Expression of Dominant Negative Mutant SHPTP2 Attenuates Phosphatidylinositol 3’-Kinase Activity via Modulation of Phosphorylation of Insulin Receptor Substrate-1*

(Received for publication, September 7, 1995, and in revised form, March 11, 1996)

Satoshi Ugi, Hiroshi Maegawa, Atsunori Kashiwagi, Masaaki Adachi, Jerrold M. Olefsky,†, and Ryuichi Kikkawa

From the Third Department of Medicine, Shiga University of Medical Science, Otsu, Shiga 520-21, J apan, the Department of Medicine, Sapporo Medical College, Sapporo 060, J apan, and the Department of Medicine, University of California, San Diego, La Jolla, California 92039

To clarify the role of protein-tyrosine phosphatase (PTPase) containing Src homology 2 regions (SHPTP2) in insulin signaling, either wild-type or mutant SHPTP2 (ΔPTP; lacking full PTPase domain) was expressed in Rat 1 fibroblasts overexpressing human insulin receptors. In response to insulin, phosphorylation of insulin receptor substrate 1 (IRS-1), IRS-1-associated PTPase activities and phosphatidylinositol (PI) 3’-kinase activities were slightly enhanced in wild-type cells when compared with those in the parent cells transfected with hygromycin-resistant gene alone. In contrast, introduction of ΔPTP inhibited insulin-induced association of IRS-1 with endogenous SHPTP2 and impaired both insulin-stimulated phosphorylation of IRS-1 and activation of PI 3’-kinase. Furthermore, decreased content of p85 subunit of PI 3’-kinase was also found in mutant cells. Consistently, the insulin-stimulated mitogen-activated protein kinase activities and DNA synthesis were also enhanced in wild-type cells, but impaired in mutant cells. Thus, the interaction of SHPTP2 with IRS-1 may be associated with modulation of phosphorylation levels of IRS-1, resulting in the changes of PI 3’-kinase and mitogen-activated protein kinase activity. Furthermore, an impaired insulin signaling in mutant cells may be partly reflected in a decreased content of p85 protein of PI 3’-kinase.

The phosphorylation state of tyrosine residue is regulated by both protein-tyrosine kinase (PTK) and protein-tyrosine phosphatase (PTPase) and is critical for cell growth, differentiation and metabolism (1, 2). Insulin binding to its receptor results in the sequential autophosphorylation of tyrosine residues in the cytoplasmic region of the receptor, stimulation of receptor PTK, then the phosphorylation of insulin receptor substrate-1 (IRS-1). IRS-1 contains several potential phosphorylation sites at tyrosine residues (3), and tyrosine-phosphorylated IRS-1 binds to phosphatidylinositol (PI) 3’-kinase and Grb2 through the association of their Src homology 2 (SH2) regions (4). SH2 domains, at first identified in Src family PTK, are regions of about 100 amino acids, and they can directly interact with proteins containing phosphotyrosine. Thus, SH2 domains are thought to play important roles in signal transduction via tyrosine phosphorylation (5, 6).

The novel non-transmembrane PTPase, SHPTP2 (7, 8) (also known as Syp (9), PTP1D (10), and PTP2C (11)), which contains a single phosphatase domain and two adjacent copies of the SH2 domains of the Torso receptor PTK. These data suggest an important role for SHPTP2 downstream of the receptor PTK in mammalian cells. In fact, activated platelet-derived growth factor and epidermal growth factor receptors bind directly to the SH2 domains of SHPTP2, leading to the phosphorylation of the PTPase on tyrosine residues, which further stimulates its catalytic activity and positively regulates mitogenic signaling of these growth factor receptors via SHPTP2-Grb2-Sos complex formation (9, 10, 13–16).

Although it has been reported that SHPTP2 also binds to tyrosine-phosphorylated IRS-1 in vitro and in vivo (13, 17–19), the precise role of SHPTP2 in insulin signal transduction remains unclear. Recently, Xiao et al., have reported that micro-injection of either anti-Syp antibody or the GST-SH2 fusion protein of Syp into Rat 1 fibroblasts over expressing human insulin receptors (HIRc) blocks insulin-stimulated DNA synthesis (20). Furthermore, Milarski et al. (21) have reported that the introduction of a catalytically inactive mutant Syp (Cys-459 → Ser) in NIH-3T3 cells leads to impairment of insulin-stimulated mitogen-activated protein (MAP) kinase activity and thymidine uptake. Milarski’s results have been confirmed by Noguchi (22) and Yamauchi (23), who showed that the introduction of either catalytically inactive (Cys-459 → Ser) or a SH2 mutant of SHPTP2 into Chinese hamster ovary cells over-expressing the human insulin receptor (CHO-IR) attenuates insulin-stimulated MAP kinase activity (22, 23), but not insulin-stimulated PI 3’-kinase activity (22). Furthermore, overex-
SHPTP2 Regulates PI 3'-Kinase Activity

pressing wild-type SHPTP2 provides no additional increment in insulin-stimulated MAP kinase (22, 23) and PI 3'-kinase activities in CHO-IR cells (22).

