Overexpression of wild-type Akt1 promoted insulin-stimulated p70S6 kinase (p70S6K) activity and affected GSK3β regulation, but did not promote insulin-stimulated GLUT4 translocation or glucose transport in L6 myotubes

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Abstract: We have developed a simple, direct and sensitive method to detect GLUT4 on the cell surface. Using this system, we found that PI3-kinase plays a key role in the signaling pathway of insulin-stimulated GLUT4 translocation. One of the down stream effectors of PI3-kinase is serine-threonine kinase Akt (protein kinase B, RAK-PK), but the involvement of Akt in insulin-stimulated GLUT4 translocation is controversial. To investigate whether Akt1 regulates insulin-stimulated GLUT4 translocation and glucose uptake in L6 myotubes, we established L6 myotubes stably expressing c-myc epitope-tagged GLUT4 (GLUT4myc) and mouse wild type (WT) Akt1. We found that overexpression of WT Akt1 promoted insulin-stimulated p70S6 kinase (p70S6K) activity and increased the basal activity of GSK3β, but did not promote insulin-stimulated GLUT4 translocation or glucose uptake. These data supported the result that Akt is not a main signaling molecule to transmit the signal of insulin-stimulated GLUT4 translocation or glucose uptake from insulin-activated PI3-kinase. J. Med. Invest. 47 : 47-55, 2000

Key words : *AKT, p70S6 kinase, GSK3β, GLUT4*

INTRODUCTION

Insulin elicits many biological responses such as cellular metabolism and gene expression. One of the most important metabolic responses induced by insulin is the stimulation of glucose uptake in muscle and adipose tissues. This effect occurs as a consequence of the translocation of GLUT4 from the intracellular pool to the cell surface (1-4). Defect of this function is thought to be one of the main causes of non-insulin-dependent diabetes mellitus (NIDDM). Therefore, elucidating the molecular mechanism of GLUT4 translocation is important for understanding the etiology of NIDDM.

To examine the mechanisms of GLUT4 translocation, we developed a sensitive and quantitative method to measure directly c-myc epitope-tagged GLUT4 (GLUT4myc) on the cell surface (5). Using this system, we found that phosphatidylinositol (PI) 3-kinase (p85/p110 heterodimer type) plays a key role in GLUT4 translocation triggered by insulin, by platelet-derived growth factor, and by epidermal growth factor in cultured cells (6-8).

Activation of PI3-kinase is essential for insulin-stimulated GLUT4 translocation and glucose uptake (7). However, the downstream mediators of PI3-kinase for GLUT4 translocation are still unknown. One candidate molecule is Akt (Protein kinase B or RAC/PK) (9-11). Akt is the cellular homologue of a viral oncogene, v-Akt, and, has therefore also been termed c-Akt (12). It has a PH (pleckstrin homology) domain in the N-terminus which binds

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to other kinases (13). This PH domain shares structural similarity with PKC (protein kinase C) isozymes and PKA (cyclic AMP-dependent protein kinase) (12). There are three isoforms of Akt ; Akt1 (PKBα), Akt 2 (PKBβ), and Akt 3 (PKBγ) (14-16).

Akt is rapidly activated by insulin and by certain growth factors (17, 18). It is fully activated by phosphorylation of two key regulatory amino acid residues, Thr^{308} and Ser^{473} (19). Phosphatidylinositol 3,4,5-triphosphate-dependent protein kinase (PDK1) phosphorylates Akt catalytic domain Thr^{308} and activates Akt (20). There are two types of constitutively active mutants of Akt ; viral Gag protein fused type (21, 22) and myristoylation signal sequence tagged type (23-25). Overexpression of these active Akt promoted p70S6 kinase activity (10, 26), GLUT4 translocation and glucose uptake (23, 24). Overexpression of wild type Akt inhibited GSK3β activity (17). However, Kitamura *et al*. reported that Akt is not required for insulin-stimulated GLUT4 translocaiton or glucose uptake (27).

Therefore, we examined the effects of overexpressed WT Akt1 on insulin-stimulated GLUT4 translocation, glucose uptake, p70 S6kinase activity, and GSK3β activity in L6-GLUT4myc myotubes.

