Biochemical and Biophysical Research Communications **260,** 265–272 (1999)
Article ID bbrc.1999.0888, available online at http://www.idealibrary.com on IDEAL®

Molecular Cloning of Rat SH2-Containing Inositol Phosphatase 2 (SHIP2) and Its Role in the Regulation of Insulin Signaling

Hajime Ishihara,* Toshiyasu Sasaoka,*,[1] Hiroyuki Hori,* Tsutomu Wada,* Hiroki Hirai,* Tetsuro Haruta,* W. John Langlois,† and Masashi Kobayashi*

**First Department of Medicine, Toyama Medical & Pharmaceutical University, Toyama 930-0194, Japan; and †Department of Medicine, University of Toronto, Toronto, Ontario M5G 2C4, Canada*

Received May 14, 1999

SH2-containing inositol 5′-phosphatase (SHIP) plays a negative regulatory role in hematopoietic cells. We have now cloned the rat SHIP isozyme (SHIP2) cDNA from skeletal muscle, which is one of the most important target tissue of insulin action. Rat SHIP2 cDNA encodes a 1183-amino-acid protein that is 45% identical with rat SHIP. Rat SHIP2 contains an amino-terminal SH2 domain, a central 5′-phosphoinositol phosphatase activity domain, and a phosphotyrosine binding (PTB) consensus sequence and a proline-rich region at the carboxyl tail. Specific antibodies to SHIP2 were raised and the function of SHIP2 was studied by stably overexpressing rat SHIP2 in Rat1 fibroblasts expressing human insulin receptors (HIRc). Endogenous SHIP2 underwent insulin-mediated tyrosine phosphorylation and phosphorylation was markedly increased when SHIP2 was overexpressed. Although overexpression of SHIP2 did not affect insulin-induced tyrosine phosphorylation of the insulin receptor β-subunit and Shc, subsequent association of Shc with Grb2 was inhibited, possibly by competition between the SH2 domains of SHIP2 and Grb2 for the Shc phosphotyrosine. As a result, insulin-stimulated MAP kinase activation was reduced in SHIP2-overexpressing cells. Insulin-induced tyrosine phosphorylation of IRS-1, IRS-1 association with the p85 subunit of PI3-kinase, and PI3-kinase activation were not affected by overexpression of SHIP2. Interestingly, although both PtdIns-(3,4,5)P3 and PtdIns(3,4)P2 have been implicated in the regulation of Akt activity *in vitro,* overexpression of SHIP2 inhibited insulin-induced Akt activation, presumably by its 5′-inositol phosphatase activity. Furthermore, insulin-induced thymidine incorporation was decreased by overexpression of SHIP2. These results indicate that SHIP2 plays a negative regulatory role in insulin-induced mitogenesis, and regulation of the Shc · Grb2 complex and of the downstream products of PI3-kinase provides possible mechanisms of SHIP2 action in insulin signaling.

SH2-containing inositol 5′-phosphatase (SHIP) was originally identified as a Shc associated protein (1–3). The importance of SHIP has been reported in diverse hematopoietic cells (1–4). SHIP is composed of an amino-terminal SH2 domain, a central phosphoinositol 5′-phosphatase activity domain, and two phosphotyrosine binding (PTB) consensus sequences and a proline rich region at the carboxyl tail (1–4). SHIP selectively hydrolyzes the 5′-phosphate from Ins(1,3,4,5)P4 and PtdIns(3,4,5)P3, which are implicated in growth factor and cytokine mediated signaling (1–4). PtdIns(3,4,5)P3 is generated by PI3-kinase whose activation is initiated by many tyrosine kinase receptors (5, 6). Since PtdIns(3,4,5)P3 is thought to act as second messenger (5, 6), dephosphorylation of PtdIns(3,4,5)P3 by SHIP may negatively regulate signals downstream of PI3-kinase. Along this line, SHIP has been implicated in the negative regulation of FcγRIIB receptor-mediated B cell proliferation and signaling in mast cells (7, 8). In addition to the catalytic role of SHIP, the SH2 domain of SHIP has been shown to interact with Shc at its phosphorylated 317-Tyr residue (9). Since phosphorylated Shc 317-Tyr residue binds to Grb2 which is important for Ras-MAP kinase activation (10, 11), competitive interaction of SHIP with Shc via the SHIP SH2 domain may reduce Ras activity resulting in negative regulation of mitogenesis.

SHIP is abundantly expressed only in hematopoietic cells (1, 2), with barely detectable expression in CHO cells and Rat1 fibroblasts, in which certain aspects of insulin signaling have been characterized (12, 13). In

[1] To whom correspondence should be addressed at First Department of Medicine, Toyama Medical & Pharmaceutical University, 2630 Sugitani, Toyama, 930-0194, Japan. Fax: 81-764-34-5025. E-mail: tsasaoka-tym@umin.ac.jp.

0006-291X/99 $30.00

spite of negligible expression of SHIP, insulin treatment increased the phosphoinositol 5′-phosphatase activity and this activity was found in anti-Shc immunoprecipitates in CHO cells (12). Furthermore, down-regulation of insulin signaling by exogenously transduced SHIP was indicated by the fact that insulin-induced Xenopus oocyte maturation and Glut4 translocation were inhibited by SHIP expression (13, 14). The inhibitory function of SHIP appeared to be dependent on its 5′-phosphatase activity toward PtdIns(3,4,5)P3 (13, 14). Taken together with these previous reports, one can speculate that a SHIP isozyme exists which modulates insulin signaling. This prompted a search for a possible SHIP isozyme involved in insulin signaling in insulin target tissues. Here we report the cloning of a novel isozyme of SHIP, designated as rat SHIP2, from rat skeletal muscle, which is one of most important target tissues of insulin. The involvement of rat SHIP2 in regulation of insulin signaling was examined by stably overexpressing rat SHIP2 into Rat1 fibroblasts overexpressing insulin receptors (SHIP2 cells).

