Insulin signaling through protein kinase Akt/protein kinase B (PKB), a downstream element of the phosphatidylinositol 3-kinase (PI3K) pathway, regulates diverse cellular functions including metabolic pathways, apoptosis, mitogenesis, and membrane trafficking. To identify Akt/PKB substrates that mediate these effects, we used antibodies that recognize phosphopeptide sites containing the Akt/PKB substrate motif (RXRXX(p)S/T) to immunoprecipitate proteins from insulin-stimulated adipocytes. Tryptic peptides from a 250-kDa immunoprecipitated protein were identified as the protein kinase WNK1 (with no lysine) by matrix-assisted laser desorption ionization time-of-flight mass spectrometry, consistent with a recent report that WNK1 is phosphorylated on Thr\(^{40}\) in response to insulin-like growth factor I. Insulin treatment of 3T3-L1 adipocytes stimulated WNK1 phosphorylation, as detected by immunoprecipitation with antibody against WNK1 followed by immunoblotting with the anti-phosphoAkt substrate antibody. WNK1 phosphorylation induced by insulin was unaffected by rapamycin, an inhibitor of p70 S6 kinase pathway but abolished by the PI3K inhibitor wortmannin. RNA interference-directed depletion of Akt1/PKB\(_{\alpha}\) and Akt2/PKB\(_{\beta}\) attenuated insulin-stimulated WNK1 phosphorylation, but depletion of protein kinase C\(_{\alpha}\) did not. Whereas small interfering RNA-induced loss of WNK1 protein did not significantly affect insulin-stimulated glucose transport in 3T3-L1 adipocytes, it significantly enhanced insulin-stimulated thymidine incorporation by about 2-fold. Furthermore, depletion of WNK1 promoted serum-stimulated cell proliferation of 3T3-L1 preadipocytes, as evidenced by a 36% increase in cell number after 48 h in culture. These data suggest that WNK1 is a physiologically relevant target of insulin signaling through PI3K and Akt/PKB and functions as a negative regulator of insulin-stimulated mitogenesis.

Protein kinase Akt/PKB\(^{1}\) is phosphorylated and activated by phosphoinositide-dependent protein kinase, downstream of PI3K, in cells stimulated by insulin or growth factors. Numerous studies have demonstrated that activation of Akt/PKB is involved in the metabolic and mitogenic functions of insulin including the regulation of gene expression, cell survival, cell growth, glucose transport, protein synthesis, and glycerogen synthesis (1–4). Akt/PKB regulates cellular functions through phosphorylation of serine and threonine residues in the motif RXRXX(p)S/T of target proteins (5–7). Multiple Akt/PKB substrates have been discovered, including transcription factor FOXO proteins (8–11), pro-apoptotic factor Bad (12), p70 kinase (13, 14), mitogenic factor Raf1 (15–17), p53 negative regulator Mdm2 (16, 17), cyclin-dependent kinase inhibitors p27kip1 (18–20) and p21cip1 (21), endothelial nitric-oxide synthase (22, 23), glycogen synthase kinase-3 (24), and the GTPase-activating proteins tuberous sclerosis complex-2 (25–27) and AS160 (28). However, it is possible that other unknown Akt/PKB effectors regulate important cellular responses to insulin.

To search for new Akt/PKB substrates, we applied a mass spectrometry-based proteomics approach to identify potential candidates enriched by immunoprecipitation with a polyclonal antibody against the phospho-Akt/PKB substrate (PAS) motif (RXRXX(p)S/T) (29). In this study, we identified the protein kinase WNK1, a newly described protein kinase lacking lysine in kinase subdomain II and implicated in controlling ion permeability (30–32). We confirmed WNK1 as a novel Akt/PKB substrate in insulin-stimulated 3T3-L1 adipocytes, extending a recent report that insulin-like growth factor I in other cells caused phosphorylation of this kinase on Thr\(^{40}\), a putative Akt/PKB substrate site (33). RNAi-mediated gene silencing of WNK1 did not alter insulin-sensitive glucose transport in cultured adipocytes, but it did significantly enhance DNA synthesis and cell growth in 3T3-L1 preadipocytes. This suggests that WNK1 may be a negative regulatory element in the insulin signaling pathway involving Akt/PKB that regulates cell proliferation.

