PTEN, but Not SHIP2, Suppresses Insulin Signaling through the Phosphatidylinositol 3-Kinase/Akt Pathway in 3T3-L1 Adipocytes

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Glucose homeostasis is controlled by insulin in part through the stimulation of glucose transport in muscle and fat cells. This insulin signaling pathway requires phosphatidylinositol (PI) 3-kinase-mediated 3'-polyphosphoinositide generation and activation of Akt/protein kinase B. Previous experiments using dominant negative constructs and gene ablation in mice suggested that two phosphoinositide phosphatases, SH2 domain-containing inositol 5'-phosphatase 2 (SHIP2) and phosphatase and tensin homolog deleted on chromosome 10 (PTEN) negatively regulate this insulin signaling pathway. Here we directly tested this hypothesis by selectively inhibiting the expression of SHIP2 or PTEN in intact cultured 3T3-L1 adipocytes through the use of short interfering RNA (siRNA). Attenuation of PTEN expression by RNAi markedly enhanced insulin-stimulated Akt and glyco-gen synthase kinase 3α (GSK-3α) phosphorylation, as well as deoxyglucose transport in 3T3-L1 adipocytes. In contrast, depletion of SHIP2 protein by about 90% surprisingly failed to modulate these insulin-regulated events under identical assay conditions. In control studies, no diminution of insulin signaling to the mitogen-activated protein kinases Erk1 and Erk2 was observed when either PTEN or SHIP2 were depleted. Taken together, these results demonstrate that endogenous PTEN functions as a suppressor of insulin signaling to glucose transport through the PI 3-kinase pathway in cultured 3T3-L1 adipocytes.

Insulin is the primary hormone that regulates glucose homeostasis, and impairment of insulin action and secretion plays a critical role in the pathogenesis of diabetes mellitus (1, 2). One of the primary metabolic responses mediated by insulin is the stimulation of glucose transport and glyco-gen synthesis in muscle and adipose tissue (3, 4). It is now established that translocation of glucose transporter 4 (GLUT4)† from intracellular compartments to the plasma membrane is mainly responsible for the insulin-stimulated increase in glucose uptake in these tissues (5–8). This process is initiated when insulin binds and activates its receptor tyrosine kinase at the cell surface. The activated insulin receptor phosphorylates the insulin receptor substrate (IRS) family of proteins on tyrosine residues. IRS proteins propagate insulin signaling to the p85 regulatory subunit of phosphatidylinositol (PI) 3-kinase, which activates the p110 catalytic subunit (3, 4). A growing number of experiments indicate that insulin-induced PI 3-kinase activation is critical for insulin-induced metabolic actions, including glucose uptake and glycogen synthesis (9–11). Thus, phosphorylation of phosphatidylinositol 4,5-bisphosphate by activated PI 3-kinase increases levels of PI(3,4,5)P3 at cellular membranes. This phosphorylated lipid is a potent modulator of the protein kinases PDK1, atypical protein kinase C, and Akt/protein kinase B (12, 13). Blockade of the PI 3-kinase signaling pathway through the use of specific inhibitors or by the expression of a dominant negative p85 regulatory subunit disrupts the insulin regulation of glucose transport and glyco-gen synthesis (14, 15). Moreover, a recent study evaluating insulin signaling in cultured adipocytes depleted of Akt2 demonstrates a requirement of Akt2 activation in insulin-regulated glucose transport and GSK-3 phosphorylation (16, 17). Consistent with a role for Akt as a positive regulator of glucose homeostasis, mice deficient in Akt2 demonstrate a strong attenuation of insulin action on liver glucosegenesis (18). Thus, the PI 3-kinase/Akt pathway is an obligatory step in the control of glucose uptake in muscle and fat and the reduction of hepatic glucose output regulated by insulin.