MATERIALS AND METHODS

Materials. Porcine insulin was the kind gift of Shimizu Pharmaceutical Co. (Shizuoka, Japan). [γ-32P]ATP (3000 TBq/mmol) and enhanced chemiluminescence reagents were purchased from Amersham Pharmacia Biotech Corp. (Uppsala, Sweden). A polyclonal and a monoclonal anti-Shc antibody, a monoclonal anti-IRS-1 antibody, a monoclonal anti-Grb2 antibody, and a monoclonal anti-p85 antibody were from Transduction Laboratories (Lexington, KY). A polyclonal anti-FLAG antibody, a polyclonal anti-MAP kinase antibody, a polyclonal anti-Akt antibody, and a monoclonal anti-phosphotyrosine antibody (PY99) were from Santa Cruz Biotechnology (Santa Cruz, CA). A polyclonal anti-phospho-specific p44/p42 MAP kinase (Thr202/Tyr204) antibody, and a polyclonal anti-phospho-specific Akt (Ser473) antibody were from New England Biolabs, Inc. (Beverly, MA). All other routine reagents were analytical grade and purchased from Sigma Chemical Co. (St. Louis, MO).

DNA amplification and molecular cloning of rat SHIP2. Two sets of degenerate oligonucleotides, coding for two stretches of conserved amino acids within various 5′-phosphatases (1–3, 15, 16), were synthesized based on the following sequences: FWFGDLNYRLD and PSWCDRILWK (sense 5′-TITGGYTIGGIGAYYTIAAYTA-3′, antisense 5′-YKCCAIARIA-YICKRTCRCACCA-3′, sense 5′-TIGGIGAYYTIAAYTAYMGIIT-3′ and antisense 5′-ARIAYI-CKRTCRCACCAISHIGG-3′). First-strand cDNA was obtained by incubating 2 μg total RNA of rat skeletal muscle with random hexamers and Superscript II reverse transcriptase according to the manufacturer's instruction (Life Technologies, Rockville, MD). The obtained cDNA was used for hot-start PCR utilizing Ampli-Wax PCR Gem (Perkin–Elmer, Foster City, CA) under the following condition: after initial denaturation for 10 min at 95°C, 30 cycles of incubation for 30 s at 95°C and 30 sec at 45°C and 1 min at 72°C, followed by a final incubation for 7 min at 72°C. PCR products were analyzed by 2% agarose gel electrophoresis. The products were subcloned into vector pCR II (Invitrogen, Carlsbad, CA) and their sequences were determined by utilizing T7 primer by dye-terminator cycle sequence method. According to the sequence data, one clone appeared to encode the rat homologue of human INPPL1 (inositol polyphosphate-like protein-1) (15). To obtain the full length rat cDNA, 5′- and 3′-rapid amplification of cDNA ends (RACE) were carried out utilizing the Marathon cDNA amplification system according to the supplier's protocol (Clontech, San Diego, CA). The primers used were: 5′-CACATTGGTCCGGACCCCA-GTTGGCTT-3′, 5′-CCCAGTTGGCTTCTGCTTGTGCCAAGC-3′, 5′-CTGCTTGTGCCAAG-CATATGTGTCTCGG-3′, 5′-CCTTCTTCTGGATGCCTGACAGGAAG-3′, 5′-CTGGGCATA-CAGGCCTATAAGCTCACC-3′, 5′-CACAGCCAGGAAATCCTCTCCATCTGG-3′, 5′-CGCT-CTCGCTGTCTCGCACCAGGAAGCTG-3′, 5′-GCTGCCATCGCGGCCTGCCCGAGCTAG-CAG-3′ (antisense) and AP1 primer and AP2 primer of the kit (sense) for 4 sets of 5′-RACE reactions including nested PCR, and 5′-GACATGGATATCCAGGAGATCCTGAAC-3′ and 5′-TACATTAGTAGGAGAGAGTTTGAGCCCCTG-3′ (sense) and AP1 primer and AP2 primer for 1 set of 3′-RACE reactions including nested PCR. Overlapping PCR products were directly sequenced. Furthermore, these PCR products were subcloned into pCR II and various DNA fragments generated by restriction enzymes were re-subcloned into pBluescript (Stratagene, La Jolla, CA), and the sequences were confirmed.

Anti-SHIP2 antibodies. Two rabbit polyclonal antibodies to rat SHIP2 were generated against synthetic polypeptides corresponding to amino-terminal amino acids 18 to 36 (APAWYHRDLSRAAAEELLA) or to carboxyl-terminal amino acids 1864 to 1878 (PLSFPPPRIRESIQE) of rat SHIP2. These two antibodies are specific to SHIP2 and no reactive with SHIP.

Cell culture, plasmid, and DNA transfection. Rat1 fibroblasts overexpressing 1×10^6 human insulin receptors per cell (HIRc) were kindly supplied by Dr. J. M. Olefsky (University of California San Diego, CA). HIRc cells were maintained in Dulbecco's modified Eagle's medium (DMEM)/F12 medium supplemented with 10% fetal calf serum (FCS) as previously described (10, 11). A wild SHIP2 fragment was subcloned into pFLAG-CMV-5b (Eastman Kodak Co., New Haven, CT) to generate SHIP2 fused FLAG at its carboxyl-terminus (pSHIP2-FLAG). To establish cell lines stably overexpressing SHIP2, 10 μg of pSHIP2-FLAG and 1 μg of pcDEB carrying a hygromycin-resistant gene were cotransfected utilizing the TransIT-LT1 transfection reagent according to the manufacturer's instructions (Pan Vera Co., Madison, WI). In brief, the cells were washed with sterile phosphate buffered saline (PBS) followed by addition of 10 ml serum-free DMEM to each 90 mm dish. Premixed TransIT reagents including DNA were then added to each dish and dishes were placed at 37°C in 5% CO_2. After 4 h incubation, the medium was changed to 10 ml fresh growth medium. At 48 h after transfection, hygromycin B (400 μg/ml) was added to the medium to select for resistant cells. Cells expressing high levels of SHIP2-FLAG were isolated by limiting dilution and then chosen by immunoblotting with anti-SHIP2 antibody.

