The microtubule network has been shown to be required for insulin-dependent GLUT4 redistribution; however, the precise molecular function has not been elucidated. In this article, we used fluorescence recovery after photobleaching (FRAP) to evaluate the role of microtubules in intracellular GLUT4 vesicle mobility. A comparison of the rate of fluorescence recovery \( t_{1/2} \), and the maximum fluorescence recovered \( F_{\text{max}} \) was made between basal and insulin-treated cells with or without nocodazole treatment to disrupt microtubules. We found that intracellular mobility of fluorescently tagged GLUT4 (HA-GLUT4-GFP) was high in basal cells. Mobility was not increased by insulin treatment. Basal mobility was dependent upon an intact microtubule network. Using a constitutively active Akt to signal GLUT4 redistribution, we found that microtubule-based GLUT4 vesicle mobility was not obligatory for GLUT4 plasma membrane insertion. Our findings suggest that microtubules organize the insulin-signaling complex and provide a surface for basal mobility of GLUT4 vesicles. Our data do not support an obligatory requirement for long range microtubule-based movement of GLUT4 vesicles for insulin-mediated GLUT4 redistribution to the cell surface. Taken together, these findings suggest a novel model in which insulin signaling targets membrane docking and/or fusion rather than GLUT4 trafficking to the cell surface.

A major physiologic action of insulin is to stimulate redistribution of the facilitative glucose transporter (GLUT4) to the cell surface, leading to enhanced glucose uptake and storage in adipose, muscle, and heart tissue (for a recent review, see Ref. 1). Defects in GLUT4 redistribution are thought to contribute to the development of insulin resistance and Type 2 diabetes (2). Understanding the cellular and molecular mechanisms of insulin-stimulated GLUT4 redistribution is critical to identifying defects that result in insulin resistance and will reveal relevant targets for treatment and prevention of Type 2 diabetes.

The pool of GLUT4 vesicles that are insulin responsive is developmentally regulated. In adipocytes, the compartment forms early in differentiation even before GLUT4 is synthesized to significant levels (3). A precise physical characterization of the insulin-responsive compartment has remained elusive. Intracellular GLUT4 resides in two or more pools overlapping in part with the recycling endosome and in what appears to be a unique specialized compartment (3–8). The fate of newly synthesized GLUT4 is controversial in that it is unclear if GLUT4 is targeted to the plasma membrane and trafficked to the insulin-responsive pool through the early endosome (9) or targeted directly to the insulin-responsive pool from the biosynthetic pool arising from the TGN (10). The insulin-responsive GLUT4 vesicle pool can form as a specialized postendocytic compartment that is separate from the general recycling endosome pool (4). Kinetic studies using antibodies and impermeable glucose analogues have demonstrated that insulin treatment both accelerates GLUT4 appearance at the plasma membrane and inhibits its retrieval from the cell surface (11–15). The increase in exocytosis could arise from a variety of regulated steps including fusion of GLUT4 vesicles with the plasma membrane, stimulation of the rate of movement of vesicles to the plasma membrane, increased fission from an endosomal precursor, or a combination of these processes.

In an attempt to characterize the molecular basis that underlies GLUT4 trafficking and membrane fusion, several laboratories have explored the role of the microtubule network in this process. In 3T3-L1 adipocytes and primary rat adipocytes it has been shown that pharmacological disruption of the microtubule network blocks insulin-dependent GLUT4 redistribution (14, 16–22). However, none of these studies clearly distinguished where the defect is occurring in the insulin signaling/GLUT4 redistribution pathway. It remains poorly defined whether there is a defect in the formation of the insulin signaling complex or in trafficking of GLUT4 vesicles for fusion with the plasma membrane. To date the favored hypothesis has been that the microtubule network may be involved in GLUT4 trafficking (22, 23).
Microtubules Mediate Insulin Signaling

