Insulin-mediated GLUT4 Translocation Is Dependent on the Microtubule Network*

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The GLUT4 facilitative glucose transporter is recruited to the plasma membrane by insulin. This process depends primarily on the exocytosis of a specialized pool of vesicles containing GLUT4 in their membranes. The mechanism of GLUT4 vesicle exocytosis in response to insulin is not understood. To determine whether GLUT4 exocytosis is dependent on intact microtubule network, we measured insulin-mediated GLUT4 exocytosis in 3T3-L1 adipocytes in which the microtubule network was depolymerized by pretreatment with nocodazole. Insulin-mediated GLUT4 translocation was inhibited by more than 80% in nocodazole-treated cells. Phosphorylation of insulin receptor substrate 1 (IRS-1), activation of IRS-1-associated phosphatidylinositol 3-kinase, and phosphorylation of protein kinase B/Akt-1 were not inhibited by nocodazole treatment indicating that the microtubule network was not required for proximal insulin signaling. An intact microtubule network is specifically required for insulin-mediated GLUT4 translocation since nocodazole treatment did not affect insulin-mediated GLUT1 translocation or adipin secretion. By using in vitro microtubule binding, we demonstrated that both GLUT4 vesicles and IRS-1 bind specifically to microtubules, implicating microtubules in both insulin signaling and GLUT4 translocation. Vesicle binding to microtubules was not mediated through direct binding of GLUT4 or insulin-responsive aminopeptidase to microtubules. A model microtubule-dependent translocation of GLUT4 is proposed.

One of the many physiologic actions of insulin is to promote glucose uptake in skeletal muscle, heart, and adipose tissue. Several lines of evidence have shown that the effect of insulin on glucose uptake in these tissues is a direct result of the recruitment of the GLUT41 facilitative glucose transporter from an intracellular vesicle pool to the plasma membrane (for recent reviews see Refs. 1 and 2). GLUT4 was first implicated as the major insulin-responsive glucose transporter when it was shown to be the predominant isofrom expressed in tissues exhibiting insulin-stimulated glucose uptake (3–6). Prior to the cloning and identification of GLUT4, it was known that insulin stimulated the redistribution of a cytochalasin D-inhibitable glucose transport activity from an intracellular vesicle pool to the plasma membrane (7, 8). It is generally accepted that the translocatable glucose transport activity is attributable to GLUT4 recruited from an intracellular location to the plasma membrane. GLUT4 recycles between the plasma membrane and intracellular compartments in both the basal and insulin-stimulated states (9–11). Insulin increases the amount of GLUT4 on the plasma membrane by stimulating exocytosis of the intracellular GLUT4 pool and by decreasing the endocytosis of the plasma membrane-associated GLUT4 protein (10, 12–17). Increased exocytosis appears to be the most important event since complete inhibition of the GLUT4 endocytic pathway in the basal state does not result in a significant accumulation of GLUT4 at the plasma membrane (18). These data support a model in which a small portion of the total pool of GLUT4 recycles in the basal state, with the majority of GLUT4 sequestered in an insulin-responsive retention pool shown to be associated with tubulovesicular structures (19).

The mechanism by which GLUT4 vesicles move from their interior location to the plasma membrane in response to insulin is poorly understood. To date, research in this area has focused largely on docking and fusion of the vesicles at the plasma membrane. SNARE (for soluble NSF attachment protein receptor) proteins have been shown to play an important role in the fusion of GLUT4 vesicles with the plasma membrane. It has been clearly demonstrated that syntaxin 4, VAMP2, and Syndet/SNAP23 are the major SNARE proteins regulating GLUT4 membrane fusion (20–26). SNARE protein assembly may be mediated by other syntaxin 4-binding proteins such as Munc18c and the newly cloned protein, Sy nip (27–31), but it is not clear that assembly of SNARE proteins is a rate-limiting step in insulin-mediated GLUT4 translocation. Measurement of GLUT4 translocation in the presence of a temperature-sensitive Munc18c mutant indicates that membrane fusion is not rate-limiting in GLUT4 translocation (32). These data suggest that mechanisms regulating the movement of vesicles from the retention pool to the plasma membrane need to be explored to determine the specific steps in GLUT4 translocation that are regulated by insulin.

