Regulation of Insulin Signaling and Glucose Transporter 4 (GLUT4) Exocytosis by Phosphatidylinositol 3,4,5-Trisphosphate (PIP$_3$) Phosphatase, Skeletal Muscle, and Kidney Enriched Inositol Polyphosphate Phosphatase (SKIP)

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Background: Insulin-mediated glucose incorporation in skeletal muscle is negatively regulated by phosphatidylinositol 3,4,5-trisphosphate (PIP$_3$) phosphatases.
Results: Among PIP$_3$ phosphatases, skeletal muscle and kidney enriched inositol polyphosphate phosphatase (SKIP) silencing increased insulin signaling in C2C12 cells.
Conclusion: SKIP is the predominant PIP$_3$ phosphatase regulating insulin signaling.
Significance: SKIP is a promising target for the regulation of insulin resistance.

The glucose transporter 4 (GLUT4) is responsible for glucose uptake in the skeletal muscle. Insulin-induced translocation of GLUT4 to the plasma membrane requires phosphatidylinositol 3-kinase activation-mediated generation of phosphatidylinositol 3,4,5-trisphosphate PIP$_3$ and subsequent activation of Akt. Previous studies suggested that skeletal muscle and kidney enriched inositol polyphosphate phosphatase (SKIP) has negative effects on the regulation of insulin signaling in the skeletal muscle cells. Here, we compared its effects on insulin signaling by selective inhibition of SKIP, SHIP2, and phosphatase and tensin homologue on chromosome 10 (PTEN) by short interfering RNA in the C2C12 myoblast cells. Suppression of SKIP significantly increased the insulin-stimulated phosphatidylinositol 3,4,5-trisphosphate levels and Akt phosphorylation. Furthermore, silencing of SKIP, but not of PTEN, increased the insulin-dependent recruitment of GLUT4 vesicles to the plasma membrane. Taken together, these results imply that SKIP negatively regulates insulin signaling and glucose uptake by inhibiting GLUT4 docking and/or fusion to the plasma membrane.

Glucose homeostasis is controlled by insulin, which stimulates glucose transport in the skeletal muscle and adipose tissues, and impairment of insulin action is the cause of type II diabetes mellitus (1). Skeletal muscle accounts for the majority of whole body insulin-induced glucose disposal by the redistribution of glucose transporter 4 (GLUT4) to the plasma membrane, and thus, regulation of insulin signaling in the skeletal muscle is critical for glucose homeostasis.

Insulin-dependent translocation of GLUT4 requires activation of the insulin receptor, which leads to activation of the phosphatidylinositol (PI)[2] 3-kinase pathway. PI 3-kinase phos-phorylates phosphatidylinositol 4,5-bisphosphate (PI(4,5)P$_2$) and increases intracellular phosphatidylinositol 3,4,5-trisphosphate (PIP$_3$) at the plasma membrane. PIP$_3$ leads to the activation of Akt and 3-phosphoinositide-dependent protein kinase (PDK1), which is required for insulin action such as the translocation of GLUT4 to the plasma membrane (2, 3). Akt is known to translocate from the cytosol to the plasma membrane, where it is phosphorylated at Thr-308 and Ser-473 (4, 5). Activation of the PI 3-kinase-Akt signaling pathway is implicated in the regulation of insulin signaling. Akt2 knock-out mice exhibit insulin resistance and diabetic phenotype (6, 7), and this signaling pathway is often diminished in Type II diabetes mellitus. Recently, AS160 (Akt substrate of 160 kDa) was identified as the Akt2 target that induces GLUT4 trafficking to the plasma membrane (8). AS160 is a Rab-GTPase-activating protein (GAP) that maintains its target Rabs in an inactive, GDP-bound form. Insulin-induced phosphorylation of AS160 mediates the docking and fusion of GLUT4 vesicles to the plasma membrane. An AS160 mutant lacking Akt phosphorylation sites inhibited GLUT4-containing vesicle translocation to the plasma membrane (9).

PIP$_3$ phosphatases hydrolyze intracellular PIP$_3$ and negatively regulate the PI 3-kinase-Akt signaling pathway. Phosphatase and tensin homologue on chromosome 10 (PTEN) hydrolyzes 3-phosphate from PIP$_3$, and studies on PTEN have revealed its implication in insulin signaling. Overexpression of PTEN in 3T3-L1 adipocytes inhibits GLUT4 translocation and glucose uptake (10). Adipose tissue-specific PTEN knock-out results in insulin hypersensitivity and resistance to streptozotocin-induced diabetes (11, 12). Several studies have shown that SHIP2 (SH2 domain-containing inositol polyphosphate phos-

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phatase) is also involved in the regulation of insulin signaling. Expression of SHIP2 attenuated insulin-induced glucose uptake in 3T3-L1 cells (13) and modestly inhibited insulin-stimulated Akt and glycogen synthase kinase 3β (GSK-3β) phosphorylation in L6 myoblast cells (14). SHIP2 knock-out mice have been reported to exhibit increased insulin-induced Akt phosphorylation and p70 S6 kinase phosphorylation, whereas they showed normal glucose homeostasis and insulin tolerance (15). It remains to be elucidated why enhanced phosphorylation of Akt does not affect the glucose uptake in SHIP2 knock-out mice.

SKIP (skeletal muscle and kidney enriched inositol polyphosphate 5-phosphatase) is a PIP_3 phosphatase that is abundantly expressed in the skeletal muscle. SKIP is localized to the endoplasmic reticulum under basal conditions and is translocated to the membrane ruffles in response to insulin, and SKIP negatively regulates insulin-dependent glucose incorporation (16). Recently, we have found that heterozygous knock-out of SKIP in mice caused increased glucose uptake in the skeletal muscle with increased insulin signaling (17).

Based on these studies of PTEN, SHIP2, and SKIP, all of the PIP_3 phosphatases are implicated in the regulation of insulin signaling. However, several important findings about the differences between the PIP_3 phosphatases have been reported. For example, PTEN, but not SHIP2, predominantly suppressed insulin signaling through the PI 3-kinase pathway by RNA interference (RNAi)-based gene silencing in 3T3-L1 adipocytes (18). In differentiating myoblast cells, SHIP2 affects the steady state levels of PIP_3, whereas PTEN moderately regulates the intracellular levels of 3-phosphoinositides and significantly regulates Akt phosphorylation (19). Despite these findings, the role of these PIP_3 phosphatases in the regulation of insulin signaling in the skeletal muscles remains unknown.