However, in our preliminary study, the introduction of mutant SHPTP2, which completely lacked the catalytic domain (∆PTP), attenuated insulin-stimulated PI 3'-kinase activity in HIRc cells. Furthermore, overexpression of wild-type SHPTP2 enhanced MAP kinase activity. The contrast of this stimulation to the inhibition observed in CHO-IR cells suggests that the influence of introduction of wild-type or mutant SHPTP2 differs between CHO cells and Rat 1 fibroblasts. Therefore, we examined insulin signaling in cells overexpressing either wild-type (WT) or ∆PTP (MT) to clarify the role of SHPTP2 in insulin signal transduction. We found that insulin-stimulated PI 3'-kinase activities were enhanced in WT cells, but attenuated in MT cells, resulting in modulation of DNA synthesis via the regulation of IRS-1 phosphorylation. This is the first direct evidence that SHPTP2 positively regulates both the insulin-stimulation of MAP kinase cascade and PI 3'-kinase pathway.

EXPERIMENTAL PROCEDURES

Materials—Porcine insulin was a gift from Novo-Nordisk Pharmar (Copenhagen, Denmark) and Eli Lilly Co. (Indianapolis, IN). Porcine insulin 125I-labeled at TyrA14 (125I-insulin; 2200 Ci/mmol), Pharam (Copenhagen, Denmark) and Eli Lilly Co. (Indianapolis, IN). Materials—Chemicals (Uppsala, Sweden). Aprotinin, phenylmethylsulfonyl fluoride (PMSF), and hygromycin B were from Wako Chemical Inc. (Osaka, Japan). All other reagents were from United Kingdom). Lipofectin reagent was from Life Technologies, Inc. (Grand Island, NY). Immobilon-P, and immunoblotting with specific antibodies (pNY20, α-RS, and α-Syp). The bios were then incubated with horseradish peroxidase-linked second antibody, followed by enhanced chemiluminescence detection according to the manufacturer's instructions.

PI 3'-Kinase Activity—PI 3'-kinase activity was assayed with either anti-IRS-1 or anti-p85 subunit antisense was measured according to the method of Okamoto et al. (33). Cells were starved for 24 h and then stimulated with 100 nM insulin for 5 min. The cells were then washed with ice-cold PBS and lysed with 250 μl of 1% Triton X-100, 50 mM sodium orthovanadate, 1 mM PMSF, 0.1 mM aprotinin, 1 μg/ml leupeptin, and then centrifuged. The supernatant (1 mg of protein) was incubated with GST-IRS-1 or GST-p85 fusion proteins for 3 h, then incubated for another 1 h with Protein G-Sepharose at 4°C. The immunoprecipitated proteins were washed three times with phosphate-buffered saline containing 1% Nonidet P-40, 100 μM sodium orthovanadate, 100 mM NaCl, 50 mM NaF, 50 μg/ml aprotinin at 4°C for 20 min. The supernatants were immunoprecipitated with GST-IRS-1 or GST-p85 fusion protein coupled to glutathione-Sepharose beads in the presence of phosphatase inhibitors for 90 min at 4°C. After extensively washing, bound proteins to either GST-SHPTP2 or GST-SH2 fusion protein were resolved by SDS-PAGE, electrophoresed to Immobilon-P, and immunoblotted with αPY20, α-RS-1, and α-Syp. The bios were then incubated with horseradish peroxidase-linked second antibody, followed by enhanced chemiluminescence detection according to the manufacturer's instructions.

Porcine insulin 125I-labeled at TyrA14 (125I-insulin; 2200 Ci/mmol), Pharam (Copenhagen, Denmark) and Eli Lilly Co. (Indianapolis, IN). Materials—Chemicals (Uppsala, Sweden). Aprotinin, phenylmethylsulfonyl fluoride (PMSF), and hygromycin B were from Wako Chemical Inc. (Osaka, Japan). All other reagents were from United Kingdom). Lipofectin reagent was from Life Technologies, Inc. (Grand Island, NY). Immobilon-P, and immunoblotting with specific antibodies (pNY20, α-RS, and α-Syp). The bios were then incubated with horseradish peroxidase-linked second antibody, followed by enhanced chemiluminescence detection according to the manufacturer's instructions.

PI 3'-Kinase Activity—PI 3'-kinase activity was assayed with either anti-IRS-1 or anti-p85 subunit antisense was measured according to the method of Okamoto et al. (33). Cells were starved for 24 h and then stimulated with 100 nM insulin for 5 min. The cells were then washed with ice-cold PBS and lysed with 250 μl of 1% Triton X-100, 50 mM sodium orthovanadate, 1 mM PMSF, 0.1 mM aprotinin, 1 μg/ml leupeptin, and then centrifuged. The supernatant (1 mg of protein) was incubated with GST-IRS-1 or GST-p85 fusion proteins for 3 h, then incubated for another 1 h with Protein G-Sepharose at 4°C. The immunoprecipitated proteins were washed three times with phosphate-buffered saline containing 1% Nonidet P-40, 100 μM sodium orthovanadate, 100 mM NaCl, 50 mM NaF, 50 μg/ml aprotinin at 4°C for 20 min. The supernatants were immunoprecipitated with GST-IRS-1 or GST-p85 fusion protein coupled to glutathione-Sepharose beads in the presence of phosphatase inhibitors for 90 min at 4°C. After extensively washing, bound proteins to either GST-SHPTP2 or GST-SH2 fusion protein were resolved by SDS-PAGE, electrophoresed to Immobilon-P, and immunoblotted with αPY20, α-RS-1, and α-Syp. The bios were then incubated with horseradish peroxidase-linked second antibody, followed by enhanced chemiluminescence detection according to the manufacturer's instructions.

