Insulin Recruits GLUT4 from Distinct Compartments via Distinct Traffic Pathways with Differential Microtubule Dependence in Rat Adipocytes*

Li-Bin Liu, Waka Omata, Itaru Kojima, and Hiroshi Shibata‡

From the Department of Cell Biology, Institute for Molecular and Cellular Regulation, Gunma University, 3-39-15 Showa-machi, Maebashi 371-8512, Japan

In the present study, we investigated the physiological significance of the microtubules in the subcellular localization and trafficking of GLUT4 in rat primary adipocytes. Morphological and biochemical analyses revealed a dose- and time-dependent disruption of the microtubules by treatment with nocodazole. With nearly complete disruption of the microtubules, the insulin-stimulated glucose transport activity was inhibited by 55%. This inhibition was concomitant with a comparable inhibition of GLUT4 translocation measured by the subcellular fractionation and the cell-surface GLUT4 labeling by trypsin cleavage. In addition, the time-course of insulin stimulation of the glucose transport activity was significantly delayed by microtubule disruption (t½ were 7 and 2.3 min in nocodazole-treated and control cells, respectively), while the rate of GLUT4 endocytosis was little affected. The impaired insulin-stimulated glucose transport activity was not fully restored to the level in control cells by blocking GLUT4 endocytosis, suggesting that the inhibition was due to the existence of a microtubule-dependent subpopulation in the insulin-responsive GLUT4 pool. On the other hand, nocodazole partially inhibited insulin-induced translocation of the insulin-regulated aminopeptidase and the vesicle-associated membrane protein (VAMP)-2 without affecting GLUT1 and VAMP-3. In electrically permeabilized adipocytes, the insulin-stimulated glucose transport was inhibited by 40% by disruption of the microtubules whereas that stimulated with GTPγS was not affected. Intriguingly, the two reagents stimulated glucose transport to the comparable level by disruption of the microtubules. These data suggest that insulin recruits GLUT4 to the plasma membrane from at least two distinct intracellular compartments via distinct traffic routes with differential microtubule dependence in rat primary adipocytes.

Insulin stimulates glucose uptake mainly by promoting subcellular redistribution of a facilitative glucose transporter isoform GLUT4 from intracellular compartments to the plasma membrane in adipocyte and skeletal/cardiac muscles (1–3). While the subcellular trafficking pathways and the molecular mechanisms by which insulin recruits GLUT4 to the plasma membrane still remain obscure, several lines of evidence have suggested that GLUT4 is associated with more than one intracellular compartment. Morphologically, studies with immunoelectron microscopy have shown that most of GLUT4 localizes intracellularly to tubulovesicular structures clustered near the stacks of Golgi and the endosomes, or scattered throughout the cytoplasm in unstimulated adipocytes and that insulin decreases GLUT4 from all the intracellular compartments (4, 5). On the other hand, many biochemical studies have indicated that insulin-responsive GLUT4 is associated with the endosomal recycling pathway and with a more specialized postendosomal compartment (for review, see Ref. 1). Additionally, more recent works have suggested that insulin recruits GLUT4 to the plasma membrane from the postendosomal vesicle-associated membrane protein (VAMP)1-2-positive compartment as well as from the cation-dependent mannose-6-phosphate receptor (CD-M6PR)-positive compartment that is dynamically shuttling between endosomes and the trans-Golgi network (TGN) (6, 7). While such a diversity of the subcellular localization of GLUT4 would hamper our understanding of the insulin-regulated trafficking of GLUT4, it implies that the transit of GLUT4 to and from such distinct subcellular compartments would be regulated by different signals and trafficking machinery (e.g. Refs. 8 and 9).

The actin and microtubule cytoskeleton networks have been implicated in the subcellular movements of the GLUT4-containing membranes. While the role of the actin filaments in insulin-induced GLUT4 translocation has been reported with relatively consistent results (10–13), the relevance of the microtubules to the insulin action has been controversial. Thus, depolymerization of the microtubules caused a complete cessation of the basal linear tracking movements of green fluorescent protein (GFP)-tagged GLUT4, and inhibited insulin-induced GLUT4 translocation and glucose uptake by 40–70% in 3T3-L1 adipocytes (14, 15). Likewise, Olson et al. (16) also showed that nocodazole treatment of 3T3-L1 adipocytes resulted in about 80% inhibition of insulin-stimulated translocation of GLUT4. In addition, the GLUT4-containing vesicles have been shown to be associated with α-tubulin and polyvinylidene fluoride microtubules under the in vivo and in vitro conditions, respectively (16, 17). Furthermore, the two molecular motor proteins, dynemin and kinesin, that direct the vesicles along the microtubules toward the minus and plus ends, respectively.

1 The abbreviations used are: VAMP, vesicle-associated membrane protein; IRAP, insulin-regulated aminopeptidase; TGN, trans-Golgi network; CD-M6PR, cation-dependent mannose 6-phosphate receptor; MeSO₃, dimethyl sulfoxide; HRP, horseradish peroxidase; PI 3-kinase, phosphoinositide 3-kinase; GTPγ-S, guanosine 5’O-(3-thiotriphosphate); H-7, 1-(5-isouquinolinesulfonyl)-2-methylpiperazine; PBS, phosphate-buffered saline; PIPES, 1,4-piperazinediethanesulfonic acid.

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‡ To whom correspondence should be addressed. Tel.: 81-27-220-8836; Fax: 81-27-220-8893; E-mail: hshibata@showa.gunma-u.ac.jp.

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have been implicated in the subcellular localization and insulin-responsive movements of GLUT4 (17, 18). These observations have strongly supported a physiological significance for the microtubules in the subcellular trafficking of GLUT4. On the other hand, more recent studies demonstrated that depolymerization of the microtubules with lower concentrations (2–3 μM) of nocodazole did not affect insulin-stimulated GLUT4 translocation and glucose uptake (19, 20). They also argued that the inhibition of glucose transport with higher concentrations (30 μM or more) of nocodazole derived from a direct inhibition with the drug of the glucose transporter activity itself.