Regulated insulin-dependent trafficking of GLUT4 vesicles from the retention pool to the plasma membrane may occur by several mechanisms. GLUT4 vesicles may pass through an endosomal intermediate en route to the plasma membrane. This is unlikely, based on data demonstrating that depletion of

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The abbreviations used are: GLUT4, adipose/muscle-specific glucose transporter; PAGIE, polyacrylamide gel electrophoresis; IRS-1, insulin receptor substrate 1; PI 3-kinase, phosphatidylinositol 3-kinase; PKB, protein kinase B; PVD, polyvinylidene fluoride; PIPES, 1,4-piperazine diethanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride; IRAP, insulin-responsive aminopeptidase; PBS, phosphate-buffered saline; DMEM, Dulbecco’s modified Eagle’s medium; TBS, Tris-buffered saline.
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The endosomal compartment does not interfere with insulin-mediated GLUT4 translocation (33, 34). Alternatively, GLUT4 vesicle trafficking may be regulated by cytoskeletal elements. GLUT4 secretory vesicles may be tethered to cytoskeletal structures in a manner similar to endoplasmic reticulum and Golgi membrane structures (35). Insulin stimulation could induce a release of tethered vesicles, allowing movement to the plasma membrane by simple diffusion, or insulin may trigger the movement of vesicles along cytoskeletal tracks as has been observed for regulated exocytosis of secretory vesicles in other cell systems (36).

Studies examining the role of the actin cytoskeleton in GLUT4 translocation in L6 myotubes, 3T3-L1 adipocytes, and rat adipocytes show that disruption of the actin cytoskeleton by either cytochalasin D or latrunculin A inhibited insulin-mediated GLUT4 translocation (37–39). In L6 myotubes, disassembly of the actin network did not prevent GLUT4 translocation by stimuli other than insulin, suggesting that the actin cytoskeleton is required for transduction of the insulin signal to GLUT4 vesicles (40). Disassembly of the actin cytoskeleton in 3T3-L1 cells and L6 myotubes did not interfere with proximal insulin signaling events but did prevent the relocalization of PI 3-kinase to GLUT4 vesicles (38, 41).

In this work, we provide evidence that the microtubule cytoskeleton is required for insulin-mediated GLUT4 translocation. We show that insulin-mediated GLUT4 translocation requires an intact microtubule network and that under basal conditions, GLUT4 vesicles associate with polymerized microtubules.

**Experimental Procedures**

**Cell Culture—**3T3-L1 fibroblasts were obtained from the American Type Culture Collection and were cultured at 37 °C in 5% CO2 and maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 25 mM glucose and 10% fetal bovine serum. Media were changed at least twice a week.

**Extraction of Monomeric and Polymeric Tubulin—**Monomeric and polymeric tubulin were differentially extracted from cells plated on 35-mm dishes according to previous methods (43). Following treatment with 33 μM nocodazole and/or 100 nM insulin for 30 min, the media were captured by microcentrifugation and resuspended in 40 μl of solubilization buffer (1% SDS, 20 mM Hepes, pH 7.5, 150 mM NaCl, 1 mM MgCl2, 0.5 mM benzamidine, 5 mM 2-mercaptoethanol, 0.1 M Tris, 0.1 M Pefabloc, pH 7.1, 1 mM MgSO4, 1 mM EDTA, 10 μM leupeptin, 10 μM aprotinin, 0.1% Triton X-100) for 30 min at 37 °C. The Triton X-100-soluble extract contained monomeric tubulin. Polymeric tubulin was then extracted from the remaining Triton X-100-insoluble material by extracting in SDS lysis buffer (25 mM Tris, pH 7.4, 0.4 M NaCl, 0.5% SDS) for 5 min at 37 °C. Aliquots from monomeric and polymeric fractions were fractionated by 10% SDS-PAGE and Western blotting with α-tubulin antibodies.

**Whole Cell Detergent Lysates—**100-nm plates of treated 3T3-L1 adipocytes were washed twice with ice-cold TBS followed by freezing in liquid nitrogen. The plates were thawed on ice and scraped into 1 ml of 1% Nonidet P-40 lysis buffer (1% Nonidet P-40, 20 mM Hepes, pH 7.4, 2 mM EDTA) containing phosphatase inhibitors (100 μM sodium fluoride, 10 μM sodium pyrophosphate, 1 mM sodium vanadate, and 1 mM sodium molybdate) and protease inhibitors (10 μM leupeptin, 10 μg/ml aprotinin, 1.5 mM pepstatin A, and 1 mM PMSF). The cells were lysed on ice for 20 min, and insoluble material was removed by microcentrifugation for 10 min at 4 °C. Protein concentrations of the detergent lysates were determined by a Bradford protein assay (Pierce) using manufacturer’s specifications.

