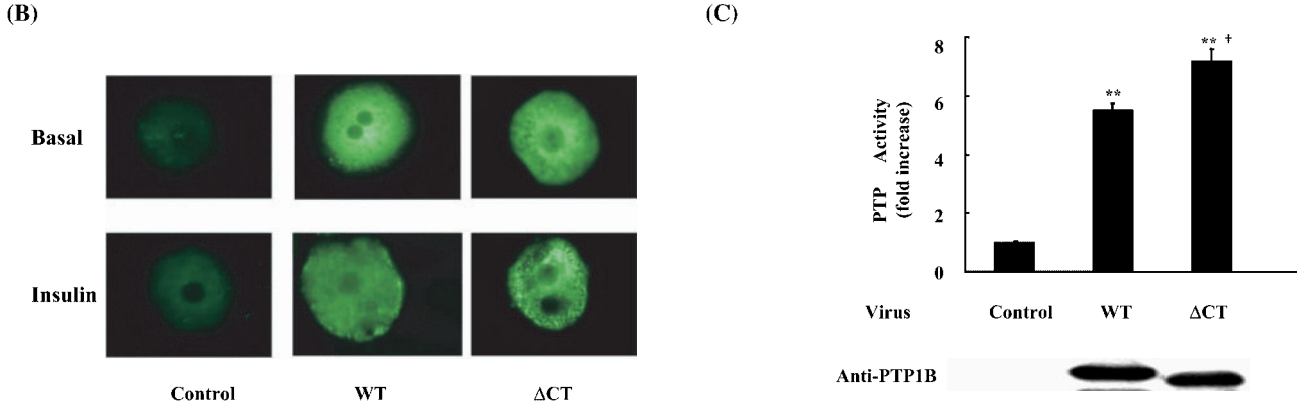


Fig. 4. **Expression of PTP1B and *in vitro* PTP activity in 3T3-L1 adipocytes and primary cultured hepatocytes.** 3T3-L1 adipocytes expressing wild-type PTP1B (WT) and PTP1BΔCT (ΔCT) were stimulated with or without 100 ng/ml insulin for 5 min, lysed, and then fractionated into cytosol and membrane fractions as described under Materials and Methods. Each fraction was analyzed by immunoblotting with anti-PTP1B antibodies (A). Ad5-PTP1BWT (WT) or Ad5-PTP1BΔCT (ΔCT) infected primary hepatocytes were incubated with anti-PTP1B antibodies. Control, Ad5-ctrl–infected cells (B). 3T3-L1 adipocytes infected with Ad5-ctrl (Control), Ad5-PTP1BWT (WT), or Ad5-PTP1BΔCT (ΔCT) at 50 MOI for 16 h were lysed and immunoprecipitated with anti-PTP1B antibodies, and then *in vitro* PTP activity was measured with pNPP as the substrate. Each PTP activity level shown is the mean ± SEM of six experiments, and the values are expressed as the fold increase above the basal activity observed for Ad5-ctrl–infected cells. **Indicates a significant difference compared to Control ($p < 0.01$). †Indicates a significant difference between Ad5-PTP1BΔCT–infected cells and Ad5-PTP1BWT–infected cells ($p < 0.05$) (C).



about 5.5-fold and 7.2-fold, respectively. Although the activity paralleled the expression level of PTP1BWT as described previously (*6*), PTP1BΔCT exhibited higher activity than PTP1BWT.

Effects of PTP1B Overexpression on Tyrosine Phosphorylation of Insulin Receptor and IRS-1, and on Association of IRS-1 with the p85 Subunit of PI 3-Kinase—In accordance with our previous report (*6*), the levels of insulin-stimulated tyrosine phosphorylation of insulin receptor and IRS-1 decreased to 53.2 ± 5.9% and 65.1 ± 11.5% of those in the control, respectively, in Ad5-PTP1BWT–infected 3T3-L1 adipocytes ($p < 0.05$) (Fig. 5). PTP1BΔCT showed similar effects to PTP1BWT as to prevention of insulin-stimulated phosphorylation of insulin receptor and IRS-1, and association of IRS-1 with the p85 subunit of PI 3-kinase (Fig. 5A–E). These inhibitory effects of PTP1BWT and PTP1BΔCT on insulin-stimulated receptor phosphorylation paralleled the expression levels of the PTP1B protein, and there was no difference between PTP1BWT and PTP1BΔCT (Fig. 6).

