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SH2-containing inositol phosphatase 2 negatively regulates insulin-induced glycogen synthesis in L6 myotubes

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Abstract

Aims/hypothesis. PI(3,4,5)P3 produced by PI3-kinase seems to be a key mediator for insulin's metabolic actions. We have recently cloned rat SHIP2 cDNA which is abundantly expressed in target tissues of insulin. Here, we clarify the role of SHIP2 possessing 5'-phosphatase activity toward PI(3,4,5)P3 in insulin signalling in the skeletal muscle.
Methods. The role of SHIP2 in insulin-induced glycogen synthesis was studied by expressing wild-type (WT)-SHIP2 and a 5'-phosphatase defective (ΔIP)-SHIP2 into L6 myotubes by means of adenovirus mediated gene transfer.
Results. The early events of insulin signalling including tyrosine phosphorylation of the insulin receptor and IRS-1, IRS-1 association with the p85 subunit, and PI3-kinase activity were not affected by expression of WT- and ΔIP-SHIP2. Although PI(3,4,5)P3 and PI(3,4)P2 are known to possibly activate a downstream molecule of PI3-kinase Akt in vitro, overexpression of WT-SHIP2 inhibited insulin-induced phosphorylation and activation of Akt. Conversely, Akt activity was increased by expression of ΔIP-SHIP2. GSK3β located downstream of Akt is an important molecule to further transmit insulin signal for glycogen synthesis in skeletal muscles. In accordance with the results of Akt, insulin-induced phosphorylation and inactivation of GSK3β, subsequent activation of glycogen synthase and glycogen synthesis were decreased by expression of WT-SHIP2, whereas these events were increased by expression of ΔIP-SHIP2.
Conclusion/interpretation. Our results indicate that SHIP2 plays a negative regulatory role via the 5'-phosphatase activity in insulin signalling, and that PI(3,4,5)P3 rather than PI(3,4)P2 is important for in vivo regulation of insulin-induced Akt activation leading to glycogen synthesis in L6 myotubes. [Diabetologia (2001) 44: 1258–1267]

Keywords SHIP2, 5'-phosphatase, insulin, PI3-kinase, Akt, glycogen synthesis.

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Abbreviations: SHIP, SH2-containing inositol 5'-phosphatase; PI3-kinase, Phosphatidylinositol 3-kinase; PI(3,4,5)P3, Phosphatidylinositol 3,4,5-triphosphate; PI(3,4)P2, Phosphatidylinositol 3,4-bisphosphate; GSK3β, Glycogen synthase kinase 3β; PP1, Protein phosphatase 1; PDK, Phosphionositide-dependent kinase

Activation of the PI3-kinase is known to be extremely important for exerting various metabolic actions of insulin [1–3]. PI(3,4,5)P3 produced by the activated PI3-kinase is thought to function as a key lipid second messenger for signalling to further downstream molecules [4–6]. Therefore, it is possible that hydrolysis of PI(3,4,5)P3 by lipid phosphatases can affect insulin signalling [7–10]. SH2-containing inositol 5'-phoshatase 1 (SHIP1) was identified as a lipid phosphatase possessing 5'-phosphatase activity to hydrolyse PI(3,4,5)P3 to PI(3,4)P2 [11, 12]. Previous reports have indicated that SHIP1 plays a negative regulatory part via the 5'-phosphatase activity in haematopoi-

etic cells [13, 14]. Involvement of the 5'-phosphatase activity in insulin signalling has also been indicated. Exogenous expression of SHIP1, but not 5'-phosphatase defective SHIP1, inhibited insulin-induced *Xenopus* oocyte maturation [7] and insulin stimulation of Glut4 translocation in 3T3-L1 adipocytes [8]. Despite these facts, the expression of SHIP1 is relatively restricted to haematopoietic cells, and it is not apparently detectable in target tissues of insulin such as skeletal muscles and fat cells [15]. These results indicate the existence of SHIP1 isozyme responsible for insulin signalling. Accordingly, we and others cloned a SHIP1 isozyme named SHIP2 and it was found to be predominantly expressed in the skeletal muscle [9, 16]. Subsequently, it was reported that targeted disruption of the SHIP2 gene in mice resulted in increased insulin sensitivity without affecting biological systems other than insulin signal [17]. Insulin-induced glycogen synthesis was enhanced in isolated soleus muscle from heterozygous SHIP2 knockout mice [17]. Since the storage of excess amounts of glucose as glycogen in the skeletal muscle is critical for insulin's maintenance of the glucose homeostasis [1, 2], determining the precise molecular mechanisms by which SHIP2 controls insulin-induced glycogen synthesis would contribute to understanding the novel regulatory role of SHIP2 in the insulin signalling pathway. Among the PI3-kinase dependent pathways, activation of Akt by the generated phospholipids is known to be required for insulin-induced glycogen synthesis [5, 6, 18, 19]. In addition, GSK3β was identified as a substrate of Akt, and inactivation of GSK3β by Akt, at least in part, resulting in the activation of glycogen synthase, which is the rate-limiting enzyme for glycogen synthesis by insulin [19, 20, 21]. Since SHIP2 possesses 5'-phosphatase activity specifically toward PI(3,4,5)P3 [22], hydrolysis of PtdIns(3,4,5)P3 by SHIP2 via its 5'-phosphatase activity could affect insulin-induced glycogen synthesis. In our study, to clarify the molecular mechanisms of SHIP2 via its 5'-phosphatase activity in the negative regulation of insulin-induced glycogen synthesis in the skeletal muscle, wild-type SHIP2 (WT-SHIP2) and 5'-phosphatase defective SHIP2 (ΔIP-SHIP2) were transiently expressed in differentiated L6 myotubes by means of adenovirus mediated gene transfer. Insulin-induced metabolic signalling leading to glycogen synthesis was compared among the transfected cells.

