Kobe J. Med. Sci., Vol. 54, No. 4, pp. E209–E216, 2008

# Insulin-stimulated Fusion of GLUT4 Vesicles to Plasma Membrane is Dependent on Wortmannin-sensitive Insulin Signaling Pathway in 3T3-L1 Adipocytes

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Received 30 July 2008/ Accepted 5 August 2008

**Key words:** GLUT4, translocation, wortmannin, class IA PI 3-kinase, 3T3-L1 adipocytes

**It is established that wortmannin which completely inhibits class IA PI 3-kinase activation abrogated the insulin-dependent translocation of GLUT4 to the plasma membrane in adipocytes and skeletal muscle. However, it was not clear which steps wortmannin inhibited during the whole translocation process of GLUT4. We have now dissected the each steps of the GLUT4 trafficking in 3T3-L1 adipocytes using exogenously-expressed GLUT4 reporter in combination with plasma membrane lawn assay. We showed that 100 nM wortmannin inhibited the fusion of GLUT4 vesicles to the plasma membrane without affecting the movement and the subsequent tethering/docking event of GLUT4 vesicles to the membrane in 3T3-L1 adipocytes. These results suggest that wortmannin-sensitive insulin signaling pathway plays a crucial role in the fusion step of GLUT4 vesicles to the plasma membrane in 3T3-L1 adipocytes.**

Insulin stimulates glucose uptake in adipose tissue and skeletal muscle by enhancing the insulin-responsive glucose transporter isoform (GLUT4)-containing vesicle movement from intracellular storage compartments to the cell surface (1). This pathway is thought to consist of several distinct steps (2,3). The first step is the movement of GLUT4 vesicles from a specialized intracellular compartment to the cell periphery. The second step is thought to include two processes: tethering and docking. Tethering means a low-affinity interaction between GLUT4 vesicles and the plasma membrane and docking is defined by assembly of the soluble *N*-ethylmaleimide-sensitive factor-attachment protein (SNAP) receptor (SNARE) complex. The final step of GLUT4 trafficking is fusion, in which the lipid bilayers of each GLUT4 vesicle and the plasma membrane merge. Several lines of studies using gene-engineered mouse models demonstrate that glucose uptake by GLUT4 in skeletal muscle and adipose tissue plays a critical role in the normal whole-body glucose homeostasis (4-7). Therefore, the characterization of the mechanism of insulin-stimulated GLUT4 translocation is important for elucidation of the pathogenesis of type 2 diabetes.

It is well accepted that wortmannin, an inhibitor of class IA PI 3-kinase inhibits the insulin-dependent externalization of GLUT4 to cell surface (8). However, it has not been clearly demonstrated which steps this lipid kinase regulates during the whole process of insulin-stimulated GLUT4 externalization in adipocytes. Therefore, the purpose of the

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present study was to demonstrate the precise steps in which wortmannin-sensitive insulin signaling pathway was involved during the entire process of insulin-stimulated GLUT4 translocation in 3T3-L1 adipocytes using the forced expression of a GLUT4 reporter tagged with both the Myc epitope and green fluorescence protein (GFP) in combination with plasma membrane lawn assay.

## MATERIALS AND METHODS

### Materials

Polyclonal antibodies to GLUT4 were kindly provided by S. W. Cushman (NIH, Bethesda, MD). Mouse monoclonal antibodies to c-Myc were from Santa Cruz Biotechnology. Rabbit polyclonal antibodies to Akt and to Akt phosphorylated on $Ser^{473}$ were obtained from Cell Signaling Technology. Wortmannin was purchased from Sigma.

