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Insulin-induced GLUT4 Movements in C2C12 Myoblasts: Evidence against a Role of Conventional Kinesin Motor Proteins

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Insulin induces translocation of the glucose transporter GLUT4 from intracellular storage compartment to the plasma membrane via complex mechanisms that require intact cytoskeletal networks. In cultured adipocytes, conventional kinesin motor proteins have been proposed to mediate GLUT4 movements on microtubules. It remains, however, unclear whether kinesin motor system plays a similar regulatory role in myocytes. We addressed this issue using C2C12 myoblasts, which have now been shown to express both heavy and light chains of conventional kinesin. In these cells, overexpression of either wild-type kinesin light chain 2 (KLC2) or its phosphorylation-defective mutant did not significantly affect insulin-stimulated translocation of exofacial Myc-tagged GLUT4-green fluorescent protein to the cell surface and its subsequent externalization. Likewise, a dominant-negative mutant of KLC2 had no marked effect on GLUT4 movements in this cell type. These results suggest that conventional kinesin is dispensable for insulin-induced GLUT4 translocation in cultured myoblasts and may thus reveal a cell-type specific role of the microtubules-based cytoskeleton in glucose transport in response to insulin.

Insulin stimulates glucose transport in muscle and fat cells by promoting translocation of the glucose transporter GLUT4 to the plasma membrane. Although this physiological process is largely mediated by the PI 3-kinase/Akt signaling pathway, it also requires intact cytoskeletal networks (12,28). Several studies have implicated microtubules-based cytoskeleton as an important structural and regulatory element for insulin-induced GLUT4 translocation (6,7,11,20,21). First, GLUT4-containing vesicles were found associated with α-tubulin, a basal component of microtubules (11). Second, inhibition of the microtubule motor proteins dynein and kinesin impaired insulin-stimulated GLUT4 translocation (6,11). Third, microtubule-depolymerizing agents (nocodazole, colchicine, and vinblastine) dispersed perinuclear GLUT4 protein and partially inhibited insulin-stimulated glucose uptake and GLUT4 translocation (6,7,20,21). Lastly, dynein was shown to function with Rab5 in regulating the endocytosis of GLUT4 (14).

In contrast, other studies have failed to support a role of microtubules in insulin-induced GLUT4 translocation. Thus, disruption or stabilization of the microtubule structure had no marked effect on insulin stimulation of GLUT4 movements (19,24). In addition, microtubule

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disruption did not alter the initial rate of GLUT4 endocytosis but prevented internalized transporter from recycling back correctly to the perinuclear region (24). Furthermore, nocodazole was shown to inhibit glucose uptake through a direct interaction with GLUT4 itself, without affecting its ability to translocate to the cell surface in response to insulin (19,24). Although these data establish microtubules as a major determinant for subcellular localization of GLUT4 endocytosed from early endosomes, they, on the other hand, cast doubt on the role of microtubules in insulin-stimulated translocation of GLUT4 to the plasma membrane.

The kinesin motor proteins mediate microtubule-dependent transport of cargo in eukaryotic cells (10,13). Conventional kinesin was discovered as the first member of the kinesin superfamily (5,27); it subsequently turned out to be a tetrameric protein consisting of two heavy chains and two light chains (3). At least three conventional kinesin heavy chain (KHC) genes (KIF5A, KIF5B, and KIF5C) and three kinesin light chain (KLC) genes (KLC1, KLC2, and KLC3) have been identified in mammalian genome (10). KLC1 and KLC2 bind either to KIF5A or to KIF5B, forming a core machinery of the kinesin motor system (22).

More recent work has demonstrated that KIF5B is highly expressed in adipocytes, and that dominant negative mutants of KLC1 blocked insulin-induced GLUT4 translocation (23). These results thus suggested that conventional kinesin is required by insulin to stimulate the movement of GLUT4-containing vesicles on microtubules. However, since the conclusion of that study was based solely on the data with cultured adipocytes, a question might arise as to whether the kinesin motor system also regulates insulin-induced GLUT4 translocation in other cell types. To address this issue, we have now examined the effects of overexpression of wild-type KLC2 as well as that of the mutant forms of this protein on GLUT4 movements in C2C12 myoblasts in response to insulin.

