Fluoromicroscopic detection of myc-tagged GLUT4 on the cell surface. Co-localization of the translocated GLUT4 with rearranged actin by insulin treatment in CHO cells and L6 myotubes

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Abstract : We earlier developed a novel method to detect translocation of glucose transporter type 4 (GLUT 4) directly, quantitatively and simply using c-MYC epitope-tagged GLUT4 (GLUT4myc) (Kanai F, Nishioka Y, Hayashi H, Kamohara S, Todaka M, Ebina Y : J Biol Chem 268 : 14523-14526, 1993). We further developed the method to visualize GLUT4myc on the cell surfac by fluorescence microscope using a highly sensitive immunochemical detection system in tissue culture cells stably expressing GLUT4myc. The translocation of GLUT4myc was observed on stimulation with insulin in 3T3-L1 adipocytes, CHO cells and L6 myotubes stably expressing GLUT4myc. Platelet-derived growth factor (PDGF), norepinephrine and bradykinin also triggered GLUT4 translocation in CHO-GLUT4myc cells stably expressing each receptor. To observe the distribution of GLUT4 and actin after insulin treatment, double staining for GLUT4myc and actin was performed. Translocated GLUT4myc on the cell surface was co-localized with rearranged actin in CHO cells and L6 myotubes. This result suggests that a correlation exists between GLUT4 translocation and actin rearrangement. J. Med. Invest. 46 : 192-199, 1999

Key words **:** *GLUT4myc translocation, actin rearrangement, fluorescence microscope*

INTRODUCTION

GLUT4 is an insulin sensitive glucose transporter expressed exclusively in adipocytes and myocytes (1, 2). Insulin-stimulated glucose uptake is caused by the translocation of GLUT4 from intracellular pools to the plasma membrane (3, 4). The intracellular signal transduction pathway from insulin stimulation to GLUT4 translocation involves the activation of insulin receptor tyrosine kinase after insulin binding, phosphorylation of insulin receptor substrate-1 (IRS-1) and activation of phosphatidylinositol 3-kinase (PI-3 kinase) by the formation of a complex with phosphorylated IRS-1 (5-7). Recently, the signaling pathway down stream of PI-3 kinase has been revealed (8-12).

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The final stages of the pathway are the trafficking of GLUT4 containing vesicles and the fusion of the vesicles with the plasma membrane (13). At this point, the process resembles that of the release of neurotransmitters, with similar molecules such as syntaxin 4, VAMP-2, SNAP-23 and Munc-18 involved in the trafficking and the fusion of GLUT4 containing vesicles (14-16). It has been reported that actin is one of the cytoskeleton proteins that contribute to endocytosis, exocytosis and vesicular transport (17-19). Not only insulin, but platelet-derived growth factor (PDGF) or epidermal growth factor (EGF) can also stimulate actin rearrangement and membrane ruffling (20-22). These stimulation pathways are associated with PI-3 kinase activation (23), but few studies have been performed to examine the relation between GLUT4 translocation and actin rearrangement. A study using cytocharasin D, an inhibitor of actin rearrangement, showed that a relation may exist between the actin network and GLUT4 translocation (24).

Using myc-tagged GLUT4 (25) and a fluores-

cence microscope, we visualized the translocated GLUT4myc on the cell surface in 3T3-L1 adipocytes, CHO cells and L6 myotubes stably expressing GLUT4myc. GLUT4myc translocated by insulin was co-localized with rearranged actin in CHO cells and L6 myotubes. This result suggests that a close correlation exists between GLUT4 translocation and actin rearrangement.

MATERIALS AND METHODS

Cells

GLUT4myc was constructed by inserting a human c-MYC epitope (14 amino acids) into the first ectodomain of GLUT4 as described previously (25). Cell lines expressing GLUT4myc and receptors (CHO-GLUT4myc, 3T3-L1-GLUT4myc, L6-GLUT4myc, CHO-GLUT4myc-PDGFR, CHO-GLUT 4 myc-α_{1b} adrenergic receptor (α_{1b}AR) and CHO-GLUT4myc-bradykinin B_2receptor (BKB_2R)) were constructed as described (26, 27). 3T3-L1-GLUT4myc cells were differentiated into adipocytes and L6-GLUT4myc cells were differentiated into myotubes as reported previously (25, 28).