Materials and methods

Materials. Human crystal insulin was provided by Novo Nordisk Pharmaceutical (Copenhagen, Denmark). [U-^{14}C]UDP-glucose (10.6 GBq / mmol) and [γ-^{32}P]ATP (111 TBq / mmol) were purchased from NEN Life Science Products (Boston, Mass., USA). Two polyclonal anti-SHIP2 antibodies were described previously [9]. A monoclonal anti-p85 subunit of PI3-kinase antibody, a monoclonal anti-GSK3β antibody, and a monoclonal anti-phosphotyrosine antibody (PY20) were from Transduction Laboratories (Lexington, Ky., USA). A polyclonal anti-IRS1 antibody was from Upstate Biotechnology (Lake Placid, N.Y., USA). A polyclonal anti-Thr^{308} phospho-specific Akt antibody, a polyclonal anti-Ser^{473} phospho-specific Akt antibody, a polyclonal anti-Ser$^{21/9}$ phospho-specific GSK3 antibody, and Akt kinase assay kit were from New England Biolabs (Beverly, Mass., USA). Enhanced chemiluminescence reagents were from Amersham Pharmacia Biotech (Uppsala, Sweden). Minimum essential medium α medium (α-MEM), MEM vitamin mixtures, and MEM amino acid solutions were from Gibco BRL (Tokyo, Japan). All other routine reagents were analytical grade and purchased from Sigma Chemical (St. Louis, Mo., USA) or Wako Pure Chemical Industries (Osaka, Japan).

Construction of adenovirus vectors. A cDNA encoding rat wild-type SHIP2 (WT-SHIP2) was described previously [9]. Phosphatidylinositol 5'-phosphatase defective mutant of SHIP2 (ΔIP-SHIP2) was generated by introducing Pro-687 to Ala, Asp-691 to Ala, and Arg-692 to Gly by utilizing QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, Calif., USA). These amino acids of SHIP2 are conserved among the known 5'-phosphatases and shown to be critical to elicit the 5'-phosphatase activity [14]. The mutagenic oligonucleotides were 5'-ACC AAT GTG GCT TCA TGG TGT GCC GGA ATT CTA TGG-3'. The obtained nucleotide sequences of ΔIP-SHIP2 were verified using dye terminator cycle sequence method. WT-SHIP2 and ΔIP-SHIP2 were subcloned into a vector pAxCAwt, and transferred to recombinant adenovirus by homologous recombination by utilizing Adenovirus Expression Vector Kit (Takara Biomedicals, Tokyo, Japan).

Cell culture and infection of adenovirus. L6 myoblasts were kindly provided by Dr. A. Klip (Hospital for Sick Children, Toronto, Canada) and were grown and passaged in α-MEM supplemented with 10% FCS. The confluent cells were used for differentiation. L6 myoblasts were differentiated in the culture medium containing 2% FCS [23]. After the cells were differentiated into L6 myotubes, WT- or ΔIP-SHIP2 was transiently expressed in differentiated L6 myotubes by means of adenovirus mediated gene transfer. A multiplicity of infection (m.o.i.) of 10–40 pfu/cell was used to transfect into L6 myotubes in α-MEM containing 2% FCS, with the virus being left on the cells for 16 h before removal. Following experiments were done approximately 24 to 48 h after initial addition of the virus.

Immunoprecipitation and Western blotting. L6 myotubes grown in six multiwell plates were serum-starved for 16 h in α-MEM. The cells were treated with 17 nmol/l insulin at 37 °C for 5 min. The cells were lysed in a buffer containing 30 mmol/l Tris, 150 mmol/l NaCl, 10 mmol/l EDTA, 0.5% sodium deoxycholate, 1% Triton X-100, 1 mmol/l phenylmethylsulfonyl fluoride (PMSF), 1 mmol/l Na_3VO_4, 0.1 μ mol/l aprotinin, 1 μ mol/l leupeptin, pH 7.4, for 15 min at 4 °C. Lysates obtained from the same number of cells were centrifuged to remove insoluble materials. The supernatants (100 μg of protein) were immunoprecipitated with various antibodies for 2 h at 4 °C. The precipitates or whole cell lysates were then separated by 7.5% or 12% SDS-PAGE and transferred onto polyvinylidene difluoride membranes (PVDM) using a Bio-Rad Transblot apparatus. The membranes were blocked in a buffer containing 50 mmol/l Tris, 150 mmol/l NaCl, 0.1% Tween 20, and 2.5% bovine serum albumin or 5% non-fat milk, pH 7.5, for 2 h at 20 °C. The membranes

were then probed with the specified antibodies for 2 h at 20 °C or for 16 h at 4 °C. After washing the membranes in a buffer containing 50 mmol/l Tris, 150 mmol/l NaCl, 0.1 % Tween 20, pH 7.5, blots were incubated with horseradish peroxidase-linked second antibody followed by enhanced chemiluminescence detection using ECL reagent according to the manufacturer's instructions (Amersham) [9].

Measurement of PI3-kinase activity. Serum-starved L6 myotubes grown in 10 cm dishes were stimulated with 17 nmol/l insulin at 37 °C for 5 min. The cells were lysed in a buffer containing 20 mmol/l Tris, 137 mmol/l NaCl, 1 mmol/l $MgCl_2$, 1 mmol/l $CaCl_2$, 0.2 mmol/l Na_3VO_4, 1 % Nonidet-P40, 10 % glycerol, 2 mmol/l PMSF, 1 μmol/l aprotinin, pH 7.6. The cell lysates were centrifuged to remove insoluble materials. The supernatants were immunoprecipitated with anti-PY20 antibody for 2 h at 4 °C. The precipitates were washed twice with Buffer A (Tris-buffered saline, 1 % Nonidet-P40, 0.1 mmol/l Na_3VO_4, 1 mmol/l dithiothreitol (DTT), pH 7.6), twice with Buffer B (100 mmol/l Tris, 500 mmol/l LiCl, 0.1 mmol/l Na_3VO_4, 1 mmol/l DTT, pH 7.6), and twice with Buffer C (10 mmol/l Tris, 100 mmol/l NaCl, 1 mmol/l EDTA, 1 mmol/l DTT, pH 7.6). The phosphorylation reaction was started by adding 20 μl PI solution containing 0.5 mg/ml PI, 50 mmol/l HEPES, 1 mmol/l NaH_2PO_4, 1 mmol/l EGTA, pH 7.6 at 20 °C, followed by adding 10 μl of the reaction mixture containing 250 μmol/l [γ-^{32}P]ATP (0.37 Mbq/tube), 100 mmol/l HEPES, 50 mmol/l $MgCl_2$, pH 7.6 for 5 min. The reaction was stopped by adding 15 μl of 8 mol/l HCl. The products were extracted by adding 130 mmol/l of chloroform and methanol (1:1) followed by centrifugation. The organic phase was removed and spotted on Silica Gel thin layer chromatography (TLC) plate (Merck). The plates were developed and dried [9]. The phosphorylated inositol was visualized by autoradiography and quantitated by the Fuji BAS 2000 image analyser (Fuji Film, Tokyo, Japan).