The effects of PTP1BΔCT expression on the downstream signaling of PI 3-kinase, such as Akt and p70S6 kinase phosphorylation, were also similar to those of PTP1BWT expression (data not shown).

Effects of PTP1B Localization on 2-Deoxyglucose Uptake and Glycogen Synthesis in 3T3-L1 Adipocytes—We measured the effects of PTP1B constructs on insulin-stimulated glucose uptake in 3T3-L1 adipocytes. As shown in Fig. 7, PTP1BΔCT inhibited glucose uptake by only about 20% compared to the control, *i.e.*, to the same level as PTP1BWT.

We also examined [^{14}C]-glucose incorporation into glycogen in 3T3-L1 adipocytes. There was no significant difference between the effects of PTP1BWT and PTP1BΔCT on glycogen synthesis (data not shown).

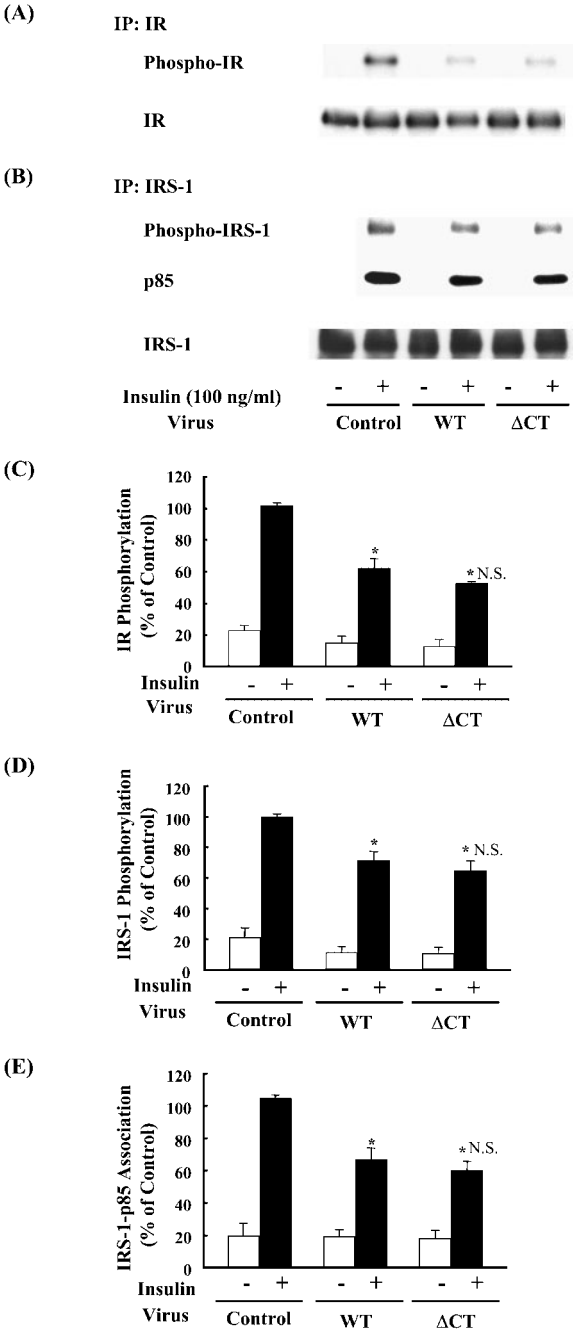


Fig. 5. **Effects of overexpression of PTP1B constructs on tyrosine phosphorylation of insulin receptor and IRS-1, and insulin-induced association of IRS-1 with p85 of PI 3-kinase.** Differentiated 3T3-L1 adipocytes were infected with Ad5-ctrl (Control), Ad5-PTP1B-WT (WT), or Ad5-PTP1BΔCT (ΔCT) at 50 MOI for 16 h. After 56-h incubation, the cells were starved for 16 h and then stimulated with or without 100 ng/ml insulin for 5 min. The cells were then lysed and immunoprecipitated (IP) with anti-insulin receptor antibodies (IR) or anti-IRS-1 antibodies (IRS-1). Immunocomplexes were analyzed by Western blotting with either phosphotyrosine antibodies (RC20H) (A and B, upper panels) or anti-p85 antibodies (B, middle panel). Each membrane was reblotted with the corresponding antibodies. Each Western blot is representative one of at least three independent experiments. The tyrosine phosphorylation level was determined with NIH Image, and then the degree of phosphorylation (count of tyrosine phosphorylation/protein amount) was calculated. Each column shows the mean ± SEM (n = 3–4) fold increase in Ad5-ctrl–infected and insulin-stimulated cells. $^{*}p < 0.05$ compared to the insulin-stimulated values for Ad5-ctrl–infected cells (C, D and E); N.S. compared to WT.