Thus there remain important issues to be addressed on the role of the microtubules in the GLUT4 trafficking and the action of nocodazole as a microtubule-depolymerizing tool. In addition, all of the studies were carried out using 3T3-L1 adipocytes, while the relevance of the microtubules to the GLUT4 trafficking has never been precisely studied in primary adipocytes. Structurally, isolated adipocyte has a spherical shape with a large diameter (usually 50–100 μm), a huge lipid droplet inside the cell, and the thin cytoplasm confined beneath the plasma membrane. Because of these morphological features, the relationship between the microtubules and GLUT4 has not been visualized in primary adipocytes. Functionally, although both GLUT1 and GLUT4 isoforms are expressed in primary adipocytes, the latter is far more abundant and virtually accounts for the major portion of the cellular glucose uptake (21). This feature makes it easier to study the effect of nocodazole on the glucose transport activity of GLUT4. Additionally, some of the observations described in 3T3-L1 adipocytes may not be applicable to primary adipocytes; for example, endothelin-1 stimulates glucose transport and GLUT4 translocation via a Gq/11-dependent stimulation of the endosomal recycling in 3T3-L1 adipocytes (22–24) whereas it inhibits the insulin effect without affecting the basal glucose transport in primary adipocytes (Refs. 25 and 26). In the present study, we reconstructed the three-dimensional images to investigate the spatial relationship between the microtubules and GLUT4 in isolated rat adipocytes. In addition, we investigated the role of the microtubules in the insulin-stimulated translocation of GLUT4. The results of our study suggested that insulin recruits GLUT4 to the cell surface from at least two distinct intracellular compartments via distinct traffic routes with differential microtubule dependence in rat primary adipocytes.

EXPERIMENTAL PROCEDURES

Materials—Nocodazole was purchased from ICN (Costa Mesa, CA) and dissolved in dimethyl sulfoxide at 20 mM (stock solution). The maximal final concentrations of nocodazole and Me2SO were 100 μM and 0.5%, respectively. The same concentration of Me2SO (0.5%) was

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Fig. 1. Effects of nocodazole on the microtubule integrity. A, dose-dependent disruption of the microtubules by nocodazole. Isolated rat adipocytes in Buffer A were incubated for 30 min without (a and e) or with 1 (b and f), 10 (c and g), or 100 (d and h) μM of nocodazole. The cells were fixed and the microtubules were visualized with anti-β-tubulin antibody as described under “Experimental Procedures.” a–d, images obtained by confocal microscopy at ×400 magnification. e–h, enlarged images of representative cells in a through d, respectively. Bars, 10 μm. B, time course of disruption of the microtubules by nocodazole. Adipocytes in Buffer A were incubated without (a) or with 100 μM nocodazole for 5 (b), 15 (c), or 30 (d) minutes at 37 °C. The cells were then fixed and immunostained for β-tubulin. Bars, 10 μm.

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H. Shibata and L. B. Liu, unpublished observations.
Addendum to the controls. The D^+-(62–85) peptide (RETQIAKNGEQS-FRVDLRTLREY) was synthesized as described previously (27), dissolved to a concentration of 1.0 mM in 0.1 M NaCl, and activated by incubation at 37 °C overnight prior to the addition to cells. Rabbit polyclonal antibodies against GLUT4 were raised as described previously (27). Rabbit polyclonal anti-GLUT1 antibody was a generous gift from Dr. Kuniaki Takata (Gunma University). Mouse monoclonal anti-β-tubulin (clone TUB 2.1) and anti-vimentin (clone V9) antibodies were from ICN Biomedicals (Aurora, OH) and Lab Vision (Fremont, CA), respectively. Rabbit anti-VAMP-2 and sheep anti-VAMP-3 antibodies were kindly provided by Masami Takahashi (Mitsubishi Kasei Institute of Life Sciences) and Jeffrey E. Pessin (University of Iowa), respectively. Rabbit polyclonal anti-insulin responsive aminopeptidase (IRAP) antibody was kindly donated by Mitsuhiro Hashimamoto (Ehime University). Alexa Fluor-labeled anti-mouse IgG and anti-rabbit IgG antibodies were obtained from Molecular Probes (Eugene, OR). Mouse monoclonal anti-phosphotyrosine antibody (clone 4G10) was purchased from Upstate (Charlottesville, VA) and Rabbit anti-phospho-c-Cbl (Tyr-774) and anti-phospho-Akt (Ser-473) antibodies were from Cell Signaling Technology (Beverly, MA). GTPγS tetralithium salt was purchased from Roche Applied Science. Endothelin-1 was from Peptide Institute, Inc. (Osaka, Japan).

Preparation of Isolated Rat Adipocytes and Permeabilization—Isolated adipocytes were prepared by the collagenase method from epidymal adipose tissues of Sprague-Dawley rats (from Charles-River, −170–220 g) (28). Unless otherwise specified, isolated cells were suspended in Buffer A (25 mM Krebs-Henseleit Hepes buffer supplemented with 40 mM bovine serum albumin (Fraction V) and 3 mM pyruvate, pH 7.4). The cells to be permeabilized by electroporation were suspended in high K^+/low Ca^2+ buffer designated as Buffer X (118.0 mM KCl, 4.74 mM NaCl, 0.38 mM CaCl_2, 1.0 mM EGTA, 1.18 mM MgSO_4, 1.18 mM KH_2PO_4, 23.4 mM Hepes/KOH, 40 mM bovine serum albumin, 3 mM pyruvate, pH 7.4). The electroporation was carried out four times in a Gene-Pulser (from Bio-Rad) set at 25 microfarads and 2 kV/cm as described previously (29).

Visualization of the Microtubules and GLUT4 in Isolated Adipocytes—For visualization of the microtubules and GLUT4 by laser confocal microscopy, cells were washed three times with Buffer A and fixed with 3% (w/v) paraformaldehyde in phosphate-buffered saline (PBS) for 1 h at room temperature. The cells were permeabilized and nonspecific binding sites were blocked in PBS containing 0.1% saponin, 1% bovine serum albumin, and 3% normal goat serum for 45 min at room temperature. The cells were then incubated with rabbit anti-GLUT4 serum (1:1000 dilution) and mouse anti-β-tubulin antibody (1:500) for 2 h at room temperature, and washed three times with PBS containing 0.1% saponin. Next, the cells were incubated with Alexa Fluor 488-conjugated anti-rabbit IgG and Alexa Fluor 568-conjugated anti-mouse IgG (1:200 dilution) for 1 h at room temperature. Finally, the cells were washed with PBS containing 0.1% saponin, mounted in 50% glycerol saturated with n-propyl gallate as an anti-bleaching reagent, and observed with an epifluorescence microscope (BX-50; Olympus, Tokyo) equipped with a laser confocal system (MRC-1024; Bio-Rad, Hemel Hempstead, UK). Captured images were processed with Bio-Rad LaserSharp software. The three-dimensional images were reconstructed from serial confocal images taken at 0.5 or 1-μm intervals along the z-axis.