Measurement of Akt activity. Measurement of Akt activity was done using Akt kinase assay kit (New England Biolabs, Beverly, Mass., USA). In brief, L6 myotubes grown in six multiwell plates were serum-starved for 16 h in α-MEM, and were treated with or without 17 nmol/l insulin at 37 °C for 10 min. The cells were harvested and lysed according to the protocol of western blot. Cell lysates were immunoprecipitated with immobilized anti-Akt antibody for 3 h at 4 °C. The precipitates were washed twice with the cell lysis buffer, twice with a kinase buffer containing 25 mmol/l Tris, 5 mmol/l β-glycerophosphate, 2 mmol/l DTT, 0.1 mmol/l Na_3VO_4, 10 mmol/l $MgCl_2$, pH 7.5. Forty μl of the pellets were suspended with 200 μmol/l ATP and 1 μg GSK3 fusion protein, and incubated for 30 min at 30 °C. Reactions were terminated by adding SDS sample buffer containing 187.5 mmol/l Tris, 6 % w/v SDS, 30 % glycerol, 150 mmol/l DTT, 0.03 % w/v bromophenol blue, pH 6.8. The samples were then separated by 12 % SDS-PAGE and transferred onto PVDM. The membranes were blocked and probed with anti-Ser$^{21/9}$-phospho-specific GSK3 antibody for 16 h at 4 °C. After washing the membranes, the blots were incubated with horseradish peroxidase-linked second antibody followed by enhanced chemiluminescence detection using ECL reagent according to the manufacturer's instructions (Amersham).

Measurement of GSK3β activity. L6 myotubes grown in 6-cm plates were serum-starved for 16 h in α-MEM, and were treated with or without 17 nmol/l insulin at 37 °C for 10 min. The cell were lysed in a buffer containing 25 mmol/l Tris, 125 mmol/l NaCl, 25 mmol/l NaF, 1 mmol/l EDTA, 1 % Triton X-100, 10 mmol/l β-glycerophosphate, 5 mmol/l sodium pyrophosphate, 1 mmol/l Na_3VO_4, 200 nmol/l okadaic acid, 1 mmol/l DTT, complete protease inhibitor mixture (Boehringer Mannheim, Germany), pH 7.4, for 15 min at 4 °C. The cell lysates were immunoprecipitated with anti-GSK3β antibody for 3 h at 4 °C. The precipitates were washed once with kinase buffer containing 25 mmol/l Tris, 10 mmol/l $MgCl_2$, pH7.5. Kinase reactions were done in a kinase buffer with 100 μmol/l [γ-^{32}P]ATP and 6.8 μmol/l phospho-GS peptide 2 (Upstate Biotechnology, Lake Placid, N.Y., USA) as the substrate. Phospho-GS peptide 2 comprises sites 3b, 3c, and phosphorylation site 4 of skeletal muscle glycogen synthase. After 20 min at 30 °C, the reactions were spotted on phosphocellulose P81 paper (Whatman), washed four times with 100 mmol/l phosphoric acid, and counted in scintillation counter [24].

Glycogen synthase assay. L6 myotubes grown in six multiwell plates were incubated in serum-deprived and glucose-deprived DMEM supplemented with 2 mmol/l pyruvate and 0.1 % BSA for 3 h. The cells were then stimulated with or without 17 nmol/l insulin in 5 mmol/l glucose containing medium for 30 min. The cells were washed three times with ice-cold PBS, and lysed with glycogen synthase extraction buffer containing 25 mmol/l Tris, 30 % glycerol, 10 mmol/l EDTA, 100 mmol/l KF, 1 mmol/l PMSF, pH 7.4. The cell lysates were centrifuged, and the supernatants were assayed in a final volume of 90 μl of glycogen synthase buffer containing 6.2 mmol/l UDP-glucose, 1 μCi/ml [U-^{14}C]UDP-glucose, 0.74 % glycogen, in the absence or presence of 6.2 mmol/l glucose 6-phosphate (G6P) at 30 °C for 30 min. Seventy μl of the samples were spotted on Whatmann GF/A filters, dried for 5 s and then placed in ice-cold 70 % ethanol. The filters were washed three times with ice-cold 70 % ethanol for 30 min, once with acetone, and air dried. [U-^{14}C]UDP-glucose incorporation into glycogen was measured by liquid scintillation counting [19, 25].

Glycogen synthesis assay. L6 myotubes grown in six multiwell plates were serum-starved with PBS containing 40 mmol/l HEPES, 0.1 % BSA, 0.5 mmol/l $MgCl_2$, 0.5 mmol/l $CaCl_2$, 2 mmol/l L-glutamine, 20 mmol/l $NaHCO_3$, MEM amino acid solution, and MEM vitamin mixture, pH 7.4 for 4 h. The medium were then replaced with incubation medium containing 5 mmol/l glucose, 1 μCi [^{14}C] glucose and stimulated with various concentrations of insulin for 2 h at 37 °C. After incubation, the cells were washed twice with cold Tris-buffered saline and solubilized with 30 % KOH solution for 30 min at 37 °C. After boiling the sample for 30 min with 4 mg carrier glycogen, glycogen was precipitated by adding ice-cold ethanol for 16 h at 4 °C. The precipitates were solubilized in PBS, and [^{14}C]glucose incorporated into glycogen was measured by liquid scintillation counting [25].

Statistical analysis. The data are represented as means ± SEM. *p* values were determined by unpaired Student's *t* test, and a *p* value of less than 0.05 was considered statistically significant.

Results

Structures of SHIP2 constructs and the expression in L6 myotubes. The structure of WT-SHIP2 and ΔIP-SHIP2 is shown (Fig. 1A). WT-SHIP2 is a 140000 M_r protein which is composed of a SH2 domain at the N-terminus, a central 5'-phosphatase catalytic domain, and a proline rich region including PTB do-