MATERIALS AND METHODS

Cells and materials

The parent cell line used in this study was L6-GLUT4myc (28, 29). The HA-tagged mouse wild-type Akt1 (30) was subcloned into a mammalian expression vector, pCXN (31). This plasmid was cotransfected into L6-GLUT4myc cells with pSV2-hph (32), a hygromycin B phosphotransferase expression plasmid using lipofectamine reagent, and selected with hygromycin B (Sigma). Two independent clones were established, #203 and #2, to avoid clonal deviations.

Cell surface anti-c-myc antibody binding assay (GLUT4myc translocation assay)

Cells in 24-well plates were incubated in 500μl of Krebs-Ringer-HEPES buffer (KRHB) (5) for 20 min at 37°C and then with given concentrations of ligands for 10 min at 37°C. GLUT4myc translocation was measured as described (29).

2-Deoxyglucose Uptake Measurement

Cells in 24-well plates were treated with given concentrations of ligands for 10 min at 37°C. 2-Deoxyglucose uptake was measured as described previously (5).

Cell lysate and Immunoprecipitation

For Akt phosphorylation and kinase assays, cells in 6 -well plates were incubated in KRH buffer for 30 min at 37°C, incubated in the absence or presence of 10^{-7}M insulin for 5 min at 37°C, lysed with buffer containing 1% Nonidet P-40, sonicated and centrifuged at 15,000 rpm for 15 min as described previously (33), and the supernatants were incubated with appropriate polyclonal antibodies for 2h at 4°C. The immunocomplexes were precipitated with protein A-Sepharose CL-4B (Amersham Pharmacia Biotech). For p70S6K, GSK3β phosphorylation and kinase assays, cells in 6 -well plates were deprived of serum for 4 h, incubated in KRH buffer for 30 min at 37°C, incubated in the absence or presence of 10^{-7}M insulin for 10 min at 37°C, lysed in a solution containing 50mM Hepes (pH7.5), 150mM NaCl, 1% TritonX-100, 20μM P-APMSF, 1mg/ml Bacitracin, 5mM EDTA, 5 mM EGTA, 1 mM Na pyrophosphate, 1 mM Na_3VO_4, and 20 mM NaF, and immunoprecipitated as described above.

Immunoblotting

Cell lysates were boiled for 5 min in Laemmli sample buffer and subjected to SDS-polyacrylamide gel electrophoresis ; the separated proteins were transferred to nitrocellulose filter, probed with the antibodies indicated below and detected as described previously (34).

Antibodies

A monoclonal antibody (9E10) against human c-myc was obtained from the American Type Culture Collection. Phosphospecific Akt (Ser473), p70S6K (Thr389), and GSK 3 α/β (Ser21/9) antibody were purchased from New England Biolabs, Inc.. Anti-Akt1, anti-p70S6K, and anti-GSK3β were prepared by immunizing rabbits with keyhole limpet hemocyanin-coupled peptides, the COOH-terminal 20 amino acids of Akt1 (CVDSERRPHFPQFSYSASGTA), COOH-terminal 31 amino acids of p70S6K (MAG VFDIDLDQPEDAGSEDELEEQQNLNESC), and NH3-terminal 23 amino acids of GSK3β (CDTNA GDRGQTNNAASASASNST).

Analysis of Akt, p70S6K, and GSK3α/β Phosphorylations

Cells lysates were separated by 7% SDS-polyacrylamide gel electrophoresis and immunoblotted with each Phosphospecific antibody as described above.

Akt, p70S6K, and GSK3β kinase assays

For Akt kinase assays, the immunoprecipitates were washed three times with washing buffer (i) (140mM NaCl, 20mM Tris-HCl (pH 8.0), 1% Nonidet P-40, and 1 mM DTT), and once with kinase buffer (50mM Tris-HCl (pH7.5), 10mM $MgCl_2$, 1mM DTT), and incubated for 30 min at 30°C in a reaction mixture containing 1μM Protein kinase inhibitor, 160μM "Crosstide"(GRPRTSSFAEG) as substrate, 50mM Tris-HCl (pH7.5), 10mM $MgCl_2$, 1mM DTT, 5μM unlabeled ATP, and 3.0μCi/μl [γ-^{32}P] ATP. The reaction was stopped by adding 10 μl of 250 mM EDTA, and 5 mM ATP, and the reaction mixture centrifuged at 15,000rpm for 1 min.. The resulting supernatant (25 μl) was spotted onto 2×2 cm P81 (Whatman) filter paper, dryed and washed 15 min× 4 times with 75 mM phosphoric acid, and once with 99.5% Ethanol for 1 min. The ^{32}P incorporation into the peptide was determined by liquid scintillation spectroscopy.