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

Cells in 24-well plates were preincubated with 500μl of Krebs Ringer Hepes (KRH) buffer (136 mM NaCl, 4.7mM KCl, 1.25mM $CaCl_2$, 1.25mM $MgSO_4$, 0.2% BSA and 20mM Hepes, pH7.4) for 30 min at 37°C and then stimulated with 250 μl of 3×10^{-7} M insulin, 50ng/ml PDGF, 1×10^{-6} M norepinephrine, 1×10^{-7} M bradykinin, or buffer alone for 10 min at 37°C. In inhibitor experiments, cells were treated with wortmannin for 10 min before stimulation. After fixation with 2% paraformaldehyde in PBS (136 mM NaCl, 2.7mM KCl, 8.1mM Na_2HPO_4 and 1.5 mM KH_2PO_4), GLUT4myc translocation was measured as previously described (26). Chemiluminescence intensity was measured by Luminescensor-JNR (ATTO, Tokyo, Japan). Translocation activity was indicated as the fold increase from control (buffer alone).

Fluorescence microscopy

Cells in 6-well plates were stimulated with ligands as described above. After fixation with 2% paraformaldhyde in PBS, single staining was performed for GLUT4myc or actin, and double staining for GLUT4myc and actin. For the GLUT4myc single staining, TSA-Direct (tyramide signal amplication system, NEN Life Science Products, Boston, MA) was used. After fixation, cells were blocked with 1ml of blocking reagent in TN buffer (0.15M NaCl and 0.1M Tris-HCl, pH 7.5) at 37°C for 30min. After blocking, cells were incubated with 30 μl of anti-MYC monoclonal antibody 9E10 (1:20 dilution, provided by ATCC, Rockville, MD) at 37°C for 30min, washed with TNT buffer (0.05% Tween 20 in TN buffer) for 5 min three times, then incubated with 30μl of horseradish peroxidase-conjugated anti mouse IgG (1:20 dilution, Biosource, Camarill, CA) at 37°C for 30min. They were then washed with TNT buffer for 5min three times. The cells were incubated with 30μl of fluorescein tyramide at room temperature for 7 min and washed with TNT buffer for 10min three times. The cells were mounted in PBS containing 80% glycerol and 2% propyl galate. For the actin single staining, cells were permeabilized with 1ml of 0.1% Triton X100 in PBS at room temperature for 10min, washed with PBS three times, and incubated at room temperature for 20 min with 1.5U/30μl of rhodamin-phalloidin (Molecular Probes, OR) in PBS. After staining, they were washed with PBS for 5min three times and mounted as described above. For the GLUT4myc and actin double staining, the procedure used for GLUT4myc was followed until the second antibody incubation. After washing, cells were incubated with 1.5U/30μl of rhodamin-phalloidin in PBS, and again washed with TNT buffer for 5min three times. They were then incubated with 30μl of fluorescein tyramide at room temperature for 7 min. Finally, the cells were washed with TNT buffer for 10min three times and mounted as described above. The cells were observed and photographed with a fluorescence microscope (Olympus, Tokyo, Japan).

RESULTS

We previously found that insulin triggers GLUT4myc translocation in 3T3-L1-GLUT4myc adipocytes, CHO-GLUT4myc cells and L6-GLUT4myc myotubes by the binding assay of anti-MYC antibody on the cell surface (25-27).

Cells were stained for GLUT4myc and actin to observe morphologic changes after insulin stimulation. Translocated GLUT4myc on the cell surface was visualized under a fluorescence microscope using a highly sensitive immunochemical detection system (TSA-Direct). GLUT4myc was translocated to the cell surface by insulin in 3T3-L1-GLUT4myc adipocytes

(L1-G4), CHO-GLUT4myc cells (CHO-G4) and L6-GLUT4myc myotubes (L6-G4) (Fig. 1 upper row shown in green). In 3T3-L1-GLUT4myc adipocytes, GLUT4myc was observed in a ring shaped pattern and was translocated uniformly to the cell surface. In CHO-GLUT4myc cells and L6-GLUT4myc myotubes, the translocation took on a fragmental pattern. The distribution of actin was observed under a fluorescence microscope using an actin binding reagent rhodamin-phaloidin. After insulin stimulation, the distribution changed to a fragmental pattern like for GLUT4myc in CHO-GLUT4myc cells and L6-GLUT4myc myotubes, while in 3T3-L1-GLUT4myc adipocytes, no actin rearrangement was readily apparent (Fig. 1, lower row shown in orange). The distribution of vimentin and tubulin did not change after insulin stimulation in any of the cell lines (results not shown).