Immunoprecipitations and Western blotting. Serum-starved cells were treated with 17 nM insulin at 37°C for the indicated times. The cells were lysed in a buffer containing 30 mM Tris, 150 mM NaCl, 10 mM EDTA, 0.5% sodium deoxycholate, 1% Triton X-100, 1 mM phenylmethyl-sulfonyl fluoride (PMSF), 1 mM Na_3VO_4, 160 mM sodium fluoride, 10 μM aprotinin, 10 μM leupeptin, pH 7.4, for 10 min at 4°C. The cell lysates were centrifuged to remove insoluble materials. The supernatants were immunoprecipitated with the indicated antibodies for 2 h at 4°C. The precipitates or the remaining supernatants were then separated by 7.5% SDS–PAGE and electrically transferred onto polyvinylidene difluoride membranes. After incubation with the specified antibody, enhanced chemiluminescence detection was performed according to the manufacturer's instructions (Amersham Pharmacia Biotech Corp.) (10, 11).

PI3-kinase activity. Serum-starved cells were stimulated with 17 nM insulin at 37°C for the indicated times. The cells were solubilized in a buffer containing 20 mM Tris, 137 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 100 μM Na_3VO_4, 1% Nonidet-P40, 10% glycerol, 2 mM PMSF, and 10 μg/ml aprotinin, pH 7.6. The cells were centrifuged to remove insoluble materials. The supernatants were immunoprecipitated with anti-IRS-1 antibody for 2 h at 4°C. The precipitates were

washed twice with Buffer A (Tris-buffered saline, pH 7.6, 1% Nonidet-P40, 100 μM Na_3VO_4, and 1 mM dithiothreitol (DTT)), twice with Buffer B (100 mM Tris, pH 7.6, 500 mM LiCl, 100 μM Na_3VO_4, and 1 mM DTT) and twice with Buffer C (10 mM Tris, pH 7.6, 100 mM NaCl, 1 mM EDTA, and 1 mM DTT). The phosphorylation reaction was started by adding 20 μl PI solution containing 0.5 mg/ml PI, 50 mM Hepes, 1 mM NaH_2PO_4, 1 mM EGTA, pH 7.6 at 20°C, followed by addition of 10 μl of the reaction mixture containing 250 μM [γ-^{32}P]ATP (0.37 Mbq/tube), 100 mM Hepes, 50 mM $MgCl_2$, pH 7.6 for 5 min. The reaction was stopped by the addition of 15 μl 8M HCl. The products were extracted by adding 130 mM of chloroform/methanol (1:1) followed by centrifugation. The organic phase was removed and spotted on Silica Gel 60 plates. The plates were developed and dried. The phosphorylated inositol was visualized by autoradiography and quantitated by the Bio-Image Analyzer (Fuji Film, Tokyo, Japan).

BrdU incorporation. 1×10^4 cells were grown in 96-multiwell culture plates and serum-starved for 24 h in DMEM. After stimulation of the cells with various concentrations of insulin for 20 h, the cells were incubated with BrdU for 2 h at 37°C. BrdU incorporation into DNA was measured using Cell Proliferation ELISA kit according to the manufacturer's instructions (Roche Diagnostics, Basel, Switzerland).

RESULTS AND DISCUSSION

Identification, structure, and overexpression of rat SHIP2. We have isolated a cDNA encoding a SHIP isozyme using a PCR based strategy in which degenerate primers were designed coding for highly conserved amino acid regions within the 5′-phosphatase catalytic domain of known inositol and phosphatidylinositol polyphosphate 5′-phosphatases. Reverse-transcription PCR on rat skeletal muscle RNA amplified a fragment of approximately 300 bp which was homologous to human INPPL1 (15) and SHIP2 (17). Human INPPL1 was cloned using a similar strategy (15), and the predicted protein product was homologous to SHIP, but did not have an N-terminal SH2 domain. Another group subsequently cloned a gene they designated as SHIP2, almost identical to INPPL1 in the catalytic domain, but differing at both the N- and C-termini from INPPL1. The predicted human SHIP2 protein had the expected N-terminal SH2 domain, and probably represents the same gene as INPPL1, with the differences possibly representing sequencing of an unspliced intron upstream of the catalytic domain, and an artificial frameshift downstream, in the INPPL1 sequence (17). Therefore, we proceeded to obtain the full length rat cDNA by a direct PCR-based cloning strategy utilizing 5′- and 3′-RACE. The deduced amino acid sequence of the cloned rat cDNA is shown in Fig. 1A. The predicted open reading frame encodes a 1183-amino-acid protein, referred to as rat SHIP2, with a predicted molecular weight of 133 kDa. The cDNA and predicted amino acid sequences of rat SHIP2 were deposited in DDBJ under Accession No. AB011439. Rat SHIP2 contains an amino-terminal SH2 domain, a central inositol polyphosphate 5′-phosphatase catalytic region, and a PTB binding consensus sequence (NPAY) and a carboxyl-terminal proline rich region for potential binding to SH3 domains (PPAPPR). This structure is similar to that of SHIP, except for the fact that SHIP contains two NPXY PTB domain binding consensus sites (1–3). In addition, rat SHIP2 is highly homologous with 92% amino acid identity to the more recently cloned human SHIP2, and has 45% identity with rat SHIP (Fig. 1B). It is of note that the sequence of rat SHIP2 differs at the carboxyl-terminus from human SHIP2 (17). The carboxyl-terminus amino acid sequence of rat SHIP2 is 74 aa shorter than that of human SHIP2. This finding was confirmed by the fact that anti-SHIP2 immunoblot of samples derived from rat and human tissues demonstrated shorter molecular weight of rat SHIP2 than that of human SHIP2