For p70S6 kinase assays, the immunoprecipitates were washed three times with buffer containing 2M LiCl, 82mM NaCl, 12mM Tris-HCl (pH 8.0), 0.58% Nonidet P-40, and 580μM DTT, and washed twice with kinase buffer (25mM MOPS (pH 7.2), 1mM DTT, 6mM $MgCl_2$, 1mM EDTA, and 0.05% Triton X-100), and incubated for 30min at 30°C in a reaction mixture (25 μl) containing 1 μM Protein kinase inhibitor, 200μM 40SR-pep20 (KRRRLASLRASYS KSESSQK) as substrate, 25mM MOPS (pH 7.2), 1mM DTT, 6mM $MgCl_2$, 1mM EDTA, and 0.05% Triton X-100, 5 μM unlabeled ATP, and 3.0 μCi/μl [γ-^{32}P] ATP. The kinase activity was determined as described above for Akt kinase assays.

For GSK3β kinase assays, the immunoprecipitates were washed once with buffer (i), as described above, washed twice with buffer (ii) (100 mM Tris-HCl (pH 7.4), 0.5M LiCl, and 1mM DTT), and washed twice with kinase buffer (20mM Hepes, 10mM $MgCl_2$, and 1mM DTT), and incubated for 30 min at 30°C in a reaction mixture (25 μl) containing 2μM Protein kinase inhibitor, 80 μM p-CREB (KRREIL SRRP(p)SYR) as substrate, 16 mM Hepes, 8 mM $MgCl_2$, 0.8mM DTT, 35μM unlabeled ATP, and 3.0 μCi/μl [γ-^{32}P]ATP. The kinase activity was determined as described above in Akt kinase assays.

RESULTS

Overexpression of WT Akt1 promoted the basal and insulin-stimulated kinase activity of Akt in L6 myotubes

We transfected HA-tagged mouse WT Akt1 into parent L6GLUT4myc cells, and established two independent stable clones, #203 and #2. To confirm the overexpression of WT Akt1, the cell lysate was immunoblotted with a polyclonal anti-Akt1 antibody (Fig. 1A) and anti-HA antibody (Fig. 1B). The exogenous mouse WT Akt1 was overexpressed at about 5-fold the level of endogenous rat Akt1 in L6GLUT4myc. Fig. 1B shows that the lysates from parent cells did not immunoreact with the HA antibody. To examine the activity of the exogenously overexpressed WT Akt1 in L6 myotubes, we assessed Akt phosphorylation (Fig. 2A) and kinase activity after insulin stimulation (Fig. 2B,C). Overexpression of WT Akt1 promoted the insulin-stimulated Akt phosphorylation of Ser 473 about 2-3 fold compared to that of parent L6GLUT4myc cells (Fig. 2A). Overexpression of WT Akt1 elevated basal Akt

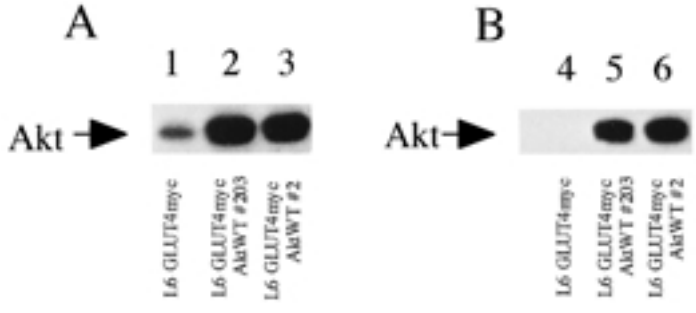

Fig. 1. Overexpression of wild-type (WT) Akt1 in L6 myotubes.
A : Cell lysates were subjected to immunoblotting with a polyclonal anti-Akt1 antibody. Lane 1 ; a band representing endogenous Akt in L6GLUT4myc cells. Lane 2 and 3 ; endogenous Akt plus exogenously overexpressed WT Akt1 in L6GLUT4mycAktWT#203 and in L6GLUT4mycAktWT#2, respectively. By scanning densitometry, the relative intensity of these bands representing Akt in lanes 1-3 was 106, 633, and 580, respectively. B : Immunoblotting with an anti-HA antibody to show exogenously overexpressed WT Akt1.

