The Tumor Suppressor PTEN Negatively Regulates Insulin Signaling in 3T3-L1 Adipocytes*

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PTEN is a tumor suppressor with sequence homology to protein-tyrosine phosphatases and the cytoskeleton protein tensin. PTEN is capable of dephosphorylating phosphatidylinositol 3,4,5-trisphosphate in vitro and down-regulating its levels in insulin-stimulated 293 cells. To study the role of PTEN in insulin signaling, we overexpressed PTEN in 3T3-L1 adipocytes 30-fold above uninfected or control virus (green fluorescent protein)-infected cells, using an adenovirus gene transfer system. PTEN overexpression inhibited insulin-induced 2-deoxyglucose uptake by 36%, GLUT4 translocation by 35%, and membrane ruffling by 50%, all of which are phosphatidylinositol 3-kinase-dependent processes, compared with uninfected cells or cells infected with control virus. Microinjection of an anti-PTEN antibody increased basal and insulin stimulated GLUT4 translocation, suggesting that inhibition of endogenous PTEN function led to an increase in intracellular phosphatidylinositol 3,4,5-trisphosphate levels, which stimulates GLUT4 translocation. Further, insulin-induced phosphorylation of downstream targets Akt and p70S6 kinase were also inhibited significantly by overexpression of PTEN, whereas tyrosine phosphorylation of the insulin receptor and IRS-1 or the phosphorylation of mitogen-activated protein kinase pathway were not affected, suggesting that the Ras/mitogen-activated protein kinase pathway remains fully functional. Thus, we conclude that PTEN may regulate phosphatidylinositol 3-kinase-dependent insulin signaling pathways in 3T3-L1 adipocytes.