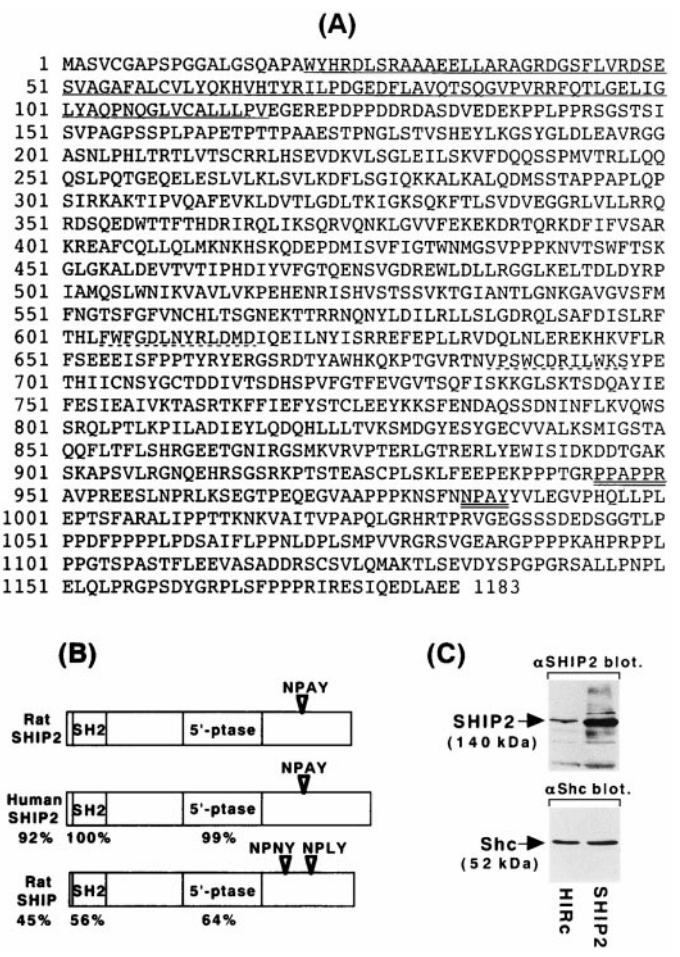

FIG. 1. Amino acid sequence of rat SHIP2, comparison of rat SHIP2 with rat SHIP and human SHIP2, and overexpression of SHIP2 in HIRc cells. (A) Deduced amino acid sequences of rat SHIP2 are shown. The underlined sequence indicates the SH2 domain, the dotted line denotes the two conserved 5′-phosphatase motifs, and the NPXY PTB domain binding consensus sequence and the target sequence for binding to the SH3 domains (PXXPXR) are doubly underlined. (B) Amino acid homology among rat SHIP2, rat SHIP, and human SHIP2. Percent identity for the indicated regions are shown. (C) Parental and SHIP2 overexpressing HIRc cells were solubilized, and expression of SHIP2 in the cell lysates were analyzed by immunoblotting with anti-SHIP2 antibody.

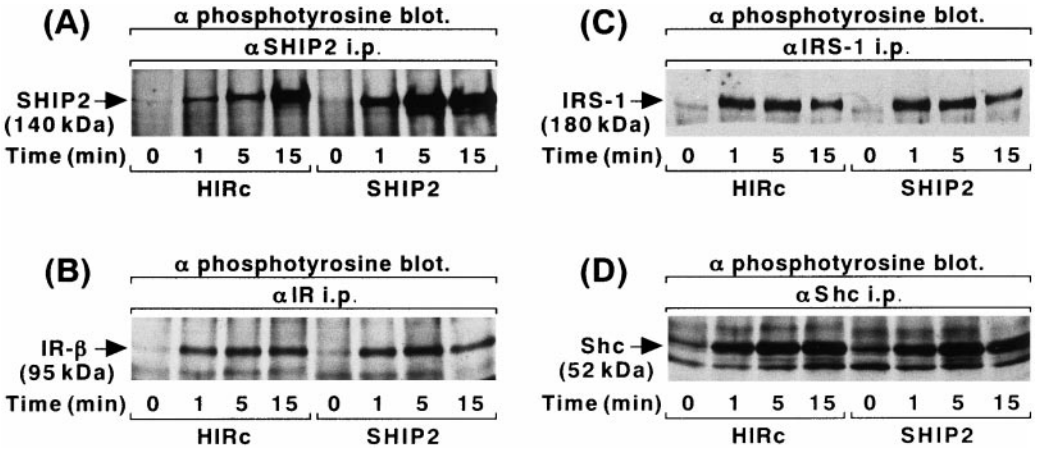

FIG. 2. Effects of SHIP2 overexpression on insulin-mediated early signaling events. Serum-starved cells were incubated with 17 nM insulin for the indicated times. The cell lysates were immunoprecipitated with the specified antibody. The immunoprecipitates were immunoblotted with anti-phosphotyrosine antibody. Tyrosine phosphorylation of SHIP2 (A), insulin receptor β-subunit (B), IRS-1 (C), and Shc (D) is shown. Results are representative of three separate experiments.

(data not shown). Further the carboxyl-terminus of rat SHIP2 is less proline-rich than that of human SHIP2, although the SH3 domain binding motif, PPAPPR, in rat SHIP2 is well conserved. To investigate the role of SHIP2 in insulin signaling, SHIP2 cDNA was stably transfected into Rat1 fibroblasts expressing insulin receptors (HIRc). From the SHIP2 transfected cell lines, a cell line expressing a 10-fold excess of SHIP2 compared with endogenous SHIP2 (designated SHIP2 cells) was chosen for further studies (Fig. 1C).