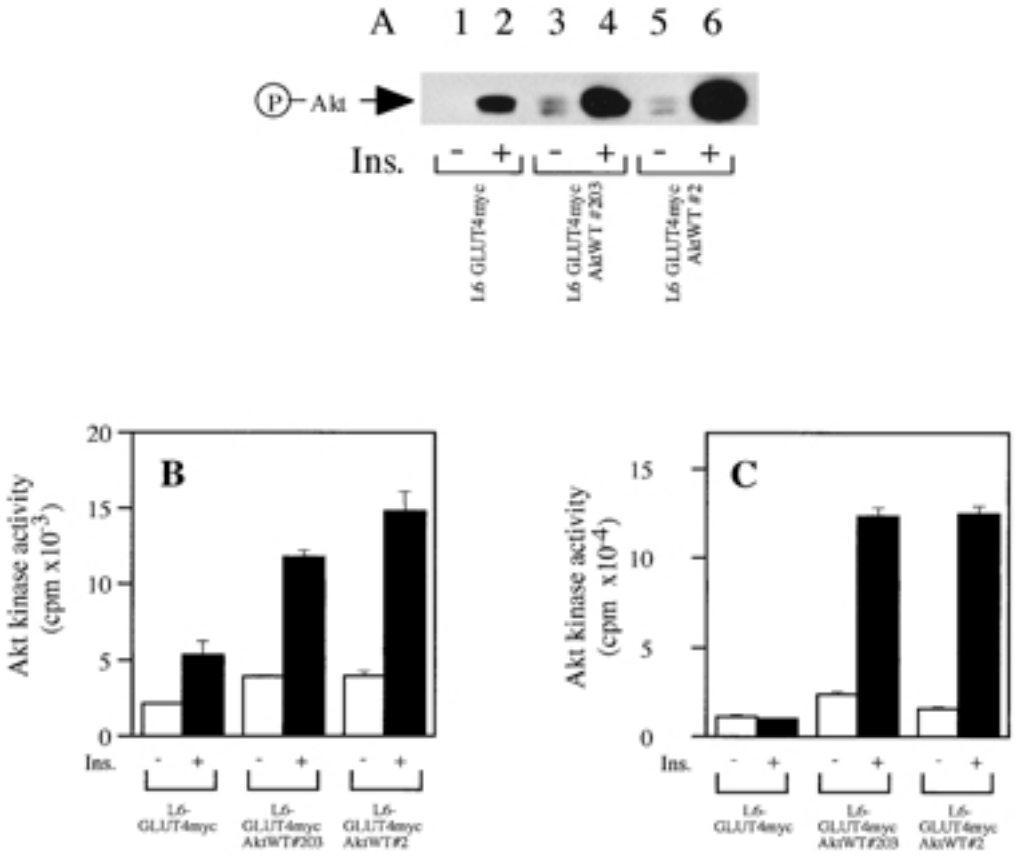

Fig. 2. Overexpression of WT Akt1 promoted the insulin-stimulated phosphorylation and kinase activity of Akt in L6 myotubes.
A : Phosphorylation of Akt in L6 myotubes. Cells were incubated in the presence or absence of 10^{-7}M insulin for 5 min at 37°C, lysed, and subjected to immnunoblotting with an anti-phospho-specific Akt antibody. By scanning densitometry, the relative intensity of the bands representing phosphorylated-Akt in lanes 2, 4, 6 was 612, 1150 and 1385, respectively.
B : Cells were incubated in the presence or absence of 10^{-7}M insulin for 5 min at 37°C, lysed, immunoprecipitated with anti-Akt1 antibody, and subjected to Akt kinase assay.
C : Cells were incubated in the presence or absence of 10^{-7}M insulin for 5 min at 37°C, lysed, immunoprecipitated with anti-HA antibody, and subjected to Akt kinase assay. Values represent means±S.E. of three determinations.

activity about 2-fold, and insulin-stimulated Akt activity about 2-3 fold after immunoprecipitation with anti-Akt1 antibody (Fig.2B). The activation of exogenously expressed Akt1 was detected after immunoprecipitation with anti-HA antibody (Fig.2C).