PI 3'-Kinase Activity—PI 3'-kinase activity was assayed with either anti-IRS-1 or anti-p85 subunit antisense was measured according to the method of Okamoto et al. (33). Cells were starved for 24 h and then stimulated with 100 nM insulin for 5 min. The cells were then washed with ice-cold PBS and lysed with 250 μl of 1% Triton X-100, 50 mM sodium orthovanadate, 1 mM PMSF, 0.1 mM aprotinin, 1 μg/ml leupeptin, and then centrifuged. The supernatant (1 mg of protein) was incubated with GST-IRS-1 or GST-p85 fusion proteins for 3 h, then incubated for another 1 h with Protein G-Sepharose at 4°C. The immunoprecipitated proteins were washed three times with phosphate-buffered saline containing 1% Nonidet P-40, 100 μM sodium orthovanadate, 100 mM NaCl, 50 mM NaF, 50 μg/ml aprotinin at 4°C for 20 min. The supernatants were immunoprecipitated with GST-IRS-1 or GST-p85 fusion protein coupled to glutathione-Sepharose beads in the presence of phosphatase inhibitors for 90 min at 4°C. After extensively washing, bound proteins to either GST-SHPTP2 or GST-SH2 fusion protein were resolved by SDS-PAGE, electrophoresed to Immobilon-P, and immunoblotted with αPY20, α-RS-1, and α-Syp. The bios were then incubated with horseradish peroxidase-linked second antibody, followed by enhanced chemiluminescence detection according to the manufacturer's instructions.

Western Blot Analysis of p85 Subunit of PI 3'-Kinase in the Cells—Western blot analysis was performed with cell lysates of CHO cells transfected with wild-type or mutant SHPTP2. The parental cells transfected with phg vector alone were used for control in our study (phg).

Insulin Binding Assay—Insulin binding to each cell line was determined as described (29). In brief, 125I-insulin binding to cells (5 × 10^3 cells) was measured at 8.3 nM insulin concentration in Eagle’s medium containing 1% bovine serum albumin and 20 mM HEPS (pH 7.6) at 4°C for 6 h.

Measurement of PTPase Activity—PTPase activity was measured with pNPP as the substrate as described by Sugimoto et al. (30). The immunoprecipitate with GST-SH2-SHPTP2 antisera was incubated with 10 μM pNPP at 30°C for 15 min in 50 μl of 50 μM 3,3-dimethylglyutarate (pH 5.6), containing 50 mM NaCl, 10 mM diithiothreitol, and 2 mM EDTA. The reactions were terminated with 950 μl of 1 n NaOH. The amount of para-nitrophenol produced was determined by measuring the absorbance at 405 nm.

Association of GST-SHPTP2 Fusion Proteins with Phosphorylated Proteins in Response to Insulin—In insulin-stimulated association of GST-SHPTP2 fusion proteins with phosphorylated proteins were analyzed according to method of Staubs et al. (31). In brief, GST fusion proteins containing either single-length SHPTP2 or only SH2 domains of SHPTP2 (∆PTP) were made as described (25, 32). To prepare whole cell lysates, cells were starved for 24 h. Then, cells were stimulated with 100 nM insulin for 1 min at 37°C. Following insulin stimulation, cell lysates (500 μg of protein) were incubated with either GST-SHPTP2 or GST-SH2 fusion protein coupled to glutathione-Sepharose beads in the presence of phosphatase inhibitors for 90 min at 4°C. After extensively washing, bound proteins to either GST-SHPTP2 or GST-SH2 fusion protein were resolved by SDS-PAGE, electrophoresed to Immobilon-P, and immunoblotted with αPY20.

Insulin-stimulated Tyrosine Phosphorylation and Association of IRS-1 with SH-PTP2—Cells were starved in serum-free Dulbecco’s modified Eagle’s medium for 24 h, then stimulated with insulin at 37°C for 5 min. Thereafter, the cells were lysed in 20 mM Tris-CI (pH 7.5) containing 1 mM EDTA, 140 mM NaCl, 1% Nonidet P-40, 1 mM sodium orthovanadate, 1 mM PMSF, 50 mM NaF, 50 μg/ml aprotinin at 4°C for 20 min. The cell lysates were centrifuged to remove insoluble materials at 15,000 × g for 20 min. The supernatants were immunoprecipitated at 4°C with αIRS-1. The bound proteins were then resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), electrophoresed to Immobilon-P, and immunoblotted with specific antibodies (αPY20, α-RS-1, and α-Syp). The bios were then incubated with horseradish peroxidase-linked second antibody, followed by enhanced chemiluminescence detection according to the manufacturer's instructions.
HICr cells and established several cell lines overexpressing cells at a concentration of 8.3 nM was 43.6 ± 1.3%.