Insulin binding to its receptor stimulates receptor auto phosphorylation activating its intrinsic tyrosine kinase activity leading to phosphorylation of cellular substrates such as Shc, IRS-1, IRS-2, IRS-3, IRS-4, and other proteins (1–5). Tyrosine phosphorylation of the IRS and Shc proteins allows them to interact with signaling molecules containing Src homology 2 domains (6), including PI3-kinase. PI3-kinase is a dual protein and lipid kinase composed of a heterodimer of a 110-kDa catalytic subunit (p110) and an 85-kDa regulatory subunit (p85) that contains two Src homology 2 domains (7). Binding of the p85 Src homology 2 domains to phosphotyrosines of the IRSs or directly to the carboxyl terminus of the IR, leads to activation of the associated p110 (8–10), which preferentially phosphorylates the D3 position of phosphatidylinositol (PtdIns), PtdIns 4-phosphate, and PtdIns 4,5-bisphosphate producing PtdIns 3-phosphate, PtdIns 3,4-bisphosphate, and PtdIns 3,4,5-P3, respectively. These PtdIns can serve as lipid second messengers that play a crucial role in the biologic actions of growth factors (11). However, the exact function of each of these PtdIns in hormone signaling is not fully defined. PI3-kinase activation is necessary for a number of insulin-stimulated effects, ranging from stimulation of glucose transport, glycogen synthesis, and membrane ruffling to mitogenesis, although the precise function of PtdIns products in eliciting these responses is currently unknown (12, 13). Insulin promotes glucose uptake in muscle and fat tissue through the translocation of the GLUT4 transporter to the plasma membrane (14), and PI3-kinase is both necessary (15) and sufficient (16) for this effect. One target of PI3-kinase is the serine/threonine protein kinase B, PKB (also called Akt), and overexpression of a constitutively active form of Akt leads to increased glucose uptake and GLUT4 translocation in 3T3-L1 adipocytes (17). Akt contains a pleckstrin homology domain at its NH2 terminus, which has been implicated in interactions with the products of PI3-kinase, PtdIns 3,4-bisphosphate, and PtdIns 3,4,5-P3 in vitro (18–20), but full activation of the kinase requires Ser/Thr phosphorylation of the protein (19, 21). Akt is activated in response to insulin and growth factors, and this activation is prevented by inhibitors of PI3-kinase (22–26). Phosphorylation of Akt is thought to be essential for its full activation (27). Insulin, like other growth factors can also induce actin filament rearrangement in various cell lines leading to stress fiber breakdown and membrane ruffling (16, 28). This latter effect requires PI3-kinase activation and, in particular, PtdIns 3,4,5-P3 formation (16, 29, 30). Stress fiber formation correlates with PtdIns 3,4-bisphosphate generation (31), and it has been suggested that stress fiber breakdown is induced by 3’-phosphorylation of 4,5-P1P2 induced by PI3-kinase. The pleotropic effects of PtdIns suggest that their synthesis must be tightly regulated. Many agonists and growth factors stimulate an increase in the level of PtdIns 3,4,5-P3, which is rapidly metabolized and inactivated by a family of 5’-phosphatases (32–34) or by a 3’-phosphatase (35, 36) to give PtdIns 3,4-bisphosphate and PtdIns 4,5-bisphosphate, respectively. Although there are several phosphoinositide phosphatases, the mechanism of regulation and particularly the degradation...
pathway of PtdIns 3,4,5-P_3 in vivo is still unclear (37, 38). The tumor suppressor-PTEN, is a 3'-phosphatase with sequence similarity to the cytoskeletal protein tensin. When overexpressed in 293 cells, it reduces insulin-induced PtdIns 3,4,5-P_3 levels by dephosphorylation, specifically at position 3 on the inositol ring, without affecting insulin-induced PI 3-kinase activation (39). The catalytically inactive mutant of PTEN causes PtdIns 3,4,5-P_3 accumulation in the absence of insulin stimulation. PTEN may therefore act in vivo as a phosphoinositide 3-phosphatase by regulating PtdIns 3,4,5-P_3 levels. The physiological substrates and functions of PTEN protein, however, remain to be elucidated. Overexpression of PTEN inhibits cell migration, whereas antisense PTEN enhances migration (40). Further, wild-type PTEN down-regulates integrin-mediated cell spreading and the formation of focal adhesions. Thus, the hydrolysis of PI 3-kinase products by PTEN may be a general mechanism for the regulation of PI 3-kinase effects (36, 37, 41, 42). In the present study, we examined the effects of PTEN on insulin signaling using an adenovirus (Ad-PTEN) engineered to express PTEN in differentiated 3T3-L1 adipocytes.

EXPERIMENTAL PROCEDURES

Materials—Porcine insulin was kindly provided by Lilly. Mouse monoclonal anti-phosphotyrosine (PY-20), mouse monoclonal anti-ERK1, anti-PTEN and the anti-GFP antibodies were purchased from Transduction Laboratories. Anti-IRS-1 and anti-human 56 Kinase (p70S6K) were from Upstate Biotechnology Inc. Phospho-specific MAP kinase and p70S6K antibodies were from New England Biolabs, Inc. The goat polyclonal AKT (C-20) antibody of human origin and horse-radish peroxidase-linked anti-rabbit, -mouse, and -goat antibodies were purchased from Santa Cruz Biotechnology. Dulbecco’s modified Eagle’s medium and fetal bovine serum were obtained from Life Technologies, Inc. All radioisotopes were obtained from NEN Life Science Products. Hoechst dye 33342 was obtained from Molecular Probes (Eugene, OR). XAR-5 film was obtained from Eastman Kodak Co. All other reagents and chemicals were purchased from Sigma.