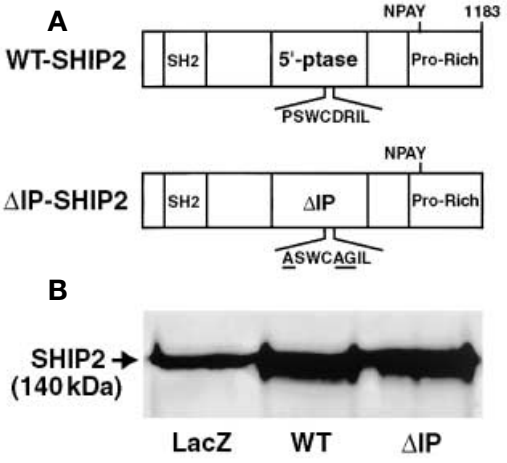

Fig. 1. Structures of SHIP2 constructs and expression in L6 myotubes. **A** Structures of wild-type SHIP2 and 5'-phosphatase defective SHIP2 are shown. **B** L6 myotubes were transfected with LacZ, WT-SHIP2, or ΔIP- SHIP2 at an m.o.i. of 40 pfu/cell. After the infection, the cells were lysed and subjected to immunoblot analysis with anti-SHIP2 antibody. Results are representative of three separate experiments

main binding consensus at the C-terminus. A 5'-phosphatase defective SHIP2 (ΔIP-SHIP2) was constructed by mutating three amino acids within the catalytic domain of SHIP2, which is highly conserved among the known 5'-phosphatases [9]. WT-SHIP2 and ΔIP-SHIP2 were transiently expressed in L6 myotubes with the use of adenovirus mediated gene transfer. Endogenous SHIP2 was observed in control L6 myocytes transfected with LacZ alone. By transfection with either WT-SHIP2 or ΔIP-SHIP2 at an m.o.i. of 40 pfu/cell, the value of WT-SHIP2 and ΔIP-SHIP2 expression was fivefold of endogenous SHIP2 amounts (Fig. 1B).

Effects of SHIP2 on insulin signallings up to PI3-kinase activation. Insulin induces tyrosine phosphorylation of the insulin receptor β-subunit and IRS-1, IRS-1 association with the p85 subunit of PI3-kinase, and PI3-kinase activation [1–3]. These signalling events are shown to be important for metabolic actions of insulin [1–3]. To investigate the role of SHIP2 in these steps of insulin signalling, we examined the effects of SHIP2 expression in L6 myotubes. Transfection with either WT-SHIP2 or ΔIP-SHIP2 did not affect insulin-induced tyrosine phosphorylation of the insulin receptor β-subunit (Fig. 2A, B), tyrosine phosphorylation of IRS-1 (Fig. 2C, D), IRS-1 association with the p85 subunit (Fig. 2E, F), and PI3-kinase activation (Fig, 2G, H). These results indicate that SHIP2 is not involved in the steps up to insulin-induced PI3-kinase activation.

Effect of SHIP2 overexpression on insulin-induced phosphorylation and activation of Akt. SHIP2 is known to possess 5'-phosphatase activity to specifically hydrolyse PI3-kinase product PI(3,4,5)P3 [22]. Since the insulin-induced increase in PI(3,4,5)P3 and PI(3,4)P2 can be modulated by SHIP2, the expression of SHIP2 could affect insulin-induced activation of Akt which is a key downstream target of PI3-kinase and has been shown to mediate insulin signalling leading to glycogen synthesis in L6 cells [19, 20]. Since Akt is primarily activated as a result of its phosphorylation [5, 6], we examined the role of SHIP2 in insulin-induced phosphorylation of Akt on Thr^{308} and Ser^{473} residues (Fig. 3A, C). Insulin induced phosphorylation of Akt on both residues in LacZ transfected control L6 myotubes. Overexpression of WT-SHIP2 decreased insulin-induced phosphorylation of Akt, whereas insulin-induced Akt phosphorylation was increased by transfection with ΔIP-SHIP2 (Fig. 3B, D). After insulin stimulation, phosphorylation contents of Akt on both Thr^{308} and Ser^{473} residues corrected for the loaded amount of Akt was significantly decreased by 30.5 ± 6.7 % and 30.7 ± 6.1 %, respectively, by expression of WT-SHIP2. In contrast, these phosphorylation contents were increased by 35.1 ± 8.0 % and 63.3 ± 3.6 %, respectively, by expression of ΔIP-SHIP2. Similar amounts of protein loaded among the samples and similar expression value of WT-SHIP2 and ΔIP-SHIP2 were confirmed by immunoblotting the cell lysates with anti-Akt antibody (Fig. 3E) and with anti-SHIP2 antibody (data not shown), respectively. We further examined the effect of SHIP2 expression on Akt activity toward GSK3 as a substrate (Fig. 4A). Insulin stimulated Akt activity by a factor of 4.2 ± 0.9 in LacZ transfected control cells. Similar to the results with Akt phosphorylation, Akt activity corrected for the protein amount was decreased by 31.2 ± 4.7 % by overexpression of WT-SHIP2, whereas it was increased by 71.6 ± 8.2 % by transfection with ΔIP-SHIP2 compared to that in LacZ transfected L6 myotubes (Fig. 4B).

Effect of SHIP2 overexpression on insulin-induced phosphorylation and inactivation of GSK3β. GSK3β is located downstream of Akt and plays an important part in insulin-induced glycogen synthesis in L6 myotubes [19, 20]. Activated Akt is known to induce phosphorylation of GSK3β resulting in inactivation of the enzymatic activity [19–21]. Since SHIP2 negatively regulates insulin-induced Akt activation, we examined the effect of SHIP2 overexpression on insulin-induced GSK3β phosphorylation by utilizing anti-$Ser^{21/9}$–phospho-specific GSK3 antibody. Insulin phosphorylated GSK3β on Ser^{9} residue in control L6 myotubes. Consistent with the results of Akt activation, insulin-induced phosphorylation of GSK3β was decreased by overexpression of WT-SHIP2, whereas it was increased by overexpression of ΔIP-SHIP2 (Fig. 5A). Thus, insulin-induced phosphorylation of GSKβ corrected for the loaded protein