Cells—
a protein kinase inhibitor, 50 mM sodium orthovanadate, 10 mM NaF, 10 mM sodium pyrophosphate, 1 mM EGTA, 1 mM PMSF, 10 μg/ml leupeptin. After a brief sonication, the cell lysates were centrifuged and 10 μl of the supernatants were assayed for kinase activity, in a final volume of 40 μl containing 1 μM protein kinase inhibitor, 50 μM ATP, 2 μCi of [γ-32P]ATP, and 20 μg of myelin basic protein at 25°C for 15 min. Twenty-five μl of the reaction was transferred onto P81 phosphocellulose paper, a 2.5-cm diameter (Whatman). The papers were washed with 180 mM phosphoric acid five times and then rinsed with 95% ethanol. Phosphorylation was quantified by scintillation counting.

BrdUrd Incorporation—The cells were grown on glass coverslips, starved for 48 h, and then stimulated with 10 μM insulin. Fifteen hours later, the cells were pulsed for 30 min with BrdUrd. The cells were fixed and stained using a monoclonal anti-BrdUrd antibody and peroxidase-linked second antibody according to the manufacturer’s recommendations.

Statistical Analysis—Data are means ± S.E. as indicated. The p values were determined by Scheffe’s multiple comparison test, and p < 0.05 was considered statistically significant.

RESULTS

The Expression of Wild-type and ΔSHPTP2 in HICr Cells—We transfected either full-length SHPTP2 cDNA or cDNA encoding only the SH2 domains (ΔPTP) of SHPTP2 into HICr cells and established several cell lines overexpressing either wild-type (WT) or ΔPTP (MT). Immunoblot with the αSyp antibody showed that WT11 expressed 5-fold increases in SHPTP2 protein when compared with pHyg cells as shown in Fig. 1A. In agreement with the results of Western blotting, total cellular PTPase activity immunoprecipitated with αGST-SH2-SHPTP2 antiserum also increased in WT11 cells as summarized in column 1 in Table I. In contrast, although the mutant clone (MT15) expressed 2–3-fold more endogenous rat SHPTP2 as shown in Fig. 1B, total PTPase activity immunoprecipitated with αGST-SH2-SHPTP2 antiserum was similar to that of pHyg cells. To evaluate the effects of overexpression of wild-type and ΔPTP on insulin signaling, we first measured the level of insulin binding. Percent insulin binding to 5 × 10^6 cells at a concentration of 8.3 nM was 43.6 ± 1.3%, 45.8 ± 1.3%, and 47.8 ± 1.0% in pHyg, WT, and MT cells, respectively, suggesting that all clones had the same numbers of insulin receptors.

Association of GST-SHPTP2 Fusion Proteins with Phosphorylated Proteins in Response to Insulin—To determine whether the overexpression of either wild-type or ΔPTP could affect the insulin-stimulated tyrosine phosphorylation of cellular proteins, we tested the phosphorylation levels of insulin receptors and IRS-1. As shown in Fig. 3A, the insulin-stimulated phosphorylation levels of insulin receptor and IRS-1 were increased in WT11 cells and decreased in MT15 cells when compared with those of pHyg cells. When the insulin-stimulated phosphorylation levels of IRS-1 were assessed using densitometric scanning, the stimulation in WT11 cells and MT15 cells was 110 and 67% of that in pHyg cells, respectively. When αRS-1-immunoprecipitates were blotted with αPY20, we confirmed that phosphorylation levels of IRS-1 were increased in WT11 cells, and decreased in MT15 cells (Fig. 3B). However, the content of IRS-1 proteins was comparable among these cells (Fig. 3C).

Since SHPTP2 was associated with IRS-1 both in vitro and in vivo as reported previously (13, 17–19), we tested whether this association could be observed in HICr cells. Cells were stimulated with insulin, and then the cell lysates were immunoğu-
SHPTP2 Regulates PI 3'-Kinase Activity

**Fig. 2.** Association of GST-SHPTP2 fusion proteins with phosphorylated proteins in response to insulin. After being starved for 24 h, cells were stimulated with 100 nM insulin for 1 min at 37°C. Following insulin stimulation, whole cell lysates (500 μg of protein) were incubated with either GST-SHPTP2 (right) or GST-SH2 (left) fusion protein coupled to glutathione-Sepharose beads for 90 min at 4°C. After extensive washing, bound proteins to either GST-SHPTP2 or GST-SH2 fusion protein were resolved by SDS-PAGE, electotransferred to membrane, and immunoblotted with αPY20.

**Fig. 3.** Insulin-stimulated phosphorylation of insulin receptor and IRS-1 in WT11, MT15, and pHyg cells. A, tyrosine-phosphorylated proteins in the cell lysate (200 μg of protein) from the basal and 10 nM insulin-stimulated cells were analyzed by Western blotting using αPY20. B, after immunoprecipitated with αIRS-1 antibody, bound proteins were analyzed by Western blotting using αPY20. Both basal and 10 nM insulin-stimulated phosphorylation levels of IRS-1 were presented. C, to quantify IRS-1 protein content, equal amount of cell lysates (40 μg of protein/line) was analyzed by Western blotting using αIRS-1 antibody.

**Fig. 4.** Association of IRS-1 with SHPTP2 in WT11, MT15, and pHyg cells. After cells were incubated with or without 100 nM insulin for 5 min, lysed, and immunoprecipitated with αIRS-1, bound proteins were resolved by SDS-PAGE. The immunoprecipitate was analyzed by Western blotting using αPy20.

 association of IRS-1 with SHPTP2 in the basal state was not consistent by different means. In contrast to WT11 cells, basal and insulin-stimulated association of IRS-1 with rat endogenous SHPTP2 was not detected in MT15 cells.