MATERIALS AND METHODS

Cells, antibodies, and reagents. C2C12 myoblasts and 293 human embryonic kidney cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (D5796, Sigma) supplemented with 10% fetal bovine serum (FBS) (Gibco-BRL). Chinese hamster ovary cells overexpressing insulin receptor (CHO-IR) were cultured in Ham's F12 medium (N6658, Sigma) supplemented with 10% FBS. Mouse monoclonal antibodies (mAbs) to kinesin heavy chain (clone H2), or to kinesin light chain (clone L1) were obtained from Chemicon; mAb to the Myc tag (9E10) and normal mouse IgG were from Santa Cruz Biothchnology; mAb to the Flag tag (M2) was from Sigma; Texas Red-conjugated sheep polyclonal antibodies to mouse IgG were from Amersham Biosciences. We generated polyclonal antibodies that react specifically with KLC2 by immunizing female rabbits with a purified glutathione *S*-transferase (GST) fusion protein containing the C-terminal region of KLC2 (nucleotides 1039-1366 of KLC2 cDNA). Insulin, forskolin, isoproterenol, and A23187 were purchased from Sigma; AICAR was from Toronto Research Chemicals Inc.

Immunoprecipitation and immunoblot analysis. Cells in one 60-mm dish were lysed on ice in 1 ml of lysis buffer [20 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.1% SDS] containing 5 mM NaF, 1 mM phenylmethylsulfonyl fluoride and aprotinin (10 μg/ml). The cell lysates were centrifuged at 10,000 × *g* for 15 min at 4°C, and the resulting supernatants were incubated for 2 h at 4°C with antibodies and for additional 1 h with protein G–Sepharose beads (20 μl of beads; Amersham Biosciences). The beads were then washed three times with lysis buffer and suspended in Laemmli sample buffer, and the associated proteins were resolved by SDS-polyacrylamide gel electrophoresis. Immunoblot analysis was performed with the ECL detection system (Amersham Biosciences).

In vitro binding assay. CHO-IR cells or C2C12 myoblasts (~4×10^5 cells per 60-mm dish) were transiently transfected with cDNAs encoding wild-type KLC2 (pCMV-Tag2C-KLC2WT) or a mutant KLC2 (pCMV-Tag2C-KLC2S575A) in which Ser^{575} was substituted by Ala (15) (kindly provided by T. Ichimura, Tokyo Metropolitan University) with the use of a LipofectAMINE PLUS Transfection Kit (Gibco-BRL). After 48 h, cells were treated with various reagents, and were lysed in 1 ml of lysis buffer [20 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40] containing 5 mM NaF, 1 mM sodium pyrophosphate, 10 mM β-glycerophosphate, 1 mM phenylmethylsulfonyl fluoride and aprotinin (10 μg/ml). Cell lysates were cleared by centrifugation and incubated for 2 h at 4°C with GST or GST fusion protein containing 14-3-3 that had been immobilized on glutathione-Sepharose beads (5 μg of protein/15 μl of packed beads; Amersham Biosciences). The beads were washed with lysis buffer and then subjected to SDS-polyacrylamide gel electrophoresis and immunoblot analysis as described above.

Preparation of adenoviruses. We first performed polymerase chain reaction (PCR) to obtain cDNAs that encode wild-type or the phosphorylation-defective mutant of KLC2 with pCMV-Tag2C-KLC2WT and pCMV-Tag2C-KLC2S575A, respectively, as the template, the sense primer 5'-TCCCCCGGGGAATTCACGACCATGGCCACGATGGTGCTTG-3', and the antisense primer 5'-AGGCCCGGGCTCAAAGTGCGGCTGTCAG-3'. A cDNA encoding KLC2 that lacks the COOH-terminal TPR domain (KLC2-ΔTPR) was also amplified by PCR with pCMV-Tag2C-KLC2WT as the template, the sense primer 5'-TCCCCCGGGGAATTCACGACCATGGCCACGATGGTGCTTG-3', and the antisense primer 5'-AGGCCCGGGCTGTCAGCTACATCTCCTC-3'. The PCR products were digested with SmaI and inserted independently into SwaI site of pAxCAwt, which contains the CAG promoter (TaKaRa). Recombinant adenoviruses were prepared by cotransfection of 293 cells according to the manufacture's protocol, and were then screened by immunoblot analysis and cloned by limiting dilution.