PTEN (phosphatase and tensin homolog deleted on chromosome 10) is a dual-function lipid and protein phosphatase that was originally identified as a tumor suppressor gene frequently mutated or deleted in a variety of human cancers (19–21). PTEN has been shown to dephosphorylate PI(3,4,5)P3 at the 3'-phosphate, leading to decreased levels of this phospholipid and simultaneous reduction of Akt activity (22, 23). In Caenorhabditis elegans, the PTEN homolog, DAF-18, acts in insulin receptor-like pathway and regulates longevity and dauer larva development (24–27). Drosophila melanogaster devoid of PTEN have abnormal high concentrations of PI(3,4,5)P3 and are viable only if the affinity of the Akt ortholog for PI(3,4,5)P3 is decreased or the mutation in the pleckstrin homology (PH) domain (28). The sufficiency of an Akt PH domain mutant to rescue PTEN-deficient Drosophila indicates that Akt is the primary target activated by increased PI(3,4,5)P3 levels. Hyperactivation of the PI 3-kinase/Akt pathway also appears to be a main result of PTEN deletion in mammalian systems (29–31). A number of studies have been published that support a role for PTEN in the negative regulation of insulin-induced metabolic actions in 3T3-L1 adipocytes (32–34). In these studies, high expression of full-length PTEN suppressed insulin-
an increase in GLUT4 translocation and glycogen synthesis in this gene leads to insulin hypersensitivity as demonstrated by within a few hours after birth (41). Heterozygous disruption of SHIP2 as a negative regulator of insulin signaling, homozygous way in 3T3-L1 adipocytes (38–40). Consistent with a role for more broadly detected than the isoform SHIP1, which is mainly

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3\text{phosphosphate of the inositol ring from PI(3,4,5)P}_3. \text{SHIP2 is}
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\text{Src homology 2-containing inositol 5'-phosphatase 2)
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\text{PTEN protein levels by densitometry are depicted in A and B. Data are presented as mean ± S.E. of three independent experiments.}
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stimulated Akt activation and glucose transport. Conversely, expression of dominant negative PTEN enhanced Akt activation (32). However, the role of PTEN is controversial as recent results derived from the expression of a dominant inhibitory PTEN protein levels by densitometry are depicted in A and B. Data are presented as mean ± S.E. of three independent experiments.

Based on the above review of the literature, definitive experiments testing the role of PTEN or SHIP2 in insulin signaling in adipocytes through depletion of these proteins have not yet been conducted. In this present investigation, we have tested the relative importance of endogenous SHIP2 and PTEN in the control of insulin induced Akt activation and 2-deoxyglucose transport in 3T3-L1 adipocytes by RNAi-based gene silencing. Depletion of PTEN markedly enhanced insulin-stimulated Akt and GSK-3α phosphorylation, as well as insulin-stimulated deoxyglucose transport in 3T3-L1 adipocytes. Surprisingly, almost complete loss of SHIP2 had no detectable effect on these insulin-regulated events. Taken as a whole, these results reveal the surprising result that PTEN, but not SHIP2, functions as a suppressor of insulin signaling to glucose transport through the PI 3-kinase — Akt pathway in cultured adipocytes.

**Experimental Procedures**

**Materials and Chemicals**—Human insulin was obtained from Lilly. Rabbit polyclonal anti-PTEN, anti-IR3-1, and mouse monoclonal anti-phosphotyrosine (clone 4G10) antibodies were from Upstate Biotechnology, Inc. Goat polyclonal anti-SHIP2 (I-20, sc-14502), mouse monoclonal anti-GSK-3α/β (0011-A, sc-7291), and anti-insulin receptor β-subunit (29B4, sc-09) were from Santa Cruz Biotechnology. Rabbit polyclonal antibodies against phospho-Akt (Ser-473), phospho-GSK-3α/β (Ser-21/9), phospho-Erk1/2, anti-Akt, and anti-Erk1/2 were from Cell Signaling Technology (Beverly, MA). Mouse anti-actin monoclonal antibody was from Sigma. Mouse anti-PI3K monoclonal antibody and the PI3K strips were from Echelon Biosciences Inc. The horseradish peroxidase-conjugated goat anti-rabbit IgG and rabbit anti-goat IgG were from Chemicon International.

**Cell Culture and Electroporation of 3T3-L1 Adipocytes**—3T3-L1 fibroblasts were grown in DMEM supplemented with 10% fetal bovine serum, 50 μg/ml streptomycin, and 50 units/ml penicillin and differentiated into adipocytes as described (16, 43). The 3T3-L1 adipocytes were transfected with siRNA duplexes by electroporation. Briefly, the adipo-
Depletion of PTEN, but not SHIP2, enhances insulin action on deoxyglucose uptake in 3T3-L1 adipocytes. Dose dependence of insulin-stimulated deoxyglucose uptake is shown. Cells were transfected with scrambled (30 nmol), PTEN (30 nmol), or SHIP2 (20 nmol) siRNA by electroporation, reseeded for 72 h, and serum-starved for 2 h. The serum-starved cells were then stimulated with insulin for 30 min at 37 °C, and deoxyglucose uptake was assayed as described under “Experimental Procedures.” Depicted is the fold insulin stimulation of glucose uptake in cells treated with scrambled siRNA or PTEN- or SHIP2-directed siRNA. Protein concentrations in each well were determined using the Bio-Rad protein assay kit and used to normalize counts. The rate of glucose transport (pmol/mg of protein/min) was then measured under each of the knockdown conditions. The data represent the average ± S.E. of four independent experiments. *, p < 0.05.