Not only insulin, but also PDGF, norepinephrine (NE) and bradykinin (BK) triggers GLUT4 translocation (25-27, 29). Immunofluorescence microscopy was performed to see the GLUT4myc translocation by each ligand in CHO GLUT4myc cells stably expressing each receptor (Fig. 2). In CHO-GLUT4myc-α_{1b}AR (CHO-G4-AR) and CHO-GLUT4myc-BK_{B_2}R (CHO-G4-BR) cells, norepinephrine and bradykinin triggered GLUT4myc translocation uniformly. In CHO-GLUT4myc-PDGFR (CHO-G4-PR) cells stimulated with PDGF, the translocation pattern of GLUT4myc was linear in shape along the cell edges. These patterns are slightly different from that for insulin stimulation (Fig. 2).

Wortmannin, a PI3-kinase inhibitor, is known as an inhibitory reagent for GLUT4 translocation and actin rearrangement (7, 23). As shown in Fig. 3, pretreatment with 10^{-7}M wortmannin abolished the insulin-induced GLUT4myc translocation. Fluorescence staining for GLUT4myc and actin was done to observe the effects of wortmannin in the CHO - GLUT4myc cells. Wortmaninn inhibited both insulin-stimulated GLUT4 translocation and actin rearrangement as shown in Fig. 4.

Distributions of GLUT4myc and actin after insulin stimulation showed similar patterns in CHO-GLUT4myc cells and L6-GLUT4 myotubes (Fig. 1). To examine the co-localization of GLUT4myc and actin, GLUT4myc and actin double staining was performed. The distribution patterns for GLUT4myc (Fig. 5, upper row) and actin (Fig 5, middle row) were almost the same. Double staining (Fig 5, lower row) showed that GLUT4myc was co-localized with actin as shown by the yellow color. In CHO cells (absence of GLUT4myc), no green for GLUT4myc was observed before or after insulin stimulation. However, actin rearrangement after insulin stimulation was observed as orange, suggesting that there was no cross reaction of staining between GLUT4myc and actin with this double staining technique (Fig. 5).

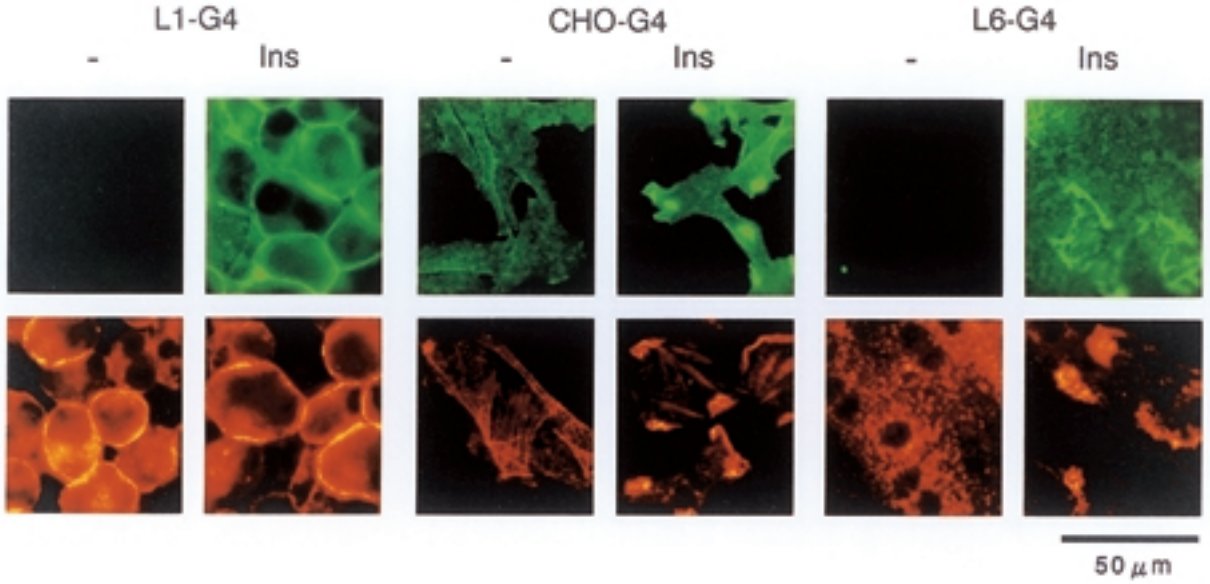

Fig.1. Immunofluorescence microscopy of insulin-stimulated GLUT4myc translocation and actin rearrangement in 3T3-L1-GLUT4myc adipocytes (L1-G4), CHO-GLUT4myc cells (CHO-G4) and L6-GLUT4myc myotubes (L6-G4).
Cells were stimulated with 3×10^{-7}M insulin (Ins) or buffer alone (-) for 10 min at 37°C after pretreatment with buffer for 30 min at 37°C. GLUT4myc localization was detected with TSA-Direct (upper row shown in green) and actin localization with rhodamine-labeled phalloidin (lower row shown in orange) as described under materials and methods.