Overexpression of WT Akt1 promotes the insulin-stimulated kinase activity of p70S6K in L6 myotubes

To examine the effect of overexpressed WT Akt1 on one of the downstream effectors, p70S6K, in L6 myotubes, we assayed p70S6K phosphorylation and the kinase activity after insulin stimulation (Fig. 3A, B). Overexpression of WT Akt1 promoted the insulin-stimulated p70S6K phosphorylation of Thr 389 about 3-fold compared to that of parent L6GLUT4myc cells (Fig.3A). The insulin-stimulated p70S6 kinase activity was promoted by the overexpression of WT Akt 1 about 2-3 fold compared to that of parent cells (Fig. 3B).

Overexpression of WT Akt1 affects the basal kinase activity of GSK 3β in L6 myotubes

GSK3β is a down stream target of Akt, and negatively regulated by Akt-dependent phosphorylation (17, 35). We assayed the phosphorylation and activity of GSK3β kinase to examine whether the exogenously overexpressed WT Akt1 affects the GSK3β activity in the insulin signaling pathway. The phosphorylations of GSK3α (Ser21) and GSK3β (Ser9) were increased after insulin stimulation, and were promoted by the overexpression of WT Akt1 in L6 myotubes (Fig. 4A). Stable overexpression of

WT Akt1 results in about a 1.5~1.7 fold increase of basal GSK3β activity relative to that of parent cells (Fig.4B) (see" Discussion "). Insulin-stimulated GSK3β kinase activity was reduced about 43% in parental cells (L6GLUT4myc) and about 28% in the cells overexpressing WT Akt1 (#203, #2) (Fig. 4B). These results indicated that overexpression of WT Akt1 affected the insulin-induced phosphorylation and the inactivation of GSK3β.

WT Akt1 overexpression did not affect the GLUT4 translocation or glucose uptake in L6 myotubes

Exogenously overexpressed Akt in L6 myotubes promoted insulin-stimulated Akt and p70S6K activities. Overexpression of WT Akt1 promoted

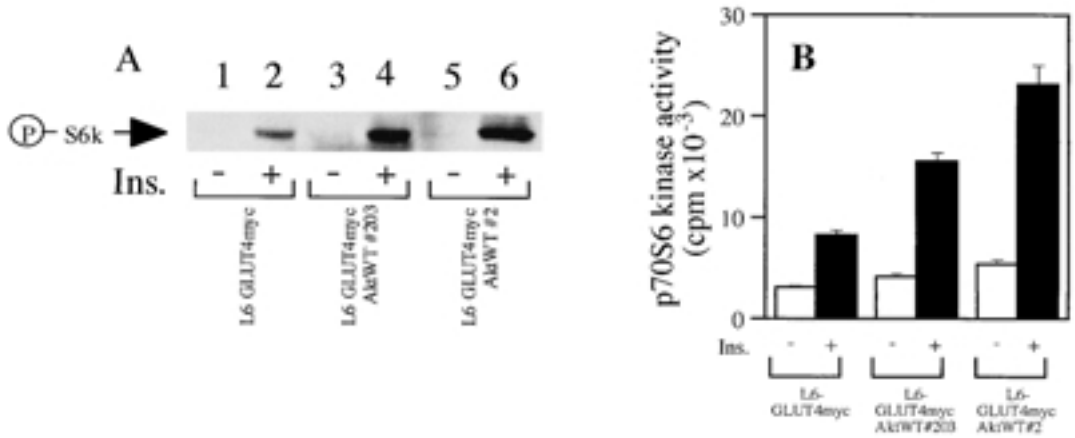

Fig. 3. Overexpression of WT Akt1 promoted the insulin-stimulated phosphorylation and kinase activity of p70S6K in L6 myotubes.
A : Phosphorylation of p70S6K in L6 myotubes. Cells were incubated in the presence or absence of 10^{-7}M insulin for 10 min at 37°C, lysed, and subjected to immnunoblotting with an anti-phospho-specific p70S6K antibody (BioLabs). By scanning densitometry, the relative intensity of the bands representing phosphorylated-p70S6K in lanes 2, 4, 6 was 569, 1577 and 1423, respectively.
B : Cells were incubated in the presence or absence of 10^{-7}M insulin for 10 min at 37°C, lysed, immunoprecipitated with anti-p70S6K antibody, and subjected to p70S6 kinase assay. Values represent means±S.E. of three determinations.