In this study, we have compared the relative effects of endogenous SKIP, PTEN, and SHIP2 on insulin signaling in C2C12 myoblast cells by using the RNAi method. We found that the suppression of SKIP markedly increased insulin-induced Akt phosphorylation, GLUT4 exocytosis, and glucose incorporation. Taken together, our results show that SKIP plays the predominant role in the regulation of insulin signaling in C2C12 cells among the PIP_3 phosphatases.

EXPERIMENTAL PROCEDURES

Materials—Antibodies for SKIP and SHIP2 raised against the C-terminal region were generated and used for immunoblotting. Antibodies specific for Akt, phospho-Akt (Ser-473), phospho-Akt (Thr-308), AS160, phospho-AS160 (Thr-642), insulin receptor β, phospho-insulin receptor β (Tyr-1134), p70 S6 kinase, phospho-p70 S6 kinase (Thr-389), GSK3β, phospho-GSK3β (Ser-9), Erk1/2, phospho-Erk1/2 (Thr-202/Tyr-204), and PTEN were purchased from Cell Signaling Technology (Beverly, MA). Anti-insulin receptor β antibody was purchased from BD Biosciences.

Transfection and RNAi—Small interfering RNA (siRNA) duplexes were purchased from Invitrogen. The following siRNA oligonucleotides corresponding to mouse SKIP, PTEN, and SHIP2 and rat SKIP and PTEN were used in this study: mouse control siRNA, 5′-GAGCAACTGCGTGTCGA-ATCTCTTA-3′; mouse SKIP siRNA, 5′-GAGTCAACGTCT-GCCTGAAGCTTTA-3′; mouse PTEN siRNA, 5′-CCAATG-TTCAGTGGCGGAACTTGCA-3′; mouse SHIP2 siRNA, 5′-GAGGTTGGAGTTACTTCCCAGTTCA-3′; rat control siRNA, 5′-TATCCGTACCACATTACTTATTTGC-3′; rat SKIP siRNA, 5′-CCATGGAGCAGTTTCTTCATGGATA-3′; and rat PTEN siRNA, 5′-ACTGCAGAGTTGCACAGT-ATCCTTT-3′.

Twenty nanomoles of control siRNA, SKIP siRNA, PTEN siRNA, and SHIP2 siRNA species were transfected into mouse C2C12 or rat L6 myoblast cells. After 24 h, cells were serum-starved for 24 h and stimulated with insulin (0–100 nM) for 10 min.

Immunoprecipitation and Western Blotting—C2C12 cells were cultured in 60-mm plates in DMEM supplemented with 10% fetal bovine serum at 37 °C in 5% CO_2. Cells were treated with insulin (0–100 nM) at 37 °C for 10 min. The cells were washed twice with Tris-buffered saline and then lysed in a lysis buffer containing 20 mM Tris-HCl, pH 7.4. 150 mM NaCl, 5 mM EDTA, 50 mM NaF, 1 mM Na_3VO_4, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml aprotinin, and 10 μg/ml leupeptin for 10 min. Lysates were centrifuged after brief sonication. The supernatants were used for Western blotting analysis and immunoprecipitation. The protein levels were quantified by densitometry.

GLUT4 Translocation Assay and Immunofluorescence—DNA encoding a GLUT4 reporter containing a GFP epitope tag at the C terminus and a Myc tag in the first extracellular loop (7xMyc-GLUT4-GFP) was provided by Dr. Harvey F. Lodish. We cloned DsRed2-tagged 7xMyc-GLUT4 cDNA into pcDNA3.1(−) expression vector (Invitrogen) and used this as a GLUT4 reporter. The GLUT4 reporter was transfected into C2C12 or L6 cells. After 24 h, the cells were serum-starved for 48 h and stimulated with insulin (10 nM). Cells were fixed with 4% paraformaldehyde, and membrane localization of GLUT4 was visualized by immunofluorescence with anti-Myc antibodies. Cells were imaged using a confocal microscope (Olympus Co., Tokyo, Japan). Cell surface GLUT4 was quantified by calculating the Myc-positive fluorescence intensity divided by the total fluorescence intensity of the cell.

Total Internal Reflection Fluorescence (TIRF) Microscopy—To measure GLUT4 translocation, C2C12 cells expressing 7xmyc-GLUT4-GFP were cultured in glass bottom dishes. TIRF images of the cells were acquired using TIRF microscopy (Olympus Co., Tokyo, Japan). For the normalization, the fluorescence in the TIRF mode was divided by the epifluorescence intensity.

Glucose Incorporation Analysis—[3]H-Labeled 2-deoxyglucose and [^{14}C]mannitol (GE Healthcare) were used to measure glucose incorporation into the C2C12 or L6 myoblast cells constitutively expressing GLUT4 as described previously (16). Cells were stimulated with insulin (10 nM) for 60 min.

High Performance Liquid Chromatography (HPLC) Analysis and Quantification of Phosphoinositides—C2C12 cells transfected with the indicated siRNA were radiolabeled with [^{32}P]orthophosphate for 2 h. Phosphoinositides were purified and deacylated, as described previously (20). Samples were separated by HPLC on a Partisphere strong anion exchange col-

umn (Whatman, Clifton, NJ). The radioactivity was measured with a Flow Scintillation analyzer (Packard, Meridian, CA).

Immunofluorescence—C2C12 cells were transfected with GLUT4 reporter. After 24 h, cells were serum-starved for 24 h and stimulated with insulin (100 nM) for 1 min. Cells were fixed with 3.7% paraformaldehyde and 0.1% glutaraldehyde, and the cell surface GLUT4 was visualized by immunofluorescence with anti-Myc antibodies. Cells were then permeabilized with 1.5% saponin for 10 min. To visualize the intracellular PIP_3, semiconductor quantum dot-655 (Qdot655)-labeled Grp1 PH domain protein was prepared. Cells were fixed with 3.7% paraformaldehyde and 0.1% glutaraldehyde and then permeabilized with 1.5% saponin for 10 min. Qdot655-labeled Grp1 PH was incubated with the cells at room temperature for 1 h. Cells were imaged by confocal microscopy.

RESULTS

SKIP and PTEN Work on Different PIP_3 Pools in C2C12 Cells— SKIP, SHIP2, and PTEN have been reported to be implicated in protection against insulin resistance in mice (12, 15, 17). In C2C12 myoblast cells, several species of PIP_3 phosphatases, including SKIP, PTEN, and SHIP2, are simultaneously expressed. Therefore, we first assessed the selective suppression of these phosphatases in C2C12 cells by using specific RNAi. Expression of SKIP was reduced by $80.0 \pm 8.5\%$ by SKIP-directed siRNA but not by control siRNA ($p = 1.39 \times 10^{-5}$, Fig. 1*A*). Approximately 80–90% suppression of PTEN or SHIP2 expression was achieved without significant change in the expression of the other phosphatases.

