Expression of a Constitutively Active Akt Ser/Thr Kinase in 3T3-L1 Adipocytes Stimulates Glucose Uptake and Glucose Transporter 4 Translocation* 

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Akt is a serine/threonine kinase that requires a functional phosphatidylinositol 3-kinase to be stimulated by insulin and other growth factors. When directed to membranes by the addition of a src myristoylation sequence, Akt becomes constitutively active. In the present studies, the constitutively active Akt and a nonmyristoylated control mutant were expressed in 3T3-L1 cells that can be induced to differentiate into adipocytes. The constitutively active Akt induced glucose uptake into adipocytes in the absence of insulin by stimulating translocation of the insulin-responsive glucose transporter 4 to the plasma membrane. The constitutively active Akt also increased the synthesis of the ubiquitously expressed glucose transporter 1. The increased glucose influx in the 3T3-L1 adipocytes directed lipid but not glycogen synthesis. These results indicate that Akt can regulate glucose uptake and metabolism.

Insulin affects many biological responses, including gene expression and cellular metabolism (1). One of the primary metabolic responses mediated by insulin is the stimulation of facilitated glucose transport in muscle and adipose tissues. Adipocytes express both the ubiquitous GLUT1 and the muscle and fat cell-specific GLUT4. Insulin regulates glucose uptake in adipocytes primarily by recruiting glucose transporters to the plasma membrane from an intracellular pool (2, 3). Although acute insulin treatment in adipocytes is associated with the translocation of both GLUT1 and GLUT4 to the cell surface, the majority of the insulin-induced increase in glucose uptake is mediated by the recruitment of GLUT4 to the cell surface. In contrast, chronic insulin treatment increases glucose uptake by increasing both the expression and translocation of GLUT1 (4, 5). However, the signaling events that mediate insulin-stimulated glucose transport and GLUT4 translocation are poorly understood.

Insulin transmits its signal intracellularly by binding to a specific receptor at the cell surface and activating the receptor’s intrinsic tyrosine kinase activity (1). The activated insulin receptor phosphorylates downstream proteins, including insulin receptor substrate 1 (6). Insulin receptor substrate 1 gets phosphorylated on multiple tyrosine residues that serve as docking sites for proteins that contain Src homology 2 domains. This multimeric complex initiates a pleiotropic array of signaling pathways emanating from the insulin receptor.

One protein that binds to insulin receptor substrate 1 is the heterodimeric phosphatidylinositol 3-kinase (PI 3-kinase) (6). The p85 regulatory subunit of PI 3-kinase binds to insulin receptor substrate 1 via two Src homology 2 domains and activates the p110 catalytic subunit (7). The p110 subunit phosphorylates phosphoinositides at the 3’-position of the inositol ring to generate phosphoinositides 3P, (3,4)P2, and (3,4,5)P3 (8). Although the exact function of PI 3-kinase is unknown, it has been implicated in a number of cellular processes, including insulin-stimulated glucose transport. Inhibition of PI 3-kinase either by the chemical inhibitors wortmannin and LY294002 or by microinjection of a glutathione S-transferase-p85 fusion protein or a dominant-negative mutant of p85 all prevent insulin-stimulated glucose transport and GLUT4 translocation in rat primary adipocytes and 3T3-L1 differentiated adipocytes (9–12). More recently, constitutively active forms of PI 3-kinase have been shown to be capable of stimulating glucose uptake in 3T3-L1 differentiated adipocytes by inducing GLUT4 translocation to the plasma membrane (13, 14).

Akt kinase is a serine/threonine kinase that has recently been shown to be activated by insulin and certain growth factors, such as platelet-derived growth factor. Several lines of evidence indicate that Akt functions downstream of PI 3-kinase (15–17). First, insulin and growth factor stimulation of Akt kinase activity is inhibited by wortmannin, a specific inhibitor of PI 3-kinase (9). Second, platelet-derived growth factor receptor mutants that fail to activate PI 3-kinase also fail to activate Akt. Finally, the dominant-negative mutant of p85 prevents the stimulation of Akt kinase activity by platelet-derived growth factor. Although the biological function of Akt kinase is currently unknown, it has been hypothesized to play a role in glucose metabolism, since glycogen synthase kinase 3, which controls glycogen synthesis, has been shown to be a substrate of Akt (18). In addition, constitutively active Akt has been shown to activate the 70-kDa S6 kinase (16).