EXPERIMENTAL PROCEDURES

Materials—Human insulin was obtained from Eli Lilly Co. Goat polyclonal anti-Akt1/PKB\(_{\alpha}\) antibody (antigen human Akt1/PKB\(_{\alpha}\) peptide near carboxyl terminus, sc-7126), horseradish peroxidase-conjugated donkey anti-goat IgG, and polyclonal anti-PKC\(_{\alpha}\iota\) (C-20, sc216) were from Santa Cruz Biotech (Santa Cruz, CA). Rabbit polyclonal antibody against Akt2/PKB\(_{\beta}\) was kindly provided by Dr. Morris J. Birnbaum (University of Pennsylvania). Polyclonal antibodies against phospho-Akt/PKB serine 473, phospho-Erk1/2, and insulin receptor substrate (IRS)-1 were from Cell Signaling Technology (Beverly, MA). Monoclonal phosphotheorysinpprotein antibody (4G10) was from Upstate (Charlottesville, VA). Polyclonal antibody against phosphorylated Akt/PKB substrate motif was generated as described previously (29).

* This work was supported by National Institutes of Health Grant DK30648 (to M. P. C.) and an American Diabetes Association Junior Faculty Award (to Z. Y. J.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
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‡ The abbreviations used are: PKB, protein kinase B; IRS, insulin receptor substrate; KRH, Krebs-Ringer HEPES; PAS, phospho-Akt/PKB substrate; PKC, protein kinase C; PI3K, phosphatidylinositol 3-kinase; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; DMEM, Dulbecco’s modified Eagle’s medium; RNAi, RNA interference; siRNA, small interfering RNA; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling; Erk, extracellular signal-regulated kinase; mTOR, mammalian target of rapamycin.
Cell Culture and Transfection of siRNAs—The 3T3-L1 preadipocytes 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 previously (34). Small interfering RNA duplexes were synthesized and purified by Dharmaco, Inc. (Lafayette, CO) and transfected into the 3T3-L1 preadipocytes and adipocytes by electroporation as described previously (35). The targeting sequences of each gene are as follows: Akt1/PKBα, AACCAGGACGAGAGGACCUUCCAUGUAG and UGCCAGACGAGGACCUUCCAUGUAG and UGCCAGAUCGAGAGGACCUUCCAUGUAG; Akt2/PKBβ, GAGAGGACCUUCCAUGUAG and UGCCAGACGAGGACCUUCCAUGUAG; PPARα (36), GAGUGAGUGUGUGAGAUAAU and GCAAAUCUGUGGUCAUAA; WNK1, AAGAGUGUAGUACGUAGUGUA and AGACUGAGGGCUUGUGUAUGA.

Immunoprecipitation and Immunoblotting—After experimental treatments, the cells were solubilized in ice-cold lysis buffer containing 50 mM HEPES (pH 7.4), 5 mM sodium pyrophosphate, 5 mM β-glycerophosphate, 10 mM sodium fluoride, 2 mM EDTA, 2 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprroxitin, 10 μg/ml leupeptin, and 1% Triton X-100. Total cell lysates of 1–2 mg of protein were immunoprecipitated with antibodies against phospho-Akt/PKB substrate (1:100 dilution) or WNK1 (1:200 dilution) for 2 h followed by incubation with 80 μl of protein A-Sepharose 6MB for 2 h at 4 °C. The beads were then washed four times with lysis buffer before boiling for 5 min in Laemmli buffer.

To detect phospho-Akt/PKB substrates, phosphorylation of Akt/PKB serine 473 and tyrosine/threonine phosphorylation of Erk1/2 total cell lysates (50 μg of protein) were resolved with SDS-PAGE and electrotransferred to nitrocellulose membranes, which were incubated with rabbit polyclonal anti-phospho-specific antibodies (1:1000 dilution) overnight at 4 °C. Primary rabbit polyclonal antibodies against Akt2/PKBα (1:1000 dilution) and PKCi/α (1:500 dilution) were used to detect their antigens using 25 μg of protein from total cell lysates. Tyrosine phosphorylation of IRS proteins was detected with monoclonal phosphotyrosine antibody (clone 4G10, 1 μg/ml). Akt1/PKBα was detected with primary goat polyclonal antibody (1:750 dilution). All the membranes were then incubated with the appropriate horseradish peroxidase-labeled secondary antibodies (1:10,000 dilution) for each 1 h at room temperature. The membranes were washed with wash buffer (phosphate-buffered saline, pH 7.4, 0.1% Tween 20) for 1 h at room temperature after incubation with each antibody before detection with ECL™ kit.