RESULTS AND DISCUSSION

Insulin-stimulated Deoxyglucose Transport Is Enhanced by siRNA-based Depletion of Endogenous PTEN but Not by Depletion of SHIP2 in 3T3-L1 Adipocytes—It has been reported that both PTEN and SHIP2 phosphatases are expressed in 3T3-L1 adipocytes (32, 38). Studies that have invoked high expression of full-length or dominant negative constructs of SHIP2 or PTEN have concluded that both phosphatases are involved in PI(3,4,5)P3 hydrolysis and function as suppressors of the insulin-stimulated PI 3-kinase/Akt pathway in these cells (32–34, 38–40). Both PTEN and SHIP2 are also expected to be negative regulators of insulin-stimulated glucose transport, based on experiments in which these proteins were expressed at high levels in cultured adipocytes (32–34, 38–40). In addition, it has been shown that SHIP2(−/−) mice exhibit hypersensitivity to insulin and more rapid glucose clearance than wild-type mice (41), suggesting that SHIP2 may be a negative regulator for glucose transport. However, more definitive approaches, such as depletion of the endogenous phosphatases, have not been used to address the function of SHIP2 and PTEN phosphatases in insulin signaling in either primary or cultured adipocytes. Thus, our first set of experiments was designed to selectively deplete endogenous SHIP2 versus PTEN proteins in cultured 3T3-L1 adipocytes. Assays were then performed to examine how the absence of each one of these phosphatases affects insulin-stimulated signaling. To specifically decrease SHIP2 or PTEN expression levels in 3T3-L1 adipocytes, we used a siRNA-mediated gene silencing technique recently applied successfully to these cells (16, 46). 3T3-L1 adipocytes were electroporated with scrambled siRNA as a control or with siRNA targeted against mouse PTEN or SHIP2 mRNAs. After 72 h, the cells were harvested, and PTEN and SHIP2 protein expression levels were assessed by immunoblotting.

As depicted in Fig. 1, the protein expression level of PTEN was reduced by ~50% in 3T3-L1 adipocytes electroporated with...
PTEN-directed siRNA but not with scrambled or SHIP2-directed siRNA (Fig. 1). Expression of SHIP2 protein was unaffected by treatment of the cultured adipocytes with PTEN-directed siRNA (Fig. 2). However, electroporation of 3T3-L1 adipocytes with SHIP2-directed siRNA caused a marked reduction of SHIP2 protein expression (about 90% depletion, Fig. 2), without a detectable change in PTEN protein level (Fig. 1). Thus, selective depletion of both PTEN and SHIP2 phosphatases can be achieved by siRNA-mediated gene silencing in cultured adipocytes.

We next conducted experiments to examine the consequences of this selective depletion of PTEN or SHIP2 protein on insulin-stimulated deoxyglucose transport in 3T3-L1 adipocytes. Attenuation of PTEN protein expression led to a marked enhancement of insulin stimulated glucose uptake in 3T3-L1 adipocytes, compared with the scrambled siRNA control (Fig. 3). This potentiation of insulin action by PTEN depletion occurred at all insulin concentrations tested. In contrast, no significant change in insulin-stimulated deoxyglucose uptake was observed in adipocytes depleted of SHIP2, compared with control cells electroporated with scrambled siRNA. Based on these results, we conclude that endogenous PTEN, but not SHIP2, functions to suppress insulin signaling to glucose transport in cultured adipocytes.