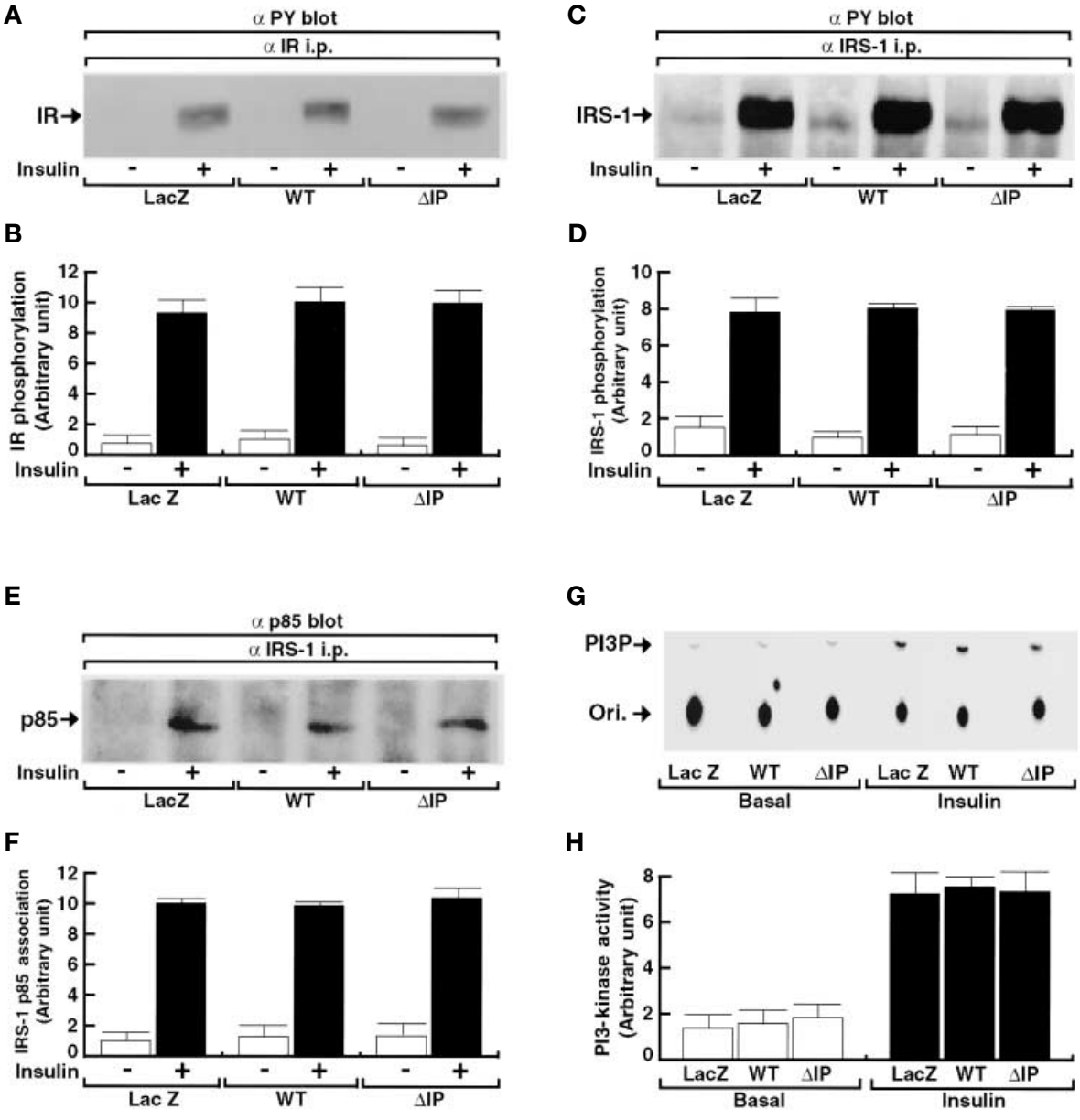

Fig. 2. Effect of SHIP2 overexpression on early events of insulin signalling in L6 myotubes. The transfected cells were serum-starved, and treated with 17 nmol/l insulin for 5 min. The cell lysates were immunoprecipitated with anti-insulin receptor (**A**), anti-IRS1 (**C**, **E**), or anti-phosphotyrosine (**G**) antibody. The precipitates were subjected to immunoblot analysis with anti-phosphotyrosine (**A**, **C**), or anti-p85 subunit (**E**) antibody. **G** The precipitates were assayed for PI3-kinase activity with PI as a substrate. The amount of phosphorylated insulin receptors (**B**), IRS-1 (**D**), IRS-1 associated with the p85 subunit (**F**), and PI(3)P (**H**) was quantitated by densitometry. Results are means ± SEM of four separate experiments

amount was significantly reduced by 30.9 ± 4.6 % by expression of WT-SHIP2 and increased by 29.1 ± 6.8 % by expression of ΔIP-SHIP2 compared to that in LacZ transfected control L6 cells (Fig. 5B). Furthermore, the effect of SHIP2 expression on the activity of GSK3β toward Phospho-GS peptide as a substrate was examined. Insulin treatment inhibited GSK3β activity by 29.0 ± 7.0 % in control L6 cells. Similar to the results of GSK3β phosphorylation, the degree of insulin-induced inactivation of GSK3β was decreased by 17.0 ± 2.5 % by expression of WT-SHIP2, whereas it was enhanced by 45.0 ± 2.0 % by expression of ΔIP-SHIP2 compared to that in LacZ transfected control L6 cells (Fig. 5C).

SHIP2 negatively regulates insulin-induced glycogen synthesis. Insulin-induced activation of glycogen synthase can be mediated by inactivation of GSK3β via its phosphorylation [19, 21]. Since SHIP2 was involved in the regulation of GSK3β, glycogen synthase activity might also be regulated by SHIP2. Therefore, we examined the effect of SHIP2 expression on insulin-induced glycogen synthase activation (Fig. 6A).