Mass Spectrometry—Proteins immunoprecipitated with PAS antibody were resolved in 5–15% gradient SDS-PAGE and visualized with a Silver-plus kit according to the procedure recommended by the manufacturer (Bio-Rad). The 250-kDa band was excised from the silver-stained gel and digested with sequencing grade porcine-modified trypsin (Promega). The tryptic peptides were analyzed by MALDI-TOF mass spectrometer. The peptide mass spectrum was acquired over a mass range 500–3500 Da. Peptide mass mapping was carried out against the National Center for Biotechnology Information data base (www.prospector.ucsf.edu). To further verify the MS-FIT identification, selected peptides were fragmented via Post-Source-Decay and searched against the National Center for Biotechnology Information data base using the MSTag program.

Deoxyglucose Uptake Assay—To detect the effect of specific gene silencing on insulin-stimulated glucose transport, [3H]deoxyglucose uptake assays were carried out in 3T3-L1 adipocytes as described previously (35). Briefly, 5 million adipocytes (day 5 of differentiation) were transfected with siRNA by electroporation, and this procedure usually leads to about 30% cell loss. The cells were reseeded for 60 h before serum starvation for 3 h with Krebs-Ringer HEPES (KRH) buffer (120 mM NaCl, 5 mM KCl, 1.3 mM CaCl2, 1.3 mM MgSO4, and 25 mM HEPES, pH 7.4) supplemented with 0.5% bovine serum albumin and 2 mM sodium pyruvate. Cells were then stimulated with insulin for 30 min at 37 °C. Glucose uptake was initiated by addition of [1,2-3H]2-deoxy-D-glucose to a final assay concentration of 100 μM. The cells were then washed four times with ice-cold phosphate-buffered saline and lysed by incubation with 0.5 ml of 10% SDS for 30 min. Macromolecules were precipitated by 20% trichloroacetic acid using 100 μl of 1 mg/ml DNA as carrier. The resulting trichloroacetic acid-insoluble pellet was washed twice with 5% trichloroacetic acid and then washed with 75% ethanol prior to dissolution in 0.4 ml of formic acid. [3H]Thymidine incorporation was determined by scintillation counting. To assess cell growth rate, the preadipocytes were transfected with designed siRNAs by electroporation and reseeded to 0.5% bovine serum albumin for 5 h. The cells were then incubated with or without insulin for 16 h in the starvation media before adding 0.1 μCi/ml [methyl-3H]thymidine (in the presence of 5 μM cold thymidine) for an additional 2 h at 37 °C. The cells were washed four times with ice-cold phosphate-buffered saline and lysed by incubation with 0.5 ml of 300 mM NaOH and 100 μl of 10% SDS for 30 min. Macromolecules were precipitated by 20% trichloroacetic acid using 100 μl of 1 mg/ml DNA as carrier. The resulting trichloroacetic acid-insoluble pellet was washed twice with 5% trichloroacetic acid and then washed with 75% ethanol prior to dissolution in 0.4 ml of formic acid. [3H]Thymidine incorporation was determined by scintillation counting.

RESULTS

RNAi-based Depletion of Akt/PKB Proteins in 3T3-L1 Adipocytes—RNAi-based Depletion of Akt/PKB Proteins in 3T3-L1 Adipocytes Inhibits Insulin-stimulated Phosphorylation of Proteins Detected by PAS Antibodies—PAS antibody has been reported to recognize the Akt/PKB substrate motif RXRRXXX/S/T in the target proteins of the kinase (28, 37, 38). However, this polyclonal antibody may also cross-react with phosphoproteins that are not the Akt/PKB substrates. In previous studies (39, 40), we tested whether insulin-stimulated protein phosphorylation in cultured adipocytes could be detected with PAS antibody. Analysis of lysates from control or insulin-treated 3T3-L1 adipocytes by SDS-PAGE and Western blotting with PAS antibody revealed multiple phosphoprotein bands (Fig. 1). The intensity of many of the insulin-sensitive bands was markedly diminished by treatment of the cells with wortmannin, indicating that their phosphorylation is downstream of the PI3K pathway.