To assess how much SHPTP2 was associated with IRS-1 after insulin stimulation, we measured total cellular SHPTP2-specific PTPase activity and studied the effects of αIRS-1 immunabsorption on the PTPase activity in these cells. The total cellular PTPase activity of SHPTP2 was not stimulated by insulin (data not shown). In response to insulin, PTPase activity was decreased due to immunabsorption by αIRS-1 antibody by 0.005 and 0.169 ΔA405 nm/15 min/10^7 cells in pHyg cells and WT11 cells, respectively, but was not significantly affected in MT15 cells as shown in Table I. The activity reductions are explained by a portion of SHPTP2 binding to IRS-1 in response to insulin. Even with more than 5-fold greater SHPTP2 activity in WT11 cells than in pHyg cells, a 6-fold higher proportion of this elevated PTPase activity was removed. These results strongly suggest that overexpression of native SHPTP2 acts to enhance insulin-facilitated IRS-1 binding of the SHPTP2 and that overexpression of the SH2 region blocks this enhancement.

PI 3'-Kinase Activity Associated with IRS-1 and p85 Subunit—In response to insulin, PI 3'-kinase is activated through IRS-1-p85 complex formation and this pathway is thought to be independent of the MAP kinase cascade. We therefore measured the PI 3'-kinase activities in these cells. As shown in Fig. 5 (A and B), the levels of PI 3'-kinase activities associated with IRS-1 in all cell lines increased in response to insulin. However, the magnitude of activation in WT11 cells was greater than that in pHyg cells (141% of pHyg cells). On the other hand, the stimulation of the PI 3'-kinase in MT cells was attenuated (60% of pHyg cells).

It is possible that ΔPTP mutant, which consists only SH2 domains, nonspecifically inhibits p85 binding to IRS-1, resulting in the impaired PI 3'-kinase activation in MT cells. To rule out this possibility, we have performed the stoichiometric analysis to determine relationships between the degree of phosphorylation of IRS-1 and degree of its association of p85 in pHyg and MT cells. In response to insulin, IRS-1 was less phosphorylated in MT cells compared to that in pHyg cells as shown in Fig. 6A. However, when association of IRS-1 with p85 in MT cells was adjusted to the value of pHyg cells, degree of tyrosine-phosphorylation of IRS-1 in MT cells was only 20% of pHyg cells, suggesting that IRS-1 more efficiently binds p85 in MT cells (Fig. 6B). These results clearly indicate that impairment of insulin-stimulated PI 3'-kinase activity in MT cells may be mainly due to decreased levels of IRS-1 phosphorylation, but not due to nonspecifically inhibition of p85 binding to IRS-1 by
FIG. 5. Insulin-stimulated PI 3'-kinase activity in WT11, MT15, and pHyg cells using HIRc cells as parent cells. A, PI 3'-kinase activities immunoprecipitated with anti-IRS-1 antisem were measured as described under "Experimental Procedures." The radioactivity of phosphatidylinositol phosphate was measured by liquid scintillation counter. PI 3'-kinase activity in each cell line was presented as mean ± S.E. of four separate experiments (*, p < 0.05 compared to the corresponding activity in pHyg cells). B, PI 3'-kinase activity in each cell line was presented as mean ± S.E. of four separate experiments (D) (*, p < 0.01 compared to the corresponding activity in pHyg cells).

We have cloned another independent cell line, HIRY/F2 cells, which overexpress either wild-type (WT1) or mutant insulin receptors were expressed (28). We have cloned HIRY/F2 cells, which overexpress either wild-type (WT1) or ΔPTP (MT6) at expression levels similar to those for HIRc cells (WT11 and MT15). In HIRY/F2 cells, PI 3'-kinase activities immunoprecipitated with αIRS-1 antisem were also decreased in MT6 cells but increased in WT1 cells as shown in Fig. 7 (A and B). Furthermore, PI 3'-kinase activity immunoprecipitated with α85 antibody was decreased in only MT6 cells but increased in WT1 cells. To study the reason for decreased total PI 3'-kinase activity, we next assessed the protein content of p85 subunit in these cell lines. As shown in Fig. 8, Western blot analysis using polyclonal α85 antibody showed that the content of p85 protein was decreased in MT cells in both HIRc and HIRY/F2 cells. Using another monoclonal p85 antibody that recognizes a different epitope, we confirmed that the protein content of the p85 subunit was decreased in MT cells, suggesting that expression of ΔPTP might induce decreased total PI3-kinase content of p85 subunits in both HIRc and HIRY/F2 cells. On the other hand, p85 protein content in WT cells was comparable to that in pHyg cells.