To study the role of these phosphatases in the regulation of insulin signaling, we measured the PIP_3 levels in the siRNA-transfected C2C12 cells. We labeled cells with inorganic ^{32}P to measure synthesized phosphoinositides. In this method, inorganic ^{32}P are efficiently incorporated into inositol ring of newly synthesized phosphoinositides. Insulin stimulation activated PI 3-kinase and caused a dramatic increase in intracellular PIP_3. In SKIP or PTEN siRNA-transfected cells, insulin treatment showed a remarkable increase in PIP_3 in 5 min compared with the control cells (145.8 ± 10.0 and $136.0 \pm 6.9\%$ higher than the control siRNA, $p = 5.0 \times 10^{-4}$ and 0.023, respectively; Fig. 1*B*), which were not altered by the silencing of SHIP2. A significant decrease in $PI(3,4)P_2$ levels was observed by the silencing of SKIP ($27.2 \pm 8.6\%$ lower than control siRNA, $p = 0.022$; Fig. 1*B*). In contrast, $PI(4,5)P_2$ levels were not altered by the silencing of SKIP, PTEN, or SHIP2 (Fig. 1*B*). These results suggest that among the PIP_3 phosphatases examined, SHIP2 is not implicated in the regulation of insulin signaling in the C2C12 cells.

We have found a difference between SKIP and PTEN in the regulation of intracellular PIP_3 content in response to insulin stimulation (Fig. 1*C*). PTEN knockdown resulted in an increase in PIP_3 levels under basal conditions. In SKIP-silenced cells, basal PIP_3 was hardly detectable. PIP_3 reaches a maximum after insulin stimulation for 1 min, and this was increased by the attenuation of PTEN. In SKIP-silenced cells, PIP_3 level was comparable with that of the control cells after 1 min (Fig. 1*C*), which remained constant even after insulin stimulation for 10 min. These results imply that endogenous SKIP and PTEN

hydrolyze PIP_3 in a temporally different manner. SKIP chiefly controls PIP_3 content in an insulin stimulation-dependent manner, whereas PTEN mainly regulates PIP_3 under basal conditions.

We used a fluorescence-labeled Grp1 PH domain (Qdot655-Grp1 PH) to visualize PIP_3 in intact C2C12 cells. Under resting conditions, PIP_3 generation was hardly detectable; however, clear accumulation of PIP_3 could be observed at the plasma membrane after insulin stimulation for 5 min (Fig. 1*D*). In these cells, PIP_3 accumulated at the membrane ruffles in response to insulin (Fig. 1*E*). Silencing of SKIP did not alter these patterns (Fig. 1*E*). By contrast, attenuation of PTEN caused an accumulation of PIP_3 throughout the plasma membrane under basal conditions (Fig. 1*E*). In these cells, accumulation of PIP_3 was observed at the membrane ruffles in response to insulin (Fig. 1*E*). In C2C12 cells treated with insulin, SKIP was co-localized with actin at the membrane ruffles (Fig. 1*F*). These results demonstrate that SKIP and PTEN hydrolyze PIP_3 in a temporally different manner. SKIP chiefly controls regulates PIP_3 levels in an insulin stimulation-dependent manner, whereas PTEN controls basal PIP_3 levels under basal conditions.
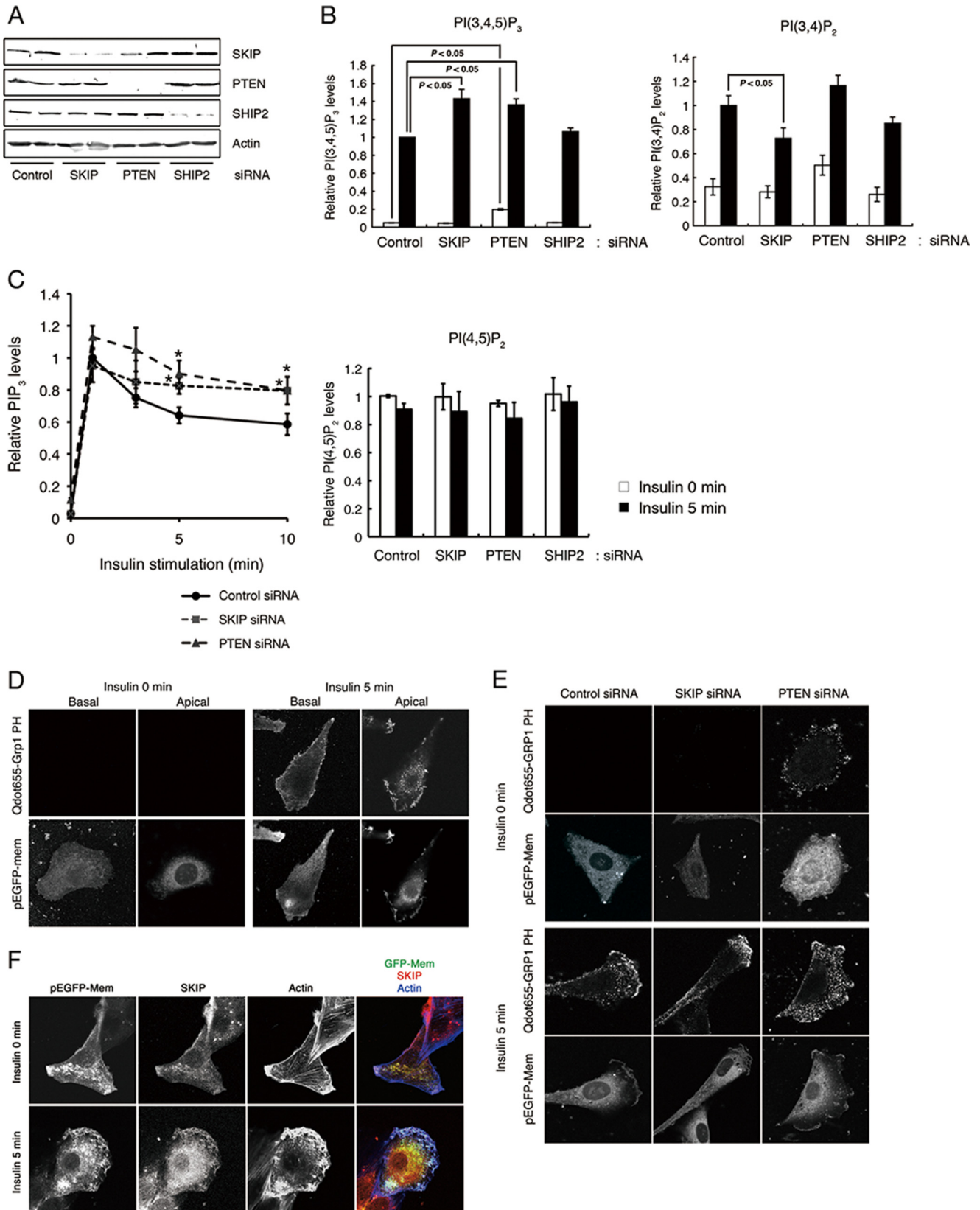
SKIP and PTEN Negatively Regulate Insulin Signaling—Akt is implicated in the regulation of insulin-induced glucose uptake and GLUT4 translocation to the plasma membrane (2). We have analyzed the insulin-induced phosphorylation of Akt when endogenous SKIP or PTEN was depleted in C2C12 cells. Suppression of SKIP caused a significant increase in Akt phosphorylation at Thr-308 and Ser-473 in response to insulin stimulation (10 nM) without affecting basal levels (Fig. 2*A*). By contrast, suppression of PTEN increased phosphorylation of Akt only at Ser-473 and not at Thr-308 under basal conditions but also increased phosphorylation of Akt at Thr-308 and Ser-473 under insulin-induced conditions (Fig. 2*A*). We next examined the consequences of selective suppression on insulin-induced activation of the downstream targets GSK-3β and ribosomal p70 S6 kinase. GSK-3β is rapidly inactivated by insulin through the phosphorylation of an N-terminal Ser-9 residue (21). Suppression of SKIP or PTEN further increased this insulin-induced phosphorylation; however, the increase was much higher in SKIP knockdown (2.24 ± 0.18-fold over control siRNA, $p = 1.9 \times 10^{-3}$) than in PTEN knockdown (1.52 ± 0.04-fold over control siRNA, $p = 5.6 \times 10^{-4}$) (Fig. 2*B*). p70 S6 kinase is also one of the downstream targets of the Akt signaling pathway (22). Insulin-induced phosphorylation of p70 S6 kinase at Thr-389 was also markedly increased by knockdown of SKIP or PTEN (2.27 ± 0.27- and 1.40 ± 0.03-fold over control siRNA, $p = 0.013$ and 0.096, respectively) (Fig. 2*C*).