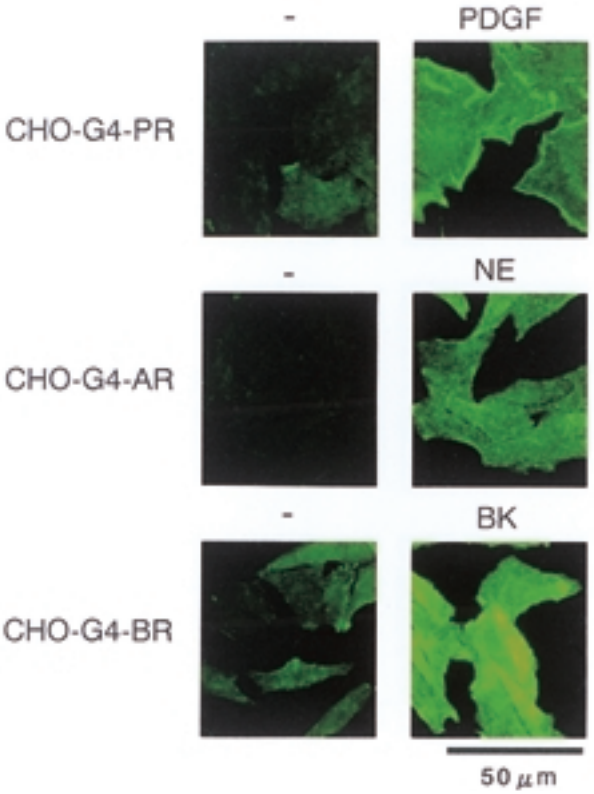

Fig.2. Immunofluorescence microscopy of PDGF-, norepinephrine (NE)-and bradykinin (BK)-stimulated GLUT4myc translocation in CHO-GLUT4myc-PDGFR cells (CHO-G4-PR), CHO-GLUT4myc-α_{1b}AR cells (CHO-G4-AR) and CHO-GLUT4myc-BKB_2R cells (CHO-G4-BR). Cells were stimulated with 50ng/ml PDGF, 1×10^{-6}M norepinephrine or 1×10^{-7}M bradykinin for 10 min at 37°Cafter pretreatment with buffer for 30 min at 37°C. GLUT4myc localization was detected with TSA-Direct (shown in green) as described under materials and methods.

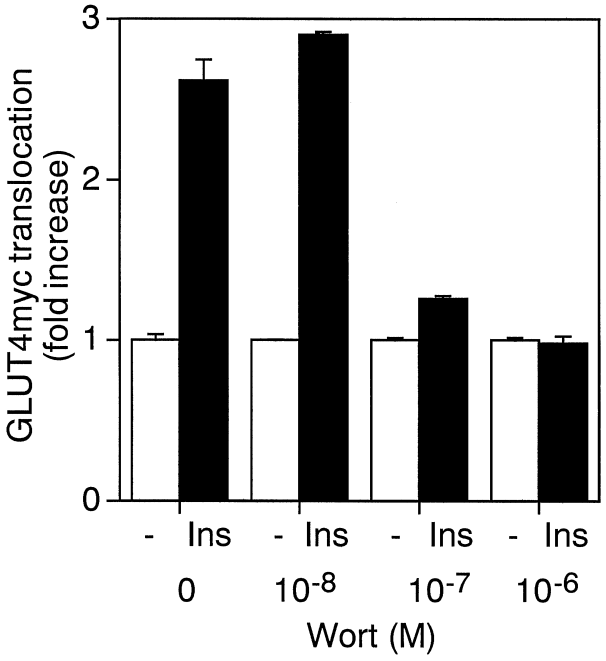

Fig.3. Effects of wortmannin on the GLUT4myc translocation in CHO-GLUT4myc cells.
Cells were treated with wortmannin (Wort) for 10 min at 37°C after pretreatment with buffer for 30 min at 37°C. Then they were stimulated with 3×10^{-7}M insulin (Ins, solid bar) or buffer alone (-, open bar) for 10 min at 37°C. Translocation activity was measured as described under materials and methods and indicated by fold increase from control (buffer alone).