We have recently demonstrated that an Akt variant lacking its pleckstrin homology domain became constitutively active on membrane targeting via the addition of the 14-amino acid src myristoylation signal (19). To further understand the role of Akt kinase in insulin signaling, especially in glucose metabolism, this constitutively active form of Akt was expressed in 3T3-L1 cells. 3T3-L1 cells represent a useful model for studies of insulin action, particularly glucose metabolism, since these...
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cells can differentiate from fibroblasts to adipocytes under appropriate culture conditions (20). Insulin treatment of differentiated 3T3-L1 cells results in a significant increase in glucose uptake associated with GLUT4 translocation to the plasma membranes and increased incorporation of glucose into lipid and glycogen, as well as increased gene expression, including expression of GLUT1.

In this report, we show that expression of the constitutively active variant of Akt kinase in 3T3-L1 cells results in spontaneous differentiation of 3T3-L1 fibroblasts into 3T3-L1 adipocytes, associated with the accumulation of increased lipid droplets. Constitutively active Akt under normal differentiation conditions causes constitutive glucose uptake that is associated with both increased GLUT1 expression, as well as increased GLUT4 translocation to the plasma membrane without increased expression. The increased glucose influx is associated with constitutive lipogenesis, whereas the insulin-stimulated glycolgen synthesis normally observed in differentiated 3T3-L1 adipocytes is inhibited in the presence of the constitutively active Akt kinase. We conclude that Akt kinase critically contributes to glucose uptake and metabolism and consequently represents a major PI 3-kinase effector in the insulin signaling pathway.

MATERIALS AND METHODS

Akt Constructs—The constructs myrAkt Δ4–129, which contains a src myristoylation signal sequence, and A2myrAkt Δ4–129, which encodes an alanine instead of a glycine at amino acid position 2 of the src myristoylation signal sequence, were as described previously (19). These constructs were cloned into the murine retrovirus pWZLneo, which bears a neomycin resistance gene, to allow for selection of infected cells.

Retroviral Infection—The Phoenix retroviral packaging cell line and the retroviral vectors pWZLneo and pWZLhgyro were gifts from Dr. Garry P. Nolan (Stanford University) (21). 3T3-L1 fibroblasts were infected with either the empty pWZLneo retroviral vector A2myrAkt Δ4–129 or myrAkt Δ4–129 as described previously (19).

To generate cell lines that express both the Akt constructs and the myc-tagged GLUT4 cDNA, the myc-tagged GLUT4 cDNA (22) was first subcloned into pWZLhgyro, which carries a hygromycin resistance gene. This plasmid was then used to infect 3T3-L1 fibroblasts that had already been infected with the different Akt constructs. The infection protocol was as noted above, except that cells exposed to the viral supernatant were not spun, and the infected cells were selected using 0.25 mg/ml hygromycin.

Cell Culture—3T3-L1 fibroblasts were maintained in Dulbecco’s modified Eagle’s-H21 medium containing 10% calf serum. To differentiate fibroblasts into adipocytes, cells were grown for 2 weeks in the absence of insulin. The media was then changed to Dulbecco’s modified Eagle’s-H21 medium containing 5% fetal bovine serum (FBS), 1 µg/ml insulin, 0.1 µg/ml dexamethasone, and 112 µg/ml isobutylmethylxanthine. After 3 days, cells were then maintained in Dulbecco’s modified Eagle’s-H21 medium containing 10% fetal bovine serum (FBS), 1 µg/ml insulin, 0.1 µg/ml dexamethasone, and 112 µg/ml isobutylmethylxanthine. After 3 days, cells were then maintained in Dulbecco’s modified Eagle’s-H21 medium containing 10% FBS, 1% penicillin-streptomycin, and 1 µg/ml insulin for 3 additional days. Finally, the media were replaced with Dulbecco’s modified Eagle’s-H21 medium containing only 10% FBS and 1% penicillin-streptomycin for at least 2 days, after which the cells were used. At least 90% of the cell population showed the adipocyte phenotype with the accumulation of lipid droplets.