**Phosphorylation and Immunoblotting—**Samples were fractionated using SDS-PAGE and transferred to PVDF membranes (Millipore) in transfer buffer (25 mM Tris, 193 mM glycine, pH 8.5) for 3–4 h at 4 °C. Membranes were blotted with insulin receptor β-subunit, IRS-1, GLUT4, IRAP, tubulin, and dynin were blocked with 7–10% dried milk and 0.3% Tween 20 in TBS. Anti-phosphotyrosine blots were blocked with 10% dried milk and 0.1% Tween 20 in TBS. Anti-phosphorylated GLUT4 monoclonal antibodies were blotted onto PVDF membranes and probed with anti-phospho-GLUT4 (Santa Cruz Biotechnology). GLUT4 antiserum was provided by Dr. Gwyn Gould, and IRAP antiserum was provided by Dr. Kostantin Kandror. GLUT1 antiserum was provided by Dr. Samuel W. Cushman. Tubulin antiserum was obtained from Sigma (TUB 2.1). Phospho-PKB/Akt-1 antibody specific for Ser-473 was obtained from New England Biolabs, and antibody against total PKB/Akt-1 was purchased from Transduction Laboratories. Immunoblots were visualized using an enhanced chemiluminescence system (Pierce) and quantified using scanning laser densitometry.

**2-Deoxyglucose Uptake—**3T3-L1 adipocytes were plated in Ham’s F-12 media containing 0.5% bovine serum albumin for 2 h at 37 °C. Nocodazole was added to a final concentration of 33 μM at designated times. Cells were then washed with KRPP buffer (5 mM Na, HPO42–, 20 mM Hepes, pH 7.4, 1 mM MgSO4, 1 mM CaCl2, 136 mM NaCl, 4.7 mM KCl, and 1% bovine serum albumin) and treated with or without additional nocodazole to prevent depolymerization of microtubules and with or without 100 nM insulin added for 20 min. Glucose transport was determined at 37 °C by incubation with 50 μM 2-deoxyglucose uptake media. Aliquots from 2-deoxyglucose uptake media were transferred to 2% DMSO Triton X-100 in the presence or absence of 10 μM cytochalasin B. The reaction was stopped after 4 min by washing the cells 3 times with ice-cold PBS. The cells were solubilized in 1% Triton X-100 at 37 °C for 30 min, and aliquots were subjected to scintillation counting or protein assay to calculate uptake as pmol/min/mg protein.

**PI 3-Kinase Assay—**Phosphatidylinositol 3-OH kinase activity was measured in IRS-1 immune complexes obtained from whole cell detergent lysates as described above. The detergent-soluble cell extract was incubated overnight with anti-IRS-1 pre-loaded on protein A-conjugated Sepharose. Immune complexes were washed and subjected to the in vitro PI 3-kinase assay as described previously using bovine phosphatidylinositol (Sigma) and γ32P]ATP as substrates (44). Phosphorylated lipids were separated by thin layer chromatography, visualized by autoradiography, and quantified by collecting and counting radiolabeled phosphatidylinositol 3-phosphate.

**Adipin Secretion—**Adipin secretion into cell culture media was measured as described previously (45). Thirty-five-mm dishes of 3T3-L1 adipocytes were treated without or with 33 μM nocodazole for 3 h in serum-free Ham's F-12 media. The cells were washed 2 times with fresh media, followed by replacement with 1 ml of Ham's F-12 media without or with 33 μM nocodazole and/or 100 nM insulin for 30 min. The media were removed, and proteins were precipitated in the presence of 10% trichloroacetic acid and deoxycholate as carrier (46). Pelleted proteins were captured by microcentrifugation and re suspended in 40 μl of 0.25 M Tris base. Protein concentration was determined using a Bradford assay (Pierce). Equivalent protein aliquots were fractionated by 10% SDS-PAGE and transferred to PVDF. Membranes were immunoblotted using an adipin antibody (provided by Dr. Jess Miner, University of Nebraska).