### Methods

Preparation of 3T3-L1 adipocytes expressing GLUT4myc7-GFP—3T3-L1 cells were obtained from American Type Culture Collection. Adipogenesis was induced in these cells as described previously (9). Platinum-E (PLAT-E) ecotropic packaging cells were transfected with the retroviral vector pMX-GLUT4myc7-GFP (kindly provided by H. F. Lodish, Massachusetts Institute of Technology, Cambridge, MA) (10) with the use of the transfection reagent FuGENE 6 (Roche Diagnostics). Culture medium containing recombinant retroviruses was harvested 48 h after the onset of transfection. For infection of the target cells, 1 ml of the retrovirus-containing medium was added to 3T3-L1 fibroblasts ($2 \times 10^5$ cells per 60-mm dish) cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, and the cells were incubated for 24 h. The efficiency of infection with the recombinant retroviruses was estimated at 60 to 80% on the basis of GFP fluorescence.

Confocal immunofluorescence microscopy—Adipocytes expressing GLUT4myc7-GFP were fixed with phosphate-buffered saline (PBS) containing 4% paraformaldehyde, washed with PBS containing 100 mM glycine, and exposed to PBS containing 5% bovine serum albumin. Externalized GLUT4myc7-GFP was visualized without cell permeabilization by indirect immunofluorescence staining with antibodies to c-Myc and phycoerythrin (PE)–conjugated goat antibodies to mouse immunoglobulin G (Jackson ImmunoResearch Laboratories). The cells were examined for PE and GFP fluorescence with a confocal laser-scanning microscope (LSM5 PASCAL version 3, Carl Zeiss).

Plasma membrane lawn assay—Translocation of GLUT4 to the plasma membrane was measured with a plasma membrane lawn assay as described previously (11). In brief, 3T3-L1 adipocytes cultured on cover slips were washed with PBS, exposed to poly-L-lysine (0.5 mg/ml) in PBS, incubated in a hypotonic solution [KHMgE buffer: 30 mM Hepes-NaOH (pH 7.5), 70 mM KCl, 5 mM $MgCl_2$, and 3 mM EGTA], and disrupted by ultrasonic treatment in KHMgE buffer supplemented with 0.1 mM phenylmethylsulfonyl fluoride and 1 mM dithiothreitol. The cells were then fixed in 4% paraformaldehyde, incubated with antibodies to GLUT4, washed three times with PBS, and incubated with fluorescein Indocarbocyanine (Cy3)–conjugated antibodies to rabbit immunoglobulin G (Amersham). After a final wash with PBS, the cover slips were mounted in FluoroGuard Antifade Reagent (Bio-Rad) and examined with a confocal laser-scanning microscope (LSM5 PASCAL version 3). In plasma membrane lawn assay in 3T3-L1 adipocytes expressing GLUT4myc7-GFP, GLUT4 localization on the plasma membrane of the disrupted cells was

determined by visualizing GFP fluorescence of GLUT4myc7-GFP with a confocal laser-scanning microscope.

Assay of 2-deoxy-D-glucose transport—Adipocytes were deprived of serum by incubation with DMEM in 12-well plates for 2 h. They were then incubated with 100 nM insulin for 20 min in 0.45 ml of KRH buffer [25 mM Hepes-NaOH (pH 7.4), 120 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 1.3 mM $CaCl_2$, 1.3 mM $KH_2PO_4$]. Glucose transport was initiated by the addition of 0.05 ml of KRH buffer containing 2-deoxy-D-[1,2-$^3$H]glucose (final concentration, 0.05 mM; 0.25 μCi) to each well, and it was terminated after 5 min by washing the cells three times with ice-cold KRH buffer. The cells were solubilized with 0.5% SDS, and the incorporated radioactivity was measured by liquid scintillation counting.

Statistical analysis—Quantitative data are presented as means ± SEM. Differences between groups were examined for statistical significance with Student's t test. A P value of $<0.05$ was considered statistically significant.

## RESULTS

***100 nM wortmannin treatment abrogated insulin-stimulated glucose uptake in 3T3-L1 adipocytes***—It is well known that 100 nM wortmannin completely inhibits class IA PI 3-kinase activation (12). Actually, we confirmed that treatment of 100 nM wortmannin completely inhibited insulin-stimulated phosphorylation of Akt in 3T3-L1 adipocytes (Figure 1A). In addition, we showed that the same concentration of wortmannin completely inhibited the insulin-stimulated glucose uptake in these cells (Figure 1B). This implies that 100 nM wortmannin completely inhibited the insulin-stimulated cell surface appearance of GLUT4 in 3T3-L1 adipocytes.