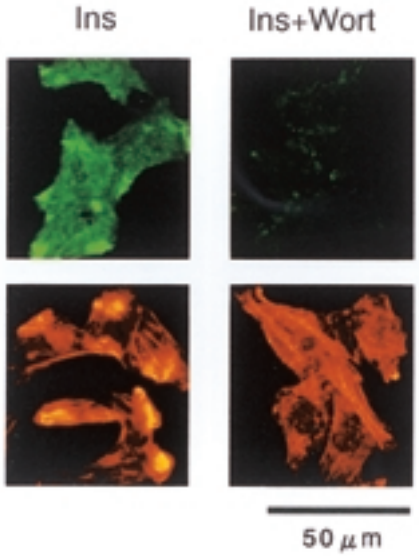

Fig.4. Immunofluorescence microscopy of insulin stimulated GLUT4myc translocation and actin rearrangement in CHO-GLUT4myc cells after treatment of wortmannin.
Cells were treated with 10^{-7}M wortmannnin or buffer alone for 10 min at 37°Cafter pretreatment with buffer for 30 min at 37°C. Then they were stimulated with 3×10^{-7}M insulin for 10 min at 37°C. GLUT4myc localization was detected with TSA-Direct (upper row shown in green) and actin localization with rhodamine-labeled phalloidin (lower row shown in orange) as described under materials and methods.

DISCUSSION

Several methods to detect GLUT4 translocated by insulin stimulation have been proposed. These include subcellular fractionation after cytocharasin B labeling, immunological detection in western blots after subcellular fractionation, photo-labeling with immunoprecipitaion and histological approaches such as immunofluorescence or immunoelectron microscopy (4, 30, 31). However, these methods do not allow for the detection of GLUT4 specifically and quantitatively. An ideal method would be the direct immunological detection of GLUT4 on the cell surface with an anti-ectodomain antibody specific for GLUT4 ; heretofore, however, no such antibody has been obtained. Therefore, we developed a simple direct sensitive method to detect GLUT4 immunologically on the cell surface (25). In this report, we further developed this method to visualize the translocated GLUT4 on the cell sur-