In this study, we have directly tested the effect of insulin on intracellular GLUT4 vesicle mobility. A common interpretation of existing data is that insulin either stimulates the rate of intracellular mobility of GLUT4 vesicles or insulin releases vesicles from a molecular tether (8, 24–27). Our results demonstrate that insulin does not significantly accelerate the rate of GLUT4 mobility. Our data suggest that GLUT4 is not tethered in the basal state, but rather is highly mobile in the cell in the absence of insulin. These data support a newer model holding that insulin governs GLUT4 redistribution by stimulating GLUT4 vesicle docking and fusion with the plasma membrane (23, 28). We also examined the role of the microtubule network in insulin-stimulated GLUT4 redistribution. Our results demonstrate that the microtubule network does play a significant role in GLUT4 mobility, but this role in mobility is not obligatory for GLUT4 redistribution to the plasma membrane. In addition, we demonstrate that the microtubule requirement in insulin-stimulated GLUT4 redistribution lies upstream of Akt/PKB activation.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—3T3-L1 fibroblasts were obtained from the American Type Tissue Culture repository (Manassas, VA), cultured at 37°C in 5% CO₂, and maintained in DMEM containing 25 mM glucose and 10% calf serum. Confluent cultures were induced to differentiate by incubation of the cells with DMEM plus 25 mM glucose, 10% fetal bovine serum, 175 mM insulin, 1 μM dexamethasone, and 0.5 mM isobutyl-1-methylxanthine. After 4 days the medium was changed to DMEM containing 25 mM glucose, 10% fetal bovine serum, and 175 mM insulin, with the incubation period continuing an additional 3 days.

**Electroporation**—100-mm plates of 3T3-L1 adipocytes 5 days postdifferentiation were washed with PBS and incubated 10 min with 2 ml of trypsin/EDTA and 2 ml of PBS with 4 mg of collagenase. Cells were resuspended and pelleted at 900 rpm for 5 min. Cells were washed with PBS and resuspended in 3 ml of DMEM containing 25 mM glucose. 0.5 ml of cell suspension was transferred to a 0.4-cm electroporation cuvette (Bio-Rad), and 45 μg of DNA was added. Cells were electroporated in a Gene Pulser II (Bio-Rad) at 0.18 kV and 950 μF. Cells were allowed to recover for 10 min at room temperature. Equivalent amounts of cell suspension and fresh medium were added together and plated on 4 chamber BD Falcon CultureSlides (BD Biosciences, Bedford, MA) or 30-mm glass bottom dishes (WillCo, Ft. Worth, TX). All experiments were conducted 18–24 h postelectroporation.

**Live Cell Imaging**—3T3-L1 adipocytes electroporated with HA-GLUT4-GFP were plated onto glass-bottomed tissue culture dishes and allowed to recover overnight. Cells were starved for 2 h at 37°C in ambient air in Leibovitz L-15 medium (Invitrogen) supplemented with 2% bovine serum albumin in serum-free F-12 Hamm’s medium with or without 33 μM or 3.3 μM nocodazole as indicated. Cells were treated with or without 100 μM insulin in serum-free F-12 Hamm’s medium for 30 min. Cells were washed twice in PBS and fixed with 3% paraformaldehyde in PBS for 20 min with no permeabilization. Paraformaldehyde-treated cells were quenched for 10 min in 0.05 M ammonium chloride in PBS. Cells were incubated with α-HA antibody (Covance), and the antibody was detected using anti-mouse IgG conjugated with Alexa 568 (Molecular Probes, Eugene, OR). The cells were analyzed by confocal microscopy using a Leica TNS confocal microscope (Deerfield, IL). Images displayed represent a maximum projection from sections and represent the entire z-axis of the cells (12 sections of ~0.5-μm separation). Image analysis and quantification of GFP and HA were performed using Leica LCS Lite software.

**Akt/PKB Activity Assay**—Total Akt/PKB activity was measured from immunoprecipitates of whole cell detergent lysates using the GSK3 fusion protein as a substrate. The enzyme assay was performed using a commercially obtained kit according to the manufacturer’s specifications (Cell Signaling, Beverly, MA). Phospho-Akt and total Akt measurements were made as described previously (35).

**Statistical Analysis**—Statistical analysis was performed using Analyze-It software for Microsoft Excel (Analyze-It Software, Ltd., Leeds, UK).