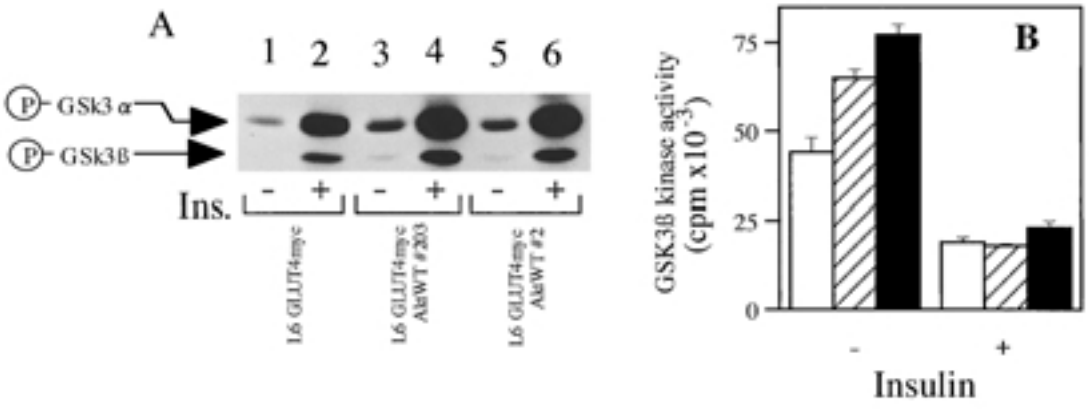

Fig. 4. Effect of WT Akt1 overexpression on the phosphorylations and activities of GSK 3β in L6 myotubes.
A : Phosphorylation of GSK3α and GSK3β in L6 myotubes. Cells were incubated in the presence or absence of 10^{-7}M insulin for 10 min at 37°C, lysed, and subjected to immnunoblotting with an anti-phospho-specific GSK3 α/β antibody. By scanning densitometry, the relative intensity of the bands representing phosphorylated-GSK3α in lanes 2, 4, 6 was 156, 225 and 224, and phospholyrated-GSK3β in lanes 2, 4, 6 was 59, 89 and 86, respectively.
B : Cells were incubated in the presence or absence of 10^{-7}M insulin for 10 min at 37°C, lysed, immunoprecipitated with anti-GSK3β antibody, and subjected to GSK3 kinase assay. L6-GLUT4myc (open bars) ; L6-GLUT4myc Akt1WT#203 (diagonally striped bars) ; L6-GLUT4myc Akt1WT#2 (closed bars). Values represent means±S.E. of three determinations.

insulin-stimulated the phosphorylation (Ser 9) and reduced the activity of GSK3β. We therefore examined insulin-stimulated GLUT4 translocation and glucose uptake in L6 myotubes overexpressing WT Akt1. Despite that exogenously overexpressed WT Akt1 promoted insulin-stimulated Akt activity, promoted p70S6K activity and reduced insulin-stimulated GSK3β activity, no significant differences of insulin-stimulated GLUT4 translocation or insulin-stimulated glucose uptake were found between L6GLUT4myc myotubes overexpressing WT Akt1 and parent L6GLUT4myc myotubes (Fig. 5A, B).