Effects of SHIP2 overexpression on proximal events in insulin signaling. Since SHIP is tyrosine-phosphorylated following cytokine stimulation in hematopoietic cells (1, 2, 9), the effect of insulin treatment on SHIP2 tyrosine phosphorylation was examined. Endogenous SHIP2 underwent tyrosine phosphorylation at 1 min and this persisted at 15 min of insulin stimulation in HIRc cells. Insulin induced tyrosine phosphorylation of SHIP2 was faster and greater amount of SHIP2 was phosphorylated in SHIP2 cells as shown in Fig. 2A. Since the SHIP2 987-Tyr residue is located within the phosphotyrosine binding consensus motif, we suspected that it might be phosphorylated. This prediction was confirmed by expressing a 987-Tyr→Phe mutant SHIP2 which demonstrated decreased tyrosine phosphorylation (data not shown). Although the role of tyrosine phosphorylation of SHIP2 is unknown at present, one can speculate that it may modulate the 5′-phosphatase activity of SHIP2, as tyrosine phosphorylation of SHIP was reported to negatively regulate the 5′-phosphatase activity (4, 18). Constitutive phosphorylation of SHIP with elevated PtdIns(3,4,5)P3 levels is observed in Bcr-Abl-transformed cells (4, 18).

Interestingly, a tyrosine phosphorylated 95-kDa protein was seen in anti-SHIP2 immunoprecipitates. The identity of this 95-kDa protein as the insulin receptor β-subunit was established by immunoblotting the anti-SHIP2 precipitates with anti-insulin receptor antibody (data not shown). Although we can not rule out the possibility that insulin receptor interacts with SHIP2 via a third molecule, SHIP2 may be a substrate of the insulin receptor.

Insulin treatment induces autophosphorylation of the insulin receptor β-subunit. The activated insulin receptor phosphorylates various substrates including IRS-1 and Shc to propagate the insulin signal downstream (19). The effect of SHIP2 overexpression on these early intracellular signaling events was examined. Basal tyrosine phosphorylation of the insulin receptor β-subunit, IRS-1, and Shc was barely detected in both parental HIRc and SHIP2 cells. In addition, overexpression of SHIP2 did not affect these early insulin signaling events, including tyrosine phosphorylation of the insulin receptor β-subunit (Fig. 2B), IRS-1 (Fig. 2C), and Shc (Fig. 2D). These results indicate that SHIP2 does not interfere with the most proximate events in the insulin signaling pathway.

Effect of SHIP2 overexpression on insulin stimulation of Ras-MAP kinase pathway. Tyrosine phosphorylated Shc binds to Grb2, which is important for Ras-MAP kinase activation in Rat1 fibroblasts (10, 11, 20, 21). SHIP was originally identified as a Shc binding protein, and has been shown to associate with Shc following cytokine and growth factor stimulation (1–3, 9, 22, 23). Since SHIP2 has a similar domain structure compared with SHIP, one can speculate that SHIP2 may also interact via its SH2 domain with 317-Tyr, and via its carboxyl-terminus tyrosine residue with the Shc PTB domain. In fact, endogenous SHIP2 underwent insulin-stimulated association with Shc, as demonstrated by co-immunoprecipitation, and this was greatly increased upon overexpression of SHIP2 (Figs.

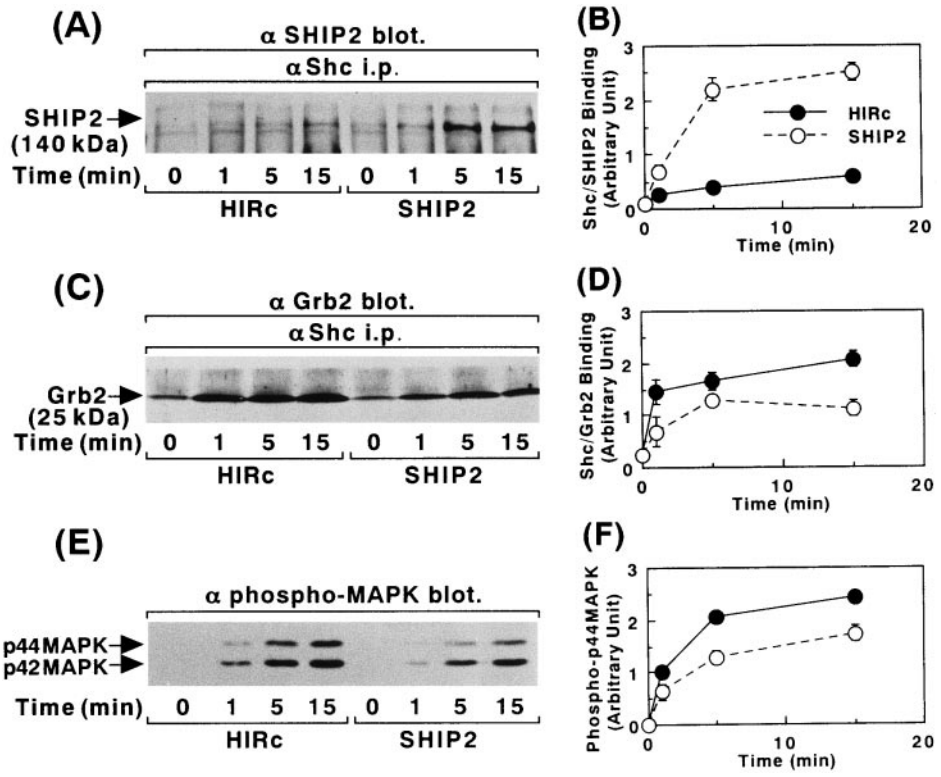

FIG. 3. Effect of SHIP2 overexpression on insulin-mediated Shc · Grb2 association and MAP kinase activation. Serum-starved cells were incubated with 17 nM insulin for the indicated times. The cell lysates were immunoprecipitated with anti-Shc antibody and the precipitates were immunoblotted with anti-SHIP2 antibody (A) or anti-Grb2 antibody (C). The amount of SHIP2 associated with Shc (B) and of Grb2 associated with Shc (D) was analyzed with densitometry. Results are the mean ± SE of four separate experiments. The cell lysates were immunoblotted with anti-phospho-specific p44/p42 MAP kinase (Thr202/Tyr204) antibody (E). The amount of phosphorylated p44 MAP kinase was analyzed with densitometry. Results are means ± SE of three separate experiments (F).