**RESULTS**

**GLUT4 Redistribution in Live 3T3-L1 Adipocytes**—Previous biochemical and kinetic studies have shown that insulin treatment causes an increase in GLUT4 exocytosis from the intracellular storage compartment to the plasma membrane (11–15, 29, 30). We tested the insulin-stimulated redistribution of GLUT4 in living 3T3-L1 adipocytes using the HA-GLUT4-GFP reporter. 3T3-L1 adipocytes expressing the HA-GLUT4-GFP construct were insulin-stimulated and monitored by confocal microscopy. Confocal images at various time points after insulin stimulation showed a decrease in fluorescence intensity in the perinuclear-localized HA-GLUT4-GFP (Fig. 1A). The concomitant increase in GLUT4 at the cell surface is not easily quantified by confocal microscopy given that HA-GLUT4-GFP is redistributing from small vesicles with a high ratio of GLUT4 to membrane lipid (31) to a large membrane pool, thus dimin-
GLUT4 mobility in 3T3-L1 adipocytes—The kinetics of GLUT4 redistribution to the plasma membrane in insulin-stimulated 3T3-L1 adipocytes have been measured by several laboratories using the measurement of an exposed epitope in the first exofacial loop of a GLUT4 reporter (4, 24, 32). The limitation of these studies is that the kinetics of exocytosis measures a combination of trafficking and membrane fusion. We sought to separate intracellular GLUT4 trafficking from GLUT4 vesicle membrane fusion by directly measuring GLUT4 mobility using fluorescence recovery after photobleaching (FRAP). FRAP has been used previously to measure dynamic protein movement in a variety of cell types (for recent reviews see Refs. 33 and 34). We define mobility as the overall change in GLUT4 distribution, not as the change in the movements of individual GLUT4 containing vesicles. 3T3-L1 adipocytes expressing HA-GLUT4-GFP were imaged on an inverted Zeiss confocal microscope at 37 °C. Discrete sections of HA-GLUT4-GFP fluorescence in one z-plane at the cell periphery were photobleached in the indicated boxed area (Fig. 2A) and allowed to recover over 8 min with image acquisition at 15-s intervals. The initial photobleach destroys both the immobile and mobile pools of the fluorescently tagged protein, and the maximum recovery is the amount of mobile fluorescent protein. A second photobleach was performed after initial recovery, and the cell was allowed to recover again. This second recovery curve represents bleaching and recovery of only the mobile fraction, giving an estimate of the size the mobile fraction as well as the relative rate of mobility of the GLUT4 reporter in the bleached region of the cell. The second curve was analyzed by transforming the data using a double-reciprocal plot to estimate the rate of fluorescence recovery (t½) and the maximum fluorescence recovered (Fmax). Because each transfected cell had variation in the fluorescence intensity, the fluorescence measurements were normalized to the initial prebleached level, assigning it a value of one. The variability in the raw fluorescence was random, likely caused by variation in reporter expression levels. However, the variation in fluorescence intensity was not dependent upon the experimental treatment applied to the cell (data not shown). These values were obtained for each of three independent experiments for a given FRAP area, and the mean and standard deviation for each condition are summarized for all experiments in Table 1.

The FRAP measurements in the cell periphery are most likely caused by a combination of movement of preformed vesicles and retrieval of plasma membrane HA-GLUT4-GFP resulting from endocytosis. We do not believe that the FRAP results from lateral diffusion of HA-GLUT4-GFP in the plasma membrane, because the fluorescence signal is diluted in this large membrane compartment and is difficult to visualize in confocal microscopy of the whole cell.

The recovery of fluorescence following both the first and second (from the new initial fluorescence) photobleach was ~90%, indicating that there is a 10% loss of signal because of either cell imaging or re-equilibration of vesicles between the mobile and immobile fraction. To confirm that the recovery plateau was reached by 265 s following the second photobleach, we carried out the recovery period for 530 s (supplemental Fig. S1). The half-time for recovery and maximal recovery was not different after 530 s compared with 265 s (Table 1). These data support that a true plateau was reached, and all subsequent experiments were carried out for 265 s to minimize the effects of cell mobility over the longer recovery period.

This linear transformation and quantification of the second recovery curve demonstrated no statistically significant difference between the half-time of recovery (t½) or the maximal fluorescence (Fmax) recovered in insulin-treated versus basal cells at the cell periphery (Fig. 2B, Table 1, and supplemental Movies M1 and M2). Experiments were also carried out over shorter time periods (3 min using 5-s intervals between image acquisition), and the results were similar to the 15-s interval experiments (data not shown).
To confirm that the recovery after photobleaching was caused by mobility of intact HA-GLUT4-GFP into the FRAP area, and not simply refolding of photobleached GFP, 3T3-L1 adipocytes were treated with paraformaldehyde before imaging to eliminate protein mobility in cells. These fixed cells did not recover fluorescence after bleaching to the same extent as control cells (Fig. 2C and supplemental Movies M3 and M4). Quantification of these experiments demonstrated that the recovery of paraformaldehyde-treated cells was unable to be fitted to a linear curve upon transformation, indicating a significant loss of the mobile pool compared with basal cells.

GLUT4 mobility in the cell interior apart from the perinuclear area of intense GLUT4-GFP fluorescence (i.e. in the cytosol) was also tested to determine if insulin affected a change in mobility in that compartment (Fig. 3A). Insulin also did not significantly affect the rate of fluorescence recovery in the cytosolic compartment (Fig. 3B, Table 1, and supplemental Movies M5 and M6).