Differential Effects of PTEN and SHIP2 Protein Depletion on Insulin-stimulated Akt Phosphorylation—Based on the dramatic differential sensitivity of insulin-stimulated deoxyglucose transport to depletion of PTEN versus SHIP2, we tested their roles in regulating the PI 3-kinase pathway. The protein kinase downstream of PI 3-kinase pathway is Akt2. Activation of the Akt protein kinases occurs by phosphorylation at a threonine (308) and a serine (473) by the protein kinase PDK1 and an unknown protein kinase, respectively (47–49). We conducted experiments to examine whether the depletion of the PTEN or SHIP2 phosphatases would affect Akt activation, as detected by its enhanced...
phosphorylation at serine 473 upon insulin stimulation of 3T3-L1 adipocytes. As seen in Fig. 4, attenuation of PTEN expression levels was associated with a significantly enhanced phosphorylation of Akt on serine 473. This enhancement was observed when either submaximal or maximal doses of insulin were employed. Similar results were obtained upon Western blotting using an anti-phospho-threonine 308 Akt antibody (data not shown). Thus, reduction of PTEN protein levels resulted in enhanced insulin-stimulation of Akt/PKB activation, consistent with previous reports that PTEN acts as a suppressor of the PI 3-kinase/Akt signaling pathway.

We also performed experiments to examine insulin-stimulated Akt activation in adipocytes depleted of endogenous SHIP2 protein. Surprisingly, as seen in Fig. 4, no effect of SHIP2 protein loss was observed on Akt serine 473 phosphorylation, either in the presence of submaximal or maximal concentrations of insulin. The dose-response relationship between the insulin concentrations used and Akt serine 473 phosphorylation were similar in cells treated with scrambled siRNA versus SHIP2 siRNA (Fig. 4). Thus, these results strongly suggest that endogenous PTEN, but not endogenous SHIP2, suppresses insulin signaling to Akt in cultured 3T3-L1 adipocytes.

We also conducted experiments to silence PTEN and SHIP2 expression using different siRNA oligonucleotides for each one of these lipid phosphatases. In these studies, 3T3-L1 adipocytes were electroporated with each siRNA, then treated with or without insulin 72 h later. PTEN and SHIP2 protein levels as well as Akt phosphorylation were analyzed by immunoblot. Similar results were obtained with these additional oligonucleotides compared with Figs. 1–4. We found decreases in PTEN or SHIP2 expression levels by 50 or 90%, respectively, and increases in the insulin stimulation of phospho-Akt in PTEN-depleted cells but not SHIP2-depleted cells (data not shown).

We then examined whether the depletion of PTEN or SHIP2 phosphatases in cultured adipocytes affect the PIP_3 levels stimulated by insulin. Cells were treated or not with insulin for 10 min and total lipids were extracted and resolved by TLC. The lipid spots corresponding to PIP_3 were eluted from the TLC plates and loaded onto a membrane, and the PIP_3 levels were detected using an anti-PIP_3 monoclonal antibody. This antibody reacts with PIP_3 with a high degree of specificity (Fig. 4B). We found that the attenuation of PTEN expression levels, but not SHIP2 depletion, was associated with a significantly enhancement of PIP_3 levels when compared with scrambled control. Thus, these data are consistent with the results depicted in Fig. 4A, where depletion of PTEN, but not SHIP2, enhances insulin stimulation of Akt phosphorylation.

To confirm the results depicted in Figs. 1–4, we next assessed the consequences of the selective attenuation of PTEN versus SHIP2 expression on a downstream target of Akt, GSK-3β. As depicted in Fig. 5, GSK-3β is phosphorylated by Akt protein kinase in response to insulin in a dose-dependent manner. Loss of 90% or more of SHIP2 protein directed by SHIP2 siRNA caused no enhancement of insulin-stimulated GSK-3β phosphorylation. These data are consistent with the lack of effect of SHIP2 depletion on Akt activation (Fig. 4). In contrast, attenuation of PTEN expression by 50% caused an enhancement of insulin-stimulated GSK-3β phosphorylation (Fig. 5). Furthermore, we found that the attenuation of PTEN expression levels, but not SHIP2 depletion, was associated with a significantly enhanced phosphorylation of p70S6 kinase on threonine 389 (data not shown). This enhancement was more marked when submaximal doses of insulin were employed (data not shown). Since GSK-3β and p70S6 kinases are downstream targets of Akt, these results are consistent with the hypothesis that PTEN, but not SHIP2, acts as a suppressor of the PI 3-kinase signaling pathway in cultured adipocytes.

Mitogen-activated protein kinases (MAPKs) do not require the PI 3-Kinase/Akt pathway to respond to insulin signaling under the conditions of our experiments. As predicted, no enhancement of insulin signaling to the MAPKs Erk-1 and Erk-2 was observed when either PTEN or SHIP2 were depleted (Fig. 6). Both submaximal and maximal concentrations of insulin caused similar phosphorylations of Erk1 and Erk2 whether or not PTEN or SHIP2 proteins were depleted in the 3T3-L1 adipocytes. This result confirms the specificity of silencing the SHIP2 and PTEN proteins, since a PI 3-kinase-independent, but insulin-dependent, signaling pathway is regulated normally in PTEN- or SHIP2-depleted 3T3-L1 adipocytes.