Cell Culture—3T3-L1 cells were grown and maintained in Dulbecco’s modified Eagle’s medium high glucose containing 50 units/ml penicillin, 50 μg/ml streptomycin, and 10% fetal bovine serum in a 10% CO_2 environment. The cells were allowed to grow 2 days postconfluence and then differentiated by addition of the same medium containing ouabain, methylxanthine (500 μM), dexamethasone (25 μM), and insulin (4 μg/ml) for 3 days and the medium containing insulin for 3 additional days. The medium was then changed every 3 days until the cells were fully differentiated, typically by 10 days. Prior to experimentation, the adipocytes were trypsinized and reseeded in the appropriate culture medium to achieve 80–90% confluence. The adipogenesis was determined by incubation with rabbit polyclonal anti-PTEN antibody (3493) (1 μg/ml) that had been raised against a synthetic peptide corresponding to the carboxyl-terminal 16 residues of PTEN (47) in PBS with 2% fetal calf serum overnight at 4 °C. The cells were then incubated with horseradish peroxidase-linked secondary antibody followed by chemiluminescence detection, according to the manufacturer’s instructions (Pierce).

Microinjection—Cells were cultured in the same medium without serum for 2 h, and microinjection of various reagents was carried out using a semiautomated Eppendorf microinjection system. All reagents were dissolved in a buffer containing 5 mM sodium phosphate (pH 7.4), 100 mM KCl. Glutathione S-transferase fusion proteins were each coinjected with 5 mg/ml sheep IgG for detection purposes. Prior to staining the cells were allowed to recover for a period of 1 h.

GLUT4 Immunostaining and Fluorescence Microscopy—The cells were stimulated with insulin for 20 min and then fixed in 3.7% formaldehyde in PBS for 5 min on ice and for 5 min at room temperature. Following washing, the cells were permeabilized and blocked with 0.1% Triton X-100 and 0.2% fetal calf serum in PBS for 10 min at room temperature. The cells were then incubated with rabbit polyclonal anti-GLUT4 antibody (3494) (1 μg/ml) that had been raised against a synthetic peptide corresponding to the carboxyl-terminal 16 residues of GLUT4 (47) in PBS with 2% fetal calf serum overnight at 4 °C. After washing and GLUT4 staining the identity of microinjected cells was determined by incubation with rhodamine-conjugated donkey anti-rabbit. The results were analyzed and photographed by immunofluorescence microscopy. Each fluorescein isothiocyanate-positive microinjected cell was evaluated for the presence of plasma membrane-associated GLUT4 staining. As in all microinjection studies, control cells were microinjected with preimmune sheep IgG and then processed in the same way as experimental injected cells.

2-Deoxyglucose Transport—The procedure for glucose transport was a modification of the methods described by Klip et al. (48). Differentiated 3T3-L1 adipocytes were starved overnight before stimulation with insulin or with insulin (17 nm) for 15 min. Cells were washed and permeabilized as above and then incubated first with anti-GFP in PBS, as indicated, for 1 h at room temperature. After washing several times with PBS, they were then incubated with rhodamine-phallolidin (0.125 mg/ml) to visualize the location of polymerized actin at the cell membrane (membrane ruffles) or stress fibers and stained with fluorescein isothiocyanate-conjugated anti-mouse to detect expressed protein in injected cells.

PTEN in Insulin Signaling

2-Deoxyglucose Transport—The procedure for glucose transport was a modification of the methods described by Klip et al. (48). Differentiated 3T3-L1 adipocytes were infected with Ad5-PTEN or Ad5-GFP (negative control) at the indicated m.o.i. for 24 h at 37 °C and grown in medium containing glucose-free medium (2%) for 48 h. Serum and glucose deprived cells (1 h at 37 °C) were incubated in a minimum essential medium in the absence (basal) or presence of 0.5 and 17 nm insulin for 1 h at 37 °C. Glucose uptake was determined in duplicate at each point after the addition of 10 μl of substrate ([2-3H]2-deoxyglucose/14Cglucose, 0.1 μCi; final concentration, 0.01 nmol/l) to provide a concentration at which cell membrane transport is rate-limiting. The value for l-glucose was subtracted to correct each sample for the contributions of diffusion.
and trapping. Data are representative of four different observations, and each value was corrected for protein content.