According to our live cell experiment, the insulin responsive compartment appeared to arise from the perinuclear region of the cell (Figs. 1 and 3C). Nonetheless, fluorescent recovery rates were unchanged between insulin and basal states in the perinuclear compartment (Fig. 3D, Table 1, and supplemental Movies 7 and 8). Shorter time scale experiments (3 min using 5-s intervals between image acquisition) were also carried out in the cytosol and perinuclear areas and were similar to the 15-s interval experiments (data not shown). Therefore, insulin stimulation showed no statistically significant change in the rate of recovery in any of the three locations tested by FRAP (Table 1).

GLUT4 Mobility in 3T3-L1 Adipocytes without a Functional Microtubule Network—Previous studies from several laboratories have established that the microtubule network is necessary for proper insulin-stimulated GLUT4 redistribution in 3T3-L1 adipocytes (14, 16−22). We sought to determine if the microtubule network was necessary for proper GLUT4 mobilization to the cell periphery as analyzed by FRAP. 3T3-L1 adipocytes were serum-starved and treated with or without 33 μM nocodazole for 2 h to completely depolymerize the microtubule network. Treatment with nocodazole caused dispersion of the perinuclear compartment, an effect that has been previously described (16, 20) (Fig. 5). Similar to above, discrete sections at the cell periphery were photobleached and allowed to recover over 15-s intervals. Treatment with 33 μM nocodazole in either the absence or presence of insulin caused a statistically significant decrease in recovery when compared with basal cells.

**TABLE 1**

| Kinetic analysis of FRAP | Average \(t_{1/2}^a\) | Average \(F_{max}^b\) |
|-------------------------|-----------------|-----------------|
|                         | s               |                  |
| **Cell periphery**      |                 |                 |
| Basal                   | 48 ± 6          | 0.45 ± 0.06     |
| Insulin                 | 50 ± 8          | 0.55 ± 0.13     |
| **Cytosol**             |                 |                 |
| Basal                   | 43 ± 15         | 0.47 ± 0.04     |
| Insulin                 | 50 ± 11         | 0.60 ± 0.08     |
| **Perinuclear**         |                 |                 |
| Basal                   | 60 ± 8          | 0.60 ± 0.18     |
| Insulin                 | 55 ± 19         | 0.52 ± 0.18     |
| **Cell periphery with 33 μM nocodazole with 100 nM insulin** | | |
| Basal                   | 39 ± 10         | 0.44 ± 0.12     |
| Insulin                 | 41 ± 6          | 0.43 ± 0.14     |
| Nocodazole              | 55 ± 23         | 0.27 ± 0.11     |
| Nocodazole & insulin    | 55 ± 46         | 0.27 ± 0.13     |
| **Cell periphery with 10 μM Latrunculin with 100 nM insulin** | | |
| Basal                   | 53 ± 16         | 0.53 ± 0.16     |
| Insulin                 | 42 ± 31         | 0.50 ± 0.20     |
| Latrunculin             | 65 ± 36         | 0.53 ± 0.14     |
| Latrunculin & insulin   | 39 ± 10         | 0.45 ± 0.04     |
| **Cell periphery with 33 μM Nocodazole with myr-Akt** | | |
| Basal                   | 57 ± 6          | 0.48 ± 0.04     |
| myr-Akt                 | 50 ± 10         | 0.50 ± 0.14     |
| Nocodazole              | 43 ± 4          | 0.20 ± 0.04     |
| myr-Akt & Nocodazole    | 51 ± 5          | 0.34 ± 0.05     |
| **Cell periphery (long time course)** | | |
| Basal                   | 31 ± 6          | 0.35 ± 0.11     |
| Insulin                 | 34 ± 7          | 0.37 ± 0.05     |

\(a\) Half time for maximum fluorescence recovery (zeroed from start of 2nd bleach).

\(b\) Maximum fluorescence recovery.