Analysis of subcellular localization of GLUT4. Retrovirus encoding GLUT4-myc7-GFP was prepared as described previously (4,17) with the use of Platinum-E (PLAT-E) ecotropic packaging cells and transfection reagent FuGENE 6 (Roche Diagnostics). C2C12 myoblasts (~2×10^5 cells per 60-mm dish) were cultured for 24 h in 10% FBS-DMEM consisting of 1 ml of media containing retrovirus. Cells expressing GLUT4-myc7-GFP were seeded on glass coverslips that had been coated with 0.015% gelatin in a 6-well plate and cultured for 16 h, which were then infected with adenoviruses for 1 h and cultured for additional 12 h. After stimulation with insulin, cells were fixed with phosphate buffered saline (PBS) containing 3% paraformaldehyde, washed with PBS containing 100 mM glycine, and incubated with PBS containing 10% FBS and 1% bovine serum albumin. Externalized GLUT4-myc7-GFP was visualized without cell permeabilization by indirect immunofluorescence staining with mAb to the Myc tag and Texas Red-conjugated sheep antibodies to mouse IgG. Cells were then examined for Texas Red and GFP fluorescence with a Zeiss Axiophot confocal laser scanning microscope (LSM5 PASCAL version 3, Carl Zeiss).

Statistical analysis. For quantitative data, the significance of differences between independent means was assessed by Student's *t* test. A *P* value of <0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Conventional kinesin motor proteins are present in C2C12 myoblasts. Although the KIF3 subfamily of kinesin-related proteins was shown to be present in C2C12 myoblasts (9), little has been reported on the expression of conventional kinesin motor proteins in these

cells. Immunoblot analysis revealed that proliferating C2C12 myoblasts express a substantial amount of KHC and KLC2 proteins (see Fig. 1). KLC2 was detected in KHC immunoprecipitates (Fig. 1A) and, conversely, KHC was detected in KLC immunoprecipitates (Fig. 1B), indicating that these two components associate with each other *in vivo*. Thus, conventional kinesin motor system is likely to be functional in C2C12 myoblasts. However, since the mAb to KHC used in this study reacts with all heavy chains (KIF5A, KIF5B, KIF5C), it remains unclear which isoform consists of conventional kinesin in this cell line.

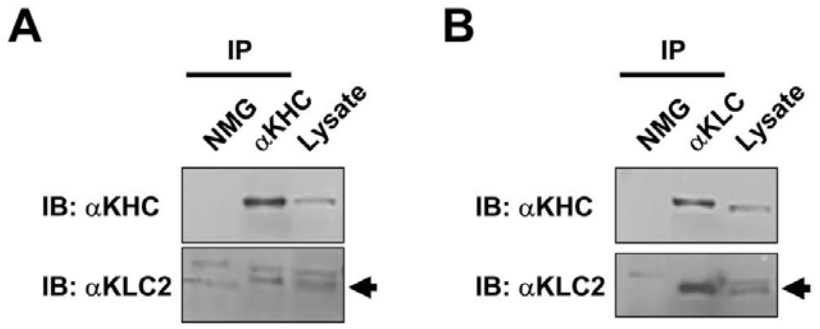


Figure 1. KLC2 interacts with the heavy chain of conventional kinesin in C2C12 myoblasts. Whole cell lysates prepared from C2C12 myoblasts were subjected to immunoprecipitation (IP) with either antibodies to kinesin heavy chain (αKHC) (A) or to kinesin light chain (αKLC) (B), or with normal mouse immunoglobulin (NMG). The resulting precipitates were subjected to immunoblot analysis (IB) with the same antibody to kinesin heavy chain (A and B, upper panels). Duplicate immunoprecipitates were subjected to immunoblot analysis with polyclonal antibodies specific for KLC2 (αKLC2) (A and B, lower panels). Cell lysates (Lysate) were also analyzed for the presence of kinesin heavy chain and KLC2. The positions of KLC2 are indicated by arrows.

The cAMP-PKA signaling pathway induces phosphorylation-dependent interaction between KLC2 and 14-3-3. Conventional kinesin was recently shown to bind 14-3-3 through KLC2 in a manner that requires phosphorylation of the latter protein on Ser^{575} (15). Since 14-3-3 regulates a variety of functions of target proteins including subcellular redistribution, conformational change and stability, and enzymatic activity (8,26), phosphorylation-dependent binding of KLC2 to 14-3-3 likely modulates kinesin-mediated vesicle transport processes.