We next examined the effect of these phosphatases on PI 3-kinase-independent signaling. Erk1/2 is not involved in PI 3-kinase-Akt signaling pathway to respond to insulin. We have previously reported that insulin-induced Erk1/2 phosphorylation is unaltered by the expression of SKIP. Silencing of neither SKIP nor PTEN affected insulin-dependent Erk1/2 phosphorylation at Thr-202/Tyr-204 (Fig. 3*A*), which was comparable with the control siRNA. Insulin-induced tyrosine phosphorylation of the insulin receptor, which is located upstream of PI 3-kinase, was not affected by the depletion of SKIP or PTEN (Fig. 3*B*).

Depletion of SKIP Markedly Enhanced Insulin-induced GLUT4-containing Vesicle Translocation and Docking/Fusion to the Plasma Membrane at the Membrane Ruffles—To examine whether the modulation of insulin signaling by SKIP was correlated with glucose homeostasis, insulin-induced GLUT4 translocation and glucose uptake were analyzed. AS160 is a Rab-GTPase required for insulin-induced GLUT4 translocation to the plasma membrane and glucose incorporation. Sup-

pression of SKIP markedly enhanced insulin-induced phosphorylation of AS160 at Thr-642 by Akt2 (1.62 ± 0.03-fold over control siRNA, $p = 1.81 \times 10^{-5}$), which was not observed by the suppression of PTEN ($p = 0.073$), implying that endogenous SKIP predominantly regulates GLUT4 translocation in C2C12 cells (Fig. 4*A*). This phosphorylation is necessary for GLUT4 transport (9). The GLUT4-DsRed2 construct was used to visualize intracellular GLUT4. In C2C12 cells, SKIP translocates from the ER to the plasma membrane in response to insulin for 10 min, where co-localization with GLUT4-DsRed2 could be observed (Fig. 4*B*).

The 7xMyc-GLUT4-GFP reporter construct was used to characterize the role of SKIP in insulin-induced GLUT4 translocation to the plasma membrane. This construct contains an exofacial Myc epitope at the first extracellular loop and EGFP at the C terminus. When the GLUT4 reporter is translocated to the plasma membrane, the Myc epitope tag faces outside the cell and can be visualized by immunofluorescence staining with anti-Myc antibody (Fig. 5*A*). Knockdown of SKIP increased the amount of cell surface GLUT4 in response to insulin in C2C12 and in L6 cells (Fig. 5*B*), but this was not altered by the suppression of PTEN. The percentage of cells exhibiting cell surface 7xMyc-GLUT4-GFP was significantly increased by the silencing of SKIP (PTEN siRNA, $44.3 \pm 5.0\%$ *versus* control siRNA, $30.0 \pm 1.5\%$ ($p = 0.07$); SKIP siRNA, $58.9 \pm 2.9\%$ ($p = 6.8 \times 10^{-3}$); data not shown), suggesting that SKIP regulates insulin-induced fusion of GLUT4 vesicles to the plasma membrane.

Regulation of insulin-induced GLUT4 exocytosis occurs at several steps, including recruitment, docking, and/or fusion of the GLUT4-containing vesicles to the plasma membrane (23, 24). These steps are dependent on PI 3-kinase activity (25). To examine whether SKIP is implicated in the regulation of GLUT4 exocytosis to the plasma membrane, TIRF microscopy was used to measure the insulin-induced relocation of 7xMyc-GLUT4-GFP (26). In this assay, GLUT4 distribution within the TIRF zone ($\sim$250 nm from the plasma membrane) could be observed (Fig. 5*A*). Attenuation of SKIP, but not of PTEN, increased the amount of GLUT4 at the cell surface of C2C12 and L6 cells (Fig. 5*B*) and within the TIRF zone of C2C12 cells (Fig. 5*C*), demonstrating that SKIP regulates the recruitment and/or docking steps of the GLUT4-containing vesicles. In these cells, 2-deoxyglucose incorporation was also increased (SKIP siRNA in C2C12 cells, 3.91 ± 0.81 nmol min^{-1} mg^{-1} protein *versus* control siRNA in C2C12 cells, 2.41 ± 0.20 nmol min^{-1} mg^{-1} protein; $p = 0.023$) (Fig. 5*D*). In contrast, PTEN depletion did not increase insulin-dependent glucose uptake (2.84 ± 0.03 nmol min^{-1} mg^{-1} protein, $p = 0.10$). These results suggest that among PIP$_3$ phosphatases, SKIP predominantly regulates insulin-dependent glucose incorporation into C2C12 cells.