3A and 3B). This association was confirmed by the fact that Shc was also found in anti-SHIP2 immunoprecipitates following insulin stimulation (data not shown). These results are in contrast to a previous report showing that insulin fails to induce Shc association with human SHIP2 in 3T3-L1 adipocytes (24). Although the reason for the difference is uncertain, it may arise from tissue specificity or experimental conditions used in the study. In our hands, insulin-induced Shc association with SHIP2 was clearly visualized by using two different SHIP2 specific antibodies in HIRc cells. Since SHIP2 binding via its SH2 domain to phosphorylated Shc 317-Tyr residue may compete with Shc binding to Grb2, one can speculate that overexpression of SHIP2 may interfere with the association of Shc with Grb2 resulting in attenuation of signaling through the Ras pathway. Indeed, overexpression of SHIP2 inhibited Shc association with Grb2 by 45 ± 2% at 15 min following insulin stimulation as shown in Figs. 3C and 3D. As a result, insulin induced MAP kinase activation detected by utilizing phospho-specific anti-MAP kinase antibody was also reduced by 37 ± 2% in SHIP2 cells (Figs. 3E and 3F). To assure equal amount of protein loaded for the study, the cell lysates were immunoblotted with anti-MAP kinase antibody (data not shown).

Effect of SHIP2 overexpression on insulin-stimulated PI 3-kinase pathway. It is well known that PI3-kinase is an important mediator of insulin-induced metabolic and mitogenic signaling (25, 26). SHIP2 was cloned based on homology with the 5′-inositol phosphatase activity region in SHIP (1–3, 15, 16), and therefore is likely a 5′-phosphatase capable of modifying the PI3-kinase pathway. We examined the effect of SHIP2 overexpression on insulin stimulation of the PI3-kinase pathway. Insulin induced IRS-1 association with the p85 regulatory subunit of PI3-kinase in a time-dependent manner in HIRc cells. Overexpression of SHIP2 did not affect insulin-mediated IRS-1 association with p85 (Fig. 4A). In addition, insulin-stimulated PI3-kinase activation was not affected by overexpression of SHIP2 (Fig. 4B). These results indicate that SHIP2 does not act upstream of insulin-induced PI3-kinase activation. Activation of PI3-kinase generates PtdIns(3,4,5)P3 *in vivo,* which is thought to function as a second messenger to signal downstream molecules (6). Akt lies downstream of PI3-

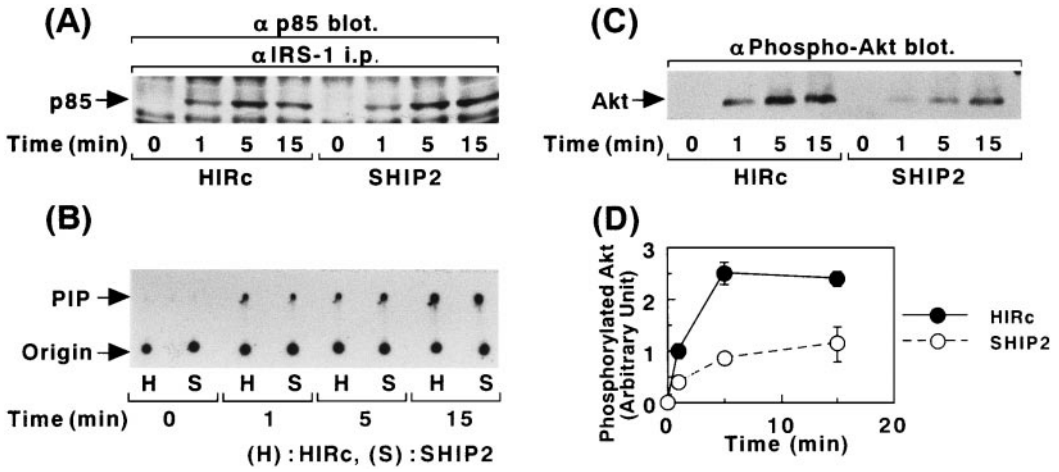

FIG. 4. Effect of SHIP2 overexpression on insulin-stimulated PI3-kinase and Akt activation. Serum-starved cells were incubated with 17 nM insulin for the indicated times. (A) The cell lysates were immunoprecipitated with anti-IRS-1 antibody and the precipitates were immunoblotted with anti-p85 subunit antibody. Results are representative of three separate experiments. (B) PI3-kinase activity was assayed as described under Materials and Methods. Results are representative of three separate experiments. (C) The cell lysates were immunoblotted with anti-phospho-specific Akt antibody. (D) The amount of phosphorylated Akt was analyzed with densitometry. Results are means ± SE of four separate experiments.

kinase (27, 28), and has been shown to play an important role in diverse biological actions of insulin including mitogenesis, glucose uptake, and glycogen synthesis (29–31). Previous studies indicated that Akt is directly activated *in vitro* by PtdIns(3,4)P2 and inhibited by PtdIns(3,4,5)P3 in the absence of PDK1 (32, 33). In contrast, activation of Akt is more dependent on PtdIns(3,4,5)P3 than PtdIns(3,4)P2 in the presence of PDK1 (34, 35). Thus, the relative role of PtdIns (3,4,5)P3 versus PtdIns(3,4)P2 in the activation of Akt is controversial *in vitro,* and unclear *in vivo.* Since Akt is activated by phosphorylation on Ser-308 and Ser-473 residues (36), insulin stimulation of Akt activity was examined by immunoblotting with a phospho-specific Akt antibody. Insulin stimulated Akt phosphorylation in a time-dependent manner. Importantly, overexpression of SHIP2 inhibited insulin-mediated Akt activation (Fig. 4C). The results are summarized in Fig. 4D. Following 15 min of insulin stimulation, Akt phosphorylation was decreased by 58 ± 4% in SHIP2 cells compared with parental HIRc cells. To assure equal amount of protein loaded for the study, the cell lysates were immunoblotted with anti-Akt antibody (data not shown). These results are consistent with a recently published report showing that SHIP inhibits Akt activation in B cells (37). Taken together, this suggests that SHIP2 down-regulates this pathway by decreasing levels of PtdIns(3,4,5)P3, and one can infer that PtdIns(3,4,5)P3 rather than PtdIns(3,4)P2 plays the more important role in *in vivo* activation.