We examined whether typical stimuli capable of affecting vesicle transport alter KLC2 binding to 14-3-3. The GST-pull down assay revealed that wild-type KLC2 (KLC2-WT), but not a phosphorylation-defective mutant of KLC2 (KLC2-S575A) in which Ser^{575} was substituted by Ala, bound to 14-3-3ζ in CHO-IR cells (Fig. 2A) or in C2C12 myoblasts (Fig. 2B), consistent with the previous report (15). Exposure of CHO-IR cells to insulin or AICAR, which promote glucose transport through the activation of Akt and AMP-activated protein kinase, respectively, did not significantly affect this binding (Fig. 2A). Treatment with the calcium ionophore A23187 had also no marked effect (Fig. 2B), although this reagent would trigger exocytosis via kinesin-driven steps of vesicle recruitment (2) and was reported to modulate insulin-induced Akt phosphorylation and GLUT4 translocation (29).

In rat and human adipocytes, forskolin and the β-adrenergic agonist isoproterenol were shown to inhibit glucose transport (18). Furthermore, catecholamines have been proposed to counter-regulate insulin-stimulated glucose transport in rat adipose cells via a

cAMP-mediated mechanism (25). We found that stimulation of C2C12 myoblasts with forskolin or isoproterenol substantially increase the amount of KLC2-WT, but not that of KLC2-S575A, associated with 14-3-3 (Fig. 2B). These results imply that the cAMP-PKA signaling pathway induces phosphorylation of KLC2 on Ser^{575} and its subsequent binding to 14-3-3, thereby altering the motor function of KHC-KLC heterotetramer. Given a major role of conventional kinesin in insulin-stimulated GLUT4 movements in adipocytes (23), this phosphorylation-dependent process might mediate the inhibitory effect of cAMP on insulin-induced GLUT4 translocation.

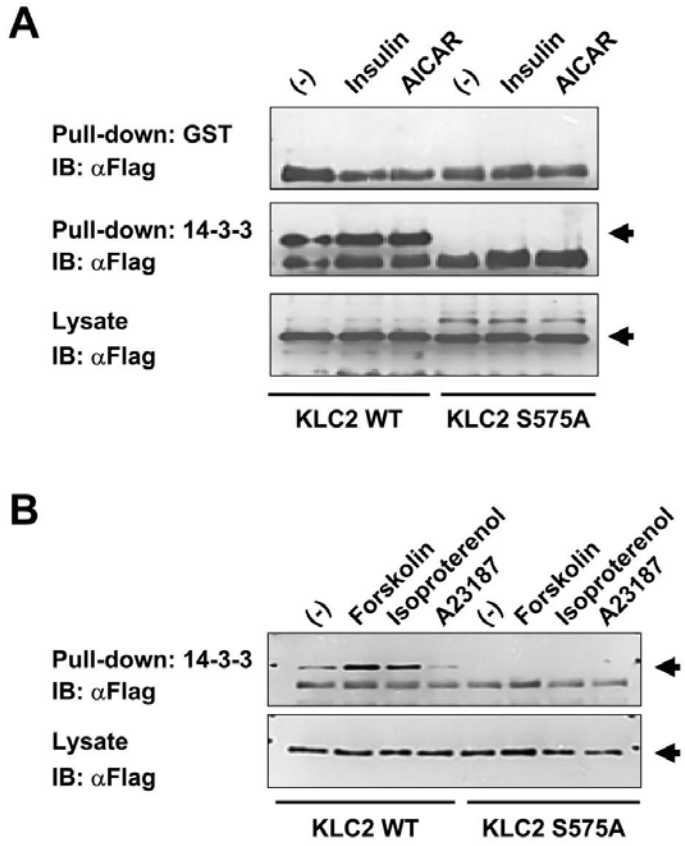


Figure 2. Effects of various stimuli on phosphorylation-dependent binding of KLC2 to 14-3-3. CHO-IR cells (A) or C2C12 myoblasts (B) were transiently transfected with 1 μg of pCMV-Tag2C encoding Flag-tagged wild-type KLC2 (WT) or a mutant KLC2 (S575A) in which Ser^{575} was substituted by Ala. Forty-eight hours after transfection, cells were deprived of serum for 15 h and then treated for 30 min either with insulin (100 nM) or AICAR (500 μM) (A), or with forskolin (50 μM), isoproterenol (10 μM), or A23187 (1 μM) (B). Whole cell lysates were prepared and incubated with GST-fusion proteins that contained (or not) 14-3-3ζ. The bound proteins were then subjected to immunoblot analysis with antibody to the Flag tag (αFlag). Cell lysates were also directly probed with the same antibody to verify the presence of equal amounts of exogenously expressed KLC2. The positions of recombinant KLC2 proteins are indicated by arrows.