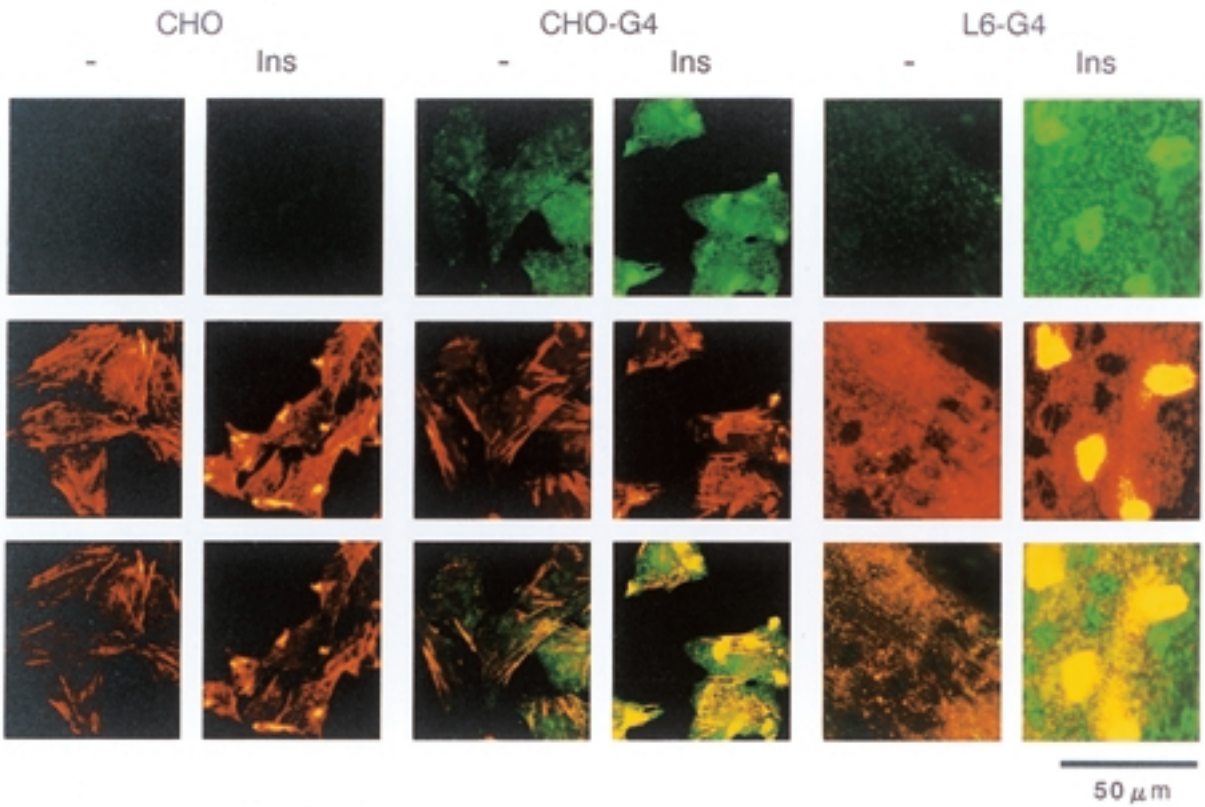

Fig.5. Immunofluorescence microscopy of co-localization of GLUT4myc with actin in CHO-GLUT4myc cells and L6-GLUT4myc myotubes.
Cells were stimulated with 3×10^{-7} M insulin (Ins) or buffer alone (-) for 10 min at 37°C after pretreatment with buffer for 30 min at 37°C. Co-localization of GLUT4myc with actin was detected with a double staining technique as described under materials and methods. Upper row, excitation for fluorescein (GLUT4myc, green). Middle row, excitation for rhodamine (actin, orange). Lower row, double exposure of both excitations (GLUT4myc+actin, yellow indicates the area of co-localization).

face under a fluorescence microscope.

After insulin stimulation, translocated GLUT4myc on the cell surface was observed by fluorescence microscope in 3T3-L1-GLUT4myc adipocytes, CHO-GLUT4myc cells and L6-GLUT4myc myotubes. Simultaneously, actin rearrangement was observed in CHO-GLUT4myc cells and L6-GLUT4myc myotubes, but not in 3T3-L1-GLUT4myc adipocytes. This may be due to difficulty in detecting the actin rearrangement because almost all of the cytoplasmic volume of adipocytes is occupied by a large lipid mass. Previous reports have not shown such an obvious change in the distribution pattern of GLUT4 after insulin stimulation in CHO cells (32, 33). Wortmannin, an inhibitor of PI-3 kinase, inhibited GLUT4myc translocation (7). We observed the inhibition of both insulin induced actin rearrangement and GLUT4myc translocation after treatment with wortmannin by fluorescence microscope.

The pattern of actin rearrangement after insulin treatment was similar to that of translocated GLUT4myc in CHO-GLUT4myc cells and L6-GLUT4myc myotubes. Cell permeabilization is needed to stain for actin with rhodamin-phalloidin (34), but this treatment is unable to specifically detect cell surface GLUT4myc. To resolve this problem, we performed actin staining after immuno-labeling of GLUT4myc. Using this double staining technique, we showed that translocated GLUT4myc on the cell surface was co-localized with rearranged actin, suggesting that a close correlation exists between GLUT4 translocation and actin rearrangement.

It has previously been reported that insulin receptors co-localized with actin (35). These results suggest that actin plays an important role in accumulating cell surface molecules, and may be directly involved in GLUT4 translocation. Studies using cytocharasin D, an inhibitor of actin rearrangement, suggested that an intact actin network is required for the correct intracellular localization of glucose transporters and for their incorporation into plasma membrane in response to insulin (22, 24). RhoD protein may have an important role in actin filament rearrangement and vesicle transport (36, 37).

Recently, a very useful technique to observe molecular dynamics in living cells was developed.

Using fusion protein of GLUT4-green fluorescent protein (GLUT4-GFP), it was shown that insulin promoted an increase in plasma membrane fluorescence as a result of a net translocation of GLUT4-GFP in CHO cells (38). Using this technique, the relation between actin rearrangement and GLUT4 translocation was studied, but none was observed in 3T3-L1 adipocytes (39). In the present study, we did not observe any obvious actin rearrangement after insulin stimulation in 3T3-L1 adipocytes. However, in CHO cells and L6 myotubes, we observed a co-localization of GLUT4myc and actin using a double staining technique.

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