**Figure 1** **A** **B**

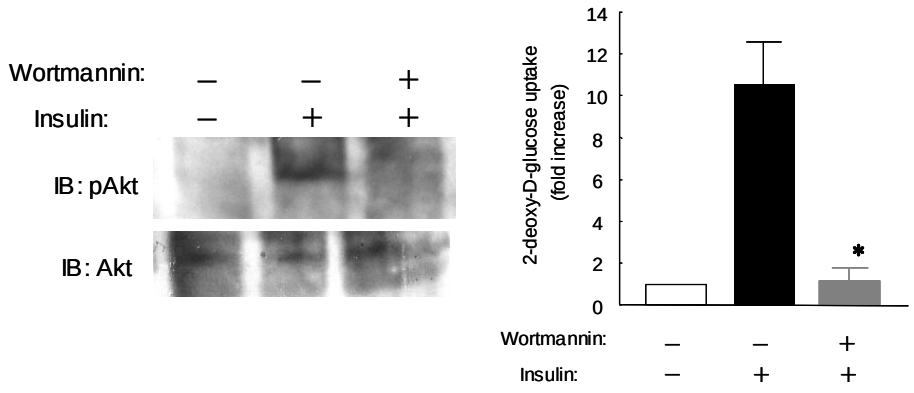



**Figure 1. 100 nM wortmannin inhibits insulin-stimulated glucose uptake in 3T3-L1 adipocytes.** *A.* 3T3-L1 adipocytes were treated with 100 nM wortmannin for 30 min and then stimulated with 100 nM insulin (or not) for 5 min, after which cell lysates were prepared and subjected to immunoblot analysis with antibodies to the Ser$^{473}$-phosphorylated form of Akt (pAkt), or to total Akt. *B.* Insulin-stimulated uptake of 2-deoxy-D-[1,2-$^3$H]glucose was measured in 3T3-L1 adipocytes treated with or without 100 nM wortmannin for 30 min. Data are means ± SEM of values from three separate experiments and are expressed as fold increase relative to the value for control cells not stimulated with insulin. *$P < 0.01$ versus the value for control cells stimulated with insulin.

***100 nM wortmannin treatment impaired insulin-stimulated externalization of GLUT4 to cell surface but not movement of GLUT4 to the plasma membrane in 3T3-L1 adipocytes—***To identify the steps of insulin-stimulated GLUT4 trafficking which were impaired by wortmannin, we prepared 3T3-L1 adipocytes that express the GLUT4 reporter GLUT4myc7-GFP, which contains seven copies of the Myc epitope in its first extracellular loop and is fused with GFP at its intracellular COOH-terminus (10). These cells allowed us to monitor both the movement of GLUT4 from intracellular storage sites to the plasma membrane by detection of GFP fluorescence at the cell periphery as well as the subsequent externalization of GLUT4 by cell surface labeling with antibodies to the Myc tag (10,13). 100 nM wortmannin did not affect the extent of insulin-stimulated GLUT4 movement to the cell periphery as estimated by the ratio of the number of cells positive for GFP fluorescence at the cell periphery to the total number of GFP-positive cells (Fig. 2A,B).