DISCUSSION

SKIP is one of the phosphoinositide 5-phosphatases that hydrolyzes PIP$_3$ to generate PI(3,4)P$_2$. SKIP is localized to ER in the absence of insulin stimulation; however, in the presence of insulin, SKIP rapidly translocates to the plasma membrane (27). We have previously reported that endogenous SKIP is abundantly expressed in the skeletal muscle and that it negatively regulates insulin-dependent membrane ruffle formation and GLUT4 translocation through PI 3-kinase-Akt signaling (17). Therefore, in addition to PTEN and SHIP2, SKIP is one of the potent regulators of insulin signaling among the PIP$_3$ phosphatases (15, 28). Therefore, to examine which of these phosphatases predominantly regulates insulin signaling in the skeletal muscle, we have compared the effect of SKIP, PTEN, and SHIP2 in insulin signaling by using C2C12 myoblast cells, which simultaneously express all of these.

Attenuation of either SKIP or PTEN, but not of SHIP2, was associated with an increase in PIP$_3$ level, indicating that SHIP2 is not implicated in the regulation of insulin signaling in these cells. Silencing of PTEN caused an increase in the basal and insulin-stimulated PIP$_3$ levels; however, the insulin-induced increase was comparable with the control cells. In contrast, insulin-induced PIP$_3$ generation was significantly increased when SKIP was knocked down, implying that SKIP and PTEN hydrolyze PIP$_3$ in different mechanisms. In these experiments, we labeled C2C12 cells with inorganic ^{32}P to measure synthesized phosphoinositides. Therefore, 3-phosphate of newly synthesized PIP$_3$ from PI(4,5)P$_2$ in response to insulin stimulation is efficiently labeled by inorganic ^{32}P in comparison with 4- or 5-phosphate. Although both SKIP and PTEN are PIP$_3$ phosphatases, they have different enzymatic activity. SKIP hydrolyzes 5-phosphate of PIP$_3$, whereas PTEN dephosphorylates 3-phosphate. Therefore, PIP$_3$ levels in insulin-stimulated C2C12 cells might be exaggerated by the silencing of PTEN compared with that of SKIP or SHIP2. Taking these effects into consideration, we conclude that among PIP$_3$ phosphatases examined, SKIP predominantly regulated PIP$_3$ levels in insulin-stimulated cells.

To visualize intracellular PIP$_3$ in intact cells, Qdot655-labeled Grp1 PH domain protein was used as a probe. Immunofluorescence by this protein showed the accumulation of PIP$_3$ at the plasma membrane after insulin stimulation for 1 min, demonstrating that this probe specifically recognizes PIP$_3$ at the plasma membrane. Knockdown of PTEN resulted in the accumulation of PIP$_3$ under basal conditions. After insulin stimulation for 5 min, PIP$_3$ generation at the membrane ruffles