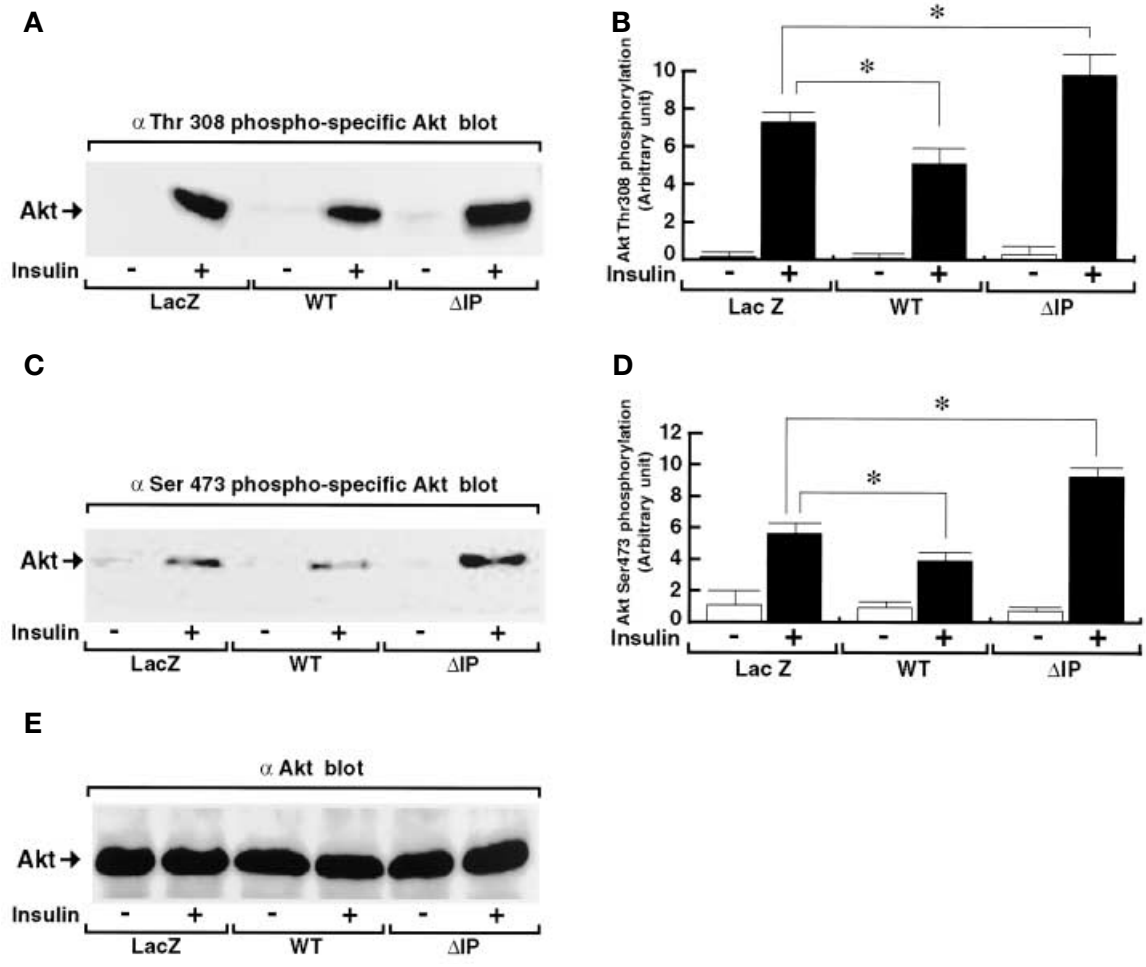

Fig. 3. Effect of SHIP2 overexpression on insulin-induced Akt phosphorylation in L6 myotubes. The transfected cells were serum-starved and treated with 17 nmol/l insulin for 5 min. The cell lysates were subjected to immunoblot analysis with anti-phospho-Thr^{308} Akt (**A**), anti-phospho-Ser^{473} Akt (**C**), or anti-Akt (**E**) antibody. The contents of phosphorylated Akt on Thr^{308} (**B**) and Ser^{473} (**D**) were quantitated by densitometry. Results are means ± SEM of five separate experiments. *$p < 0.05$ vs Akt phosphorylation in LacZ transfected control cells by Student's *t* test

Treatment with insulin resulted in 2.8 ± 0.4-fold increase in glycogen synthase activity in control cells. Overexpression of WT-SHIP2 inhibited insulin-induced glycogen synthase activation by 30.2 ± 6.4 %. In contrast, insulin-stimulated glycogen synthase activity was increased by transfection with ΔIP-SHIP2 by 45.0 ± 12.0 % compared to that in LacZ transfected L6 myotubes. Because glycogen synthase is the key enzyme for insulin-induced glycogen synthesis [1, 2, 19, 26], we examined further the effect of SHIP2 on insulin-induced glycogen synthesis by measuring [^{14}C]glucose incorporation into glycogen (Fig. 6B). Insulin stimulated [^{14}C]glucose incorporation into glycogen in a dose-dependent fashion and 17 nmol/l insulin stimulation resulted in 2.5 ± 0.3-fold increase in [^{14}C]glucose incorporation into glycogen in control L6 myotubes. Consistent with the results of glycogen synthase, overexpression of WT-SHIP2 decreased insulin-induced [^{14}C]glucose incorporation into glycogen, whereas it was increased by expression of ΔIP-SHIP2. At 17 nmol/l insulin concentration, [^{14}C]glucose incorporation into glycogen was inhibited by 26.1 ± 5.5 % by expression of WT-SHIP2, whereas it was enhanced by 90.5 ± 13.5 % by expression of ΔIP-SHIP2 compared to that in the control L6 cells.

Discussion

In contrast to the restricted expression of SHIP1 in haematopoietic cells, SHIP2 is known to be expressed in a wide range of tissues [15, 16, 27]. Abundant expression of SHIP2 is seen in target tissues of insulin including skeletal muscles [16, 27]. Importantly, targeted disruption of SHIP2 gene in mice showed increased insulin sensitivity [17]. As biological systems other than insulin signalling were not affected by the disruption based on the histological analysis, SHIP2 seems to be an essential negative regulator relatively specific to insulin signalling [17]. Further-

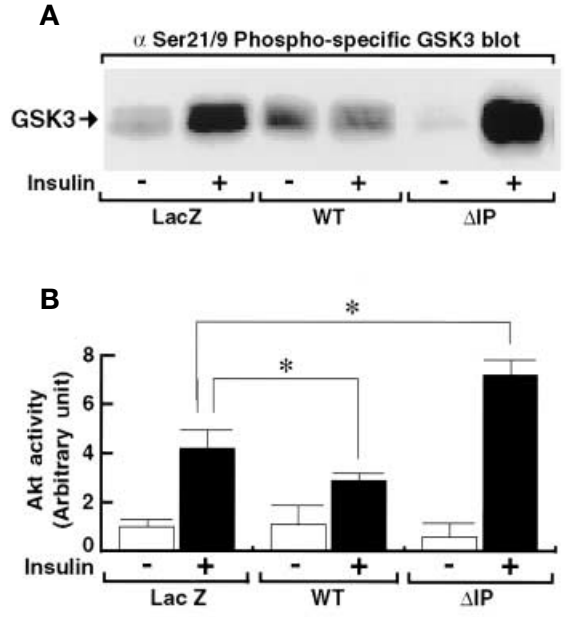

Fig. 4. Effect of SHIP2 overexpression on insulin-induced Akt activation in L6 myotubes. **A** Insulin-induced Akt activation in the transfected cells was assayed with GSK3 as a substrate. The activated Akt was analysed by immunoblot analysis with anti-$Ser^{21/9}$-phospho-specific GSK3 antibody. **B** The contents of phosphorylated GSK3 were quantitated by densitometry. Results are means ± SEM of four separate experiments. $*p < 0.05$ vs insulin-stimulated Akt activity in LacZ transfected control cells by Student's *t* test