Overexpression of KLC2 proteins in C2C12 myoblasts failed to affect insulin-induced GLUT4 movements. We reasoned that, if this scenario holds true, then overexpression of KLC2-S575A, which is refractory to phosphorylation-mediated interaction

with 14-3-3, would be expected to enhance insulin-induced GLUT4 translocation. In contrast, the COOH-terminal deletion mutant of KLC2 (KLC2-ΔTPR), which is structurally equivalent to KLC-L176 (23) and therefore would act in a dominant-negative manner, could have an opposite effect. To test these possibilities, we generated adenoviral vectors encoding KLC2-S575A or KLC2-ΔTPR as well as wild-type KLC2 as a control (Fig. 3A). Immunoblot analysis revealed that each of the three recombinant KLC2 proteins was successfully overexpressed in C2C12 myoblasts (Fig. 3B).

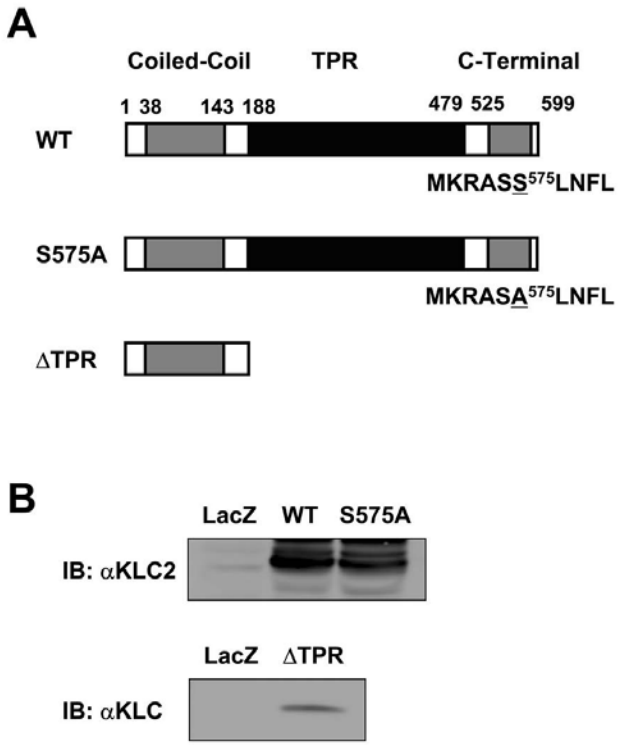


Figure 3. Preparation of adenoviruses encoding wild-type or mutant KLC2 proteins. (A) Structures of wild-type (WT) or phosphorylation-defective (S575A) and COOH-terminal deletion (ΔTPR) mutants of KLC2. The relative NH_2- and COOH-termini are indicated by adjacent numbers. (B) C2C12 myoblasts were infected with adenoviruses encoding wild-type KLC2, the two types of mutant KLC2, or β-galactosidase (LacZ) at a multiplicity of infection (MOI) of 5 plaque-forming units (PFU)/cell. Twenty-four hours after infection, whole cell lysates were prepared and subjected to immunoblot analysis with antibodies specific for KLC2 (upper panel) or for kinesin light chain (lower panel).

We then performed immunofluorescence analysis to determine the effects of these recombinant KLC2 proteins on insulin-induced changes in subcellular localization of GLUT4-myc7-GFP that had been introduced into C2C12 myoblasts via a retrovirus vector. This GLUT4 reporter construct contains 7 Myc epitope tags in its first extracellular loop and is fused with green fluorescent protein (GFP) at its intracellular COOH-terminus (4), allowing us to monitor insulin-induced movement of GLUT4 to the plasma membrane by measurement of GFP fluorescence at the cell periphery and to monitor GLUT4 externalization by measurement of cell surface labeling with antibodies to the Myc tag.