RESULTS

Expression of PTEN and GFP Proteins in 3T3-L1 Adipocytes—Differentiated 3T3-L1 adipocytes were infected with recombinant adenoviruses expressing PTEN and GFP proteins at 5, 15, 30, and 60 m.o.i. (7.55 × 108 particles/ml) for 24 h, and protein expression was examined 72 h later by Western blotting. Specific bands appeared at ~60 and ~28 kDa corresponding to PTEN and GFP, respectively. The proteins were expressed in a viral dose-dependent manner (Fig. 1, lanes 2–5), with ~30-fold higher expression levels at 60 m.o.i. (lane 5), compared with uninfected control cells (lane 1).

Effect of PTEN on the Phosphorylation State or Activity of Insulin-signaling Proteins: IR/IRS-1 Phosphorylation and PI 3-Kinase Activity—We evaluated adenovirally mediated expression of PTEN protein on insulin-stimulated tyrosine phosphorylation of IR and IRS-1. As shown in Fig. 2, no differences in the extent of insulin-induced tyrosine phosphorylation of the insulin receptor or IRS-1 were detected in cell lysates from uninfected 3T3-L1 adipocytes compared with those expressing PTEN. We further determined PI 3-kinase activity associated with IRS-1 in cells overexpressing PTEN (Fig. 3). In control cells, insulin led to a 15–20-fold increase in IRS-1-associated PI 3-kinase activity, which was unaltered by expression of either the PTEN or the GFP(Ctrl) proteins. These latter results confirm that PTEN functions downstream of PI 3-kinase.

AKT and p70S6K—Akt, a serine/threonine protein kinase, is activated upon insulin stimulation in a PI 3-kinase-dependent manner. Akt phosphorylation on both Thr-308 and Ser-473 is directly dependent on the levels of intracellular PtdIns 3,4,5-P3, the key product of PI 3-kinase activity (49, 50). To investigate whether PI 3-kinase signaling is modulated by PTEN expression, we assessed Akt activation in whole cell lysates derived from uninfected and PTEN- or GFP-expressing insulin-stimulated 3T3-L1 adipocytes. Insulin stimulation of control cells resulted in a marked activation of Akt, as determined by Western blot analyses, using a phosphospecific AKT antibody that detects pAKT only when phosphorylated at Thr-473 (Fig. 4A, upper panel, lane 2). Akt activation was also determined by retarded migration of the enzyme on SDS-PAGE followed by Western blotting with anti-Akt antibody (Fig. 4A, lower panel, lane 2). PTEN expression resulted in a ~50% inhibition of insulin-stimulated AKT activation (Fig. 4A, lane 6), as assessed by both AKT phosphorylation and AKT gel shift.

p70S6K is another protein kinase downstream of PI 3-kinase, and activation of this enzyme was determined by Western blot analysis of whole cell lysates with a phospho-specific p70S6K antibody. As shown in Fig. 4B, stimulation with insulin resulted in activation of p70S6K (Fig. 4B, upper panel, lane 2), which was not affected by overexpression of GFP (lane 4). In PTEN-expressing cells an ~50% inhibition of insulin-induced p70S6K activation (Fig. 4B, upper panel, lane 6) was observed. Comparable results were obtained when p70S6K activation was assessed by retarded migration on SDS-PAGE using anti-p70S6K antibody.

MAP Kinase—To examine the specificity of the effect of PTEN on PI 3-kinase-mediated insulin signaling, we also examined the status of MAP kinase (ERK 1/2) activation. This was accomplished in uninfected, Ad-GFP-infected, and Ad-PTEN-infected cells by assessing MAP kinase activation using a phospho-specific MAP kinase antibody (Fig. 5, upper panel). Insulin increased MAP kinase activation in uninfected and Ad-GFP-infected cells by ~20–25-fold. PTEN expression did not inhibit insulin-induced phosphorylation of either p44 or p42 MAP kinase (Fig. 5, upper panel, sixth lane). Expression of
MAP kinase, as assessed by Western blotting with a polyclonal anti-Erk-1 antibody that recognizes both nonphosphorylated and phosphorylated forms, demonstrated that protein levels were not altered in any of the cell groups (Fig. 5, lower panel).