Akt Kinase Assay—The Akt kinase assay and immunoblotting were performed as described previously (17), except that two wells of a six-well plate were combined and used for each treatment.

Membrane Isolation and Immunoblotting to Detect GLUT1, GLUT4, and CAAT/Enhancer-binding Protein Expression—Crude membrane fractions were prepared essentially as described previously (27). Differentiated 3T3-L1 adipocytes were washed two times with cold phosphate-buffered saline and scraped into 100 µl of sonication buffer (5 mM EDTA, 250 mM sucrose, 25 mM HEPES, 50 mM KF, 1 mM PMSF, 0.5% NP-40, 0.5 µM calyculin A, 0.1 µM phosphatase and protease inhibitors, 5 mM MgCl₂, and 50 mM NaF, 10 µg/ml soybean trypsin inhibitor). The cells were sonicated three times for 1 min each and centrifuged at 550 x g for 5 min at 4 °C. The low speed supernatant was centrifuged for 1 h in an airfuge. To detect GLUT1 and GLUT4 expression, the face of the pellet was rinsed with 100 µl of sonication buffer and then resuspended in 100 µl of sonication buffer for 2 min. The crude membranes were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose, as described by Haspel et al. (27). GLUT1 and GLUT4 were detected using isofom-specific antibodies (Charles River Pharmaservices) following the manufacturer’s protocols.

To detect CAAT/Enhancer-binding protein α, equal amounts of total cell lysates, determined using the Bradford protein assay (Bio-Rad), were separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and immunoblotted with anti-CAAT/Enhancer-binding protein α antibodies (Santa Cruz Biotechnology) according to the manufacturer’s instructions.

GLUT4 Translocation Assays—Plasma membrane sheets were prepared by the method of Fang et al. (28) with minor modifications. 3T3-L1 fibroblasts grown on glass coverslips were differentiated into adipocytes at least 2 weeks prior to preparing sheets. Adipocytes were then incubated for 1.75 h at 37 °C in Leibovitz’s L-15 medium (Life Technologies, Inc.) containing 0.2% bovine serum albumin. Insulin was present in selected wells during the last 15 min of this incubation. Cells were then washed once with ice-cold buffer A (100 mM NaCl, 50 mM HEPES, pH 7.3) and once with ice-cold buffer B (100 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 1 mM phenylmethylsulfonyl fluoride, and 20 mM HEPES, pH 7.2) and then left in buffer B for sonication. Cells were sonified for 1 s on a Sonics and Materials VXC-400 Sonifier with a 1-inch diameter probe (model CV26) placed 1 cm above the coverslip (amplitude, 80%; pulse, 0.1 s on, 0.9 s off). Adherent plasma membrane sheets were washed twice in phosphate-buffered saline containing 0.88 mM Ca²⁺ and 0.49 mM Mg²⁺, fixed in 3% paraformaldehyde for 10 min, and processed for indirect immunofluorescence using affinity-purified anti-GLUT4 antisera and rhodamine-conjugated secondary antibodies as described (29). Sheets were additionally stained with FITC-conjugated phosphatidylethanolamine (Avanti Polar Lipids) at a final concentration of 50 ng/ml.

The amount of glucose transporter on the plasma membrane was quantitated by digital image processing as described previously (30). To ensure that quantitation was performed in an unbiased manner, fields of sheets were selected based solely on their staining with FITC-conjugated phosphatidylethanolamine. Images of both the FITC- and rhodamine-stained sheets were acquired, and the regions to be quantitated were marked in the image captured with the FITC filter. These regions were then transferred to the image captured using the rhodamine filter, and the average pixel brightness of each marked sheet was determined.