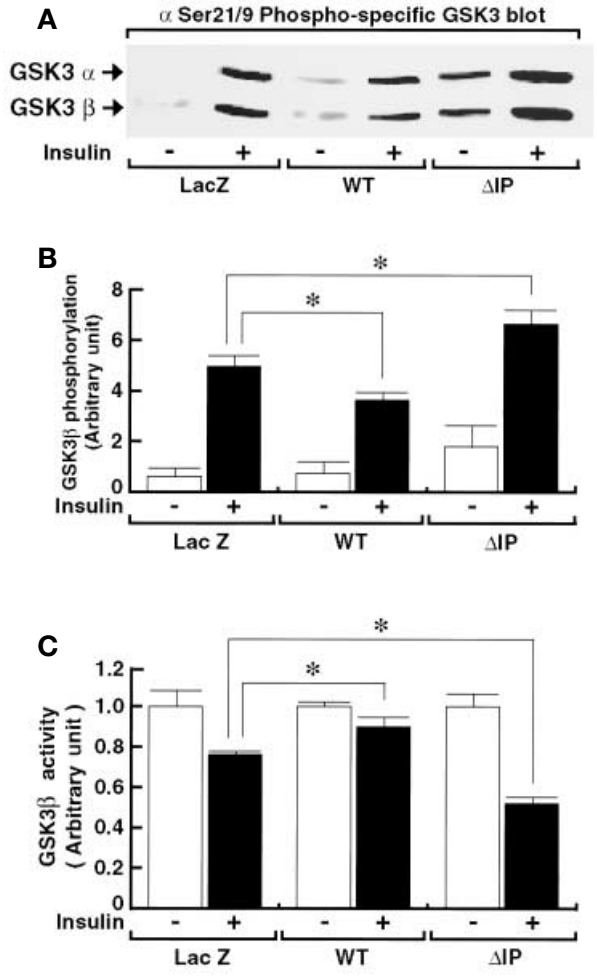

Fig. 5. Effect of SHIP2 overexpression on insulin-induced GSK3β phosphorylation and inactivation in L6 myotubes. The transfected cells were serum-starved, and treated with 17 nmol/l insulin for 5 min. **A** The cell lysates were subjected to immunoblot analysis with anti-$Ser^{21/9}$-phospho-specific GSK3 antibody. **B** The contents of phosphorylated GSKβ were quantitated by densitometry. **C** The cell lysates were assayed for GSK3β activity with phospho-GS peptide 2 as a substrate. Results are means ± SEM of four separate experiments. $*p < 0.05$ vs GSK3β phosphorylation (**B**) or GSK3β inactivation (**C**) in LacZ transfected control cells by Student's *t* test

more, studies with isolated soleus muscles from heterozygous SHIP2 knockout mice showed increased glycogen synthesis in response to insulin [17]. Since the skeletal muscle is an important target tissue of insulin in the regulation of glucose homeostasis [1–3], clarification of the molecular mechanisms by which SHIP2 controls insulin-induced glycogen synthesis is important for understanding the novel molecular events that negatively regulate insulin signalling. Activation of glycogen synthase by its dephosphorylation is known to be a key step in insulin-induced glycogen synthesis [1, 2, 19, 21]. Our results indicate that SHIP2 is involved in the negative regulation of insulin-induced glycogen synthase activation followed by glycogen synthesis in L6 myotubes, because insulin-induced glycogen synthase activation and glycogen synthesis were decreased by expression of WT-SHIP2 and increased by catalytically defective ΔIP-SHIP2. One can speculate that insulin also promotes glycogen synthesis by accelerating the rate of glucose entry into the cell in addition to glycogen synthase activation in skeletal muscles. Along this line, insulin-induced 2-deoxyglucose uptake was decreased by expression of WT-SHIP2 and increased by expression of ΔIP-SHIP2 in L6 myotubes (data not shown), indicating that SHIP2 is also involved in the negative regulation of insulin-induced glucose uptake.

Our results show that insulin-induced tyrosine phosphorylation of the insulin receptor β-subunit and IRS-1, IRS-1 association with the p85 subunit of PI3-kinase, and PI3-kinase activation were not affected by expression of either WT-SHIP2 or ΔIP-SHIP2. In addition the 5'-phosphatase activity of SHIP2 does not affect early insulin signalling events up to PI3-kinase activation, and these findings are consistent with the results seen in Rat1 fibroblasts [9]. Activation of PI3-kinase is critically important for insulin-induced glycogen synthase activation [1, 2]. Akt is one of the downstream target molecules of PI3-kinase, which is known to phosphorylate GSK3β resulting in its inactivation [19–21]. Because the activated GSK3β phosphorylates glycogen synthase, inactivation of GSK3β by Akt leads to the activation of glycogen synthase [19–21]. Alternatively,

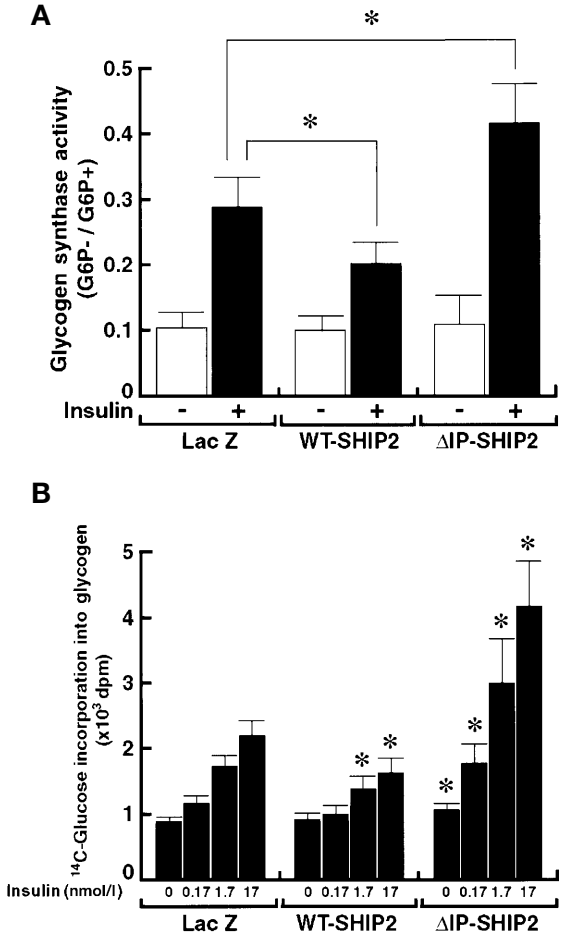

Fig. 6. Effect of SHIP2 overexpression on insulin-induced glycogen synthase activation and glycogen synthesis in L6 myotubes. **A** Insulin-induced glycogen synthase activation in the transfected cells is shown. Results are means ± SEM of percentage of glycogen synthase index (% GSI) from four separated experiments. **B** Insulin-induced glucose incorporation into glycogen in the transfected cells is shown. Results are means ± SEM of six separate experiments. *$p < 0.05$ vs insulin-stimulated GS activity (**A**) or glycogen synthesis at respective concentrations of insulin (**B**) in LacZ transfected control cells by Student's *t* test