Analysis of GLUT4 Translocation by Confocal Microscopy—After incubation without or with nocodazole for 30 min at 37 °C, cells were treated without or with 10 nM insulin for 30 min, and then immunostained with anti-GLUT4 antibody as described above. Confocal images from 100 cells were obtained for each condition, and the individual cells were graded according to the continuity of the GLUT4 fluorescence signals at the peripheral rim of the cell. Thus, cell scored 0 without any continuous GLUT4 signal, 1 with continuity less than the third of the circumference, 2 with continuity between one- and two-thirds, and 3 with more than two-thirds.

Extraction and Measurement of Polymerized and Unpolymerized Tubulin—Polymerized and unpolymerized tubulin were separated as described by Breitfeld et al. (30) with a slight modification. After incubation without or with nocodazole for 30 min at 37 °C, the cells were washed with PBS and incubated with extraction buffer (2 mM glycerol, 100 mM MgSO_4, 1 mM EGTA, 0.1% Triton X-100, 0.1 mM PIPES/Na, pH 6.9, 0.5 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 5 μg/ml pepstatin A, 10 μg/ml aprotinin) for 30 min at 37 °C. At the end of
incubation, cells were centrifuged at 500 \( \times g \) for 30 s. The resulting infranatant below the cell cake, which contains monomeric tubulin, was incubated with SDS lysis buffer (0.4 M NaCl, 0.5% SDS, and 25 mM Tris/Cl, pH 7.4) for 5 min at room temperature, and boiled for 3 min before centrifugation (at 15,000 \( \times g \)) for 2 min. The supernatant was subjected to SDS-polyacrylamide gel electrophoresis and immunoblotting with anti-\( \beta \)-tubulin antibody. Unextracted polymerized tubulin associated with the cells was recovered by incubation with SDS lysis buffer for 5 min at 37 °C, followed by centrifugation for 30 s at 500 \( \times g \). The infranatant was boiled for 3 min and centrifuged at 15,000 \( \times g \) for 2 min. The supernatant was subjected to SDS-polyacrylamide gel electrophoresis and immunoblotting. The immunoblots were semi-quantified by using NIH image software.

Measurement of 3-O-Methyl-D-glucose Uptake—The cellular glucose transport activity was estimated by measuring the rate of 0.1 mM 3-O-methyl-D-glucose uptake by the oil flotation method as described previously (29).

Subcellular Membrane Fractionation and Immunoblotting—The plasma membrane and low density microsomal (LDM) fractions were prepared by differential and sucrose density gradient centrifugation as described previously (27). Proteins in the plasma membrane and LDM fractions were separated on SDS-polyacrylamide gels and transferred to a polyvinylidene difluoride membrane (from Millipore). The polyvinylidene difluoride membrane was blocked with solution containing 5% milk and \( \beta \)-tubulin (b, f, j, and n). The three-dimensional images were reconstructed from serial confocal images taken along the z-axis as described under “Experimental Procedures.” c, g, h, and o, merged images; d, h, l, and p, enlarged images of representative cells in c, g, h, and o, respectively.

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![Reconstructed three-dimensional images of the subcellular localization of GLUT4 and the microtubules](Image 238x401 to 563x737)

**FIG. 3.** Reconstructed three-dimensional images of the subcellular localization of GLUT4 and the microtubules. After incubation in the absence (a–d and i–l) or presence (e–h and m–p) of 100 \( \mu M \) nocodazole for 30 min at 37 °C, adipocytes in Buffer A were incubated for an additional 20 min without (a–d and e–h) or with (i–l and m–p) 10 \( \mu M \) insulin. The cells were then fixed and subjected to immunostaining for GLUT4 (a, e, i, and m for plasma membrane and LDM; b, f, j, and n for plasma membrane and low density microsomal), rabbit anti-IRAP (1:1000 dilution), rabbit anti-GLUT4 (1:1000 dilution), rabbit anti-GLUT1 (1:1000 dilution), rabbit anti-GLUT3 (1:1000 dilution), rabbit anti-GLUT4 (1:1000 dilution), and a sheep anti-VAMP-3 (1:1000 dilution) antibodies overnight at 4 °C. The membrane was washed and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 h at room temperature. Following an extensive wash, the blots were visualized by using ECL Western blotting System (Amersham Biosciences).

**Cell Surface GLUT4 Labeling and Measurement of GLUT4 Endocytosis**—GLUT4 in the plasma membrane was labeled by limited proteolysis with trypsin as described by Czech and Buxton (31) with a slight modification. Briefly, adipocytes in Buffer A were stimulated with 10 \( \mu M \) insulin for 20 min at 37 °C, and then incubated for 15 min in the presence of 3 mM potassium cyanide in order to inhibit GLUT4 recycling by deprivation of metabolic energy. Then the cells were treated without or with 100 \( \mu M \) nocodazole for 30 min. TPCK-treated trypsin (a final concentration of 1 mg/ml) was added to the cells for the last 20 min of nocodazole treatment. For measurement of the amount of GLUT4 in the plasma membrane, soybean trypsin inhibitor was added to a final concentration of 2 mg/ml at the end of the incubation, and the cells were washed three times with STE buffer (250 mM sucrose, 10 mM Tris/Cl and 1 mM EDTA/Na, pH 7.4), homogenized with a Dounce glass homogenizer, and subjected to subcellular membrane fractionation as described above. For measurement of GLUT4 endocytosis, the trypsin-treated cells were washed three times with Buffer A after the addition of soybean trypsin inhibitor (2 mg/ml), resuspended in fresh Buffer A and incubated for 0 (immediately washed), 5, 10, or 20 min at 37 °C. At the end of the incubation, the cells were washed three times with STE buffer, homogenized, and subjected to subcellular fractionation and immunoblotting with anti-GLUT4 antibody.