The cell surface anti-myc antibody binding assay was performed as described previously (22). The myc-tagged GLUT4 construct was a gift from Dr. Yousuke Ebina (University of Tokushima, Tokushima, Japan), and the anti-myc monoclonal antibody 9E10 was obtained from Babco. In each experiment, control incubations were performed with an extra-neomycin monoclonal antibody followed by the secondary antibody. The nonspecific binding from these control cells was subtracted from the values obtained with the anti-myc antibodies.

RESULTS

Akt Kinase Activity in 3T3-L1 Fibroblasts and Adipocytes—3T3-L1 fibroblasts were infected with a murine retrovirus that encodes either a constitutively active Akt, a control Akt, or the empty retroviral vector. Infected cells were selected using neomycin, and all experiments were conducted using a pool of infected cells. The constitutively active Akt lacks its pleckstrin homology domain (amino acids 4–129) but has an src myristoylation signal sequence (amino acids 4–129). Its amino terminus is mutated such that myrAkt Δ4–129 and a hemaglutinin epitope tag at the carboxyl terminus. The control Akt construct is essentially the same construct as myrAkt Δ4–129, except that the myristoy-
lated glycine at the second amino acid position is converted to alanine (A2myrAkt Δ4–129).

The kinase activities of myrAkt Δ4–129 and A2myrAkt Δ4–129 were measured in undifferentiated 3T3-L1 fibroblasts and differentiated adipocytes. After serum starving the cells, they were either left untreated or treated with insulin. Akt was immunoprecipitated using an antibody directed against the hemaglutinin tag, and the precipitates were assayed in vitro for kinase activity using myelin basic protein as a substrate. The kinase activity of A2myrAkt Δ4–129 increased in response to insulin approximately 14-fold in the fibroblasts and 51-fold in the adipocytes (Fig. 1). In contrast, the kinase activity of myrAkt Δ4–129 was significantly elevated even in the absence of insulin in both the fibroblasts and adipocytes, and the activity was not further stimulated by the addition of insulin (Fig. 1). The level of protein expression of A2myrAkt Δ4–129 was comparable with that of myrAkt Δ4–129, and both proteins were equally expressed in the fibroblasts and adipocytes, as determined by immunoblotting using an antibody directed against the hemaglutinin tag (data not shown).

Constitutively Active myrAkt Δ4–129 Causes Spontaneous Differentiation of 3T3-L1 Fibroblasts into Adipocytes—3T3-L1 fibroblasts normally differentiate into adipocytes when confluent monolayers are incubated for 3 days in media containing FBS, insulin, isobutylmethylxanthine, and dexamethasone, followed by 3 days in media containing FBS and insulin alone. After a final 2-day incubation in only FBS-containing media, differentiation is considered complete, as defined by the appearance of the adipocyte phenotype, especially the accumulation of visible lipid droplets.

We studied the effect of Akt on 3T3-L1 differentiation. Confluent monolayers of 3T3-L1 cells previously infected with either the empty retroviral vector, A2myrAkt Δ4–129, or myrAkt Δ4–129 were exposed to media containing only FBS for 8 days. As a positive control, infected monolayers were exposed in parallel to the standard differentiation protocol. Fresh FBS-containing media was added to those cells being treated with media alone when the hormone supplement was changed following the drug-induced differentiation protocol. After 8 days, differentiation was evaluated by noting the extent of accumulation of lipid droplets, as determined by oil red O staining (23). As expected, the 3T3-L1 cells infected with the empty retroviral vector did not differentiate in the presence of media alone, but they were capable of differentiating in response to normal hormone treatment (Fig. 2). The cells expressing the constitutively active Akt and exposed only to media also expressed CAAT/enhancer-binding protein α, a transcription factor that is a marker of adipocyte differentiation (31), at levels comparable with those of cells differentiated by hormonal induction (data not shown). The A2myrAkt Δ4–129 infected 3T3-L1 cells did undergo some differentiation in the presence of media alone but to a much lesser extent than the cells expressing the constitutively active Akt (Fig. 2). Cells expressing an Akt that con-