Role of SHIP2 in insulin-stimulated mitogenesis. As overexpression of SHIP2 was found to modulate insulin stimulation of both the Ras-MAP kinase and PI3-kinase pathways, the biological actions of insulin may be affected by SHIP2. We examined the effect of SHIP2 overexpression on insulin induced mitogenesis (Fig. 5). Insulin stimulated BrdU incorporation in a dose-dependent manner with an ED_{50} value of 2.4 ± 0.3 nM in parental HIRc cells. Overexpression of SHIP2 resulted in marked decrease of insulin responsiveness by 61 ± 4%. Insulin sensitivity was also reduced with an ED_{50} value of 4.2 ± 0.3 nM in SHIP2 cells. Increasing evidence suggests that SHIP acts as a negative regulator of signaling in hematopoietic cells. Expression of SHIP in myeloid cells led to inhibition of the cell growth (1). Hemopoietic progenitors lacking

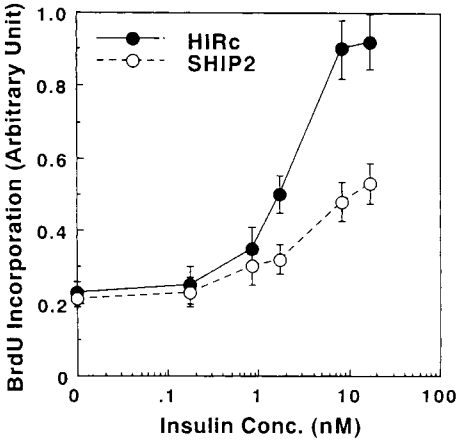

FIG. 5. Effect of SHIP2 overexpression on insulin-mediated BrdU incorporation. Serum-starved cells were incubated with the indicated concentrations of insulin. BrdU incorporation in HIRc (○) and SHIP2 (●) cells was assayed as described under Materials and Methods. Dose–response curves for insulin stimulation of BrdU incorporation are shown. Results are means ± SE of three separate experiments.

SHIP are hyper-responsive to multiple cytokines (38). Although expression of SHIP is restricted to hematopoietic cells (1, 2), a broader range of human SHIP2 expression has been reported including heart, skeletal muscle, and placenta by Northern blot analysis (15, 17). Therefore, it is logical to speculate that SHIP2 may function as a negative regulator of insulin and growth factor-mediated proliferation in these cells.

Insulin activation of PI3-kinase plays a key role in the metabolic actions of insulin. Specific blockade of PI3-kinase utilizing a dominant negative mutant of PI3-kinase or pharmacological inhibitors, such as wortmannin and LY294002, inhibited metabolic actions of insulin including glucose uptake and glycogen synthesis (26, 39, 40). Since it is apparent that regulation of the PI3-kinase product PtdIns(3,4,5)P3 is a key determinant of the metabolic actions of insulin (5, 6), it is reasonable to hypothesize that SHIP2-induced dephosphorylation of PtdIns(3,4,5)P3 and production of PtdIns(3,4)P2 could modulate insulin's metabolic signaling. The role of SHIP2 in the metabolic actions of insulin in appropriate target tissues is currently under investigation.

In summary, we have cloned a SHIP isozyme, referred to as rat SHIP2, from rat skeletal muscle, which has a 5′-inositol phosphatase domain. SHIP2 appears to play a novel role in insulin signaling by down-regulating both the Ras-MAP kinase and PI3-kinase pathways. As the result, it seems to play an inhibitory role with respect to insulin-mediated cell proliferation, and it is interesting to speculate that it may be an important in regulating insulin's metabolic actions, as many of them require the downstream products of PI3-kinase.

ACKNOWLEDGMENT

This work was supported in part by a Grant-in-Aid for Encouragement of Young Scientists from the Ministry of Education, Science, Culture, and Sports in Japan (to T.S.).