## Figure 2 A

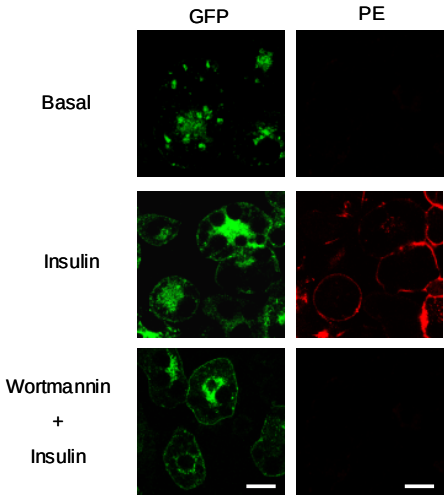
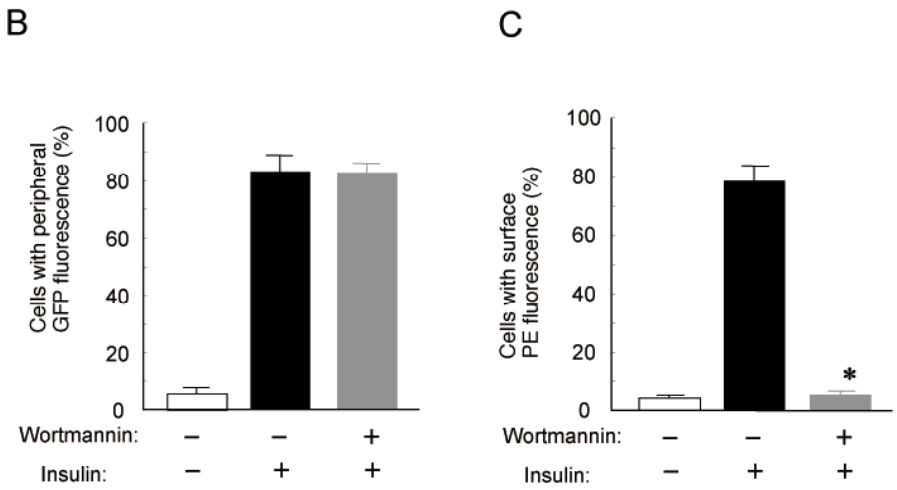



**Figure 2. 100 nM wortmannin inhibited insulin-stimulated GLUT4 externalization to cell surface but not movement to the plasma membrane in 3T3-L1 adipocytes.** *A*, 3T3-L1 adipocytes expressing GLUT4*myc*7-GFP were treated with 100 nM wortmannin and 30 min later were stimulated (or not) with 100 nM insulin for 20 min, fixed, and subjected to indirect immunofluorescence staining with antibodies to the Myc tag and PE-conjugated secondary antibodies (red fluorescence) in order to detect externalized GLUT4 (PE). Movement of GLUT4 vesicles to the cell periphery was detected on the basis of GFP fluorescence (green) on examination of the cells by confocal microscopy (GFP). Scale bar, 20 μm. *B*, Movement of GLUT4 to the cell periphery in *A* was quantitated by determination of the percentage of GFP-positive cells that manifested GFP fluorescence at the cell periphery. *C*. GLUT4 externalization was quantitated by determination of the percentage of GFP-positive cells that exhibited PE fluorescence at the cell surface. Data are means ± SEM of values from three separate experiments, with 80 to 100 GFP-positive cells being examined in each experiment. **P* < 0.01 versus the corresponding value for control cells stimulated with insulin.



In contrast, cell surface labeling of the GLUT4 reporter with antibodies to the Myc tag revealed that the insulin-induced appearance of GLUT4 at the cell surface was completely inhibited by treatment with 100 nM wortmannin (Figure 2A,C). These results suggested that GLUT4 vesicles either are localized just near the plasma membrane without tethering/docking to it or are localized on the plasma membrane in the tethered/docked state but without fusion to it in wortmannin-treated adipocytes stimulated with insulin.