![Fig. 1. Kinase activity of Akt variants in 3T3-L1 fibroblasts and adipocytes.](image)

![Fig. 2. Effect of constitutively active myrAkt Δ4–129 on differentiation of 3T3-L1 fibroblasts into adipocytes.](image)
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Fig. 3. Effect of constitutively active myrAkt Δ4–129 on glucose uptake and glucose transporter expression in differentiated 3T3-L1 adipocytes. A, uptake of 2-[3H]deoxyglucose was measured in the absence (−) or presence (+) of insulin using differentiated 3T3-L1 cells that had been infected with either empty vector, A2myrAkt Δ4–129 (A2myr), or myrAkt Δ4–129 (myr). The non-specific background measured in cells exposed to cytochalasin B in the absence of insulin was subtracted from all values. The results shown are means ± S.E. (bars) of three independent experiments. B, total membranes (50 μg of protein/lane) prepared from control-infected (vector), A2myrAkt Δ4–129 (A2myr), and myrAkt Δ4–129 (myr) were loaded on a 10% SDS-polyacrylamide gel, transferred to nitrocellulose, and immunoblotted with anti-GLUT1 or anti-GLUT4 antisera, as indicated. The data presented are representative of three independent experiments.

Constitutively active myrAkt Δ4–129 causes increased GLUT4 translocation to the cell surface—The rate of glucose uptake into adipocytes is influenced not only by the total quantity of glucose transporters expressed but also by the distribution of transporters between the plasma membrane and intracellular compartments. To determine whether the increase in basal glucose uptake observed in 3T3-L1 adipocytes expressing myrAkt Δ4–129 was also due to increased translocation of GLUT4 to the cell surface, plasma membrane sheets were prepared to analyze endogenous cell surface glucose transporter expression (28). In the plasma membrane sheet assay, 3T3-L1 cells differentiated on glass coverslips are sonicated to remove the cytoplasmic contents, thereby leaving an extremely pure preparation of plasma membranes attached to the coverslips, with the cytoplasmic surfaces available for antibody binding. Control adipocytes and adipocytes infected with either the empty retroviral vector A2myrAkt Δ4–129 or myrAkt Δ4–129 were incubated in the absence or presence of insulin for 15 min and then used to prepare plasma membrane sheets. Control adipocytes and adipocytes expressing A2myrAkt Δ4–129 both showed little GLUT4 plasma membrane staining in the absence of insulin, and the GLUT4 staining increased in response to insulin (Fig. 4A). In contrast, plasma membrane sheets prepared from cells expressing myrAkt Δ4–129 showed a 10-fold increase in basal GLUT4 staining in the absence of insulin compared with sheets prepared from either control adipocytes or adipocytes expressing A2myrAkt Δ4–129, and there was little effect of insulin on these cells (Fig. 4A). The ability of myrAkt Δ4–129 to stimulate GLUT4 translocation in the absence of insulin correlates with the increased basal glucose uptake observed in the adipocytes expressing myrAkt Δ4–129.