**Measurement of Tubulin Associated with GLUT4-containing Membranes**—The LDM fractions were prepared as described above and incubated with 5 \( \mu l \) of anti-GLUT4 antiserum and 20 \( \mu l \) (bed volume) of protein A-Sepharose (Amersham Biosciences) in STE buffer for 2 h at 4 °C. The beads were then washed three times with STE buffer. The proteins associated with the immunoadsorbed membranes were solubilized with 1% (v/v) Nonidet P-40, and subjected to immunoblotting with anti-\( \beta \)-tubulin antibody.

Detection of Phosphorylated Proteins—Adipocytes in Buffer A were washed with STE buffer and homogenized with a Dounce glass homogenizer in SDS sample buffer (62.5 mM Tris/Cl, pH 6.8, 2% SDS, 10% glycerol, 1 mM Na2VO4) (for phosphotyrosine and phospho-c-Cbl) or homogenizing buffer (50 mM HEPES/Na, pH7.5, 100 mM KCl, 10% glycerol, 0.2 mM EDTA, 2 mM EGTA, 1 mM diithiothreitol, 1 mM microcystin-LR, 1 \( \mu g/ml \) pepstatin A, 20 KIU/ml aprotinin, 1 \( \mu g/ml \) leupeptin, 0.2 mM phenylmethylsulfonyl fluoride, 10 \( mM \) NaF, 50 \( mM \) \( \beta \)-glycerophosphate) (for phospho-Akt). After centrifugation for 2 min at 3,000 \( \times g \), the infranatant fraction below the fat was subjected to SDS-polyacrylamide gel electrophoresis and immunoblotting with anti-phosphotyrosine, anti-phospho-c-Cbl (Tyr-774) or anti-phospho-Akt (Ser-473) antibodies.

Phosphodiesterase Assay—Phosphodiesterase was assayed as described previously (32) by incubation of the enzyme with 0.1 \( mM \) ATP.
[H]cAMP (PerkinElmer Life Sciences) for 5 min at 30 °C. Adipocytes were incubated for an additional 30 min without or with 10 nM insulin. The cells were then fixed and immunostained for GLUT4. Confocal images from a total of 100 cells were taken and graded according to the intensity of the GLUT4 signals at the cell surface as described under “Experimental Procedures.” A, representative images of the cells treated with none (a), insulin (b), nocodazole (c), or nocodazole plus insulin (d). Bars, 20 μm. B, effects of insulin on the cell surface localization of GLUT4 in control (left panel) and nocodazole-treated (right panel) cells. Shaded columns, without insulin; closed columns, with insulin.

RESULTS

To investigate the physiological role of the microtubules in the subcellular trafficking of GLUT4, we first examined the effect of nocodazole, a microtubule-depolymerizing reagent, on the integrity of the microtubules in isolated rat adipocytes by morphological and biochemical methods. Because the cytoplasm of isolated adipocytes is generally confined to a thin rim beneath the plasma membrane due to the presence of a lipid droplet, we obtained confocal images near the cell surface for observation of the microtubules. As shown in Fig. 1, the microtubules were observed as filamentous networks developed in the cytoplasm. Treatment with nocodazole for 30 min at 37 °C resulted in a dose-dependent disappearance of these filamentous signals (Fig. 1A). A significant portion of the microtubules was disrupted with nocodazole at 1 μM, and the disruption seemed nearly complete at 100 μM. With this concentration of nocodazole, we examined the time course of the disruption of the microtubules. As depicted in Fig. 1B, nocodazole caused a time-dependent disruption of the microtubules, with a nearly...
complete loss of the filamentous signals at 30 min.

These morphological data were corroborated by biochemical measurement of the polymerized tubulin. As illustrated in Fig. 2, the amount of polymerized tubulin decreased in a dose-dependent manner by nocodazole treatment for 30 min at 37 °C, with a concomitant increase in the amount of tubulin monomer. Approximately 50% of the polymerized tubulin was lost with nocodazole at 1 μM, and the effect of nocodazole was maximal at 100 μM.

Previous studies have shown that disruption of the microtubules resulted in dispersion of the perinuclear GLUT4 compartments in 3T3-L1 adipocytes (14, 17, 20), while the consequence of microtubule depolymerization on the subcellular localization of GLUT4 has never been studied in primary adipocytes. To investigate the spatial relationship between the microtubules and GLUT4, we reconstructed three-dimensional images from serial confocal sections of double-immunostained cells. Fig. 3 illustrates the two-dimensional projections of the reconstructed three-dimensional images. The three-dimensional images revealed the microtubules as reticular filamentous network developed throughout the cytoplasm in the basal cells (Fig. 3b). GLUT4, on the other hand, were shown as punctate signals distributed throughout the cytoplasm with intense accumulation around the nuclei (Fig. 3a). Some, but not all of the GLUT4 signals were colocalized with the microtubules (Fig. 3, c and d, arrowheads), suggesting association of the GLUT4-containing membranes with the microtubules. Insulin stimulation did not cause any significant alterations in the distribution of microtubules (Fig. 3j), while the cell surface became diffusely labeled with the GLUT4 signals, with concomitant decrease in the punctate and perinuclear signals in the cytoplasm, suggesting translocation of GLUT4 to the cell surface (Fig. 3i). In addition, the association of GLUT4 with the microtubules apparently decreased in insulin-stimulated cells (Fig. 3, k and l). Nocodazole treatment caused nearly completely disruption of the microtubules throughout the cytoplasm, resulting in more random distribution of GLUT4 in the cytoplasm (Fig. 3c). In addition, the perinuclear GLUT4 signals became moderately dispersed although they were still in the vicinity of the nuclei. In such microtubule-disrupted cells, insulin stimulation apparently caused subcellular redistribution of GLUT4, but the insulin-induced surface labeling with GLUT4 and decrease in the cytoplasmic punctate GLUT4 signals were not so consistent among the cells as in control (nocodazole-untreated) cells (Fig. 3m).