***100 nM wortmannin treatment did not affect insulin-stimulated GLUT4 vesicle tethering/docking to the plasma membrane in 3T3-L1 adipocytes***—To resolve the above question, we performed plasma membrane lawn assay with 3T3-L1 adipocytes with antibodies specific for the COOH-terminal domain of GLUT4. Because, this assay was useful to determine whether GLUT4 vesicles were directly tethered/docked to the plasma membrane or not, irrespective of the distance between GLUT4 vesicles and the plasma membrane (14). We confirmed that insulin induced the localization of GLUT4 to the plasma membrane (Fig. 3A,B). Furthermore, we found that the 100 nM wortmannin treatment did not affect the insulin-stimulated localization of GLUT4 to the plasma membrane significantly (Fig. 3A,B). This implies that GLUT4 vesicles are tethered or docked to the plasma membrane in wortmannin-treated 3T3-L1 adipocytes stimulated with insulin. In addition, we also performed plasma membrane lawn assay in 3T3-L1 adipocytes expressing GLUT4*myc*7-GFP to confirm whether wortmannin exerted the same effects to exogenously-expressed GLUT4 reporter. Again, we found that 100 nM wortmannin did not affect the insulin-stimulated localization of exogenously-expressed GLUT4 reporter to the plasma membrane, either (Fig. 3C,D). Taken together, two kinds of plasma membrane lawn assay showed that GLUT4 vesicles were localized just on the plasma membrane in the tethered or docked state in wortmannin-treated adipocytes stimulated with insulin. Our data show that 100 nM wortmannin inhibited the fusion step of GLUT4 vesicles to the plasma membrane but not the movement and the subsequent tethering/docking step.

## DISCUSSION

100 nM wortmannin inhibited the insulin-stimulated production of phosphatidylinositol-3,4,5-trisphosphate (PI(3,4,5)$P_3$) but not phosphatidylinositol-3-phosphate (PI3P) (15). Ishiki et al. showed that PI(3,4,5)$P_3$ is important for arrival and fusion of GLUT4 vesicle to plasma membrane and PI3P is important for arrival (16). Semiz et al. showed that 100 nM wortmanin did not cause the strong inhibition of insulin-induced movement of GLUT4 vesicle to plasma membrane, although it strongly inhibited the externalization of GLUT4 to cell surface in 3T3-L1 adipocytes (17). These data suggest that insulin signal pathway mediated by wortmannin-sensitive PI(3,4,5)$P_3$ signaling pathway mainly regulates the fusion step of GLUT4 vesicles with plasma membrane. Similar results were reported by van Dam et al. that fusion of GLUT4 vesicles with plasma membrane, but not redistribution of GLUT4 vesicles toward cell cortex can be the major Akt-dependent step in insulin regulation of glucose uptake (18). However, in these studies the tethering/docking step and fusion step were not clearly dissected in the analysis. In the current study, we dissected the GLUT4 trafficking to the three steps, movement to the plasma membrane, tethering/docking to it and the fusion step. We showed that 100 nM wortmannin-sensitive insulin signaling pathway is important mainly for the fusion step rather than the movement and the subsequent tethering/docking step of GLUT4 to plasma membrane. In general, wortmannin inhibits all mammalian class I, II, and III PI 3-kinase activity (19). However, studies using dominant negative forms of p85 subunit revealed that class IA PI 3-kinase is the major isoform which mediates GLUT4 trafficking by insulin (20,21).