To identify Akt/PKB-specific targets in insulin-stimulated
3T3-L1 adipocytes, we compared the insulin-stimulated protein phosphorylations detectable by PAS antibody in samples from cells transfected with the scrambled, nonspecific siRNA versus siRNA duplexes directed against mouse Akt1/PKBa plus Akt2/PKBβ. As shown in Fig. 1 (bottom panels), both Akt1/PKBa and Akt2/PKBβ protein levels were significantly reduced in cells transfected with siRNA species against Akt1/ PKBα plus Akt2/PKBβ for 48 h as compared with the cells transfected with the scrambled siRNA. Interestingly, depletion of both Akt1/PKBα and Akt2/PKBβ significantly reduced phosphorylation of several proteins (indicated by arrowheads in Fig. 1), suggesting that Akt/PKB protein kinases are required for insulin-induced phosphorylation of those proteins. It is possible that those proteins are potential physiological target substrates of Akt/PKB in the adipocytes. However, insulin-induced phosphorylation of several other proteins (indicated by asterisks in Fig. 1) was not altered by the loss of Akt1/PKBα and Akt2/PKBβ, suggesting that those insulin-sensitive phosphoproteins are not substrates of Akt/PKB.

Identification of a 250-kDa Phosphoprotein as Protein Kinase WNK1—To identify potential Akt/PKB targets, total cell lysates from control and insulin-stimulated 3T3-L1 adipocytes were immunoprecipitated with PAS antibody and separated by SDS-PAGE, followed by silver staining. As shown in Fig. 2A, the 250-kDa protein was recognized as a PAS antibody band in total lysates from control or insulin-stimulated adipocytes. The 250-kDa protein was excised and analyzed by tandem mass spectrometry peptide mapping in two separate experiments. The tryptic peptide mass fingerprinting of this protein was identical to human and rat protein kinase WNK1 (30–32). This was confirmed by tandem mass spectrometry peptide mapping in two separate experiments. Second, a Blast search for the mouse homologue within the Celera data base identified the mouse WNK1 as similar to rat and human WNK1 in the National Center for Biotechnology Information data base. Motif scanning in the Scansite (www.scansite.mit.edu) identified that WNK1, from all three species, had a conserved putative Akt phosphorylation motif at the amino-terminal region (RRRRH). In addition, depletion of both Akt1/PKBα and Akt2/PKBβ abolished insulin-stimulated phosphorylation of the 250-kDa protein in total lysates from adipocytes (indicated by the top arrowhead in Fig. 1). To confirm that the 250-kDa protein was indeed WNK1 and was phosphorylated in insulin-stimulated cells, PAS antibody was used to immunoprecipitate endogenous phosphoproteins from 3T3-L1 adipocytes, and the enriched pool was immunoblotted with rabbit polyclonal antibody against the amino-terminal peptide of mouse WNK1. As shown in Fig. 3, left panel, the amount of WNK1 precipitated by PAS antibody was dramatically increased in insulin-stimulated cells as compared with control adipocytes, whereas pretreatment of the cells with wortmannin completely demolished the effect of insulin. In addition, immunoprecipitation was also carried out with anti-WNK1 antibody followed by immunoblotting with PAS antibody. As expected, higher phosphorylation of WNK1 detected by PAS antibody was also observed in insulin-stimulated cells, although equal amounts of WNK1 were present in control and insulin-stimulated cells (Fig. 3, bottom right panel). Again, wortmannin totally inhibited insulin-stimulated WNK1 phosphorylation. These data confirm that insulin stimulates PAS-detectable phosphorylation of WNK1 through a wortmannin-sensitive PI3K pathway in adipocytes. A similar conclusion was reached in another study, which was published after we collected the data presented in Figs. 1–3, with respect to insulin-like growth factor I signaling (33). This published study (33) showed that the regulated phosphorylation site on WNK1 is Thr360.

Insulin Induces WNK1 Phosphorylation through the Akt/PKB Pathway—Because insulin signaling through the PI3K pathway leads to the activation of multiple downstream serine/threonine kinases in addition to Akt/PKB, including atypical PKC, mTOR, and p70 S6 kinase, we further investigated whether any of those kinases may be required for insulin-induced WNK1 phosphorylation. In this study, rapamycin, a specific inhibitor of mTOR, was used to examine whether the...
siRNA (40 nmol), Akt1/PKB atypical protein kinase C, is required for insulin-stimulated WNK1 phosphorylation. The adipocytes (day 4) were transfected with the scrambled Akt1/PKB were then treated with or without insulin (100 nM) for 15 min, and cell lysate samples (1 mg of protein) were immunoprecipitated with WNK1 or WNK1 antibody (second panel). Total cell lysate samples (50 µg of protein) were also separated by SDS-PAGE and blotted with antibodies against phospho-Akt/PKB (threonine 473, third panel) or phospho-pp70 S6 kinase (threonine 389, bottom panel). B and C, Akt/PKB, but not atypical protein kinase C, is required for insulin-stimulated WNK1 phosphorylation. The adipocytes (day 4) were transfected with the scrambled siRNA (40 nmol), Akt1/PKBα and Akt2/PKBβ siRNAs (20 nmol each), or PKCα siRNA (40 nmol) for 60 h before serum starvation for 6 h. The cells were then treated with or without insulin (100 nM) for 15 min, and cell lysate samples (1 mg of protein) were immunoprecipitated with WNK1 antibody and immunoblotted with PAS or WNK1 antibodies. Total cell lysate samples (50 µg of protein) were also used for immunoblotting with Akt1/PKBα, Akt2/PKBβ, or atypical protein kinase C antibodies. Wnk, wortmannin; Rap, rapamycin.