**Microtubule Binding Assay—**Microtubules and associated proteins were polymerized in vitro as described previously (47). Briefly, 3T3-L1 adipocytes (day 8–10 post-differentiation) were serum-starved in Ham’s F-12 media for 3 h and then treated without or with 100 nM insulin for...
specified times. Cells from two 100-mm plates per treatment) were scraped into 500 μl of microtubule stabilizing buffer (5 mM MgSO₄, 5 mM EGTA, 35 mM PIPES, pH 7.1, and 142 mM sucrose) containing 1 mM dithiothreitol, protease inhibitors (10 μg/ml leupeptin, 10 μg/ml aprotinin, 0.5 mM benzamidine, 5 μg/ml pepstatin A, 0.5 mM PMSF), and phosphatase inhibitors (100 mM NaF, 1 mM sodium vanadate, and 1 mM molybdate). Cells were homogenized at 4 °C by passage through a 26-guage needle. Homogenates were centrifuged at 100,000 x g for 10 min to obtain the post-nuclear supernatant. The post-nuclear supernatant was subjected to a 40,000 x g centrifugation using an SW60 rotor (Beckman) for 1 h. The supernatant was recovered, and protein concentration was adjusted to 4 mg/ml with homogenization buffer. Taxol (1 mM stock solution in Me₂SO) was added to a final concentration of 20 μM, and samples were incubated at 4 °C for 1 h. Polymerized microtubules and associated proteins were pelleted by centrifugation at 16,000 x g for 30 min using an SW60 rotor (Beckman). In some experiments, 1% Triton X-100 was added to the 40,000 x g supernatant prior to addition of taxol to solubilize membranes. The 16,000 x g taxol-stabilized pellets were resuspended in homogenization buffer and protein concentration measured by Bradford assay. The 16,000 x g taxol-stabilized pellets were analyzed by SDS-PAGE and transferred to PVDF membrane and Western blotting.

RESULTS

Nocodazole Treatment Inhibits Insulin-mediated GLUT4 Translocation—To determine whether an intact microtubule cytoskeleton is necessary for insulin-mediated GLUT4 translocation, we measured GLUT4 translocation in cells treated with 33 μM nocodazole. This concentration depolymerizes greater than 95% of microtubules in other cell types (43). To confirm that depolymerization of the microtubule network occurred in 3T3-L1 adipocytes under this treatment, we compared monomeric and polymeric tubulin levels in cells treated with or with 33 μM nocodazole for 3 h by Western blot analysis. Monomeric tubulin was extracted in 0.1% Triton X-100 extraction buffer plus protease inhibitors at 37 °C for 15 min. After the Triton X-100-soluble fraction was removed, polymeric tubulin was extracted with buffer containing 0.4 mM NaCl and 0.5% SDS. An equivalent portion of each extract was fractionated by SDS-PAGE, and samples were probed with an anti-β-tubulin antibody (Fig. 1A). Nocodazole treatment shifted greater than 95% polymeric tubulin to the monomeric form (Fig. 1A, compare lanes 2 and 4). In control cells, approximately two-thirds of the total tubulin was polymeric, and the remaining third was monomeric (Fig. 1A, compare lanes 1 and 2).

After establishing the efficacy of nocodazole treatment for depolymerization of the microtubule network in 3T3-L1 adipocytes, we examined insulin-mediated GLUT4 translocation under identical treatment conditions. Insulin-dependent GLUT4 translocation to the plasma membrane was assessed by measuring GLUT4 levels in purified plasma membrane fractions prepared by sonication of monolayers of 3T3-L1 adipocytes (23, 48). Quantification of protein levels in this fraction by Western blot analysis provides an estimate of the accumulation of GLUT4 in the plasma membrane of an entire plate of cells. Cells were treated for 3 h with 33 μM nocodazole followed by treatment without or with 100 nM insulin for 30 min. Isolated plasma membrane fragments were solubilized and analyzed by Western blot. Basal levels of GLUT4 in plasma membrane sheets were not affected by nocodazole treatment (Fig. 1B, lanes 3 and 4). In contrast, nocodazole inhibited recruitment of GLUT4 to the plasma membrane following insulin treatment (p < 0.005) (Fig. 1B, lanes 7 and 8). Quantification of four independent experiments by laser densitometry is shown in Fig. 1C.

In the previous experiment, insulin-mediated GLUT4 translocation was measured after 3 h of nocodazole treatment; however, a time course experiment revealed that complete depolymerization of microtubules in 3T3-L1 cells occurred between 15 and 30 min of nocodazole treatment (data not shown). To confirm that the effects of nocodazole on insulin-mediated GLUT4 translocation occurred at early time points paralleling microtubule depolymerization, we measured GLUT4 translocation after 20 min of nocodazole treatment (Fig. 2A). Cells were treated with nocodazole for 20 or 120 min prior to insulin treatment and plasma membrane sheets prepared as described above. Results of two independent time course experiments are shown in Fig. 2A. Densitometric analysis of the Western blot reveals a 4-fold increase in GLUT4 protein following insulin treatment in the absence of nocodazole (Fig. 2A, lanes 1–4). In contrast to control conditions, the insulin-mediated GLUT4 translocation was increased 1.4- and 1.6-fold, respectively, in cells treated for 20 and 120 min with nocodazole (Fig. 2A, lanes 5–12). Thus, the effects of nocodazole on insulin-mediated GLUT4 translocation do not likely result from progressive changes in the cell that develop overtime in the presence of nocodazole.