Insulin-stimulated MAP Kinase Cascade—To assess the changes in insulin signaling in the cells expressing wild-type and ΔPTP, we measured insulin-stimulated MAP kinase activity. MAP kinase activity in the basal state was identical among these cell lines and increased depending upon the insulin concentration in all cell lines. In WT11 cells, insulin-stimulated MAP kinase activity was enhanced compared with that of pHyg cells. Enhanced insulin action was mainly due to responsiveness (percent over basal; 600% in WT11 cells and 290% in pHyg cells, p < 0.01) with a smaller increase in insulin sensitivity (ED50 value 2.1 nM in WT11 cells and 3.2 nM in pHyg cells). On the other hand, in MT15 cells, MAP kinase activity was attenuated compared with that in pHyg cells (percent over basal 180% in MT cells, p < 0.01). Similar results were obtained in HIRY/F2 cell lines (data not shown).

Insulin-stimulated DNA Synthesis—We examined insulin-stimulated DNA synthesis by measuring the incorporation of BrdUrd in both HIRc (Fig. 9A) and HIRY/F2 (Fig. 9B) cells and found that the percentage of BrdUrd-labeled cells was increased in WT cells and decreased in MT cells when compared with those in pHyg cells in both cell lines, even though the percentage of labeled cells was comparable in the basal state among these cell lines.

As shown in Fig. 10, we found that insulin-stimulated PI 3'-kinase activity was well correlated with IRS-1 phosphorylation levels in WT and MT cells (r = 0.953, p < 0.05). Furthermore, changes in PI 3'-kinase activity were well correlated with those in DNA synthesis (r = 0.986, p < 0.01), as well as in the case of MAP kinase (r = 0.91, p < 0.05) in HIRc cells.

**DISCUSSION**

We have established cell lines overexpressing either wild-type or mutant SHPTP2 (ΔPTP) originated from HIRc and HIRY/F2 cells. In response to insulin, IRS-1-associated SHPTP2 activity in WT11 cells was significantly greater than that in pHyg cells. On the other hand, SHPTP2 activities bound to IRS-1 in MT15 cells was not affected by insulin treatment.
Thus, introduction of \( \Delta \)PTP inhibited the association of IRS-1 with native endogenous SHPTP2 and overexpression of wild-type SHPTP2 enhanced its association with IRS-1 compared to that in pHyg cells in response to insulin.

Does the change in association between SHPTP2 and IRS-1 modulate insulin signaling? In our current study, we found that both insulin-stimulated levels of tyrosine phosphorylation of insulin receptors and IRS-1 were increased in WT cells and decreased in MT cells. Thus, we speculate that SHPTP2 potentiates the phosphorylation levels of IRS-1 and regulates the insulin signal through either the inhibition of an undefined PTPase(s) or the activation of undefined PTK(s). As shown in Fig. 2, in addition to IRS-1 and insulin receptor \( \beta \) subunit, two undefined tyrosine-phosphorylated proteins bound to SH2 domains of SHPTP2 in response to insulin. One was a 115-kDa tyrosine-phosphorylated protein and thought to be a SHPTP2-binding protein as reported in several studies (21–23, 35). The other high molecular weight tyrosine-phosphorylated protein (pp135) remains undefined. Although the precise roles of these proteins for regulation of insulin signaling are unclear, they may be substrate(s) for SHPTP2. Further characterization of these proteins may have important implications for clarifying the role of SHPTP2 in the signal transduction of insulin.

However, previous studies (21–23) reported that introduction of dominant negative mutant SHPTP2 did not modulate the phosphorylation level of IRS-1. Noguchi et al. (22) showed that introduction of negative SHPTP2 inhibited Ras-MAP kinase pathway but that the phosphorylation of IRS-1 was unchanged. In the present study, our \( \Delta \)PTP mutant consists only
Fig. 10. Relationship between phosphorylation of IRS-1 and PI 3'-kinase activity (A) and relationship between PI 3'-kinase activity and DNA synthesis among pHyg, MT11, and WT15 cells (B). A, magnitude of increment in phosphorylation of IRS-1 and PI 3'-kinase activity in response to insulin in WT11 and MT15 cells were divided by those in pHyg cells, respectively. B, magnitude of increment in PI 3'-kinase activity and DNA synthesis in WT11 and MT15 cells in response to insulin were divided by those in pHyg cells. Similar relationships were observed in HIRF2 cells expressing wild-type and ΔPTP. Data represent means ± S.E. of 4–5 separate experiments.

SH2 domains of SHPTP2 and tertiary structure of its SH2 domains may differ from those of native SHPTP2 and their catalytically inactive mutant (Cys → Ser). Therefore, it may be important to rule out the possibility that this SH2 mutant nonspecifically interacts with other SH2 binding sites rather than endogenous SHPTP2 binding sites, leading to inhibit functions of other SH2-containing molecules. However, SH2 domains of SHPTP2 are reported to behave differently compared with those of PTP1C in vitro (25) and in vivo (36), even though they have 60–70% of homology in SH2 regions of both PTPases. Moreover, overexpression of wild-type SHPTP2 induces the opposite effects when compared with MT cells. Furthermore, we could not find any qualitative difference in the profile of binding proteins between GST fusion proteins containing full-length SHPTP2 and ΔPTP as shown in Fig. 2. Thus, we speculate that ΔPTP mutant interfere with native SHPTP2 to associate endogenous SHPTP2 binding sites including IRS-1. In the present study, we confirmed that the introduction of mutant SHPTP2 attenuated insulin-stimulated MAP kinase activity as reported (21–23). However, we also found that ΔPTP impaired the insulin-stimulated activation of PI 3'-kinase associated with IRS-1 in MT cells. Furthermore, we found that insulin-stimulated PI 3'-kinase activity was well correlated with IRS-1 phosphorylation levels that was evaluated using three cell lines, as shown in Fig. 10A. Thus, we speculate that changes in phosphorylation levels of IRS-1 might be associated with both changes in PI 3'-kinase and MAP kinase activities in HIRc cells.