FIGURE 1. **Attenuation of SKIP and PTEN increased PIP$_3$ levels in C2C12 cells.** *A*, C2C12 myoblast cells were transfected with control, SKIP, PTEN, or SHIP2 siRNA (20 nmol) and cultured for 48 h. Lysates (50 μg) were used for Western blot analysis. *B*, silencing of SKIP increased intracellular PIP$_3$ levels in the insulin-stimulated cells. C2C12 cells were transfected as described and labeled with [^{32}P]orthophosphate for 2 h. Serum-deprived cells were either unstimulated (0 min) or stimulated (5 min) with insulin (10 nM). Phosphoinositides were extracted, deacylated, and applied to a strong anion exchange column, as described under "Experimental Procedures." The relative amounts of PIP$_3$ (*PI(3,4,5)P$_3$*), PI(3,4)P$_2$, and PI(4,5)P$_2$ are shown. All data are presented as the mean $\pm$ S.E. (*error bars*). *C*, measurement of the PIP$_3$ levels in the insulin-stimulated C2C12 cells transfected with control-, SKIP-, or PTEN-directed siRNA (20 nmol). *, $p < 0.05$ (*t* test). *D*, intracellular PIP$_3$ could be observed by immunofluorescence with the Qdot655-labeled Grp1 PH domain. Shown is immunofluorescence of PIP$_3$ in the C2C12 cells expressing pEGFP-Mem constructs to visualize the plasma membrane. Qdot655-labeled Grp1 PH domain protein was used to visualize the intracellular PIP$_3$ at the plasma membrane. *E*, knockdown of SKIP promoted PIP$_3$ accumulation at the membrane ruffles. PIP$_3$ localization was observed in the SKIP or PTEN siRNA-transfected C2C12 cells. *F*, immunofluorescence of endogenous SKIP in C2C12 cells expressing pEGFP-Mem constructs. F-actin was visualized by Alexafluor647-labeled phalloidin. *White* indicates regions of co-localization of SKIP and F-actin at the plasma membrane.
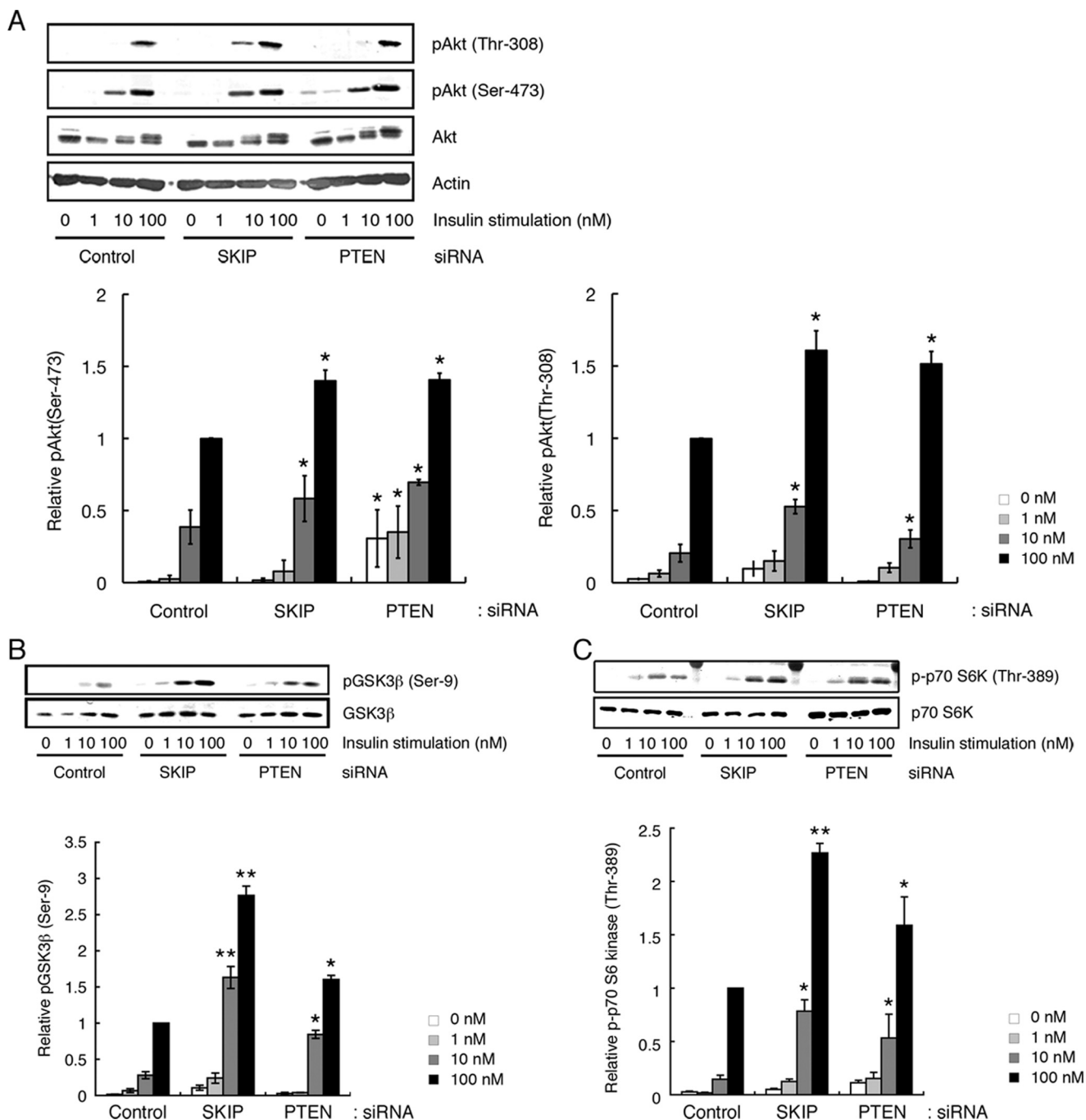
FIGURE 2. **Specific regulation of insulin signaling by the suppression of SKIP.** *A*, increased Akt phosphorylation by the attenuation of SKIP and PTEN. C2C12 cells were transfected as described above and treated with insulin (0–100 nM) for 10 min. Cells were harvested with lysis buffer, and the lysates were separated by SDS-PAGE. The protein levels of Akt and phosphorylated Akt at Thr-308 or Ser-473 were detected by Western blot analysis. The amount of phosphorylated proteins was quantified by densitometry. All values are presented as mean ± S.E. *, $p < 0.05$ (*t* test). *B*, silencing of SKIP increased insulin-stimulated GSK3β phosphorylation. C2C12 cells were transfected with the indicated siRNAs and stimulated with several concentrations of insulin (0–100 nM) for 10 min. Phosphorylation of GSK3β at Ser-9 was measured by Western blot analysis. Values are presented as mean ± S.E. (*error bars*). *, $p < 0.05$; **, $p < 0.01$ (*t* test). *C*, increased insulin-induced p70 S6 kinase phosphorylation by attenuation of SKIP. C2C12 cells were transfected with the indicated siRNAs and stimulated with several concentrations of insulin (0–100 nM) for 10 min. Phosphorylation of p70 S6 kinase at Thr-389 was measured by Western blot analysis. Values are presented as mean ± S.E. *, $p < 0.05$; **, $p < 0.01$ (*t* test).

could clearly be observed in SKIP-silenced C2C12 cells as well as in control cells. In contrast, silencing of PTEN resulted in the generation of PIP_3 throughout the plasma membrane. These results imply that SKIP and PTEN work on spatially different PIP_3 pools in a single cell. It is important to note that spatiotemporal regulation of PIP_3 is governed by distinct PIP_3 phosphatases.

PIP_3-dependent activation of Akt is necessary for insulin-dependent GLUT4 translocation to the plasma membrane and glucose uptake. Both SKIP and PTEN knockdown resulted in increased Akt phosphorylation. Silencing of PTEN expression resulted in enhanced Akt phosphorylation at Ser-473, but not at Thr-308, in the absence of insulin stimulation. These phosphorylations were mediated by different kinases. The Ser-473 posi-
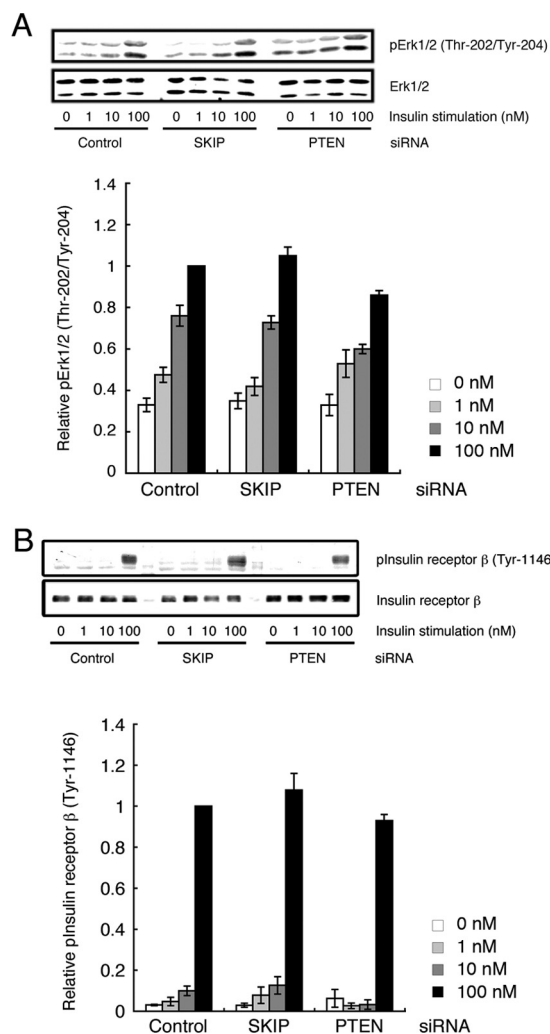
FIGURE 3. **Suppression of SKIP did not affect insulin-induced insulin receptor and Erk1/2 activation.** C2C12 cells transfected with specific siRNAs were stimulated with insulin (0–100 nM) for 10 min. Insulin-induced tyrosine phosphorylation of Erk1/2 (*A*) and insulin receptor β (*B*) was determined by Western blot analysis and quantified by densitometry. All values are presented as mean ± S.E. (*error bars*).