In a complementary experiment, we measured insulin-mediated 2-deoxyglucose uptake in 3T3-L1 adipocytes treated for 0,
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A Nocodazole: 0 min 20 min 120 min
Insulin: + + + + + + + + + + + + + + + + + + + + +

B

2-Deoxyglucose uptake (pmol/minute/mg protein)

Control Nocodazole (20 min) Nocodazole (120 minute)

FIG. 2. Insulin-mediated GLUT4 translocation is inhibited to the same extent after short and long exposure to nocodazole. GLUT4 accumulation in purified membrane sheets was measured following 20 min of treatment with 100 nM insulin in cells pretreated for 0, 20, or 120 min in the presence of 33 μM nucodazole. A shows results from two independent experiments. Insulin-mediated 2-deoxyglucose uptake was measured following 20 min of treatment with 100 nM insulin in cells pretreated for 0 (control), 20, or 120 min in the presence of 33 μM nucodazole. B shows the mean and standard error from 4 independent experiments performed in duplicate. Data were analyzed by analysis of variance to compare insulin-mediated 2-deoxyglucose uptake between treatment group. Asterisk indicates $p < 0.01$ comparing control and nucodazole-treated cells.

Nucodazole Treatment Does Not Affect Proximal Insulin Receptor Signaling—Nucodazole treatment of cells significantly reduces microtubule-based vesicle transport; however, it has also been shown to elicit effects on the cell including inhibition of protein synthesis, disruption of plasma membrane subdomains, and changes in some types of cellular signaling (49). Any of these perturbations could lead to changes in early insulin signaling events. To determine whether nucodazole interferes with GLUT4 translocation by decreasing steady state levels of relevant proteins, we measured the levels of insulin receptor, IRS-1, and GLUT4 protein in nucodazole-treated cells. 3T3-L1 adipocytes were incubated with 33 μM nucodazole for 3 h and then treated without or with 100 nM insulin for 20 min at 37 °C. Nucodazole did not alter steady state protein levels of IRS-1, insulin receptor β-subunit, or GLUT4 protein (data not shown).

The first event in insulin signaling is the activation of the intrinsic tyrosine kinase activity of the insulin receptor upon ligand binding. This leads to both mitogenic and metabolic effects of the cascade. Another early event in insulin signaling is the tyrosine phosphorylation of intracellular insulin receptor substrates of the IRS family (50). Phosphotyrosine residues on the IRS isoforms act as binding sites for specific SH2 domain molecules, including phosphatidylinositol 3-OH kinase (PI-3-kinase) (50, 51). Activation of PI-3-kinase is required for most, if not all, intracellular actions of insulin, including GLUT4 translocation (52). In both rat adipocytes and 3T3-L1 adipocytes, PI-3-kinase binds predominantly to IRS-1 (53, 54). The PI-3-kinase activity immunoprecipitated with either anti-IRS-1 or anti-phosphotyrosine antibodies is increased in both intracellular membranes and cytosol following insulin stimulation (53–56).

Tyrosine-phosphorylated proteins were solubilized in 1% Nonidet P-40 and cell lysates and then analyzed by immunoblotting with anti-phosphotyrosine antibody. Insulin treatment increased tyrosine phosphorylation of proteins corresponding in size to the β-subunit of the insulin receptor and IRS-1. Insulin-mediated tyrosine phosphorylation of these proteins was not affected by nucodazole treatment (Fig. 3A).

To determine whether immediate downstream signaling events other than tyrosine phosphorylation were modified by nucodazole treatment, we assayed the IRS-1-associated PI-3-kinase activity. Control and nucodazole-treated cells were treated without or with 100 nM insulin for 30 min. IRS-1 complexes were immunoprecipitated from 1% Nonidet P-40 cell extracts and assayed for PI-3-kinase activity under standard conditions (48). IRS-1-associated PI-3-kinase activity increased 16-fold under insulin treatment (Fig. 3B). Nucodazole treatment did not significantly alter PI-3-kinase activity associated with IRS-1 ($p = 0.7$) in either basal or insulin-treated cells. These data indicate that an intact microtubule system is not required for insulin-dependent association of PI-3-kinase with phosphorylated IRS-1.