mTOR-p70 S6 kinase pathway is involved in WNK1 phosphorylation in cultured adipocytes. As shown in Fig. 4A, incubation of the adipocytes with rapamycin prior to insulin treatment did not alter insulin-induced phosphorylation of Akt/PKB and WNK1, detected by phospho-Akt/PKB Ser473-specific antibody and PAS antibody, respectively. However, rapamycin effectively blocked insulin-stimulated threonine 389 phosphorylation of p70 S6 kinase mediated by mTOR (Fig. 4A). In contrast, wortmannin inhibited not only phosphorylation of threonine 389 of the p70 S6 kinase but also phosphorylation of Akt/PKB and WNK1. These data indicate that the mTOR-p70 S6 kinase pathway is not involved in insulin-stimulated WNK1 phosphorylation.

We next tested whether protein kinases Akt/PKB and atypical protein kinase C, both downstream of the PI3K pathway, are required for the phosphorylation of WNK1. In these studies, siRNA duplexes against Akt1/PKBα and Akt2/PKBβ or PKCα, the major form of atypical PKC in the adipocytes, were electroporated into 3T3-L1 adipocytes (31, 32). As shown in Fig. 4B, depletion of both Akt/PKB proteins by siRNA almost abolished insulin-stimulated phosphorylation of WNK1 detected by PAS antibody, whereas equal amounts of WNK1 were precipitated by WNK1 antibody. However, RNAi-directed depletion of PKCα had no effect on insulin-stimulated WNK1 phosphorylation (Fig. 4C). These data strongly suggest that insulin induces phosphorylation of WNK1 through an Akt/PKB protein kinase-specific pathway.

FIG. 5. WNK1 Is Not Involved in Feedback Regulation of Upstream Signaling by Insulin Receptor. 3T3-L1 adipocytes were transfected with scrambled siRNA (20 nmol) or WNK1-directed siRNA (20 nmol) for 48 h followed by serum starvation for 4 h. The cells were incubated with or without insulin (100 nmol) for 16 h and stimulated again with or without insulin (100 nmol) for 5 min before the cells were harvested as described under “Experimental Procedures.” Total cell lysate was used for immunoblotting with antibodies against WNK1, IRS-1, phospho-Akt/PKB (Ser473), phospho-Erk1/2, and phospho-tyrosine (4G10).

siRNA-mediated knockdown of WNK1 by ~90% did not alter the phosphorylation states of IRS-1, Akt/PKB, and Erk1/2 in mitogen-activated protein kinases in the cultured adipocytes incubated with insulin for 5 min, as detected by the respective phosphoprotein antibodies. Depletion of WNK1 also did not block the decrease in IRS-1 protein level induced by long-term treatment (16 h) of the cells with insulin. Therefore, our data suggest that WNK1 is not involved in the regulation of insulin signaling upstream of Akt/PKB and Erk1/2 mitogen-activated protein kinases.

WNK1 Is Not Required for Insulin-Stimulated Glucose Transport in Adipocytes—It is well established that insulin stimulates glucose transport at least partially through the Akt/PKB pathway (35, 45–50). Using synthetic siRNA duplexes to silence WNK1 gene expression, we tested whether WNK1 is required for this action of insulin in 3T3-L1 adipocytes. Although ~90–95% of the WNK1 protein was depleted by the specific siRNA for WNK1 (Fig. 6A), insulin-stimulated deoxyglucose uptake was not significantly altered (Fig. 6B). These data suggest that WNK1 is not required for insulin-stimulated glucose transport in adipocytes.
WNK1 Is a Negative Regulator of Insulin-stimulated Cell Proliferation—We further tested whether WNK1 was required for the mitogenic effect of insulin in cultured cells. 3T3-L1 preadipocytes were transfected with either the scrambled or WNK1-directed siRNAs for 24 h, followed by serum starvation for 24 h and assessment of insulin-stimulated DNA synthesis. Surprisingly, depletion of WNK1 resulted in a 58% increase of basal incorporation of [3H]thymidine into DNA as compared with the cells transfected with scrambled siRNA, even though the same numbers of cells were seeded in each case (Fig. 7A). Upon stimulation with insulin at 1, 10, and 100 nM for 16 h, WNK1-depleted cells showed increases in [3H]thymidine incorporation of 80%, 110%, and 90%, respectively, as compared with the cells transfected with scrambled siRNA.