Although ΔPTP may act by nonspecifically inhibiting IRS-1 and p85 subunit of PI 3'-kinase in MT cells, this seems unlikely based on the following. First, our SH2 protein was not able to bind a GST-IRS-1 fusion protein that includes binding motif for p85 of PI 3'-kinase and no SHPTP2 binding site (17). Furthermore, p85 was more efficiently bound to phosphorylated IRS-1 in MT cells, when compared with that in pHyg cells (Fig. 6). Considering these data together, we believe that decreased levels of phosphorylation of IRS-1 may cause impairment of its association with p85, resulting in decrease in PI 3'-kinase activity rather than unphysiological inhibition of IRS-1 association with p85 of PI 3'-kinase. Therefore, the reason for this discrepancy between Noguchi’s study and ours still remains unclear. However, one can speculate that different cell lines (CHO-IR versus HIRc) may respond differently to SHPTP2 depending on its concentration in the cells.

Interestingly, the content of the p85 subunit of PI 3'-kinase was significantly decreased in MT cells, and its content in WT cells was comparable with that of pHyg cells. Since the protein content of IRS-1 (Fig. 3C) was not decreased in MT cells compared with that in pHyg cells, the decrease in protein content of p85 subunit might be specific. The decrease in p85 content in MT cells was confirmed in two different parent cell lines. Therefore, it may be possible that SHPTP2 regulates expression of a p85 subunit of PI 3'-kinase. Similarly, Hausdorff et al. (37) reported that microinjection of either GST-SH2 fusion protein or anti-SHPTP2 antibody into 3T3-L1 adipocytes inhibited insulin-stimulated expression of Glut1 and that SHPTP2 was necessary for insulin-stimulated expression of Glut1 protein (37). They speculate that this may be caused by inhibition of p21ras activation (38). However, McGuire et al. (39) have reported that expression of p85 is not affected in NIH3T3 cells after a 40-h treatment with lovastatin, which inhibits farnesylation of p21ras, suggesting that p21ras does not modulate the expression of p85 (39). Although the mechanism for regulation of p85 expression remains unclear, it has been reported that differentiation of preadipocytes to adipocytes leads to increased levels of the expression and phosphorylation of insulin receptor and IRS-1, and resulted in increased expression of p85 (40). Therefore, based upon these data, SHPTP2 may modulate expression of p85 via activation of insulin signaling.

In conclusion, overexpression of SHPTP2 enhanced the insulin-stimulated activities of both MAP kinase and PI 3'-kinase pathway through increased IRS-1 phosphorylation and introduction of dominant negative mutant SHPTP2 (ΔPTP) attenuated these pathways through impaired IRS-1 phosphorylation. This is the first direct evidence that levels of PI 3'-kinase activity positively regulate the stimulation of both MAP kinase cascade and PI 3'-kinase pathway via modulation of phosphorylation of IRS-1 in Rat 1 fibroblasts expressing human insulin receptors.

Acknowledgments—We are grateful to Dr. J. Miyazaki (Tokyo University) for the gift of pCAGGS expression vector, Dr. M. Kasuga (Kobe University) for the gift of monoclonal anti-p85 antibody, and Dr. T. Sasaki (Toyama Medical and Pharmaceutical University) for valuable discussions.

REFERENCES

1. Cantly, L. C., Auger, K. R., Carpenter, C., Duckworth, B., Graziani, A., Kapeller, R., and Soltosf, S. (1991) Cell 64, 281–302
2. Fisher, E. H., Charbonneau, H., and Tonk, N. K. (1991) Science 253, 401–406
3. Shi, X. J., Rothenberg, P., Kohn C. R., Backer, J. M., Araki, E., Wilden, P. A., Cahill, D. A., Giddstein, B. J., and White, M. F. (1991) Nature 352, 73–77
4. Kahn, C. R. (1994) Diabetes 43, 1066–1084
5. Kurokawa, C. R., Anderson, D., Moran, M. F., Ellis, C., and Pawson, T. (1993) Science 252, 668–674
6. Dawson, T., and Gish, G. D. (1992) Cell 71, 359–362
7. Freeman, R. J., Plutzky, J., and Neel, B. G. (1992) Proc. Natl. Acad. Sci. U.S.A. 89, 11239–11243
8. Adachi, M., Sekiya, M., Miyahdi, T., Matsuno, K., Hinoda, Y., Imai, K., and Yachi, A. (1992) FEBS Lett. 314, 335–339
9. Feng, G. S., Hui, C. C., and Pawson, T. (1993) Science 259, 1607–1611
10. Vogel, W., Lammers, R., Huang, J., and Ulrich, A. (1993) Science 259, 1611–1614
11. Ahmad, S., Banville, D., Zhao, Z., Fisher, E. H., and Shen, S.-H. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 2197–2201
12. Perkins, L. A., Larsen, I., and Perrimon, N. (1992) Cell 70, 225–236
13. Lechleider, R. J., Freeman, R. M., Jr., and Neel, B. G. (1993) J. Biol. Chem. 268, 13434–13438
14. Kazlauskas, A., Feng, G. S., Pawson, T., and Valius, M. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 6939–6943
15. Bennett, A. M., Tang, T. L., Sugimoto, S., Walsh, C. T., and Neel, B. G. (1994) Proc. Natl. Acad. Sci. U.S.A. 91, 7335–7339
16. Lechleider, R. J., Sugimoto, S., Bennett, A. M., Kashishian, A. S., Cooper, J. A., Shoelson, S. E., Walsh, C. T., and Neel, B. G. (1993) J. Biol. Chem. 268, 24178–24181
17. Ugi, S., Maegawa, H., Olefsky, J. M., Shigeta, Y., and Kashiwagi, A. (1994) FEBS Lett. 340, 210–214
18. Kuhne, M. R., Pawson, T., Lienhard, G. E., and Feng, G. S. (1993) J. Biol. Chem. 268, 11479–11481
19. Sun, X. J., Crimmins, D. L., Myers, M. J., Miralpeix, M., and White, M. F. (1993) Mol. Cell. Biol. 13, 4718–4721
20. Xiao, S., Rose, D. W., Sasaki, T., Maegawa, H., Burke, T. R. J., Rolle, P. P.,
SHPTP2 Regulates PI 3'-Kinase Activity