DISCUSSION

The translocation of signaling molecules to the plasma membrane is critical for mediation of insulin signaling. We previously reported that membrane targeting of PI 3-kinase and PDK-1 stimulated downstream molecules, even though overexpression of the wild-type protein did not (*8*, *9*). Thus, intracellular localization and compartmentalization of PTPs may also play important roles in determination of their activities and substrate specificities (*10*). It was previously reported that PTP1B is anchored to the ER via the 35 COOH-terminal amino acids and is activated after release into the cytosol through truncation of its COOH-terminus (*12*). Moreover, it was demonstrated that calpain-induced cleavage within the COOH-terminus of PTP1B caused relocation of the enzyme from membranes to the cytosol and triggering of its tyrosine phosphatase activity (*13*). Furthermore, we reported that high-glucose conditions increased the PTP1B content in the cytosolic fraction (*4*). On the other hand, a recent report noted that PDGF receptor

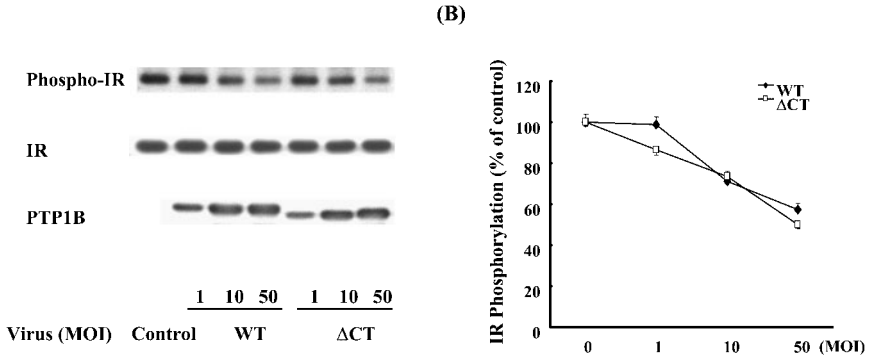


Fig. 6. **Dose-dependent effects of PTP1B expression on insulin receptor dephosphorylation.** Cells were infected with Ad5-ctrl (Control), Ad5-PTP1BWT (WT), or Ad5-PTP1BΔCT (ΔCT) at the indicated MOI for 16 h, and then simulated with 100ng/ml insulin for 5 min, lysed, and analysed by SDS-PAGE, following by Western Blotting with phospho-tyrosine antibodies or PTP1B antibodies, and reblotted with anti-IR antibodies. Control, Ad5-ctrl–infected cells (A). The tyrosine phosphorylation level was determined with NIH Image, and then the degree of phosphorylation (count of tyrosine phosphorylation/protein amount) was calculated. The graph shows the mean ± SEM (n = 3) fold increase in Ad5-ctrl–infected cells (B).

was internalized after stimulation and dephosphorylated by PTP1B at the ER, the maximum effect being observed at 30 min (*14*). Thus, the molecular mechanism by which PTP1B can regulate this signal transduction remains unclear.