The effect of WNK1 depletion on cell growth of 3T3-L1 preadipocytes was further examined by counting cell numbers. Equal numbers of these preadipocytes were seeded onto plates after the cells were electroporated with scrambled or WNK1-directed siRNA, as indicated by phase-contrast images taken at 3 h after reseeding (Fig. 7B). Interestingly, RNAi-induced depletion of WNK1 resulted in the increase of total cell number by 21% and 36% after the cells were reseeded for 24 and 48 h, respectively, as compared with the cells transfected with the scrambled siRNA at the same time point (Fig. 7, B and C). However, the effect of WNK1 depletion on cell number was only increased by 12% at 72 h after siRNA transfection (Fig. 7C). This may be due to contact inhibition after growing in normal media for 3 days. In addition, the effect of siRNA-induced WNK1 knockdown on cell apoptosis was tested with the TUNEL assay. There were no detectable apoptotic cells grown in media containing 10% fetal bovine serum for 48 h after transfection with either WNK1 or the scrambled siRNAs. When the cells were serum-starved for 24 h, the percentage of apoptotic cells (~10%) was not significantly different between WNK1 siRNA- and scrambled siRNA-transfected cells. These data suggest that WNK1 regulates cell proliferation rather than programmed cell death.

It has been reported that WNK1 may positively regulate the activity of the Na⁺-Cl⁻ cotransporter at least in kidney epithelial cells (51, 52). It is possible that knockdown of WNK1 by siRNA may regulate DNA synthesis by altering the activities of these channel proteins. To address this question, we tested whether blocking these channels mimics the effect of WNK1 siRNA on DNA synthesis. Both basal and insulin-stimulated [3H]thymidine incorporation were not significantly affected by either hydrothiazide, a selective blocker of Na⁺-Cl⁻ cotransporter (data not shown), suggesting that WNK1 may negatively regulate DNA synthesis independent of its potential action on channel functions.

DISCUSSION

In attempt to identify new Akt/PKB substrates from insulin-stimulated 3T3-L1 adipocytes, antibody against the phospho-Akt/PKB-specific substrate motif was used to immunoprecipitate phosphoproteins, and tryptic peptides from the enriched proteins were analyzed by MALDI-TOF mass spectrometry. Among several potential candidates, the protein kinase WNK1 was identified as an Akt/PKB substrate in cultured adipocytes stimulated by insulin (Fig. 2). Our data further confirmed that insulin indeed induces PAS antibody-detectable phosphorylation of WNK1 isolated from adipocytes by immunoprecipitation with antibody against mouse WNK1 (Fig. 3). This insulin-induced phosphorylation of WNK1 was inhibited by wortmannin, a PI3K inhibitor, but not by rapamycin, an mTOR inhibitor (Fig. 4). Furthermore, siRNA-induced gene silencing of Akt1/PKBα and Akt2/PKBβ together, but not of PKCα, almost completely eliminated insulin-stimulated WNK1 phosphorylation (Fig. 4). Our data strongly suggest that WNK1 is an endogenous Akt substrate in insulin-sensitive adipocytes. In addition, amino acid sequence motif searching with Scansite showed that WNK1 contains potential Akt/PKB phosphoryla-

**FIG. 6.** WNK1 is not required for insulin-stimulated glucose transport. 3T3-L1 adipocytes were transfected with scrambled siRNA (20 nmol) and WNK1 siRNA (20 nmol) for 60 h followed by serum starvation in KRB buffer supplemented with 0.5% bovine serum albumin and 2 mM sodium pyruvate for 3 h. Basal and insulin-stimulated [3H]deoxyglucose uptake was assessed as described under “Experimental Procedures.” A, immunoblotting of WNK1 in total lysate samples from the cells transfected with scrambled and WNK1-directed siRNAs. B, comparison of insulin-stimulated glucose uptake by adipocytes transfected with scrambled siRNA and cells transfected with scrambled siRNA. Data are presented as the mean ± S.D. of four independent experiments.