We next examined whether the depletion of PTEN or SHIP2 phosphatases in cultured adipocytes affect the insulin stimulation of IR or IRS-1 tyrosine phosphorylation. As depicted in Fig. 6A, no differences in IR or IRS-1 tyrosine phosphorylation stimulated by insulin were detected in cultured adipocytes depleted of PTEN or SHIP2, when compared with scrambled RNAi. The lack of effect of the PTEN or SHIP2 knockdowns on the activation of IR is also consistent with their lack of effect on insulin stimulation of MAPK phosphorylation, as shown in Fig. 6B. Together, these results are consistent with the notion that PTEN negatively regulates insulin action on glucose transport through the PI 3-Kinase/Akt pathway in cultured adipocytes.

In agreement with our present results, Butler et al. (50) demonstrated that inhibiting PTEN levels by injection of antisense oligonucleotides into mice increased insulin-stimulated Akt phosphorylation in the liver. Moreover, antisense-mediated inhibition of PTEN expression normalized glucose concentration in both db/db and ob/ob mice, improved insulin sensitivity in db/db mice, and lowered insulin concentrations dramatically in ob/ob mice. In addition, Stiles et al. (30) have recently reported that liver-specific deletion of PTEN results in increased insulin sensitivity in the liver and improved overall glucose tolerance. Finally, muscle-specific deletion of PTEN results in enhanced insulin sensitivity in soleus muscle and protection from high fat diet-induced obesity (51). These results strongly support a role of endogenous PTEN in the down-
regulation of insulin signaling related to glucose homeostasis. Our results definitively extend the impact of these studies to deoxyglucose transport regulation in cultured adipocytes, consistent with studies using expression of wild-type or dominant inhibitory constructs of PTEN (32–34).

Although it was surprising that depletion of SHIP2 failed to affect insulin-stimulated deoxyglucose transport in cultured adipocytes (Fig. 3), our results do not conflict with any previously published work describing depletion of SHIP2 in either primary or cultured adipocytes. Adipose tissue from SHIP2(/−/−) mice was not evaluated (41), even though glucose tolerance in the whole animals was increased. Interestingly, our results are consistent with a recent finding using myeloma cells in which Akt activity in response to growth factors was not affected by the loss of endogenous SHIP2 (52). PTEN acts to dephosphorylate the 3′ position of PI(3,4,5)P3, while SHIP2 catalyzes hydrolysis of the 5′-phosphate of this polyphosphoinositide. It is possible that the resulting product of the latter reaction, phosphatidylinositol 3,4-bisphosphate, plays a critical role in inducing Akt activation by binding to the PH domain of Akt (53, 54). It is also possible that SHIP2 only regulates PI(3,4,5)P3 levels in specific compartments or microdomains at the plasma membrane, as opposed to a global regulation of this lipid throughout the plasma membrane in 3T3-L1 adipocytes. In this regard, an insulin-induced recruitment of SHIP2 to the plasma membrane of adipocytes has been reported (39). Further experiments will be necessary to identify the function of SHIP2 in fat cells.

A growing number of PIs and PI regulators are being implicated in the regulation of insulin-mediated signaling pathways (55). These include the PI(3)P phosphatase myotubularin (56), the 5′-phosphatase SKIP (skeletal muscle- and kidney-enriched inositol phosphatase) (57, 58), and the Type II PI(5)P 4-kinase (59). Interestingly, SKIP may perform a similar func-
tion as PTEN in skeletal muscle, heart, and kidney (57, 58). Interference of SKIP expression by antisense oligonucleotides dramatically enhanced Akt, but not MAPK, phosphorylation in response to insulin. SKIP overexpression markedly inhibited insulin-induced GLUT4 translocation and negatively regulates insulin-induced glucose uptake and glycogen synthesis in L6 myocytes. These effects are similar to those caused by PTEN, but not SKIP2, expression in 3T3-L1 adipocytes.

In summary, our results indicate that endogenous PTEN plays a key role as a negative regulator of insulin signaling through the PI 3-kinase pathway. The actions of endogenous PTEN include attenuating insulin-mediated stimulation of glucose transport in 3T3-L1 adipocytes and likely extend to other physiological processes regulated by Akt protein kinases in adipocytes.

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