\(c\) Significantly different from basal at the \(p < 0.05\) level using one-way analysis of variance.
important for GLUT4 mobility, but that this mobility is not insulin-dependent. Therefore, we tested the ability of myr-Akt to stimulate GLUT4 redistribution in the absence of a functional microtubule network. The myr-Akt construct allows us to bypass proximal insulin signaling and test whether the microtubule network is required upstream or downstream of Akt/PKB activation. We have previously used this approach to determine that the functional requirement for the actin network in GLUT4 redistribution lies upstream of Akt/PKB activation (35). 3T3-L1 adipocytes were treated with two different concentrations of nocodazole to test the microtubule requirement. A high dose of 33 μM nocodazole was used to depolymerize the microtubule network or a low dose of 3.3 μM nocodazole was used to inhibit microtubule network dynamics (37). GLUT4 redistribution was measured and quantified using a HA-GLUT4-GFP reporter molecule as described previously (35). In this assay, exposure of the HA epitope in the extracellular loop of GLUT4 is a measure of GLUT4 fusion with the plasma membrane. In control cells, either 100 nM insulin stimulation or myr-Akt expression increases HA staining of the exposed epitope on the cell surface (Fig. 5). Insulin-stimulated cells treated with 33 μM nocodazole showed no significant increase in HA staining at the cell surface (Fig. 5). In contrast, 33 μM nocodazole treatment did not inhibit HA staining at the cell surface in cells expressing myr-Akt (Fig. 5). A lower dose of 3.3 μM nocodazole gave results that were similar to 33 μM nocodazole (supplemental Fig. S2). Given that myr-Akt is present in the cells for 24 h, it is possible that nocodazole was ineffective in diminishing GLUT4 at the cell surface because the GLUT4 fusion machinery was fully activated before nocodazole addition. To control for that possibility, we stimulated cells with insulin for 30 min prior to addition of nocodazole to similarly establish a new steady state of GLUT4 redistribution to the plasma membrane. Addition of insulin 30 min before the addition of 33 μM nocodazole treatment also resulted in inhibition of HA-GLUT4-GFP redistribution consistent with a nocodazole-dependent inhibition of the insulin signal (supplemental Fig. S3).

Pharmacological Inhibition of the Microtubule Network Blocks Akt Activation in 3T3-L1 Adipocytes—Because the expression of myr-Akt overcomes the pharmacological inhibition of the microtubule network, we next determined whether nocodazole treatment inhibited insulin-stimulated Akt activation. Previously, we showed that depolymerization of the

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**FIGURE 3.** Cytosolic and perinuclear storage compartment FRAP of GLUT4-GFP in 3T3-L1 adipocytes. A, representative cell depicting FRAP area (boxed area) defined as cell cytosol. B, quantification of cytosolic FRAP in 3T3-L1 adipocytes expressing GLUT4-GFP stimulated with or without 100 nM insulin. Fluorescence intensity inside photobleached region was quantified and normalized to initial fluorescence. Curves represent three independent experiments. Each independent experiment is the average of 4 – 6 cells per condition. C, double-reciprocal transformation of 2nd recovery curve in B used to generate maximum fluorescence recovery and half-time for fluorescence recovery reported in Table 1. D, representative cell depicting FRAP area (boxed area) defined as perinuclear region. E, quantification of perinuclear storage compartment FRAP in 3T3-L1 adipocytes expressing GLUT4-GFP stimulated with or without 100 nM insulin. Fluorescence intensity inside photobleached region was quantified and normalized to initial fluorescence. Curves represent three independent experiments. F, double-reciprocal transformation of 2nd recovery curve in E used to generate maximum fluorescence recovery and half-time for fluorescence recovery reported in Table 1. The error bars represent the S.E. calculated for each time point from the three independent experiments.

We tested the effect of a constitutively active Akt, myr-Akt, in basal and nocodazole-treated 3T3-L1 adipocytes. This constitutively active form of Akt stimulates GLUT4 redistribution in the absence of insulin (35, 36). Overexpression of this construct did not alter HA-GLUT4-GFP recovery compared with cells expressing the empty vector control (Fig. 4E). These data indicate that the increase in plasma membrane GLUT4 seen with myr-Akt expression is not caused by an increase in GLUT4 vesicle movement. The addition of 33 μM nocodazole significantly decreased HA-GLUT4-GFP recovery at the cell periphery (p < 0.05) consistent with nocodazole reducing the size of the mobile GLUT4 reporter pool. The Fmax recovered in the nocodazole-treated cells expressing myr-Akt did not reach statistical significance when compared with myr-Akt alone. The half-time of recovery (t1/2) was unchanged in all conditions.