Figure 3

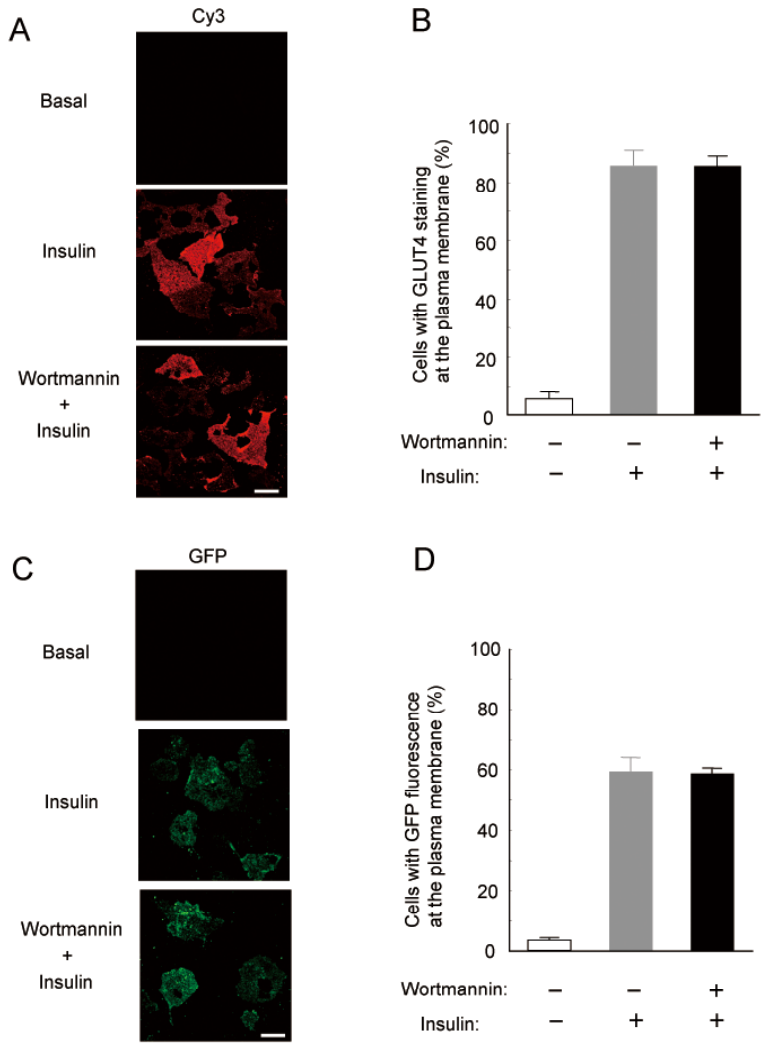



**Figure 3. 100 nM wortmannin did not affect insulin-stimulated GLUT4 vesicle tethering/docking to the plasma membrane in 3T3-L1 adipocytes.** *A.* 30 min after the treatment with 100 nM wortmannin, 3T3-L1 adipocytes were stimulated (or not) with 100 nM insulin for 20 min and then disrupted by ultrasonic treatment. The plasma membrane fragments were subjected to immunofluorescence analysis with antibodies to GLUT4 (red fluorescence). Scale bar, 20 μm. *B*, GLUT4 localization at the plasma membrane in *A* was quantitated by determination of the percentage of cells in which Cy3 fluorescence was detected at the plasma membrane. Data are means ± SEM of values from three separate experiments, with 60 cells being examined in each experiment. *C.* 30 min after the treatment with 100 nM wortmannin, 3T3-L1 adipocytes expressing GLUT4*myc*7-GFP were stimulated (or not) with 100 nM insulin for 20 min and then disrupted by ultrasonic treatment. The localization of GLUT4 on plasma membrane was analysed by detecting the green fluorescence originated from GLUT4*myc*7-GFP. *D.* GLUT4 localization at the plasma membrane in *C* was quantitated by determination of the percentage of cells in which GFP fluorescence was detected at the plasma membrane. Data are means ± SEM of values from three separate experiments, with 60 cells being examined in each experiment.

Therefore, these findings suggest that the movement of GLUT4 vesicles towards the cell cortex and the tethering/docking of them to plasma membrane may be mainly regulated by class IA PI-3 kinase-independent insulin signal pathway and the fusion can be regulated mainly by class IA PI-3 kinase-dependent insulin signal pathway in 3T3-L1 adipocytes.

## ACKNOWLEDGEMENTS

We thank H. F. Lodish for pMX-GLUT4*myc*7-GFP; S. W. Cushman for antibodies to GLUT4; as well as S. Shigeta for technical assistance. This work was supported by a grant for the Intellectual Cluster Formation Project from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (MEXT) to M.K.; a grant for the 21st Century COE Program "Center of Excellence for Signal Transduction Disease: Diabetes Mellitus as a Model" from MEXT to M.K.; and a Grant-in-Aid for Creative Scientific Research from MEXT to M.K.

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