As an alternative method to detect GLUT4 translocation to the cell surface, we used a GLUT4 cDNA in which the c-myc epitope had been inserted into the first external loop (22). This myc-tagged GLUT4 cDNA was subcloned into a retroviral vector that also carried hygromycin resistance. Cells that had been previously infected with either an empty vector, A2myrAkt Δ4–129, or myrAkt Δ4–129 and selected using neomycin were now infected with the recombinant GLUT4 construct and selected using hygromycin. All subsequent experiments were con-
To quantify the distribution of GLUT4 at the plasma membrane, the infected adipocytes were treated in the presence or absence of insulin for 25 min. The amount of myc-tagged GLUT4 at the cell surface was measured by incubating cells with an anti-myc antibody that was then detected using a 125I-labeled secondary antibody. Control-infected adipocytes and adipocytes expressing A2myrAkt Δ4–129 showed a 2-fold increase (average of three experiments) in cell surface myc-tagged GLUT4 in the presence of insulin compared with untreated cells, consistent with previously published data using this epitope-tagged construct (22). The basal amount of myc-tagged GLUT4 present at the cell surface in adipocytes infected with myrAkt Δ4–129 was consistently elevated compared with that in the control-infected cell lines (Fig. 4B). The basal amount of transporter in control adipocytes represented 47% (average of three experiments) of the maximum amount of insulin-stimulated transporter at the cell surface. In contrast, the basal amount of transporter at the cell surface in adipocytes expressing myrAkt Δ4–129 represented 88% (average of three experiments) of the maximum amount of insulin-induced cell surface transporter. Thus, both the plasma sheet assay and analysis of the epitope-tagged GLUT4 protein reveal that the constitutively active kinase myrAkt Δ4–129 stimulates GLUT4 translocation to the cell surface.

Constitutively Active myrAkt Δ4–129 Causes Constitutive Lipogenesis and Prevents Insulin-induced Glycogen Synthesis—The metabolic effects stimulated by insulin include lipogenesis and glycogen synthesis. Since constitutively active Akt stimulated glucose uptake and resulted in spontaneous differentiation of infected 3T3-L1 cells characterized by the accumulation of lipid droplets, we sought to determine the metabolic fate of the increased glucose taken up by adipocytes expressing myrAkt Δ4–129. To measure lipid synthesis, differentiated adipocytes infected with either the empty retroviral vector or myrAkt Δ4–129 were incubated in the presence or absence of insulin for 15 min prior to adding D-[3H]glucose for 1 h. Lipid synthesis was determined by counting the fraction of radioactive material in the cell lysates, which was extracted into the organic phase of the scintillant. Lipid synthesis increased in response to insulin almost 9-fold (n = 3) in control-infected adipocytes and almost 6-fold (n = 3) in adipocytes expressing A2myrAkt Δ4–129 (Fig. 5). The constitutive lipogenesis observed in 3T3-L1 cells infected with myrAkt Δ4–129 could not be attributed to constitutive activation of acetyl-CoA carboxylase, since this activity was the same in lysates of cell lines infected with either the empty vector or myrAkt Δ4–129 (data not shown).

Glycogen synthesis was also measured in the infected 3T3-L1 cell lines, since insulin also stimulates the conversion of glucose into glycogen in 3T3-L1 adipocytes, and, moreover, Akt has been shown to directly modulate the activity of glycogen synthase kinase-3, which plays a role in glycogen synthesis. Cells were treated with insulin for 15 min before adding D-[3H]glucose. After 1 h, the amount of glycogen synthesized was determined by scintillation counting of precipitated glycogen. Glycogen synthesis measured in control 3T3-L1 adipocytes infected with an empty retroviral vector increased almost 71-fold in response to insulin (Fig. 6). In cells infected with myrAkt Δ4–129, the basal level of glycogen synthesis was only slightly increased in comparison with cells infected with the empty vector, and, most surprisingly, insulin stimulated glycogen synthesis only 2-fold in these cells (Fig. 6). In contrast, in cells infected with the A2myrAkt Δ4–129, insulin was still capable of stimulating glycogen synthesis. The metabolic effects of insulin-induced glycogen synthesis were diminished in adipocytes expressing myrAkt Δ4–129 compared with control-infected adipocytes.