**FIG. 7.** Depletion of WNK1 enhances insulin-stimulated cell proliferation. A, 3T3-L1 preadipocytes were transfected with scrambled siRNA (20 nmol) and WNK1 siRNA (20 nmol) by electroporation and reseeded onto 6-well plates, and insulin-stimulated [3H]thymidine incorporation assays were carried out as described under “Experimental Procedures.” Data are presented as the mean ± S.D. of three independent experiments. B and C, 3T3-L1 preadipocytes transfected with scrambled or WNK1 siRNAs were reseeded onto 6-well plates, and cell numbers were counted at least 10 frames of images at each time point for each experiment. Data are presented as the mean ± S.D. of three independent experiments (C). *p < 0.05; **p < 0.01; cells transfected with WNK1 siRNA versus cells transfected with the scrambled siRNA.
tion sites. Whereas we were using gene-specific siRNA as a tool to analyze WNK1 function in insulin-sensitive cells, data published by Vitari et al. (33) showed that insulin-like growth factor I stimulated, in a PI3K-dependent manner, the phosphorylation of human WNK1 at Thr60. Threonine 60 lies within a RRRRPT60P peptide sequence in WNK1, a Akt/PKB substrate motif that is conserved in human, mouse, and rat WNK1 proteins. Basically, our phosphoproteomics-based approach and the motif search-based approach used by Vitari et al. (33) led to the same conclusion that WNK1 is an endogenous substrate of Akt/PKB in insulin- and insulin-like growth factor I-sensitive cells.

WNK1 is a cytoplasmic protein kinase with no lysine in its kinase domain II (30, 53). It regulates, possibly through the related WNK4 protein kinase, the activity of thiazide-sensitive Na⁺-Cl⁻ cotransporter mediating NaCl reabsorption and K⁺ secretion in the kidney tubule (51, 52, 54). It has been reported that “gain of function” mutations of the WNK1 gene lead to the increase of its expression and phenotypical type II pseudohyperkalaemia, which is characterized by hyperkalemia and hypertension (31). More recently, a study by Lee et al. (55) demonstrated that WNK1 phosphorylates synaptotagmin 2 and thus may modulate its membrane binding and function in vesicle fusion processes. However, it is unknown how WNK1 is regulated in cells. Our data suggest that WNK1 is phosphorylated via the PI3K/Akt/PKB pathway in 3T3-L1 cells in response to insulin stimulation. In attempt to determine whether phosphorylation of WNK1 alters its kinase activity, we compared in vitro phosphorylation of myelin basic protein, histone H1, and histone H3 induced by WNK1 immunoprecipitates from control and insulin-stimulated 3T3-L1 adipocytes and found no difference in phosphorylation of these substrates (data not shown). Perhaps they are poor substrates for WNK1. In addition, we examined WNK1 distribution in cellular fractions and found that WNK1 is mainly distributed in cytosol, whereas very little is detectable in plasma membranes and low density microsomes by immunoblotting. WNK1 distribution was not altered in cells treated with insulin (data not shown). It is possible that phosphorylation of WNK1 alters its interaction with other proteins rather than translocation between different cellular compartments.

It has been suggested that activation of the PI3K/Akt/PKB pathway is involved in feedback regulation of insulin receptor signaling and is required for both the metabolic and mitogenic actions of insulin such as glucose transport and DNA synthesis (35, 45–50, 56–59). In this study, we further characterized the function of WNK1 in 3T3-L1 preadipocytes and differentiated adipocytes using the approach of RNAi-mediated gene-specific silencing of WNK1. Our data show that depletion of WNK1 does not alter phosphorylation of IRS proteins, Akt/PKB, or mitogen-activated protein kinases induced by short-term (5-min) insulin stimulation or the decrease in IRS protein levels induced by long-term (16 h) insulin incubation (Fig. 5). These data suggest that WNK1 is not involved in feedback regulation of upstream signaling of the insulin receptor. Our data show that siRNA-induced knockdown of WNK1 does not alter insulin-stimulated deoxyglucose uptake in adipocytes (Fig. 6), suggesting that WNK1 is not required for insulin receptor signaling to glucose transport, although it is a downstream effector of Akt/PKB.