**Inhibition of GLUT4 Translocation**—We analyzed GLUT4 localization by immunofluorescent staining of GLUT4 in adenovirally infected cells expressing the PTEN protein, as well as in cells microinjected with PTEN neutralizing antibodies.

Cells infected with Ad-PTEN or Ad-GFP (Fig. 6) were treated with or without insulin for 20 min. Cells expressing the protein of interest were fixed and stained for GLUT4 localization as previously reported (51, 52). Unstimulated cells display typical staining in basal conditions and after insulin stimulation. Summary data are given as bar graphs, and each bar represents the means ± S.E. of three experiments. Open bars represent basal GLUT4 translocation in the presence of 0.5 nM insulin, and black bars represent values for 17 nM insulin. PTEN inhibited insulin-induced GLUT4 translocation at submaximal insulin concentrations by about 50% and by about 70% at maximal insulin concentrations. *p < 0.05; **p < 0.01 versus control.

**Glucose Transport Inhibition**—Cells were infected with Ad-GFP or Ad-PTEN, and 2-DG uptake was measured 4 days later. As seen in Fig. 7, 0.5 and 17 nM insulin stimulated 2-DG uptake, and this was not affected by Ad-GFP infection. PTEN expression inhibited insulin stimulated 2-DG uptake, with a greater negative effect observed at 0.5 nM insulin. Expression of PTEN also led to a decrease in basal 2-DG uptake. Thus, the effects of Ad-PTEN and Ad-GFP on glucose transport were comparable with the effects on GLUT4 translocation.

**Microinjection of PTEN Antibody**—The above results show that exogenous PTEN expression inhibits insulin-stimulated...
after treatment with insulin (17 nM). In the basal state, cells exhibited membrane ruffles, and this increased to 70–80% rearrangement. Cells were infected with Ad-PTEN or Ad-GFP at the indicated m.o.i. for 24 h at 37 °C and grown in medium containing heat-inactivated serum (2%) for 96 h. Serum- and glucose-deprived cells were incubated in α-minimum essential medium in the absence (basal) or presence of 0.5 and 17 nm insulin for 1 h at 37 °C. Cells were then washed with glucose free medium, and 2-deoxyglucose uptake was measured. Each measurement was performed in duplicate. Infection of differentiated 3T3-L1 adipocytes with Ad-PTEN resulted in a viral dose-dependent inhibition of insulin stimulated incorporation of 2-deoxyglucose transport with ~77% inhibition observed at submaximal (0.5 nm) insulin concentration and ~42% inhibition at 17 nm insulin. Control Ad-GFP virus had no effect on glucose transport. Data are representative of four different observations ± S.E., and each value was corrected for protein.

GLUT4 translocation and glucose uptake, most likely by decreasing the amount of intracellular PtdIns 3,4,5-P$_3$. To assess the role of endogenous PTEN in this aspect of insulin action, we utilized single cell microinjection of anti-PTEN antibodies using methods previously described (50–52). As quantitated by immunofluorescent GLUT4 staining, insulin leads to a severalfold increase in GLUT4 translocation to the plasma membrane, and this is unaffected by injection of nonimmune IgG (Fig. 8). Injection of anti-PTEN antibody (5 mg/ml) leads to a 2.5-fold increase in GLUT4 translocation in the absence of insulin treatment. This stimulatory effect was also seen in the presence of insulin, because the effects of submaximal (0.17 nm) and maximal (17 nm) insulin concentrations were greater in PTEN antibody-injected cells. Interestingly, treatment of cells with wortmannin (100 nm) completely inhibited stimulation of GLUT4 translocation by PTEN antibody injection and by insulin, consistent with the idea that the stimulatory effect of the PTEN antibody was due to inhibition of PTEN function causing an increase in intracellular PtdIns 3,4,5-P$_3$ levels.