Insulin treatment activates protein kinase B (PKB) activity through a PI-3-kinase-dependent mechanism (57). The pleckstrin homology domain on the N terminus of PKB interacts with the phospholipid product of PI-3-kinase (PI-3,4,5-P3). This interaction induces a conformational change in PKB that permits phosphorylation of PKB at two regulatory sites (Thr-308 and Ser-473), activating the kinase (58). To determine whether nucodazole treatment interfered with activation of PKB, we measured phosphorylation of PKB at serine 473 in cell lysates using a phospho-PKB-specific antibody recognizing the phosphoserine. The ratio of phospho-PKB to total PKB was determined from the densitometric analysis of the Western blots. The ratio in control cells for three independent experiments was 112 ± 1.2 (mean ± S.D.) compared with a ratio 92 ± 6.9 in nucodazole-treated cells (Fig. 3C). This 18% reduction in the ratio of phospho-PKB to total PKB was statistically significant ($p < 0.02$); however, the physiologic significance of this decrease is not certain. Nucodazole treatment did not prevent insulin-mediated phosphorylation of PKB at Ser-473.

Nucodazole Does Not Effect Insulin-mediated Adipsin Secretion or GLUT4 Translocation—To determine whether depolymerization of the microtubule network interferes specifically with insulin-mediated GLUT4 translocation, we examined other insulin-mediated exocytosis pathways that are independent of the GLUT4 pathway. First, we examined the effects of nucodazole treatment on insulin-mediated secretion of adipin. Recently, two groups have shown that insulin stimulates a 2–3-fold increase in adipin secretion through an ARF6-dependent pathway that passes through an endosomal intermediate on its way to the plasma membrane (34, 45). We measured adipin secretion into culture media over a 30-min incubation period in control and nucodazole-treated cells with-
out or with 100 nM insulin (Fig. 4). Quantification of three independent experiments is shown in Fig. 4B. Insulin treatment caused a 3-fold increase in adipsin secretion in both control and nocodazole-treated cells, indicating that an intact microtubule network is not required for this process.

GLUT1 is also recruited to the plasma membrane in response to insulin (59, 60). Two lines of evidence suggest that the GLUT1 pathway is independent of the GLUT4 pathway. First, intracellular vesicles containing GLUT1 are largely distinct from vesicles containing GLUT4 (59, 60). Second, accumulation of GLUT1 in the plasma membrane is not dependent on the syntaxin 4 SNAP receptor, which is required for GLUT4 translocation (23). To determine whether insulin-dependent GLUT1 translocation was inhibited by nocodazole treatment, the plasma membrane sheets were stained for GLUT4 (see Fig. 1B) and were next probed for GLUT1 (Fig. 5A). In contrast to its effect on GLUT4, nocodazole treatment had no effect on insulin-mediated GLUT1 translocation.

**Taxol Treatment Inhibits Insulin-mediated GLUT4 Translocation**—It is possible that the effect of nocodazole on GLUT4 translocation was independent of its effects on the microtubule cytoskeleton, but rather due to some unrelated cytotoxic effect. To test this, we treated the cells with 12 μM taxol to stabilize the microtubule network. In vivo polymerization of microtubules by taxol has been shown to inhibit vesicular exocytosis in...
GLUT4 Vesicles Associate with Microtubules in Vitro—

Although our examination of insulin-signaling pathways was not exhaustive, it was sufficient to conclude that nocodazole treatment did not affect proximal insulin signaling events at the cell surface or decrease steady state levels of proteins known to be involved in proximal insulin signaling. A plausible explanation consistent with our observations is that destabilization of the microtubule network interferes with vesicle translocation and/or with later events in insulin signaling that lead to translocation. To begin to examine these possibilities, we performed an in vitro microtubule binding assay (47) to determine whether GLUT4 vesicles associate directly with microtubules.