12602

Shoelson, S. E., and Olefsky, J. M. (1994) J. Biol. Chem. 269, 21244–21248
21. Milarski, K. L., and Saltiel, A. R. (1994) J. Biol. Chem. 269, 21239–21243
22. Noguchi, T., Matozaki, T., Horita, K., Fujioka, Y., and Kasuga, M. (1994) Mol. Cell. Biol. 14, 6674–6682
23. Yamauchi, K., Milarski, K. L., Saltiel, A. R., and Pessin, J. E. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 664–668
24. Yonezawa, K., Ueda, H., Hara, K., Nishida, K., Ando, A., Chavanieu, A., Matsuda, H., Shii, K., Yokono, Y., Calas, B., Grigorescu, F., Dhand, R., Gout, I., Otsu, M., Waterfield, M. D., and Kasuga, M. (1992) J. Biol. Chem. 267, 25958–25965
25. Maegawa, H., Ugi, S., Ishibashi, O., Tachikawa, I. R., Takahara, N., Tanaka, Y., Takagi, Y., Kikkawa, R., Shigeta, Y., and Kashiwagi, A. (1993) Biochem. Biophys. Res. Commun. 194, 208–214
26. Niwa, H., Yamamura, K., and Miyazaki, J. (1987) Gene (Amst.) 63, 193–200
27. McClain, D. A., Maegawa, H., Lee, J., Dull, T. J., Ullrich, A., and Olefsky, J. M. (1987) J. Biol. Chem. 262, 14663–14671
28. Takata, Y., Webster, N. J., and Olefsky, J. M. (1991) J. Biol. Chem. 266, 9135–9139
29. Maegawa, H., Ide, R., Hasegawa, M., Ugi, S., Egawa, K., Iwanishi, M., Kikkawa, R., Shigeta, Y., and Kashiwagi, A. (1995) J. Biol. Chem. 270, 7724–7730
30. Sugimoto, S., Wandlest, T. J., Shoelson, S. E., Neel, B. G., and Walsh, C. T. (1994) J. Biol. Chem. 269, 13614–13622
31. Staubs, P. A., Reichart, D. R., Saltiel, A. R., Milarski, K. L., Maegawa, H., Berhanu, P., Olefsky, J. M., and Seeley, B. L. (1994) J. Biol. Chem. 269, 27186–27192
32. Maegawa, H., Ugi, S., Adachi, M., Hinoda, Y., Kikkawa, R., Yachi, A., Shigeta, Y., and Kashiwagi, A. (1994) Biochem. Biophys. Res. Commun. 199, 780–785
33. Okamoto, M., Hayashi, T., Kono, S., Imoue, G., Kubota, M., Okamoto, M., Kuzuya, H., and Imura, H. (1993) Biochem. 290, 327–333
34. Takagi, Y., Kashiwagi, A., Maegawa, H., Kikkawa, R., and Shigeta, Y. (1995) Atherosclerosis 113, 19–27
35. Yamauchi, K., Ribon, V., Saltiel, A. R., and Pessin, J. E. (1995) J. Biol. Chem. 270, 17716–17722
36. Tang, T. L., Freeman, R. M., Jr., O’Reilly A. M., Neel, B. G., and Sokol, S. Y. (1995) Cell 80, 473–483
37. Hausdorff, S. F., Bennett, A. M., Nell, B. G., and Birnbaum, M. J. (1995) J. Biol. Chem. 270, 12965–12968
38. Hausdorff, S. F., Frangioni, J. V., and Birnbaum, M. J. (1994) J. Biol. Chem. 269, 21391–21394
39. McGuire, T. F., Corey, S. J., and Sebti, S. M. (1993) J. Biol. Chem. 268, 22227–22230
40. Saad, M. J. A., Folli, F., Araki, E., Hashimoto, N., Csermely, P., and Kahn, C. R. (1994) Mol. Endocrinol. 8, 545–557