In cells expressing β-galactosidase, GFP fluorescence was preferentially observed at the perinuclear region under basal conditions (Fig. 4A). Insulin stimulation significantly increased the ratio of cells exhibiting GFP fluorescence at the cell periphery as well as that of cells positive for Myc immunoreactivity on the cell surface (Fig. 4A). These results indicate that insulin induces GLUT4 translocation to the plasma membrane and its subsequent externalization in C2C12 myoblasts. Quantitative analysis, however, revealed that these effects of insulin were not substantially altered by overexpression of either wild-type KLC2 or the two types of mutant KLC2 (Fig. 4B; data not shown). Thus, in contrast to 3T3-L1

adipocytes, in which conventional kinesin motor proteins critically regulate insulin-stimulated GLUT4 movements on microtubules (23), C2C12 myoblasts do not appear to utilize these motor proteins to recruit GLUT4 in response to insulin. This notion might be consistent with a recent report showing that disruption of microtubules in rat skeletal muscle does not inhibit insulin-stimulated glucose transport (1).

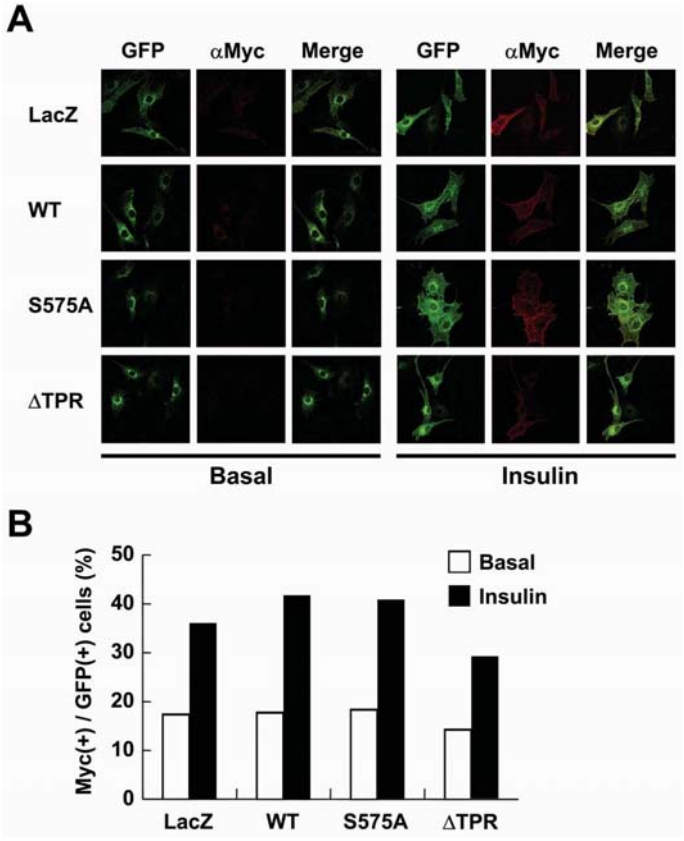


Figure 4. Lack of effect of KLC2 overexpres sion on insulin-induced GLUT4 movements. (A) C2C12 myoblasts stably expressing GLUT4-myc7-GFP were infected with adenoviruses as indicated at a MOI of 5 PFU/ cell. The cells were stimulated with insulin (100 nM) for 30 min, fixed, and subjected to indirect immunofluorescence staining with antibody to the Myc tag (αMyc) and Texas Red-conjugated secondary antibodies in order to detect externalized GLUT4 (red). GLUT4 translocation to the plasma membrane was detected by GFP fluorescence (green) on analysis of the cells by confocal microscopy and was quantitated by determination of the percentage of GFP-positive cells that manifested GFP fluorescence at the cell periphery. Original magnifycation, 630×. (B) GLUT4 externalization in experiments similar to that shown in A was quantitated by determination of the percentage of GFP-positive cells that exhibited Texas Red fluorescence at the cell surface. Data are representative mean values from 3 independent experiments, with 500 GFP-positive cells being examined in each experiment.

Although our results may argue against a contribution of conventional kinesin to insulin-induced GLUT4 translocation in cultured myoblasts, we can not exclude the possibility that other members of the kinesin superfamily participate in this process. Indeed, KIF3, a heterotrimeric motor protein containing KIF3A and KIF3B, has been shown to interact with Rab4 to mediate GLUT4 translocation in response to insulin (16). Further studies are thus required to elucidate physiological roles of kinesin and microtubules-based cytoskeleton in insulin-induced GLUT4 translocation in myocytes.

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