Tubulin was polymerized in vitro by addition of taxol to a post-40,000 × g supernatant from 3T3-L1 cells homogenized in microtubule stabilization buffer without detergent. The 40,000 × g supernatant was obtained by centrifuging the post-nuclear supernatant at 40,000 × g, and this fraction consisted of low density vesicles including GLUT4 vesicles and cytosolic proteins including tubulin dimers (47, 56). Addition of 20 μM taxol at 4 °C polymerized ~60% of tubulin (data not shown). Polymerized tubulin was pelleted from the taxol-treated 40,000 × g supernatant by slower speed centrifugation at 16,000 × g. Tubulin appeared in the 16,000 × g pellets only in the presence of taxol (Fig. 7, lane 1). The 16,000 × g supernatant (obtained from the 16,000 × g spin of the 40,000 × g supernatant) (Fig. 7, lane 5) depicts a proportional loading of the supernatant from samples not treated with taxol to indicate the level of each protein in the 40,000 × g supernatant prior to taxol treatment. The 16,000 × g pellet was probed for associated proteins by Western blot analysis. IRS-1, GLUT4, and the insulin-responsive aminopeptidase (IRAP), a protein that exclusively colocalizes with GLUT4 vesicles (61), were associated with the 16,000 × g pellets, and this association was dependent on taxol (Fig. 7, lane 2). The β-subunit of the insulin receptor was not detected in this pellet even though insulin receptor was present in the 40,000 × g supernatant (Fig. 7, compare lanes 2 and 5). Insulin receptor was not present the 16,000 × g pellets, demonstrating that vesicles containing the insulin receptor do not bind microtubules. Together, these results indicate that our assay for vesicle binding to polymerized microtubules is specific.

Insulin treatment did not affect the polymerization of tubulin; however, IRS-1, GLUT4, and IRAP bindings to polymerized tubulin were decreased in response (Fig. 7, lanes 3 and 4). The decrease in GLUT4 and IRAP binding to polymerized microtubules in response to insulin is consistent with data indicating a flux from intracellular membrane compartments to the plasma membrane. In contrast to GLUT4, the abundance of IRS-1 in the 40,000 × g supernatant was the same in basal and insulin-treated cells (data not shown). Thus, the decreased association of IRS-1 with polymerized tubulin from the insulin-treated 40,000 × g supernatant indicates that insulin treatment decreases the affinity of IRS-1 for microtubules.

To determine whether microtubule-associated IRS-1 was phosphorylated in response to insulin, the 16,000 × g pellets and 16,000 × g supernatant from cells were probed with anti-phosphotyrosine antibody. IRS-1 appearing in the 16,000 × g pellets was tyrosine-phosphorylated (Fig. 8, lanes 4 and 5) following insulin treatment. Phosphorylated IRS-1 levels were similar in the 16,000 × g pellets and the 16,000 × g supernatant (Fig. 8, compare lanes 4 and 5 with lanes 7 and 8), indicating that phosphorylation state of IRS-1 does not affect its binding to polymerized tubulin.

Characterization of GLUT4 Vesicle Binding to Microtubules In Vitro—In vitro microtubule binding was performed in a detergent-free cell homogenate; it was therefore possible that the association of GLUT4 vesicles with microtubules was mediated either directly through GLUT4, through IRAP, or through an undetermined vesicle-associated protein. To determine whether GLUT4 or IRAP bind to microtubules through direct protein interactions, we performed the microtubule bind-
Insulin stimulation of GLUT4 requires the presence of microtubules in vitro. We demonstrated that destabilization of the microtubule cytoskeleton completely inhibits GLUT4 translocation to the plasma membrane. This observation strongly implies that recruitment of GLUT4 vesicles to the plasma membrane, rather than docked and awaiting fusion, implies that an insulin-induced signal triggers the movement of storage vesicles to the plasma membrane after which fusion occurs. Movement of intracellular vesicles to the plasma membrane, as opposed to regulation membrane fusion, has recently been shown to be a rate-limiting step in insulin-stimulated GLUT4 translocation (32). It is of considerable interest to understand movement of GLUT4 vesicles in response to insulin at the molecular level. Our studies indicate that the microtubule cytoskeleton plays a fundamental role in this process.

We demonstrated that destabilization of the microtubule cytoskeleton completely inhibits GLUT4 translocation to the plasma membrane. This observation strongly implies that recruitment of GLUT4 vesicles to the plasma membrane surface requires an intact microtubule network. A reasonable assumption, based on known functions of microtubules in other secretory pathways, is that microtubules act as a filament upon which the GLUT4 vesicle moves to its cell surface location (36). Movement of vesicles between cellular compartments can occur solely by membrane fusion and fission events, but an acute response to insulin utilizing regulated fusion would require that vesicles be prelocked at the target membrane. This does not appear to be the case with GLUT4 vesicles, since insulin stimulates the recruitment of GLUT4 vesicles from varied locations within the cell.
Disruption of the microtubule network is a profound alteration of a major cellular structure that may have global effects on cellular processes and vesicular trafficking. To control for this possibility, we studied other insulin-mediated vesicular trafficking pathways independent of GLUT4 exocytosis. Neither the insulin-mediated secretion of adipisin nor translocation of GLUT1 were found to be affected by disruption of the microtubule network. Thus, the microtubule network is required for a subset of membrane trafficking events in these cells, and this subset includes insulin-mediated GLUT4 exocytosis.