Such inconsistency in the extent of the insulin effect among nocodazole-treated cells led us to further analyze the GLUT4 distribution in the microtubule-deficient state. As we and others previously reported, insulin-stimulated rat adipocytes showed characteristic continuous GLUT4 signals at the cell surface on confocal planes due to GLUT4 translocation to the plasma membrane, whereas they were found in the perinuclear region and in punctate spots distributed throughout the cytoplasm in the basal cells (12, 34). Hence, we obtained confocal images from a total of 100 cells for each condition, and individual cells were graded according to the length of the continuous
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Fig. 9. Effects of nocodazole on insulin signals. A and B, effects of nocodazole on insulin-dependent tyrosine phosphorylation. Adipocytes in Buffer A were treated without or with nocodazole (100 μM) for 30 min at 37 °C. Then the cells were stimulated without or with insulin (10 nM) for 20 min (A) or 1 min (B). At the end of incubation, cells were washed and solubilized in SDS sample buffer. The proteins in the lysates were separated on SDS-polyacrylamide gel and immunoblotted with anti-phosphotyrosine antibody (A) or anti-phospho-c-Cbl antibody (B). C, effect of nocodazole on insulin-stimulated protein kinase B phosphorylation. Adipocytes in Buffer A were incubated without or with nocodazole (100 μM) for 30 min and then stimulated without or with insulin (10 nM) for 20 min. At the end of incubation, the cells were washed with STE and homogenized in homogenizing buffer as described under “Experimental Procedures.” The lysates were subjected to SDS-polyacrylamide gel electrophoresis and immunoblotting with anti-phospho-Akt (Ser-473) and anti-Akt antibodies. D, effect of nocodazole on insulin-dependent stimulation of phosphodiesterase. Adipocytes in Buffer A were incubated for 30 min without or with nocodazole (100 μM) and stimulated without (open columns) or with insulin (2 nM) (closed columns) for 15 min. The phosphodiesterase activity was assayed as described under “Experimental Procedures.” Results are the means ± S.E. (n = 3–6). NCZ, nocodazole.

that rat adipocytes have well developed networks of the vimentin-containing intermediate filaments throughout the cytoplasm. Disruption of the microtubules with nocodazole, however, had little effect on the distribution of the vimentin-containing intermediate filaments.

To evaluate the impaired insulin effect in microtubule-disrupted cells, we next examined the effects of nocodazole on the insulin-stimulated glucose transport and GLUT4 translocation. As shown in Fig. 6, nocodazole inhibited the insulin-stimulated glucose transport activity in a dose-dependent manner without affecting the basal transport activity. However, there were discernible differences between the effects of nocodazole on the integrity of the microtubules (Figs. 1A and 2) and on the insulin-stimulated glucose transport activity. First, the half-maximal concentration of nocodazole (about 1 μM) to depolymerize the microtubules had little effect on the insulin-stimulated glucose transport activity. Second, the maximal concentration of nocodazole (100 μM), which caused nearly complete disruption of the microtubules, inhibited the insulin-stimulated glucose transport activity by only 55%. Such discrepancies in the nocodazole-sensitivity led us to examine whether the inhibition of the glucose transport activity was associated with inhibition of GLUT4 translocation in nocodazole-treated cells. As illustrated in Fig. 7, the subcellular membrane fractionation assay showed that insulin-induced GLUT4 translocation was partially (by 47%) inhibited by treatment with the maximal concentration of nocodazole. In another assay, we measured the amounts of the cell surface GLUT4 by trypsin cleavage, which generates a 35-kDa fragment of the transporter (27, 31). As shown in Fig. 8, insulin stimulation caused a 5-fold increase in the trypsin-cleaved 35-kDa fragment of GLUT4 in the plasma membrane fraction. This insulin effect was inhibited by 65% in nocodazole-treated cells, suggesting that insulin-induced insertion of GLUT4 into the plasma membrane was actually inhibited in microtubule-disrupted cells. Thus, it seemed that the integrity of the microtubules is required for insulin to fully stimulate GLUT4 translocation and glucose transport although the insulin action would not be affected unless a considerable portion (50% or more) of the microtubules is disrupted.

To exclude the possibility that nocodazole inhibited the in-
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Insulin action by any mechanisms unrelated to the microtubules, we investigated the effects of nocodazole on the insulin signaling as well as on the glucose transporter activity itself. Immunoblot analyses of tyrosine-phosphorylated proteins in cell lysates showed that insulin stimulated tyrosine phosphorylation of proteins corresponding in size to IRS-1/2 (180–190 kDa), the β-subunit of the insulin receptor (95 kDa) and IRS-3 (60 kDa) (Fig. 9A, arrows). Insulin-mediated tyrosine phosphorylation of these proteins was not affected by nocodazole treatment (Fig. 9A). Likewise, nocodazole treatment did not affect insulin-induced tyrosine phosphorylation of c-Cbl, a component of the PI 3-kinase-independent signaling pathway involved in GLUT4 translocation (35) (Fig. 9B, arrow). In addition, we did not see any inhibitory effects of nocodazole on the insulin-induced phosphorylation of protein kinase B/Akt (Fig. 9C) or activation of the insulin-sensitive cAMP phosphodiesterase, a downstream target of protein kinase B/Akt (36) (Fig. 9D). Furthermore, as shown in Fig. 10, neither nocodazole (100 μM) nor wortmannin (100 nM) inhibited the glucose transport activity in KCN-treated cells that had been stimulated with insulin, whereas phloretin, a direct inhibitor of glucose transporters, significantly inhibited the cellular glucose uptake, indicating that nocodazole was without effect on the glucose transporter activity itself. Thus, it seemed unlikely that the inhibition of the insulin effect on glucose transport in nocodazole-treated adipocytes derived from attenuation of the proximal insulin signals or direct inhibition of the glucose transporter activity.