Data are representative of four different experiments. For each set of conditions, six images, each containing 5–12 cells, were captured. All cells in a given image were quantitated. B, antibody binding was measured in 3T3-L1 adipocytes infected with myc-tagged GLUT4 and either empty vector, A2myrAkt Δ4–129 (A2myr), or myrAkt Δ4–129 (myr). Differentiated adipocytes were deprived of serum for 3 h and then treated in the absence (−) or presence (+) of 100 nM insulin for the last 15 min. Plasma membrane sheets were prepared by sonication and subjected to affinity-purified anti-GLUT4 antisera. The amount of GLUT4 on the plasma membrane was quantitated using digital image processing. The data are representative of four different experiments. For each set of conditions, six images, each containing 5–12 cells, were captured. All cells in a given image were quantitated. B, antibody binding was measured in 3T3-L1 adipocytes infected with myc-tagged GLUT4 and either empty vector, A2myrAkt Δ4–129 (A2myr), or myrAkt Δ4–129 (myr). Differentiated adipocytes were deprived of serum for 3 h and then treated in the absence (−) or presence (+) of 100 nM insulin for 25 min. Cells were then incubated with anti-myc antibody followed by 125I-labeled anti-mouse immunoglobulin. Nonspecific binding of the secondary antibody was subtracted from all values. Values represent means ± S.E. (bars) of three independent experiments performed in duplicate.

Fig. 4. Effect of constitutively active myrAkt Δ4–129 on GLUT4 translocation to the plasma membrane. A, plasma membrane sheets for immunofluorescence were prepared from control-infected 3T3-L1 adipocytes (Empty vector), adipocytes expressing A2myrAkt Δ4–129 (A2myr), or adipocytes expressing myrAkt Δ4–129 (myr). Adipocytes were serum-deprived for 1.75 h and then treated in the absence (−) or presence (+) of 100 nM insulin for the last 15 min. Plasma membrane sheets were prepared by sonication and subjected to immunofluorescence using affinity-purified anti-GLUT4 antisera. The amount of GLUT4 on the plasma membrane was quantitated using digital image processing. The data are representative of four different experiments. For each set of conditions, six images, each containing 5–12 cells, were captured. All cells in a given image were quantitated. B, antibody binding was measured in 3T3-L1 adipocytes infected with myc-tagged GLUT4 and either empty vector, A2myrAkt Δ4–129 (A2myr), or myrAkt Δ4–129 (myr). Differentiated adipocytes were deprived of serum for 3 h and then treated in the absence (−) or presence (+) of 100 nM insulin for the last 15 min. Plasma membrane sheets were prepared by sonication and subjected to immunofluorescence using affinity-purified anti-GLUT4 antisera. The amount of GLUT4 on the plasma membrane was quantitated using digital image processing. The data are representative of four different experiments. For each set of conditions, six images, each containing 5–12 cells, were captured. All cells in a given image were quantitated. B, antibody binding was measured in 3T3-L1 adipocytes infected with myc-tagged GLUT4 and either empty vector, A2myrAkt Δ4–129 (A2myr), or myrAkt Δ4–129 (myr). Differentiated adipocytes were deprived of serum for 3 h and then treated in the absence (−) or presence (+) of 100 nM insulin for the last 15 min. Plasma membrane sheets were prepared by sonication and subjected to immunofluorescence using affinity-purified anti-GLUT4 antisera.
of stimulating glycolysis synthesis approximately 18-fold. Thus, constitutively active Akt in differentiated 3T3-L1 adipocytes prevents the insulin-mediated increase in glycolysis synthesis. Measurement of glycolysis synthase activity in cell lysates showed that this enzyme was not constitutively activated in 3T3-L1 adipocytes expressing myrAkt Δ4–129 (data not shown).

DISCUSSION

Inhibitors of the specific lipid kinase PI 3-kinase have been observed to block the ability of insulin to stimulate various biological responses, including glucose uptake (9–12) and activation of the 70-kDa S6 ribosomal protein kinase (32). It has also been recently shown that constitutively active PI 3-kinase is sufficient to stimulate glucose uptake and translocation of GLUT4 in 3T3-L1 adipocytes and to activate the 70-kDa S6 kinase (13, 14, 33). However, it remains unclear how activation of PI 3-kinase leads to these subsequent biological responses.