Membrane Ruffling—We have previously shown that insulin stimulates membrane ruffling in 3T3-L1 adipocytes and that the signaling pathway mediating this biological effect is dependent on PI 3-kinase activation (52, 53). Therefore, we studied the effect of PTEN expression on insulin-induced membrane ruffling. Cells were infected with Ad-PTEN or Ad-GFP for 72 h, following which cells were starved for 24 h, stimulated with insulin for 15 min, and then fixed and stained for actin rearrangement.

As seen in Fig. 9, in the basal state, <10% of the cells exhibited membrane ruffles, and this increased to 70–80% after treatment with insulin (17 nm). In the basal state, cells expressing GFP or PTEN have very few membrane ruffles, similar to unstimulated control cells. Expression of PTEN inhibited insulin-induced membrane ruffling by 46 ± 4%, compared with control, p < 0.01 (Fig. 9).

DISCUSSION

PTEN is a recently identified tumor suppressor gene that shares sequence identity with a family of protein-tyrosine phosphatases and tensin (55, 56). Deletions and mutations within the PTEN gene have been observed in several cancer cell types and tumor cell lines. PTEN is capable of dephosphorylating both phospho-tyrosine and phospho-serine/threonine containing synthetic substrates in vitro (57). In addition, PTEN has been shown to dephosphorylate position D-3 of PtdIns 3,4,5-P$_3$, an important second messenger involved in insulin and growth
factor signaling. Indeed, PI 3-kinase activation and subsequent 3’ phosphoinositide formation is required and, in some cases, sufficient to trigger many of the biological actions of insulin.

In the current studies, we examined the possible effects of PTEN expression on the activity or phosphorylation status of various intermediates in the insulin signaling pathway. Our results show that adenovirus-mediated expression of the 3’ phosphatidyl Inisotol phosphatase, PTEN, in 3T3-L1 adipocytes, significantly inhibits insulin-induced GLUT4 translocation, glucose uptake, and membrane ruffling, all of which are dependent on PI 3-kinase activity. In addition, inhibition of endogenous PTEN by microinjection of anti-PTEN antibodies leads to enhanced GLUT4 translocation. However, not all of the actions of insulin were inhibited, because stimulation of MAP kinase was unaffected by PTEN overexpression, indicating selectivity of certain actions of insulin for the PI 3-kinase mechanism.

Insulin regulation of glucose homeostasis involves the ability of the hormone to stimulate translocation of GLUT4 glucose transporters to the cell surface, thus increasing the rate of glucose uptake into muscle and adipose tissue. It has been clearly demonstrated that PI 3-kinase activation is both necessary and sufficient for these actions (26, 28). Thus, PI 3-kinase inhibitors, such as wortmannin and LY294002, as well as dominant/negative mutants of the p85 subunit of PI 3-kinase, all inhibit GLUT4 translocation as well as glucose uptake (25), whereas constitutively active mutants of the catalytic p110 subunit of PI 3-kinase stimulate GLUT4 translocation and glucose transport (26, 28). Interestingly, the inhibitory effects of wortmannin on insulin-stimulated glucose transport can be overcome by addition of membrane-permeable PtdIns 3,4,5-P_3 to cells (58), providing evidence for a role of this lipid messenger in mediating PI 3-kinase actions on glucose transport. Along these lines, we have recently shown that microinjection of a 5’ phosphotidylinositol phosphatase SHIP, which is specific for PtdIns 3,4,5-P_3, also inhibits GLUT4 translocation (59). Our current data show that PTEN expression inhibits insulin-induced GLUT4 translocation, as well as glucose uptake. Because PTEN has been shown to decrease hormone-stimulated intracellular PtdIns 3,4,5-P_3 levels (36, 37), these results argue strongly for the important role played by PI 3-kinase-generated PtdIns 3,4,5-P_3 in this key action of insulin. Taken together, these results clearly show the importance of PtdIns 3,4,5-P_3 for this process, and this raises the question as to how does PtdIns 3,4,5-P_3 mediate GLUT4 translocation.