Interestingly, siRNA-induced depletion of WNK1 significantly enhances thymidine incorporation as well as the cell proliferation rate in insulin- and serum-stimulated 3T3-L1 preadipocytes (Fig. 7). The increase in cell proliferation is not due to the changes in programmed cell death because there was no significant difference in the number of cells undergoing apoptosis, as determined by the TUNEL assay, between the preadipocytes transfected with WNK1 and those transfected with the scrambled siRNAs (data not shown). We also carried out cell cycle analysis by flow cytometry and did not find differences in the percentages of cells in different cell cycle stages between the preadipocytes maintained with normal cell culture medium (10% fetal bovine serum in DMEM) for 48 h after transfection and the scrambled and WNK1-directed siRNAs (data not shown). It is possible that depletion of WNK1 may enhance cell proliferation rate without selectively affecting a particular phase of the cell cycle.

Akt activation by growth factors has been reported to be an important signaling element that regulates cell growth by protecting cells from apoptosis and enhancing cell proliferation. Multiple studies have suggested that Akt is involved in the degradation of tumor suppressor P53 through phosphorylation of MDM2 (16, 17). Akt/PKB also phosphorylates and induces protein degradation of cyclin inhibitors such as p27Kip1 and p21Cip1 (18–21). Moreover, it has been reported that Akt/PKB may also enhance the meiotic G2-M-phase transition through phosphorylation and down-regulation of Myt1, a member of the Wee family of protein kinases (61). In this study, we observed that Akt/PKB phosphorylates WNK1 in insulin-sensitive cells, whereas depletion of WNK1 in 3T3-L1 preadipocytes causes an increase of insulin-stimulated cell proliferation, suggesting that WNK1 may be another downstream target of Akt/PKB involved in the regulation of cell growth. It is likely that WNK1 may also regulate cell growth in other types of proliferating cells. However, further study is required to test this possibility. Further study is also required to understand the biochemical and cellular mechanisms by which WNK1 regulates cell proliferation.

In summary, using a proteomics approach and a phosphopeptide-specific antibody, we identified the protein kinase WNK1 as an Akt/PKB substrate. We further confirmed that insulin induces WNK1 phosphorylation through the PI3K/Akt/PKB pathway in cultured 3T3-L1 adipocytes. Depletion of WNK1 by siRNA did not alter insulin-stimulated glucose uptake in the adipocytes, whereas insulin- and serum-stimulated cell proliferation was significantly enhanced. Our data strongly suggest for the first time that the novel Akt/PKB substrate WNK1 is not involved in insulin-induced glucose transport but is a negative regulator of cell growth.

Acknowledgments—We appreciate many helpful discussions with Drs. Silvia Corvera, Stephen Doxsey, and Deirdre Lawe. We thank Dr. Morris J. Birnbaum for providing Akt2/PKBβ antibody and Jane Erickson for excellent assistance with the manuscript.

REFERENCES

1. Luo, J., Manning, B. D., and Cantley, L. (2003) Cancer Cell 4, 257–261
2. Brazil, D. P., Yang, Z.-Z., and Hemmings, B. A. (2004) Trends Biochem. Sci. 29, 233–242
3. Whiteman, E. L., Cho, H., and Birnbaum, M. J. (2002) Trends Endocrinol. Metab. 13, 444–451
4. Saltiel, A. R., and Kahn, C. R. (2001) Nature 414, 799–806
5. Alessi, D. R., Caufield, F. B., Andjelkovic, M., Hemmings, B. A., and Cohen, P. (1996) FEBS Lett. 399, 333–338
6. Obata, T., Yaffe, M. B., Leparc, G. G., Maegawa, H., Kashwagi, A., Kikkawa, R., and Cantley, L. C. (2000) J. Biol. Chem. 275, 36108–36115
7. Yaffe, M. B., Leparc, G. G., Iai, J., Obata, T., Volinia, S., and Cantley, L. C. (2001) Nat. Biotechnol. 19, 348–353
8. Brunet, A., Bonni, A., Zoncu, M. J., Lin, M. Z., Joo, P., Hu, L. S., Anderson, M. J., Arden, K. C., Blenis, J., and Greenberg, M. E. (1999) Cell 96, 867–868
9. Kops, G. J., de Ruiter, N. D., De Vries-Smits, A. M., Powell, D. R., Bos, J. L., and Burgering, B. M. (1999) Nature 398, 630–634
10. Rena, G., Guo, S., Cichy, S. C., Unterman, T. G., and Cohen, P. (1999) J. Biol. Chem. 274, 17179–17183
11. Biggs, W. H., III, Meisenhelder, J., Hunter, T., Vavenne, W. K., and Arden, K. C. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 7421–7426