FIGURE 4. **SKIP regulates insulin-induced AS160 phosphorylation.** *A*, effect of attenuation of SKIP in insulin-stimulated AS160 phosphorylation. C2C12 cells were transfected with the indicated siRNAs and stimulated with several concentrations of insulin (0–100 nM) for 10 min. Phosphorylation of AS160 at Thr-642 was measured by Western blot analysis and quantified by densitometry. Values are presented as mean ± S.E. (*error bars*). *, $p < 0.05$ (*t* test). *B*, co-localization of SKIP with GLUT4 at the membrane ruffles. C2C12 cells expressing GFP-SKIP and DsRed-GLUT4 were stimulated with insulin for 10 min. Cells were visualized by confocal microscopy. *Enlarged images* of *boxed areas* are shown in the *lower panels*. *Yellow* indicates regions of co-localization of SKIP and GLUT4 (*arrowheads*). *Scale bar*, 20 nm.

tion is phosphorylated by mammalian target of rapamycin complex 2 (mTORC2), which precedes the phosphorylation of the Thr-308 position by PDK1 (4, 5). A number of reports have shown that growth factors increase mTORC2 activity, albeit through unknown signaling events. PDK1 is localized at the cytosol under basal conditions. In response to insulin stimulation, it is translocated to the plasma membrane, where it binds to PIP_3 through the PH domain. In PTEN-silenced cells, we found a slight increase in PIP_3 levels under basal conditions, which is enough for the activation of mTORC2. However, the amount is not enough for the activation of PDK1 and the Akt phosphorylation at Thr-308. The study using 3T3-L1 adipocytes showed that suppression of PTEN did not affect basal PIP_3 and Akt phosphorylation (18). This discrepancy may be derived from the residual expression level of PTEN; in their study, ~50% of PTEN protein was still expressed, whereas we achieved ~90% suppression of PTEN expression in our study. Attenuation of SKIP protein did not affect basal Akt phosphorylation but did significantly increase insulin-dependent Akt
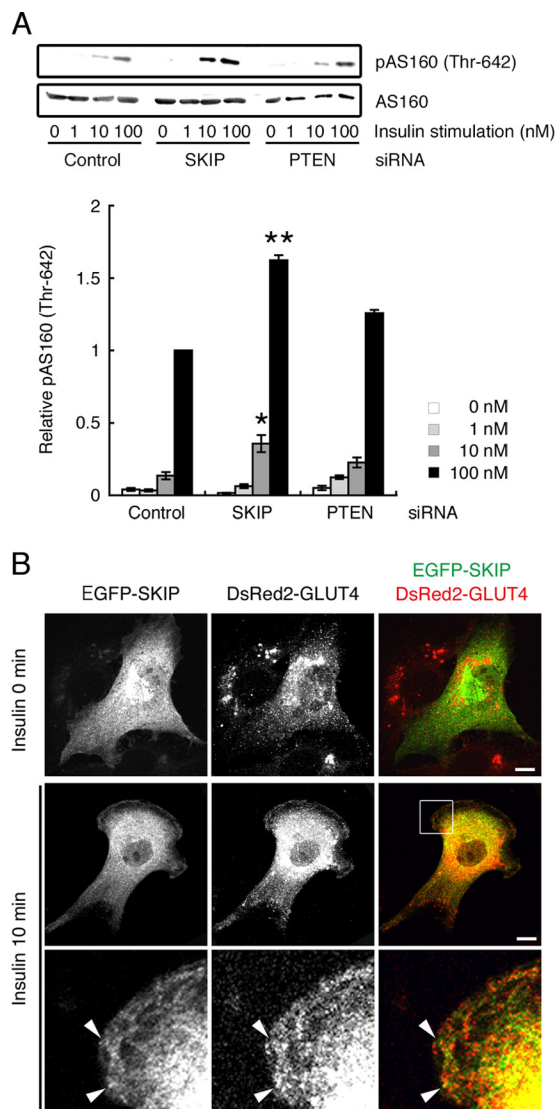
phosphorylation. We have previously reported that suppression of SKIP by the specific antisense oligonucleotides significantly increased insulin-induced Akt phosphorylation (16). Phosphorylation of the other downstream effectors, namely, GSK3β and p70 S6 kinase, was also markedly increased by the silencing of SKIP. These results suggest that SKIP might predominantly regulate insulin signaling in C2C12 cells.