In the next series of experiments, we investigated the mechanisms of inhibition of insulin-induced GLUT4 translocation in mictotubule-disrupted cells. Since the amount of GLUT4 on the plasma membrane depends on the balance between exocytotic recruitment and endocytosis of the transporter, the inhibition of insulin-induced GLUT4 translocation in microtubule-deficient cells may derive from altered recycling rate of GLUT4 in the insulin-responsive pool; either slowed exocytosis or accelerated endocytosis of GLUT4. Another possibility is that the insulin recruits GLUT4 from two distinct compartments with different microtubule dependence in the traffic to the cell surface; one is totally dependent on the microtubules in trafficking to the plasma membrane while another is less or not dependent on the microtubule integrity. The GLUT4 in the former compartment would not be able to translocate to the plasma membrane in the absence of the microtubules, resulting in a decrease in the maximal insulin effect.

To elucidate these points, we measured the time course of insulin stimulation of the glucose transport activity in nocodazole-treated cells. As shown in Fig. 11, the time-course of insulin stimulation of the glucose transport activity was significantly delayed in microtubule-disrupted cells (t1/2 were 7 and 2.3 min in nocodazole-treated and control cells, respectively). This delay was not brought about simply as a consequence of a decline in the maximal transport activity because a reduction of the insulin concentration from 10 to 0.3 nM resulted in an ~50% decrease in the maximal glucose transport activity without a significant alteration in the time course of activation.

Next, we examined the rate of GLUT4 endocytosis in nocodazole-treated cells. As depicted in Fig. 12, the time-course of endocytosis of the trypsin-cleaved 35-kDa fragment of GLUT4 was little affected by nocodazole treatment. These data suggested that the delay in the insulin activation of the glucose transport activity was not caused by accelerated endocytosis but due to slowed exocytosis of GLUT4.

Thirdly, we tested if the impaired maximal glucose transport activity would be restored by inhibition of GLUT4 endocytosis to the same level as in control cells. To this end, we used the MHC class-I antigen-derived peptide, Dκ(62–85), which has been shown to nearly completely inhibit GLUT4 endocytosis (27, 37). As shown in Fig. 13, inhibition of GLUT4 endocytosis resulted in a marked enhancement of the insulin-stimulated glucose transport activity in control cells, consistent with previous studies (37, 27). In nocodazole-treated cells, the insulin-stimulated glucose transport activity was enhanced by Dκ(62–85) peptide, but was never restored to the same level as in control cells. These data suggest that the inhibition of the insulin-stimulated glucose transport and GLUT4 translocation in microtubule-deficient cells was not caused solely by altered recycling rate of GLUT4 in the whole insulin-responsive pool. Instead, these data indicate the existence of a microtubule-de-
Fig. 12. Effect of nocodazole on the time course of GLUT4 endocytosis. Adipocytes in Buffer A were stimulated with 10 nM insulin for 20 min at 37 °C. The cells were then incubated with 3 mM potassium cyanide for 15 min, followed by incubation without or with nocodazole (100 μM) for an additional 30 min. Then TPCCK-treated trypsin was added to the cells at a final concentration of 1 mg/ml, and incubation was continued for 20 min. At the end of the incubation, soybean trypsin inhibitor (final concentration, 2 mg/ml) was added to the incubation buffer and the cells were washed with Buffer A. The cells were resuspended in fresh Buffer A and incubated for 0 (immediately washed), 5, 10, or 20 min at 37 °C. At the end of the incubation, the cells were washed with STE buffer, homogenized, and then subjected to subcellular fractionation. The LDM fractions were subjected to SDS-polyacrylamide gel electrophoresis and immunoblotting with anti-GLUT4 antibody as described under “Experimental Procedures.” A, representative immunoblot data. B, relative amounts of the 35-kDa fragments of GLUT4 in the LDM fraction. The relative intensities of the 35-kDa bands were quantified as described under “Experimental Procedures.” Results are the means ± S.D. of three determinations. Open circles, without nocodazole; closed circles, with nocodazole.

Fig. 13. Inhibition of GLUT4 endocytosis does not restore the glucose transport activity in nocodazole-treated cells. After incubation without (○) or with (●) 100 μM of nocodazole for 30 min at 37 °C, adipocytes in Buffer A were incubated with 10 nM insulin for 15 min. At the end of incubation, 50 μM Dk-(62–85) peptide was added, and the cells were incubated for an additional 30 min. The glucose transport activity was assayed at the indicated time points. Results are the means ± S.E. (n = 3–6).

Fig. 14. Effects of nocodazole of insulin-induced translocation of GLUT1, IRAP, and VAMPs. After incubation for 30 min in the absence or the presence of 100 μM nocodazole, adipocytes in Buffer A were incubated for an additional 30 min with 10 nM insulin. At the end of incubation, the cells were washed with STE buffer, homogenized, and subjected to subcellular fractionation and immunoblotting for GLUT1, IRAP, VAMP-2, and VAMP-3. PM, plasma membrane fractions; LDM, low density microsomal fractions.

pendent subpopulation in the insulin-responsive GLUT4 pool.

To characterize the microtubule-dependent and independent membrane traffic, we investigated the effects of nocodazole on insulin-induced translocation of other membrane-associated proteins. As shown in Fig. 14, nocodazole had minor effects on the insulin-stimulated translocations of GLUT1 and VAMP-3/
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Fig. 15. Effects of nocodazole on the microtubules and the glucose transport activity stimulated with insulin or GTPγS in electrically permeabilized cells. A, effects of nocodazole on the microtubules in electrically permeabilized cells. Adipocytes in Buffer X were electrically permeabilized as described under “Experimental Procedures” and incubated for 30 min at 37 °C without (a) or with 1 (b) or 10 (c) μM nocodazole. Then the cells were washed and the microtubules were visualized with anti-β-tubulin antibody. The three-dimensional images were reconstructed from 15 sequential confocal images taken at 1-μm intervals along the z-axis. B, effects of nocodazole on the glucose transport activity stimulated with insulin or GTPγS in electrically permeabilized adipocytes. Adipocytes in Buffer X were electrically permeabilized and incubated for 30 min at 37 °C with the indicated concentrations of nocodazole. Then the cells were stimulated without (○) or with insulin (100 nM) (●) or GTPγS (1 mM) (▲) for 20 min. At the end of incubation, the cellular glucose transport activity was assayed. Results are the means ± S.E. (n = 3–6).