REFERENCES

1. Lioubin, M. N., Algate, P. A., Tsai, S., Carlberg, K., Aebersold, R., and Rohrschneider, L. R. (1996) *Genes Dev.* **10,** 1084–1095.
2. Damen, J. E., Liu, L., Rosten, P., Humphries, R. K., Jefferson, A. B., Majerus, P. W., and Krystal, G. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 1689–1693.
3. Kavanaugh, W. M., Pot, D. A., Chin, S. M., Deuter-Reinhard, M., Jefferson, A. B., Norris, F. A., Masiarz, F. R., Cousens, L. S., Majerus, P. W., and Williams, L. T. (1996) *Curr. Biol.* **6,** 438–445.
4. Osborne, M. A., Zenner, G., Lubinus, M., Zhang, X., Songyang, Z., Cantley, L. C., Majerus, P., Burn, P., and Kochan, J. P. (1996) *J. Biol. Chem.* **271,** 29271–29278.
5. Toker, A., and Cantley, L. C. (1997) *Nature* **387,** 673–676.
6. Jiang, T., Sweeney, G., Rudolf, M. T., Klip, A., Traynor-Kaplan, A., and Tsien, R. Y. (1998) *J. Biol. Chem.* **273,** 11017–11024.
7. Ono, M., Bolland, S., Tempst, P., and Ravetch, J. V. (1996) *Nature* **383,** 263–266.
8. Ono, M., Okada, H., Bolland, S., Yanagi, S., Kurosaki, T., and Ravetch, J. V. (1997) *Cell* **90,** 293–301.
9. Liu, L., Damen, J. E., Hughes, M. R., Babic, I., Jirik, F. R., and Krystal, G. (1997) *J. Biol. Chem.* **272,** 8983–8988.
10. Sasaoka, T., Ishihara, H., Sawa, T., Ishiki, M., Morioka, H., Imamura, T., Usui, I., Takata, Y., and Kobayashi, M. (1996) *J. Biol. Chem.* **271,** 20082–20087.
11. Ishihara, H., Sasaoka, T., Ishiki, M., Takata, Y., Imamura, T., Usui, I., Langlois, W. J., Sawa, T., and Kobayashi, M. (1997) *J. Biol. Chem.* **272,** 9581–9586.
12. Guilherme, A., Klarlund, J. K., Krystal, G., and Czech, M. P. (1996) *J. Biol. Chem.* **271,** 29533–29536.
13. Vollenweider, P., Clodi, M., Martin, S. S., Imamura, T., Kavanaugh, W. M., and Olefsky, J. M. (1999) *Mol. Cell. Biol.* **19,** 1081–1091.
14. Deuter-Reinhard, M., Apell, G., Pot, D., Klippel, A., Williams, L. T., and Kavanaugh, W. M. (1997) *Mol. Cell. Biol.* **17,** 2559–2565.
15. Hejna, J. A., Saito, H., Merkens, L. S., Tittle, T. V., Jakobs, P. M., Whitney, M. A., Grompe, M., Friedberg, A. S., and Moses, R. E. (1995) *Genomics* **29,** 285–287.
16. Jefferson, A. B., and Majerus, P. W. (1995) *J. Biol. Chem.* **270,** 9370–9377.
17. Pesesse, X., Deleu, S., De Smedt, F., Drayer, L., and Erneux, C. (1997) *Biochem. Biophys. Res. Commun.* **239,** 697–700.
18. Sattler, M., Salgia, R., Shrikhande, G., Verma, S., Choi, J. L., Rohrschneider, L. R., and Griffin, J. D. (1997) *Oncogene* **15,** 2379–2384.
19. White, M. F., and Kahn, C. R. (1994) *J. Biol. Chem.* **269,** 1–4.
20. Sasaoka, T., Rose, D. W., Jhun, B. H., Saltiel, A. R., Draznin, B., and Olefsky, J. M. (1994) *J. Biol. Chem.* **269,** 13689–13694.
21. Sasaoka, T., Draznin, B., Leitner, J. W., Langlois, W. J., and Olefsky, J. M. (1994) *J. Biol. Chem.* **269,** 10734–10738.
22. Lamkin, T. D., Walk, S. F., Liu, L., Damen, J. E., Krystal, G., and Ravichandran, K. S. (1997) *J. Biol. Chem.* **272,** 10396–10401.
23. Tridandapani, S., Kelley, T., Pradhan, M., Cooney, D., Justement, L. B., and Coggeshall, K. M. (1997) *Mol. Cell. Biol.* **17,** 4305–4311.
24. Habib, T., Hejna, J. A., Moses, R. E., and Decker, S. J. (1998) *J. Biol. Chem.* **273,** 18605–18609.
25. Jhun, B. H., Rose, D. W., Seely, B. L., Rameh, L., Cantley, L., Saltiel, A. R., and Olefsky, J. M. (1994) *Mol. Cell. Biol.* **14,** 7466–7475.
26. Sharma, P. M., Egawa, K., Huang, Y., Martin, J. L., Huvar, I., Boss, G. R., and Olefsky, J. M. (1998) *J. Biol. Chem.* **273,** 18528–18537.
27. Burgering, B. M. T., and Coffer, P. J. (1995) *Nature* **376,** 599–602.
28. Franke, T. F., Yang, S.-II., Chan, T. O., Datta, K., Kazlauskas, A., Morrison, D. K., Kaplan, D. R., and Tsichlis, P. N. (1995) *Cell* **81,** 727–736.
29. Kohn, A. D., Summers, S. A., Birnbaum, M. J., and Roth, R. A. (1996) *J. Biol. Chem.* **271,** 31372–31378.
30. Cross, D. A. E., Alessi, D. R., Cohen, P., Andjelkovich, M., and Hemmings, B. A. (1995) *Nature* **378,** 785–789.
31. Ueki, K., Yamamoto-Honda, R., Kaburagi, Y., Yamauchi, T., Tobe, K., Burgering, B. M. T., Coffer, P. J., Komuro, I., Akanuma, Y., Yazaki, Y., and Kadowaki, T. (1998) *J. Biol. Chem.* **273,** 5315–5322.
32. Franke, T. F., Kaplan, D. R., Cantley, L. C., and Toker, A. (1997) *Science* **275,** 665–668.

33. Klippel, A., Kavanaugh, W. M., Pot, D., and Williams, L. T. (1997) *Mol. Cell. Biol.* **17,** 338–344.
34. Stokoe, D., Stephens, L. R., Copeland, T., Gaffney, P. R. J., Reese, C. B., Painter, G. F., Holmes, A. B., McCormick, F., and Hawkins, P. T. (1997) *Science* **277,** 567–570.
35. Stephens, L., Anderson, K., Stokoe, D., Erdjument-Bromage, H., Painter, G. F., Holmes, A. B., Gaffney, P. R. J., Reese, C. B., McCormick, F., Tempst, P., Coadwell, J., and Hawkins, P. T. (1998) *Science* **279,** 710–714.
36. Alessi, D. R., Andjelkovic, M., Caudwell, B., Cron, P., Morrice, N., Cohen, P., and Hemmings, B. A. (1996) *EMBO J.* **15,** 6541–6551.
37. Aman, M. J., Lamkin, T. D., Okada, H., Kurosaki, T., and Ravichandran, K. S. (1998) *J. Biol. Chem.* **273,** 33922–33928.
38. Helgason, C. D., Damen, J. E., Rosten, P., Grewal, R., Sorensen, P., Chappel, S. M., Borowski, A., Jirik, F., Krystal, G., and Humphries, R. K. (1998) *Genes Dev.* **12,** 1610–1620.
39. Okada, T., Kawano, Y., Sakakibara, T., Hazeki, O., and Ui, M. (1994) *J. Biol. Chem.* **269,** 3568–3573.
40. Yamamoto-Honda, R., Tobe, K., Kaburagi, Y., Ueki, K., Asai, S., Yachi, M., Shirouzu, M., Yodoi, J., Akanuma, Y., Yokoyama, S., Yazaki, Y., and Kadowaki, T. (1995) *J. Biol. Chem.* **270,** 2729–2734.