Insulin stimulates glucose uptake into the skeletal muscle cells, which requires GLUT4 translocation to the plasma membrane. Upon insulin stimulation, GLUT4 undergoes exocytosis and is targeted to the plasma membrane. AS160, a Rab-GAP, is one of the downstream effectors of Akt and is necessary for this translocation (29). SKIP silencing significantly increased the
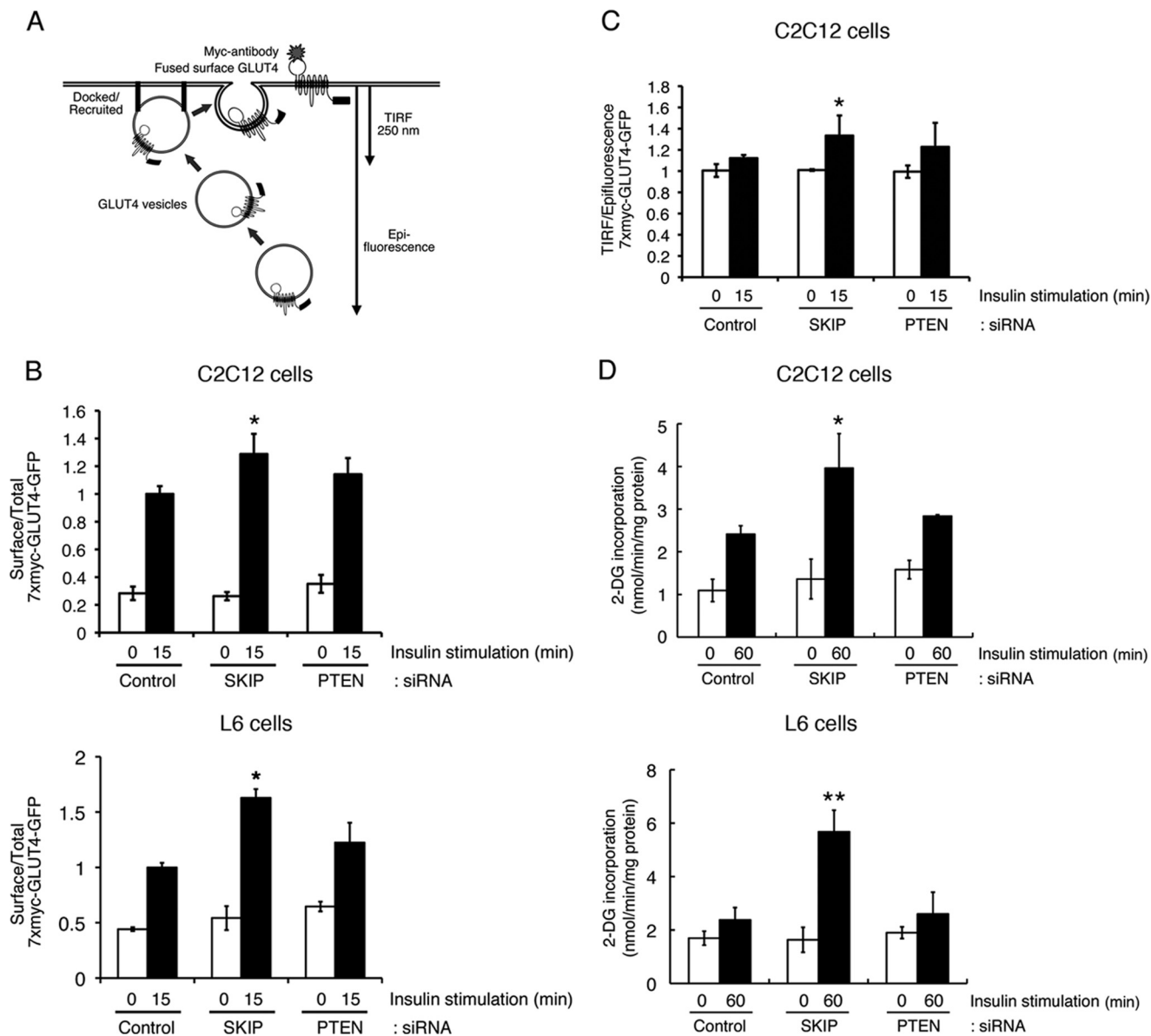
FIGURE 5. **SKIP regulates insulin-induced exocytosis of GLUT4 and glucose uptake.** *A*, schematic model of the GLUT4 translocation assay. GLUT4 reporter fluorescence in the basal or insulin-stimulated cells was measured in the epifluorescence and TIRF modes. Translocation of GLUT4 at the cell surface was measured by immunofluorescence with anti-Myc antibody. *B*, SKIP negatively regulated insulin-dependent GLUT4 translocation. Surface-to-total distribution of GLUT4 reporter was analyzed in C2C12 or L6 cells expressing GLUT4 reporter. Cell surface GLUT4 reporter was analyzed by immunofluorescence with anti-Myc antibody. Cells were stimulated with insulin (100 nM) for 15 min. *, $p < 0.05$ (*t* test). *C*, SKIP negatively regulated docking and/or fusion steps of GLUT4 translocation in C2C12 cells. Quantification of GLUT4 at the plasma membrane was performed by TIRF microscopy. For each experiment, the data are normalized to the control siRNA-transfected cells. *, $p < 0.05$ (*t* test). *D*, insulin-dependent glucose uptake was increased by suppression of SKIP. C2C12 or L6 cells transfected with control-, SKIP-, or PTEN-directed siRNAs in combination with GLUT4 reporter were subjected to a 2-deoxyglucose (*2-DG*) uptake assay, as described under "Experimental Procedures." Cells were stimulated with insulin (10 nM) for 60 min. *, $p < 0.05$ (*t* test). *Error bars*, S.E.

phosphorylation of AS160, which was unaltered by the attenuation of PTEN. GLUT4 exocytosis in the skeletal muscle cells is dependent on the actin cytoskeletal rearrangement occurring at the membrane ruffles. SKIP is known to accumulate at the membrane ruffles in response to insulin stimulation, and it negatively regulates membrane ruffle formation (27). Immunofluorescence of C2C12 cells showed the co-localization of SKIP and GLUT4 at the membrane ruffles, suggesting the regulatory role of SKIP in GLUT4 exocytosis. Attenuation of SKIP resulted in a significant increase in the insulin-induced GLUT4 localization to the surface of the plasma membrane, without affecting the basal state. In contrast, silencing of PTEN did not increase

the amount of GLUT4 at the cell surface. Consistent with these results, insulin-dependent glucose uptake was markedly increased by the attenuation of SKIP but not by the silencing of PTEN.

Insulin-dependent GLUT4 exocytosis includes several steps, including recruitment, docking, and/or fusion of GLUT4-containing vesicles to the plasma membrane, all of which are dependent on the PI 3-kinase signaling pathway. Among these steps, AS160 is implicated in the regulation of recruitment step (30, 31). Knockdown of SKIP increased the amount of GLUT4-containing vesicles within the TIRF zone, suggesting that SKIP at least regulates the recruitment step. In contrast, PTEN

knockdown increased the amount of cell surface GLUT4 under basal conditions. It was previously reported that in 3T3-L1 adipocytes, microinjection of anti-PTEN antibody increased plasma membrane-associated GLUT4, even in the absence of insulin stimulation (10). Taken together, SKIP predominantly regulates insulin-dependent GLUT4 translocation, whereas PTEN mainly regulates GLUT4 localization at the plasma membrane under basal conditions. SHIP2 is unlikely to regulate insulin signaling in C2C12 cells.

In summary, our results indicate that endogenous SKIP is predominantly involved in the regulation of insulin signaling among PIP$_3$ phosphatases. SKIP, but not PTEN or SHIP2, negatively regulated insulin-induced Akt phosphorylation, GLUT4 translocation, and glucose uptake in C2C12 cells. Therefore, these data indicate that SKIP may serve as a promising pharmaceutical target aimed at the treatment of diabetes.

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