We demonstrated that GLUT4 vesicles specifically bind polymerized tubulin in vitro, implying that this interaction occurs in vivo. In the in vitro microtubule binding assay, we demonstrated that GLUT4 vesicle binding to microtubules was specific, since insulin receptor-containing vesicles did not associate with polymerized tubulin under conditions in which GLUT4 vesicles associate with polymerized tubulin (Fig. 9). Thus the microtubule network may act as a scaffold upon which GLUT4 storage vesicles are tethered awaiting insulin stimulation and/or a track upon which they travel after a signal has been transduced to the vesicles.

Our data suggest that the microtubule network may provide a link between the insulin-signaling pathway and GLUT4 vesicle translocation. Although we demonstrated that proximal signaling events through activation of PKB were not affected by destabilization of microtubules in vivo (with nocodazole treatment), it is possible that the pathway(s) of the insulin-signaling cascade leading specifically to GLUT4 vesicle recruitment is localized to microtubules. In support of this hypothesis, we observed that IRS-1 specifically bound to polymerized tubulin (Fig. 9). Interestingly, recent work indicates that IRS-1 is bound to cytoskeletal elements, but the specific cytoskeletal components have not yet been identified (56). Our data suggest that microtubules may be an in vivo binding site for IRS-1.

Other studies have investigated the role of the actin cytoskeleton in insulin-mediated GLUT4 translocation. Disruption of the actin cytoskeleton using either cytochalasin D or latrunculin A partially inhibited GLUT4 translocation in 3T3-L1 cells (38) and completely disrupted this process in rat adipocytes (39). Although a direct association between GLUT4 vesicles has not yet been demonstrated, experimental evidence suggests that an intact actin cytoskeleton is required for recruitment of PI 3-kinase activity to the GLUT4 vesicle (38). It has not yet been established that recruitment of PI 3-kinase to GLUT4 vesicles is required for insulin-mediated GLUT4 translocation. In a recent study, it was shown that targeting of constitutively active PI 3-kinase to GLUT4 vesicles is not sufficient on its own to redistribute GLUT4 vesicles to the plasma membrane (64); however, that does not prove that it is not necessary for translocation. It is of considerable interest to determine whether the microtubule cytoskeleton in addition to the actin cytoskeleton is involved in the insulin-mediated recruitment of PI 3-kinase to GLUT4 vesicles.

There are numerous potential roles for the microtubule network in insulin-mediated GLUT4 translocation. One possibility is that the microtubule network may provide a supporting structure that tethers intracellular GLUT4 vesicles in the basal state. Insulin signaling would release the vesicles allowing them to move to the plasma membrane by simple diffusion. If this were correct, complete depolymerization of the microtubule network would be expected to result in accumulation of GLUT4 at the plasma membrane in the basal state. We did not observe a change in plasma membrane GLUT4 levels in basal cells treated with nocodazole; however, we cannot preclude the possibility that GLUT4 vesicles were docked at the plasma membrane, but not fused, in the nocodazole-treated cells. Alternatively, the microtubule network may provide a supporting structure upon which GLUT4 vesicles travel to the plasma membrane. It is possible that GLUT4 vesicles are tethered to microtubules in vivo in the basal state and that insulin promotes association of the GLUT4 vesicle with a microtubule motor protein. Support of this model requires the identification of a microtubule motor protein that is regulated by insulin and associates with GLUT4 vesicles. There are numerous examples of vesicular trafficking along microtubule filaments. For instance, the regulation of bile secretion is a regulated exocytotic process in liver cells that has been shown to require an intact microtubule system (65). This system resembles GLUT4 translocation in that the bile-containing vesicles are recruited to the plasma membrane upon stimulation of cells with an appropriate ligand.

In the present study, we provide functional and biochemical data demonstrating that insulin-mediated GLUT4 translocation is dependent on the microtubule cytoskeleton in 3T3-L1 adipocytes. The role of the microtubule network in this process is under investigation. We demonstrated that GLUT4 vesicles bind polymerized tubulin in vitro, and this suggests that the microtubule network may act to scaffold and compartmentalize GLUT4 vesicles. Our data also suggest that the microtubule network may provide a structure for the insulin-signaling cascade to interface directly with GLUT4 vesicles and stimulate their exocytosis. Further studies focusing on the role(s) of the microtubule cytoskeleton in insulin-mediated GLUT4 translocation are currently in progress.

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