GLUT4 Trafficking and Microtubules

To further confirm the notion that the insulin recruits GLUT4 to the plasma membrane from distinct compartments with differential microtubule dependence, we compared the sensitivity to nocodazole of the insulin- and GTPγS-stimulated glucose transport in electrically permeabilized adipocytes. Previous studies have shown that both insulin and GTPγS stimulate glucose transport and GLUT4 translocation (38, 39, 27), GTPγS selectively stimulates recycling via the endosomal recycling system (9). Since we previously observed increased sensitivity to H-7, a protein kinase inhibitor, by electroporation of rat adipocytes suspended in high-K+/low-Ca2+ buffer (29), we first examined the effect of nocodazole on the microtubules in electrically permeabilized cells. As shown in Fig. 15, electrically permeabilized cells showed higher sensitivity to nocodazole compared with intact cells; the microtubules were completely disrupted with nocodazole at 10 μM (Fig. 15A). At this concentration of nocodazole, the insulin-stimulated glucose transport activity was inhibited by 40%, whereas the GTPγS-stimulated glucose transport activity was not affected (Fig. 15B). Intriguingly, insulin and GTPγS stimulated the glucose transport activity to the comparable level in the absence of the microtubules. Thus, it seemed that the GTPγS recruits GLUT4 to the cell surface presumably from the endosomal recycling system by a microtubule-independent mechanism whereas insulin recruits GLUT4 to the cell surface from at least two distinct compartments with differential microtubule dependence. In addition, such differential sensitivity to nocodazole between the effects of insulin and GTPγS provides further evidence that nocodazole does not inhibit insulin-stimulated glucose transport by direct inhibition of the transporter activity.

We also examined the subcellular localization of GLUT4 and the microtubules in GTPγS-stimulated cells. The reconstructed three-dimensional images depicted that GTPγS like insulin caused diffuse labeling of the cell surface with the GLUT4 signals both in the control (Fig. 16A, e and f) and nocodazole-treated cells (Fig. 16A, g and h). However, compared with insulin-stimulated cells (see Fig. 3), there remained more punctate GLUT4 signals in the cytoplasm with GTPγS stimulation, some of which were still associated with the microtubules (Fig. 16A, e and f). To further assess the differences between the effects of insulin and GTPγS, we measured the amount of tubulin associated with the GLUT4-containing membranes immunoisolated from insulin- or GTPγS-stimulated cells. As illustrated in Fig. 16B, β-tubulin was present in the immunoisolated GLUT4-containing membranes in the basal state. Significantly, insulin but not GTPγS markedly reduced the association of β-tubulin with the GLUT4-containing membranes. These results are consistent with our morphological data and the observation that insulin causes dissociation of α-tubulin from the GLUT4-containing membranes in the insulin-sensitive fractions in 3T3-L1 adipocytes (17). Our data also support the notion that the two reagents recruit GLUT4 by distinct mechanisms with differential microtubule dependence.
GLUT4 Trafficking and Microtubules

The results of the present study showed that insulin recruits GLUT4 to the plasma membrane from at least two distinct intracellular compartments via distinct traffic routes with differential microtubule dependence in rat primary adipocytes. First, microtubule disruption with nocodazole resulted in only a partial (about 50%) inhibition of the insulin-stimulated glucose transport (Fig. 6) and GLUT4 translocation (Figs. 7 and 8). Importantly, the impaired insulin effect was not fully restored to the level in control cells by inhibition of GLUT4 endocytosis (Fig. 13), suggesting that the inhibition did not derive solely from alterations in the recycling rate of the whole insulin-responsive GLUT4. Instead, our data clearly indicate the existence of two GLUT4 subpopulations; one is unable to translocate to the cell surface in response to insulin in the absence of the microtubules while another is not or less microtubule-dependent.

Second, although the latter subpopulation of GLUT4 can undergo insulin-induced translocation to the plasma membrane, the time course of insulin activation of the glucose transport activity was markedly (~3-fold) delayed by disruption of the microtubules (Fig. 11). Since the rate of GLUT4 endocytosis was not significantly affected (Fig. 12), it is likely that the delay originated from slowed exocytosis of this subpopulation of GLUT4. The apparent slowdown of insulin-induced GLUT4 exocytosis could be interpreted in two ways. One interpretation is that insulin recruits these two subpopulations of GLUT4 via distinct routes with different traffic kinetics, and a faster traffic route is more susceptible to and eliminated by microtubule disruption, unveiling another slower traffic pathway that is independent of the microtubules. Alternatively, in addition to the microtubule-dependent route, another routes may also be affected at some step(s) by disruption of the microtubules, causing a slowdown of GLUT4 exocytosis. In either case, our data show the existence of two distinct GLUT subpopulations that take distinct traffic routes to the cell surface with differential microtubule dependence.

Third, in electically permeabilized adipocytes, the insulin-stimulated glucose transport was inhibited by 40% by disruption of the microtubules whereas that stimulated with GTPγS was not affected (Fig. 15). Intriguingly, both insulin and GTPγS stimulated the glucose transport activity to the comparable level in the absence of the microtubules. In the light of previous observations that GTPγS selectively stimulates recycling via the endosomal system, these data suggest that insulin recruits GLUT4 from the endosomal and non-endosomal compartments, and the former may correspond to the GLUT4 subpopulation that is not or less dependent on the microtubules and the latter to the microtubule-dependent one. This notion also seems consistent with the results that insulin-stimulated translocation of GLUT1 and VAMP-3 but not that of IRAP and VAMP-2 was insensitive to microtubule disruption (Fig. 14) as well as with the observation by other investigators that insulin-induced translocation of the transferrin receptor was not affected by microtubule disruption (14). It is unclear at present to what subcellular compartment(s) the microtubule-dependent GLUT4 subpopulation localizes, but since insulin-stimulated translocation of IRAP and VAMP-2 was partially inhibited by microtubule disruption, GLUT4 seems to share the compartment(s) with IRAP and VAMP-2.