glycogen synthase could be activated by PP1 which is also known to be localized downstream of PI3-kinase [25, 26]. Insulin-induced activation of PP1 dephosphorylates glycogen synthase, resulting in the activation of glycogen synthase [25, 26]. Thus, previous studies indicate two possible dephosphorylation mechanisms by which glycogen synthase is activated [19–21, 25, 26]. However, the relative importance of Akt-GSK3β versus PP1 in insulin-induced glycogen synthase activation seems to be dependent on cell types [19, 26]. Of note, Akt-GSK3β is considered to be the key pathway in the regulation of insulin-induced glycogen synthase activation in L6 myotubes [19, 20].

Akt becomes active by its phosphorylation on both Thr^{308} and Ser^{473} residues by PDK1 and unknown kinase tentatively referred to PDK2, respectively [28, 29]. Although PI(3,4,5)P3 generated from PI(4,5)P2 by the activated PI3-kinase is thought to be a key lipid second messenger to exert the biological action of insulin, it is also known that insulin stimulation increases the cellular amount of PI(3,4)P2 as well as PI(3,4,5)P3 [5, 6, 30]. Previous studies suggested that Akt was activated by PI(3,4)P2 but not by PI(3,4,5)P3 in the absence of PDK1 [18]. In contrast, in the presence of PDK1, Akt activation was preferentially induced by PI(3,4,5)P3 rather than PI(3,4)P2 [5, 6, 28]. Thus, the involvement of these phospholipids in the activation of Akt activity was considered to be complicated in vitro. However, studies have clearly shown that insulin-induced Akt activation by its phosphorylation on both Thr^{308} and Ser^{473} residues was inhibited by overexpression of WT-SHIP2 in L6 myotubes. Together with the fact that SHIP2 possesses 5'-phosphatase activity specifically toward PI(3,4,5)P3 [22, 31], SHIP2 seems to modulate insulin signalling by hydrolysing the PI3-kinase products PI(3,4,5)P3 via the 5'-phosphatase activity in L6 myotubes. In addition, our results suggest that PI(3,4,5)P3 has a greater role than PI(3,4)P2 for insulin-induced Akt activation, although it is known that both PI(3,4,5)P3 and PI(3,4)P2 bind with high affinity to the pleckstrin homology (PH) domain of Akt leading to the recruitment of Akt to be phosphorylated on the plasma membrane [5, 6, 18, 28, 29]. This idea is supported by a recent report showing that reduction of endogenous SHIP2 protein expression by an antisense oligonucleotide approach resulted in increased Akt activity in HeLa cells [31]. Expression of catalytically inactive SHIP2, ΔIP-SHIP2, resulted in enhanced phosphorylation and activation of Akt, whereas early signalling events of insulin up to PI3-kinase activation were not affected by the expression. Thus, ΔIP-SHIP2 seems to function with a dominant negative manner toward endogenous SHIP2 by possibly competing with the generated phosphoinositides by the activated PI3-kinase, although its precise mechanisms are not clear. The activated Akt phosphorylates GSK3β resulting in its inactivation [19–21]. Our results show that overexpression of WT-SHIP2 decreased insulin-induced GSK3β phosphorylation and the degree of insulin-induced inhibition of GSK3β activity, whereas they were enhanced by expression of ΔIP-SHIP2. We therefore conclude that SHIP2 is involved in the physiologically negative regulation of insulin-induced Akt-GSK3β signalling leading to glycogen synthesis via the 5'-phosphatase activity in L6 myotubes.

Inhibition of PI3-kinase activity by using pharmacological inhibitors or dominant negative forms of PI3-kinase is known to efficiently inhibit insulin-induced glycogen synthesis [32–33], whereas our results

on WT-SHIP2 expression showed the partial inhibition of insulin-induced Akt activation leading to glycogen synthesis. The reason why WT-SHIP2 overexpression only partially inhibited insulin-stimulated activation of the downstream effector of PI3-kinase is uncertain. However, we speculate that the degree of the inhibition of insulin-induced Akt activation might not be complete, because PI(3,4)P2 is possibly involved, although it has a lesser role than PI(3,4,5)P3, in the activation of Akt. Along this line, it is known that both PI(3,4,5)P3 and PI(3,4)P2 bind with high affinity to the pleckstrin homology (PH) domain of Akt leading to the recruitment of Akt to be phosphorylated on the plasma membrane [5, 6, 18, 28, 29]. Alternatively, there are redundant pathways that regulate the PI3-kinase product PI(3,4,5)P3. It is known that PTEN possesses 3'-phosphoinositol phosphatase activity toward PI(3,4,5)P3 [34]. Studies with the cells derived from PTEN-deficient mice showed increased PI(3,4,5)P3 content and increased Akt activity [35]. In addition, overexpression of wild-type PTEN inhibited insulin-induced Akt activity in 3T3-L1 adipocytes [10]. Thus, PTEN is an alternative lipid phosphatase to possibly hydrolyse PI(3,4,5)P3, at least in part, in certain types of cells. However, PTEN does not seem to be the physiologically important lipid phosphatase in L6 cells, because PTEN is not expressed in skeletal muscles [10]. Although our results are consistent with the recent report showing that insulin-induced glycogen synthesis was only partly enhanced in isolated soleus muscle from heterozygous SHIP2 knockout mice [17], the possible existence of another lipid phosphatase(s) for the hydrolysis of PI(3,4,5)P3 in L6 myotubes remains to be determined.

In summary, we have clarified the molecular mechanism by which SHIP2 is involved in the negative regulation of insulin signalling leading to glycogen synthesis in L6 myotubes. Our results suggest that SHIP2 controls insulin-induced glycogen synthase, at least in part, by negatively regulating Akt-GSK3β pathway via SHIP2 5'-phosphatase activity. As the molecular mechanism of SHIP2 5'-phosphatase mediated negative regulation, Akt seems to be preferentially activated by PI(3,4,5)P3 rather than by PI(3,4)P2.

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