**GLUT4 Redistribution in 3T3-L1 Adipocytes Expressing myr-Akt without a Functional Microtubule Network—**Results from the FRAP studies suggest that the microtubule network is important for GLUT4 mobility, but that this mobility is not insulin-dependent. Therefore, we tested the ability of myr-Akt to stimulate GLUT4 redistribution in the absence of a functional microtubule network. The myr-Akt construct allows us to bypass proximal insulin signaling and test whether the microtubule network is required upstream or downstream of Akt/PKB activation. We have previously used this approach to determine that the functional requirement for the actin network in GLUT4 redistribution lies upstream of Akt/PKB activation (35). 3T3-L1 adipocytes were treated with two different concentrations of nocodazole to test the microtubule requirement. A high dose of 33 μM nocodazole was used to depolymerize the microtubule network or a low dose of 3.3 μM nocodazole was used to inhibit microtubule network dynamics (37). GLUT4 redistribution was measured and quantified using a HA-GLUT4-GFP reporter molecule as described previously (35). In this assay, exposure of the HA epitope in the extracellular loop of GLUT4 is a measure of GLUT4 fusion with the plasma membrane. In control cells, either 100 nM insulin stimulation or myr-Akt expression increases HA staining of the exposed epitope on the cell surface (Fig. 5). Insulin-stimulated cells treated with 33 μM nocodazole showed no significant increase in HA staining at the cell surface (Fig. 5). In contrast, 33 μM nocodazole treatment did not inhibit HA staining at the cell surface in cells expressing myr-Akt (Fig. 5). A lower dose of 3.3 μM nocodazole gave results that were similar to 33 μM nocodazole (supplemental Fig. S2). Given that myr-Akt is present in the cells for 24 h, it is possible that nocodazole was ineffective in diminishing GLUT4 at the cell surface because the GLUT4 fusion machinery was fully activated before nocodazo...
**DISCUSSION**

It has been well established that insulin treatment increases the amount of GLUT4 transporters present in the plasma membrane of 3T3-L1 adipocytes, although the extent of the increase varies depending on method used (4, 38–40). Live cell imaging of insulin-stimulated 3T3-L1 adipocytes expressing HA-GLUT4-GFP confirms that there is a decrease in fluorescence intensity in the perinuclear region. This likely corresponds to a subsequent increase in the amount of HA-GLUT4-GFP at the cell surface. The intensity of the fluorescence observed at the cell surface is not directly

Microtubule network using 33 μM nocodazole does not significantly alter insulin signaling through phosphorylation of Akt/ PKB at Ser473 (21); however, Akt activity was not measured. Therefore, Akt activity was measured by an in vitro kinase assay as previously described (35). Treatment of 3T3-L1 adipocytes with insulin caused a statistically significant increase in Akt activity immunoprecipitated from whole cell lysates (p < 0.05). This increase was completely blocked by treatment with either

![Image](50x382 to 299x734)

FIGURE 4. Effect of 33 μM nocodazole on cell periphery FRAP of GLUT4-GFP-expressing 3T3-L1 adipocytes. A, quantification of cell periphery FRAP in 3T3-L1 adipocytes expressing GLUT4-GFP stimulated with or without 100 nM insulin in the presence or absence of 33 μM nocodazole (Noc). Fluorescence intensity inside photobleached region was quantified and normalized to initial fluorescence. Curves represent three independent experiments. Each experiment is the average of 4–6 cells per condition. B, double-reciprocal transformation of 2nd recovery curve in A used to generate maximum fluorescence recovery and half-time for fluorescence recovery reported in Table 1. C, quantification of cell periphery FRAP in 3T3-L1 adipocytes expressing GLUT4-GFP stimulated with or without 100 nM insulin in the presence or absence of 10 μM latrunculin (Lat). Fluorescence intensity inside photobleached region was quantified and normalized to initial fluorescence. Curves represent three independent experiments. Each experiment is the average of 4–6 cells per condition. D, double-reciprocal transformation of 2nd recovery curve in C used to generate maximum fluorescence recovery and half-time for fluorescence recovery reported in Table 1. E, quantification of cell periphery FRAP in 3T3-L1 adipocytes expressing GLUT4-GFP and either control plasmid or myr-Akt (myr-Akt) in the presence or absence of 33 μM Noc. Fluorescence intensity inside the photobleached region was quantified and normalized to initial fluorescence. Curves represent three independent experiments. F, double-reciprocal transformation of 2nd recovery curve in E used to generate maximum fluorescence recovery and half-time for fluorescence recovery reported in Table 1. The error bars represent the S.E. calculated for each time point from the three independent experiments.