In the present study, we overexpressed a phosphatase-inactive PTP1BC/S in 3T3-L1 adipocytes by means of adenovirus-mediated gene transfer to determine how PTP1B could interact with these receptors. We observed that insulin maximally stimulated the association of its receptor with PTP1B at 5 min, while PDGF showed the maximum effect at 30 min (Fig. 1B). These findings suggest the possibility that PTP1B dephosphorylates these receptors through different mechanisms. Consistent with this hypothesis, dansylcadaverine inhibited the association of PDGF receptor with PTP1B, but did not inhibit that between insulin receptor and PTP1B. Thus, it is possible that insulin stimulates translocation of PTP1B from the ER to the plasma membrane, where PTP1B dephosphorylates insulin receptor. In the case of PDGF receptor,

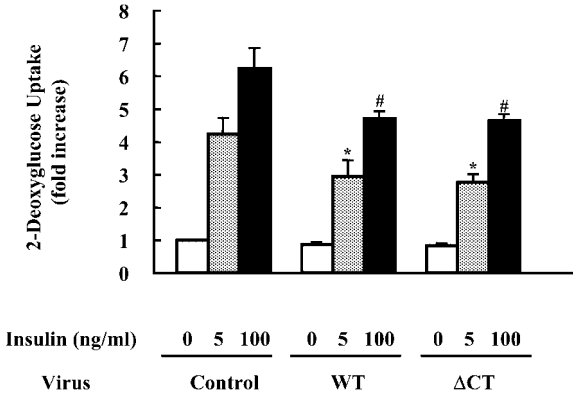


Fig. 7. **Effect of PTP1B expression on 2-deoxyglucose uptake in 3T3-L1 adipocytes.** Differentiated 3T3-L1 adipocytes infected with Ad5-PTP1BWT or Ad5-PTP1BΔCT at 50 MOI were stimulated with insulin at the indicated concentrations (ng/ml) for 60 min. Each column shows the mean ± SEM (n = 3) fold increase compared to Ad5-ctrl–infected un-stimulated cells. $^{*}p < 0.05$ and $p < 0.05$ compared to the 5 ng/ml and 100 ng/ml insulin-stimulated values for Ad5-ctrl–infected cells (Control), respectively.

it became internalized and is dephosphorylated by PTP1B at the ER surface.

To clarify the importance of anchoring of PTP1B to the ER, we overexpressed wild-type PTP1B (PTP1BWT) or cytosolic form PTP1B (PTP1BΔCT), which is truncated at its COOH-terminus, in 3T3-L1 adipocytes. We confirmed their localization by subcellular fractionation, as shown in Fig. 4A. We were able to detect PTP1B translocation after insulin stimulation on Western blotting, even though the level was very low. Furthermore, portions of PTP1BWT and PTP1BΔCT moved to the plasma membrane after insulin stimulation, as judged on immunohistochemical analysis. We hypothesized that COOH-terminal truncation might enhance the activity of PTP1B, and the phosphatase activity of PTP1BΔCT was significantly increased as compared with that of PTP1BWT. If translocation of PTP1B from the ER to the cytosol is important, PTP1BΔCT should inhibit insulin signaling more potently. However, PTP1BΔCT showed identical effects to those of PTP1BWT in this study. One possible explanation is that all PTP1BWT molecules are released into the cytosol after insulin stimulation, and thus there are no differences between PTP1BWT and PTP1BΔCT. In that case, PTP1BΔCT should act more potently under the basal conditions. However, no significant effects on glucose transport and glycogen synthesis were observed under the basal conditions. Moreover, PTP1BΔCT migrated to a position corresponding to a lower molecular weight than did PTP1BWT on SDS-PAGE, but PTP1BWT did not migrate to one corresponding to a lower molecular weight after insulin stimulation, although we cannot rule out the possibility that a small portion of PTP1B was released without being detected. Thus, we cannot conclude that translocation from the ER to the cytosol is essential for the inhibitory effect of PTP1B on the insulin action observed in this study. Localization at the plasma membrane may be more important.

In summary, our data indicate that PTP1B negatively regulates insulin signaling through dephosphorylation of insulin receptor without receptor internalization into the ER, and that ER anchoring of PTP1B is not essential for inhibition of insulin signaling, suggesting that PTP1B may be translocated from the ER to the plasma mem-

brane by insulin. On the other hand, PDGF receptor presumably becomes internalized and is dephosphorylated by PTP1B at the ER.

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