To explore this question, we examined the effects of PTEN on the activity or phosphorylation status of various intermediates in the insulin signaling pathway. AKT/PKB is a serine/threonine kinase lying downstream of PI 3-kinase that has been implicated in stimulation of GLUT4 translocation. Our data show that expression of PTEN in 3T3-L1 adipocytes causes an inhibition of insulin stimulated AKT phosphorylation. Because the activity of PI 3-kinase itself is not affected by PTEN expression, this inhibitory effect appears to occur downstream of PI 3-kinase activation. Thus, it is likely that inhibition of AKT phosphorylation by PTEN expression is caused by a reduction in PtdIns 3,4,5-P_3 levels that are required for AKT activation. This is consistent with previous findings showing that AKT activity is reduced by PTEN. Thus, embryonic fibroblasts derived from mice expressing mutated inactive PTEN exhibit elevated levels of PtdIns 3,4,5-P_3 in response to platelet-derived growth factor treatment resulting in increased AKT activity (41). Similar results showing PTEN inhibition of AKT phosphorylation have been reported in other systems (42, 60).

Recent studies indicate that PIP_2 and PtdIns 3,4,5-P_3 can bind to the pleckstrin homology domain of AKT stimulating enzymatic activity (18, 19). However, full activation requires phosphorylation of AKT at threonine 308 and serine 473. An AKT kinase (3 phosphoinositide-dependent protein kinase 1 or PKD1) has been cloned that phosphorylates threonine 308 of AKT, and the activity of PKD1 is dependent on the presence of PIP_2 or PtdIns 3,4,5-P_3. Phosphorylation of serine 473 is mediated by PKD2, which is also PI 3-kinase-dependent (50). Therefore, the current view is that PIP_2 and PtdIns 3,4,5-P_3 are important for AKT activation, which would explain the effects of PTEN on AKT. Although AKT has been implicated as a mediator of insulin-stimulated GLUT4 translocation, this suggestion is by no means conclusive. Thus, expression of membrane-targeted AKT constructs in 3T3-L1 adipocytes (17), primary adipocytes (61), and L6 myotubes (62) stimulates GLUT4 translocation, and it has been reported that a dominant inhibitory AKT construct has a modest effect to block GLUT4 translocation. On the other hand, mutant AKT with alanine substituted for threonine 308 and serine 473 behaves as a dominant inhibitory protein in both Chinese hamster ovary cells and 3T3-L1 adipocytes, inhibiting protein synthesis, but these constructs do not inhibit insulin stimulated GLUT4 translocation or glucose transport (63). Thus, the precise role of AKT in stimulation of GLUT4 translocation remains to be determined.

Protein kinase Cα is another serine/threonine kinase downstream of PI 3-kinase that is activated by PtdIns 3,4,5-P_3 in a wortmannin sensitive manner (64). Recent reports have suggested that protein kinase Cα may play a role in mediating signals from PtdIns 3-kinase to glucose transport.

Regardless of the exact mechanism whereby PtdIns 3,4,5-P_3 leads to stimulation of GLUT4 translocation, another important observation in this study relates to the activity of endogenous PTEN. Consistent with our findings on exogenously expressed PTEN, we also conducted experiments involving single cell microinjection of anti-PTEN antibodies. These microinjected antibodies would be expected to interfere with endogenous PTEN, and the results of these experiments showed that PTEN antibody injection led to a marked stimulation of basal GLUT4 translocation, as well as potentiation of the effects of insulin on GLUT4 translocation. Furthermore, we found that these stimulatory effects of anti-PTEN antibodies were completely inhabitable by treatment of the cells with wortmannin. Thus, the PTEN antibody is capable of stimulating GLUT4 translocation through a PI 3-kinase mechanism, consistent with the view that it is inhibiting endogenous PTEN activity. This finding is consistent with the view that endogenous PTEN is a physiological regulator of the efficiency of GLUT4 translocation, most likely by modulating intracellular levels of PtdIns 3,4,5-P_3 at key subcellular locations. Although PTEN is ubiquitously expressed and can function as a tumor suppressor, our studies raise the possibility that PTEN may also modulate the partition of GLUT4 molecules between intracellular vesicular and plasma membrane sites. As such, endogenous PTEN may also prove to be important in the physiologic regulation of insulin action.