![Image](314x413 to 563x734)

FIGURE 5. Effect of myr-Akt on HA-GLUT4-GFP redistribution in 3T3-L1 adipocytes treated with 33 μM nocodazole. A–F, 3T3-L1 adipocytes were transfected with the HA-GLUT4-GFP reporter and either control plasmid (A, B, D, and E) or myr-Akt (C and F). 24 h later, cells were left untreated (A–C) or treated with 33 μM nocodazole (D–F) for 2 h. Panels E and F were insulin-stimulated for 30 min, and then all samples were fixed and stained as described under “Experimental Procedures.” GFP (green) fluorescence is a measure of total reporter expression. HA epitope staining (red) is a measure of reporter at the cell surface. G, quantification of the fluorescence intensity ratio of HA staining to GFP from three independent experiments (mean ± S.E.). Under each condition, at least 30 cells were quantified. The asterisk denotes statistical difference (p < 0.05) between control and nocodazole-treated cells stimulated with insulin.

33 or 3.3 μM nocodazole (Fig. 6, A and B). Both concentrations of nocodazole significantly (p < 0.05) inhibited phosphorylation of Akt at Thr308, but had no effect on phosphorylation of Ser473 (Fig. 6, C and D). These data suggest that microtubules play a role in regulating activity of PDK1. Based on the fixed cell experiments and biochemical analysis, a requirement for the microtubule network appears to exist upstream of full Akt activation.
Our results are consistent with an insulin-dependent step at the point of docking and or fusion. Insulin treatment did not change the maximum fluorescence recovered, which indicates that the mobile pool of GLUT4 is not different in basal cells compared with insulin-treated cells (Figs. 2 and 3 and Table 1). Thus insulin exerts its effect on a step other than vesicle mobility. This conclusion correlates with other lines of investigation. For example, using time-lapse total internal reflection microscopy to closely examine the effect of insulin on movement directly at or right underneath the plasma membrane, Lizunov et al. (23) demonstrated that the rate of vesicle movement in both basal and insulin-stimulated conditions are essentially the same. Insulin promotes docking and fusion of GLUT4-containing vesicles at the cell surface and not an increase in vesicle movement (23). Docking and fusion are likely to be individual steps mediated by distinct insulin-mediated signals. The latter hypothesis is supported by the observation of docked GLUT4 vesicles at the plasma membrane of either L6 myotubes (26) or 3T3-L1 adipocytes (25) in insulin-treated cells that were pretreated with wortmannin to inhibit PI 3-kinase activity. Biochemical evidence suggests that insulin signaling changes the plasma membrane to facilitate vesicle fusion. Kounamanov et al. (28) demonstrate insulin-dependent activation of components of the PM, but neither the cytosol nor GLUT4 vesicles, is required to support an in vitro membrane fusion reaction. In addition, electron and light microscopy studies have suggested that the perinuclear compartment and the plasma membrane are actually relatively close to one another in adipocytes, suggesting that GLUT4 vesicles do not necessarily need to be mobilized over long distances to contact cell surface fusion machinery (41).

Work from other laboratories have suggested that insulin stimulates two kinetic steps, intracellular sorting of GLUT4 from the endosomal compartment and exocytosis of the specialized insulin-dependent compartment (8, 14). Given that the kinetic sorting step was found to be reversible (14), it is possible that only one step (exocytosis) is directly regulated by insulin, and that the sorting step is pulled forward by the exocytosis of the specialized compartment. The biochemical step that is regulated by insulin in the exocytosis was not identified, but these data are entirely consistent with the regulated step occurring at the plasma membrane mediating docking and fusion. Using a similar kinetic analysis, Gover et al. (24) determined that the maximum surface exposed GLUT4 was directly dependent on the dose of insulin used for stimulation. These findings were interpreted to mean that insulin released quantal units of tethered intracellular GLUT4 vesicles. Alternatively, these results could be interpreted to mean that there is an insulin-dependent activation of specific fusion sites on the plasma membrane that are limiting for GLUT4 vesicle exocytosis. In the absence of a biochemical mechanism, both interpretations are plausible. Two lines of evidence argue against the tethered vesicle model of Gover et al. (24). First, the entire intracellular pool of GLUT4 recycles to the cell surface in the basal state indicating that the intracellular vesicles are dynamically retained as opposed to statically retained (14, 42). Second, our data demonstrate that there is no insulin or Akt-dependent change in the
Microtubules Mediate Insulin Signaling

mobile pool of GLUT4 vesicles suggesting that the intracellular vesicles are not tethered.