One hypothesis to explain how activated PI 3-kinase transmits its signal is that the lipid products generated by PI 3-kinase activate other signaling molecules (8, 34). In particular, various Ser/Thr kinases have been proposed to be regulated by the phosphoinositide 3-phosphates produced by this enzyme. Most recently, a specific Ser/Thr kinase called Akt has been shown to be regulated by PI 3-kinase, either by direct binding of the lipid products to the Akt pleckstrin homology domain (15) or indirectly by increased Ser/Thr phosphorylation of Akt (16, 19). Therefore, in the present studies we examined whether Akt could modulate glucose metabolism.

We expressed a constitutively active form of Akt (called myrAkt Δ4–129) in 3T3-L1 cells, which can be induced to differentiate into adipocytes. This Akt variant lacks the pleckstrin homology domain but contains the src myristoylation sequence. In prior studies, this form of the enzyme was shown to be constitutively active when expressed in NIH 3T3 fibroblasts and to be capable of activating the 70-kDa S6 kinase (19). In addition, we expressed a mutant form of this enzyme, which lacks the myristoylated glycine at the second amino acid position (called A2myrAkt Δ4–129). In agreement with our prior data in NIH 3T3 cells (19), the enzymatic activity of the myristoylated form of the enzyme was constitutively active in the absence of insulin in 3T3-L1 adipocytes, whereas the A2myrAkt Δ4–129 mutant enzyme was regulated by insulin.

Most importantly, the constitutively active Akt was found to stimulate glucose uptake in 3T3-L1 adipocytes to essentially the same levels as those observed after insulin treatment of either control 3T3-L1 cells or cells expressing A2myrAkt Δ4–129. Since prior studies have shown that glucose uptake in 3T3-L1 cells can be regulated by either an induction of glucose transporter protein (primarily GLUT1) or by translocation of the GLUT4 protein to the plasma membrane, we examined the effect of the constitutively active Akt on these two processes. Expression of myrAkt Δ4–129 was found to increase the levels of GLUT1 approximately 55% in the 3T3-L1 adipocytes but to have no significant effect on GLUT4 expression in these cells. In addition, expression of the constitutively active Akt was found to stimulate translocation of the GLUT4 transporters to the plasma membrane. Translocation of the GLUT4 transporters to the cell surface was confirmed in the present studies using two independent approaches. The translocation of an expressed myc-tagged GLUT4 molecule was measured directly using an anti-myc antibody, and translocation of endogenous GLUT4 was determined using the plasma membrane sheet assay. Results from both of these approaches clearly indicate that the constitutively active Akt can mediate the translocation of the GLUT4 transporters to the plasma membrane.
reproduce the ability of insulin to stimulate glycogen synthesis in these cells. Rather, expression of the constitutively active Akt inhibited the ability of insulin to stimulate an increase in glycogen synthesis. One explanation for these findings is that persistent activation of the glycogen synthesis pathway by constitutively active Akt resulted in desensitization of this metabolic pathway.

The ability of the constitutively active Akt to cause GLUT4 translocation to the plasma membrane could be explained by its previously described ability to activate the 70-kDa S6 kinase (16). However, inhibitors of this enzyme, most notably rapamycin, have been shown to have no affect on the insulin-stimulated translocation of GLUT4, indicating that this enzyme is not involved in the translocation process (28). Since the 70-kDa S6 kinase has been implicated in the differentiation of 3T3-L1 cells (35), it is possible that the ability of the constitu-
nently active Akt to induce GLUT4 translocation of the 3T3-L1 cells may be explained by studies that have indicated that the src myristoylation signal may alone be sufficient to result in some membrane association (36). Support of this hypothesis comes from the finding that expression of an Akt lacking this peptide sequence did not induce the 3T3-L1 cells to differentiate.

In summary, the present studies have shown that expression of a constitutively active Akt can induce glucose uptake in 3T3-L1 adipocytes in two ways, by inducing the translocation of GLUT4 to the plasma membrane and by increasing the levels of GLUT1. Both of these responses can also be induced by insulin, supporting the hypothesis that Akt may play a role in mediating insulin-stimulated biological responses.

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