On the other hand, while there is a controversy on the mechanism of action of nocodazole especially in 3T3-L1 adipocytes (14, 15, 16, 19, 20), our results show that nocodazole has no direct inhibitory effect on the glucose transporter activity (Fig. 10). Under our experimental conditions, the maximal concentration (100 μM) of nocodazole to depolymerize the microtubules did not inhibit the glucose transport activity in ATP-deprived cells that had been stimulated with insulin. This was further supported by the observation that insulin and GTPγS showed different sensitivity to nocodazole in stimulation of glucose transport in electically permeabilized cells (Fig. 15B). Our results are seemingly inconsistent with recent reports that lower concentrations (2–3 μM) of nocodazole disrupt the microtubules without affecting insulin-induced GLUT4 translocation in 3T3-L1 adipocytes (19, 20). The authors argued that nocodazole directly inhibits the glucose transporter...
activity rather than GLUT4 translocation. We do not rule out the possibility that nocodazole is a more potent inhibitor of GLUT1 than GLUT4 because 3T3-L1 adipocytes express a considerable amount of GLUT1 in addition to GLUT4 while rat adipocytes mainly express the latter isoform. It has been reported that the sensitivity to indinavir is different among the GLUT isoforms (40). It is also possible that the difference may derive from the experimental methods for measuring the effect of nocodazole on the glucose transporter activity. While we measured the initial uptake rate of 3-O-methylglucose at 37 °C in the absence of ATP (Fig. 9), both groups measured the time-dependent accumulation of 2-deoxyglucose at 4 °C. The latter reflects and does not discriminate two metabolic steps of 2-deoxyglucose; transport and phosphorylation by glucokinase with the consumption of ATP. In addition, it was previously shown in rat adipocytes that lowering of the temperature activates the glucose transport activity and GLUT4 translocation in an ATP-dependent manner, suggesting incomplete arrest of the subcellular membrane movements at low temperature (41). Further study will be needed to elucidate these points. Our data are consistent with the notion that nocodazole inhibits the insulin action by interfering with the GLUT4 trafficking rather than the activity of glucose transporter. It is unlikely that nocodazole inhibited insulin-induced GLUT4 translocation by affecting the insulin signaling since it had little effects on the insulin stimulation of tyrosine phosphorylation of the proximal signaling proteins (Fig. 9, A and B), protein kinase B/Akt (Fig. 9C), the low Kₚ cAMP phosphodiesterase (Fig. 9D) and translocation of GLUT1 and VAMP-3 (Fig. 14).

While our data thus demonstrated that the microtubules play a significant role in insulin-induced GLUT4 translocation, one issue remains to be addressed that there was an apparent discrepancy between the effects of nocodazole on the microtubules and the insulin-stimulated glucose transport (Figs. 2 and 6). The insulin-stimulated glucose transport was not affected until a considerable portion (50% or more) of the microtubules was disrupted. A similar discrepancy was observed by other investigators in 3T3-L1 adipocytes (19). This makes a remarkable contrast to the close correlation between the actin filament integrity and the insulin action (12). Since GTP-γ-S-stimulated glucose transport was little affected with nocodazole (Fig. 15B), it seems that insulin-induced recruitment of the non-endosomal microtubule-dependent GLUT4 subpopulation is not obstructed until a considerable portion (50% or more) of the microtubules is disrupted. One possible explanation is that there may occur a subcellular shift of GLUT4 from the microtubule-dependent to the independent (or less dependent) compartments by treatment with lower concentrations of nocodazole. In this respect, the observation by Shigematsu et al. (20) seems intriguing. Their study showed a time-dependent accumulation of GLUT4 beneath the plasma membrane after the addition of a low concentration (3 μM) of nocodazole without alteration in the perinuclear localization of the transporter. They also showed that the GLUT4 compartments confined just beneath the plasma membrane were responsive to insulin in the absence of the microtubules, but unable to be sorted back to the perinuclear compartments. Thus, such a subcellular shift of GLUT4 may obscure the inhibitory effect of nocodazole on insulin-induced GLUT4 translocation and cause an apparent insensitivity to nocodazole. Alternatively, disruption of the microtubules with lower concentrations of nocodazole may potentiate the insulin action on GLUT4 translocation by unknown mechanisms. While it has long been known that disruption of the microtubules promotes actin stress fibers formation and changes in cell morphology (e.g. Ref. 42), it was recently reported that GEF-H1, a microtubule-associated Rho guanine nucleotide exchange factor is activated by microtubule disruption (43). Additionally, microtubule breakdown may stimulate other cellular signaling pathways involved in GLUT4 translocation (for review see Ref. 44). Since nocodazole did not stimulate the basal glucose transport activity (Fig. 6), such mechanisms do not seem to directly activate GLUT4 translocation, but may facilitate the insulin-stimulated trafficking of GLUT4 to the plasma membrane. Although the microtubules have been implicated in endocytic pathways (45, 46), it is unlikely that microtubule disruption augmented the insulin effect by inhibition of GLUT4 endocytosis since nocodazole had little effect on the rate of GLUT4 endocytosis (Fig. 12). Finally, there still remains a possibility that nocodazole affected the GLUT4 trafficking by microtubule-unrelated mechanisms. Other methods to manipulate the microtubules may help to clarify this point.

Since our original manuscript was submitted, Semiz et al. (47) reported that conventional kinesin KIF5B is required for insulin-stimulated GLUT4 translocation. Their data are consistent with our conclusions that the microtubules play an indispensable role in insulin-induced movement of GLUT4 to the plasma membrane.

In summary, the present study provides important insights into the physiological significance of the microtubules in the subcellular localization and trafficking of GLUT4 in primary adipocytes. Our results showed that there are two distinct GLUT4 subpopulations with differential microtubule dependence in trafficking to the plasma membrane. The microtubule-independent (or less dependent) GLUT4 subpopulation seems to localize to the endosomal system, while the compartment(s) to which the microtubule-dependent subpopulation localizes remains to be elucidated. The GLUT4 distribution among the compartments may affect the amplitude and kinetics of the cellular response to insulin when the microtubules are disrupted. With the unique morphological and functional features, the distribution and transit of GLUT4 among the intracellular compartments may be more dependent on the microtubules in primary adipocytes than in other types of cells including 3T3-L1 adipocytes. Further dissection of the trafficking pathways of GLUT4 is apparently necessary to elucidate the physiological role of the microtubules in the insulin action.

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Insulin Recruits GLUT4 from Distinct Compartments via Distinct Traffic Pathways with Differential Microtubule Dependence in Rat Adipocytes
Li-Bin Liu, Waka Omata, Itaru Kojima and Hiroshi Shibata

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