It has been demonstrated that GLUT4 vesicles appear to make long range movements in association with microtubules. Insulin increases the frequency of these movements, but not the overall velocity (22). This insulin-dependent movement of GLUT4 occurred independent of PI 3-kinase activation. This model predicts an obligatory role for microtubules in GLUT4 redistribution to facilitate arrival of vesicles at the cell surface. Our studies of GLUT4 mobility in cells lacking a functional microtubule network suggest that microtubules facilitate GLUT4 vesicle trafficking but not in an insulin-dependent fashion. This role for microtubules in GLUT4 vesicular trafficking is specific as loss of the actin network had no effect on GLUT4 mobility. The reduction in the available mobile pool of GLUT4 in nocodazole-treated cells seen in the FRAP quantification may be explained by the dispersion of the perinuclear compartment in cells. The reduction in coordinate inward movement on microtubules may be dependent on dynein motors, and this movement appears to be required to form the perinuclear compartment (16).

The microtubule network has been shown to be necessary to get full GLUT4 redistribution in response to insulin stimulation (14, 16–22). The role of the microtubule network plays has remained controversial since certain methodologies used to measure profound inhibition of GLUT4 translocation do not reveal a role for microtubules (20, 43). However, in these studies, examination of GLUT4 redistribution was performed using indirect immunofluorescence of plasma membrane sheets. This technique differs from the redistribution assay described in the current manuscript. In the plasma membrane sheet assay it is more difficult to quantify intermediate changes in GLUT4 redistribution because there is no method for measuring the proportion of GLUT4 that has redistributed to the cell surface. The redistribution assay in the current article indicates that there is not a total inhibition of GLUT4 redistribution, but rather a decrease in the proportion GLUT4 that redistributes to the plasma membrane. This suggests that microtubules may not play an obligatory role, but rather a regulatory role of some nature. In the current study we sought to determine if the microtubule network plays a role that is upstream or downstream of Akt/PKB activation in a similar way that was used to determine if the actin network requirement was upstream or downstream of Akt (35). Using this approach, we were able to determine that a block of insulin-stimulated GLUT4 redistribution by pharmacological inhibition of the microtubule network lies upstream of Akt/PKB. In the previous study, disruption of actin completely inhibited GLUT4 redistribution and in the current study, nocodazole partially inhibited GLUT4 redistribution (35). Interestingly, both studies demonstrated a complete inhibition of insulin-mediated Akt activity. A possible interpretation of these data is that the actin cytoskeleton plays a more comprehensive role in organizing the insulin signaling complex than the microtubule network, or Akt-independent signaling is also mediated by the actin network. Whereas insulin-dependent Akt-independent signaling to GLUT4 redistribution has not been biochemically described, it is known that some insulin-mediated GLUT4 redistribution persists after genetic ablation of Akt (44, 45), suggesting the existence of an Akt-independent pathway. The reduction in insulin stimulated GLUT4 redistribution caused by nocodazole treatment was completely overcome by the expression of the myr-Akt construct. This suggests that the previously described microtubule requirement is upstream of Akt activation.

Previous biochemical analysis of the effect of microtubule depolymerization on insulin signaling demonstrated that the insulin receptor β-subunit, IRS, PI 3-kinase activation, and Ser473 phosphorylation of Akt/PKB do not require an intact microtubule network in 3T3-L1 adipocytes (21, 46). However, data here demonstrates that Akt enzyme activity was not activated significantly above basal levels when adipocytes were treated with nocodazole. Therefore, the microtubule requirement in insulin-stimulated GLUT4 redistribution is likely between PI 3-kinase activation and full activation of Akt/PKB. This may occur at the level of PDK1 activation as Thr308 phosphorylation is significantly reduced in nocodazole-treated cells. We cannot eliminate the possibility that nocodazole treatment effects insulin receptor trafficking, but expression of a dominant negative dynamin mutant does not affect insulin signaling, suggesting that the effects of insulin receptor trafficking on insulin receptor signaling are minimal (47).

In summary, the data presented in this article demonstrate that insulin does not accelerate mobility of intracellular GLUT4-containing vesicles in 3T3-L1 adipocytes. Consistent with the observations outlined above from other laboratories, it is likely that the important target of insulin action lies at the level of the plasma membrane to mediate docking and fusion. Furthermore, the mobility of GLUT4 vesicles in the basal state is in part due to a microtubule-dependent process. The microtubule-dependent mobility is not obligatory for GLUT4 redistribution given that expression of constitutively active Akt will support GLUT4 redistribution in the absence of an intact microtubule network. It is possible that the microtubule network may play a role similar to the actin network in organizing the insulin signaling complex at the plasma membrane to facilitate membrane fusion (35). Further study will be required to understand the molecular basis for this proposed role of the microtubule network in insulin signaling.

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