DISCUSSION

Overexpression of a constitutively active mutant of Akt promoted GLUT4 translocation and glucose uptake (21, 23, 24), while overexpression of a dominant negative mutant of Akt inhibited the translocation and uptake (23, 36). However, Kitamura *et al.* reported that overexpression of a constitutively active mutant of Akt and dominant negative mutant of Akt using an adenovirus expression system did not affect insulin-stimulated GLUT4 translocation or glucose uptake (27). Therefore, the relationship between Akt and insulin-stimulated GLUT4 translocation and glucose uptake is still controversial. Furthermore, the effects of the dominant negative mutant of Akt, in which at two phosphorylation sites (Thr^{308} and Ser^{473}) the residue was changed to alanine, are also controversial (25, 27, 36). We established stable CHO cells which overexpressed the mutant Akt at ten- fold the level of endogenous Akt, but a 10- fold overexpression was not sufficient to inhibit endogenous insulin-stimulated Akt activation (data not shown). Stable overexpression of functional dominant negative mutant Akt might be difficult because Akt is a mediator of cell survival which prevents apoptosis (37). Therefore, we overexpressed wild type Akt in this study. Although Akt negatively regulated the activity of GSK3β by the phosphorylation of Ser 9, the stable overexpression of WT Akt1 increased basal activity of GSK3β about 1.7-fold compared to parent L6GLUT4myc cells (Fig. 4B). The same results were obtained in CHO clones (data not shown). The inconsistency of these results may be explained by the functions of Akt. There are several effectors of Akt and the regulation of GSK3β activity might be quite complicated. We found that overexpression of WT Akt1 in L6GLUT4myc affected downstream effectors, such as p70S6 kinase and GSK3β, but not GLUT4 translocation or glucose uptake. These results, however, do not indicate whether Akt1 is a mediator of insulin-stimulated GLUT4 translocation and glucose uptake or not. Exogenous expression of Akt1 does not affect the insulin-stimulated GLUT4 translocation or glucose uptake, because the level of endogenous Akt1 is sufficient mediate this effect. This is not necessarily the case, however. An approximately 2-fold increase in Akt activity was observed in L6GLUT4myc cells after stimulation

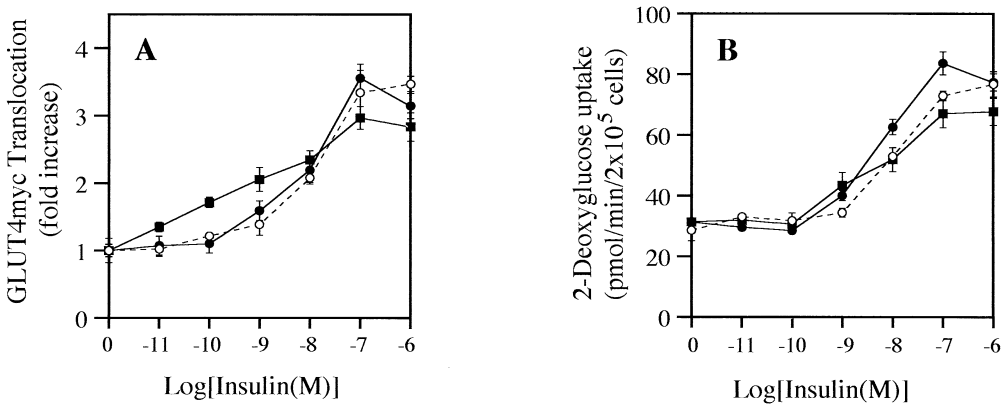

Fig. 5. Effect of WT Akt1 overexpression on the insulin-stimulated GLUT4 translocation and glucose uptake in L6 myotubes.
L6 myoblasts in 24-well plates were differentiated into L6 myotubes by incubating with low serum, as described under MATERIALS AND METHODS, and incubated with various concentrations of insulin for 10 min at 37°C. The GLUT4myc translocation (A) and glucose uptake (B) were measured as described under MATERIALS AND METHODS. ○, parent L6-GLUT4myc cells; ■, L6-GLUT4myc Akt1 WT #203; ●, L6-GLUT4myc Akt1 WT #2. Values represent means±S.E. of six determinations.

by insulin (Fig.2 B). The basal Akt activities in the cells overexpressing WT Akt1 were two-fold higher than the basal Akt activity of the parent cells (L6 GLUT4myc) (Fig.2B). However, there was no effect on the basal level of GLUT4 translocation or glucose uptake (Fig.5 B).

Our results indicated that Akt is not a main signaling molecule to transmit between PI3-kinase and insulin-stimulated GLUT 4 translocation.

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