Actin cytoskeletal rearrangement is another biologic effect of insulin, and previous studies have shown that it is dependent on PI 3-kinase activity (53). After ligand binding, one observes a rapid breakdown of actin stress fibers, followed by the appearance of membrane ruffles. Our current data show that expression of PTEN largely inhibits insulin-stimulated membrane ruffling in 3T3-L1 adipocytes, indicating the necessity of PtdIns 3,4,5-P_3 for this process. This is consistent with previous studies indicating that PtdIns 3,4,5-P_3 can participate in the activation of the small GTP-binding protein Rac, which then mediates membrane ruffling (53, 54).

Not all insulin action pathways were inhibited by PTEN.
expression, because insulin stimulated MAP kinase activation was unaffected. MAP kinase is activated by a signaling cascade involving Raf and MEK kinases, and the role of PI 3-kinase, if any, in the Ras/RAP kinase pathway has been controversial. In any event, our results are consistent with previous in vitro studies showing that MAP kinases are not direct substrates for PTEN phosphatase (22, 57, 60) and also suggest that the effects of PTEN block certain biologic effects of insulin (GLUT4 translocation, glucose transport, membrane ruffling, AKT phosphorylation, and p70S6 kinase phosphorylation), but not others (i.e. IR, IRS-1, and MAP kinase phosphorylation) within the same cells, are specific and not related to a toxic or nonspecific effect on the cells.

In summary, our results show that overexpression of PTEN negatively regulates PI 3-kinase signaling leading to inhibition of insulin induced serine/threonine phosphorylation of AKT and p70S6 kinases, as well as inhibition of GLUT4 translocation, glucose transport, and membrane ruffling. In addition, our results are consistent with the view that endogenous PTEN may be a physiological regulator of GLUT4 translocation and insulin action. Lastly, our results indicate that the lipid messenger PtdIns 3,4,5-P3 plays a critical role in these biological actions.

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REFERENCES

1. Myers, M. G., Jr., and White, M. F. (1995) Trends Endocrinol. Metab. 6, 209–215
2. Sun, X. J., Wang, L.-M., Zhang, Y., Yenush, L., Myers, M. G., Jr., Glasheen, E., Backer, J. M., Myers, M. G., Jr., and White, M. F. (1995) J. Biol. Chem. 270, 5045–5054
3. Waters, S. B., and Pessin, J. E. (1996) J. Biol. Chem. 271, 27291–27294
4. Lavan, B. E., Lane, W. S., and Lienhard, G. E. (1997) J. Biol. Chem. 272, 11439–11443
5. Lavan, B. E., Fantin, V. R., Chang, E. T., Lane, W. S., Keller, S. R., and Lienhard, G. E. (1997) J. Biol. Chem. 272, 11439–11443
6. White, M. F., and Kahn, C. R. (1994) J. Biol. Chem. 269, 1–4
7. Staubs, P. A., Reichart D. R., Saltiel, A. R., Milarski, K. L., Maegawa, H., and Rosen, J. P. (1996) Trends Endocrinol. Metab. 7, 343–47
8. Backer, J. M., Myers, M. G., Jr., Sun, X.-J., Chin, D. J., Shoelson, S. E., Kohn, A. D., Takeuchi, F., and Roth, R. A. (1996) J. Biol. Chem. 272, 5071–5075
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