Perinuclear Localization and Insulin-Responsiveness of GLUT4 Requires Cytoskeletal Integrity in 3T3-L1 Adipocytes.

Adilson Guilherme, Masahiro Emoto, Joanne M. Buxton, Sahana Bose, Rosanna Sabini, William E. Theurkauf, John Leszyk and Michael P. Czech.

Program in Molecular Medicine and Departments of Biochemistry and Molecular Biology and Molecular Genetics and Microbiology, University of Massachusetts Medical School, Worcester, MA 01605.

*Corresponding author

Address correspondence to:
Dr. Michael P. Czech
Program in Molecular Medicine
373 Plantation Street
Worcester, MA 01605
Phone: 508 856-2254
FAX: 508 856-1617
E-mail: Michael.Czech@umassmed.edu

+This work is supported by National Institutes of Health Grant DK 30898 (M.P.C.) and the Proteomics and Electron Microscopy Core Facilities of the Diabetes and Endocrinology Research Center Funded by the National Institutes of Health DK (32520).
Running Title

*Microtubules and Glut4 Localization*
SUMMARY

The GLUT4 glucose transporter resides mostly in perinuclear membranes in unstimulated 3T3-L1 adipocytes and is acutely translocated to the cell surface in response to insulin. Using a novel method to purify intracellular GLUT4-enriched membranes, we identified by mass spectrometry the intermediate filament protein vimentin and the microtubule protein α-tubulin as components of these membranes. Immuno-electron microscopy of the GLUT4-containing membranes also revealed their association with these cytoskeletal proteins. Disruption of intermediate filaments and microtubules in 3T3-L1 adipocytes by microinjection of a vimentin-derived peptide of the helix initiation 1A domain caused marked dispersion of perinuclear GLUT4 to peripheral regions of the cells. Inhibition of the microtubule-based motor dynein by brief cytoplasmic acidification of cultured adipocytes also dispersed perinuclear GLUT4 and inhibited insulin-stimulated GLUT4 translocation to the cell surface. Insulin-sensitivity was restored as GLUT4 was again concentrated near the nucleus upon recovery of cells in physiological buffer. These data suggest that GLUT4 trafficking to perinuclear membranes of cultured adipocytes is directed by dynein and is required for optimal GLUT4 regulation by insulin.
INTRODUCTION

Insulin regulates the cellular uptake of glucose in adipose tissue and muscle by acutely controlling the number of glucose transport proteins present in the cell surface membrane (1-4). The major insulin-responsive sugar transporter, GLUT4, recycles in endosomal and exocytic membranes of these cells (5-7), and is mostly sequestered within intracellular membranes in the unstimulated state (8-10). Insulin acts primarily by enhancing the exocytosis of GLUT4, but the hormone also appears to inhibit endocytosis as well (5-7). Recent studies have revealed that the insulin-regulated intracellular membranes containing GLUT4 are specialized and appear to exclude some other cycling proteins such as the transferrin receptor and the GLUT1 glucose transporter (11, 12). These latter proteins are present at the cell surface in higher abundance than GLUT4 in unstimulated cells, and move through a rapid constitutive endosomal recycling pathway (13, 14). Complicating our understanding of the interrelationships between these trafficking systems are findings suggesting that GLUT4 also partially co-localizes with endosomal membranes containing the transferrin receptor and GLUT1 (12, 15, 16). Because normal glucose homeostasis in humans is dependent upon the dynamics of GLUT4 membrane trafficking, intensive efforts have been directed at understanding the basis for the unique recycling characteristics and regulation of GLUT4.

Isolation of the intracellular membranes of adipocytes and muscle that are enriched in GLUT4 has been achieved using immuno-adsorption procedures with immobilized antibodies raised against GLUT4 (17, 18). These membranes are likely a mixture of endosomal membrane fractions as well as the specialized insulin-responsive membranes. Many proteins that reside in the intracellular membranes containing GLUT4 have been identified (for review, see ref 19). Such membrane preparations have been analyzed by both biochemical and immunological methods and contain receptors that transport various ligands within cells such as the IGF-
II/mannose-6-phosphate receptor (17) as well as several other transmembrane proteins, including IRAP (20), amine oxidase (21) and sortilin (22). Proteins that associate with GLUT4-containing membranes also include several that are thought to function in mechanisms of membrane trafficking such as VAMP2 (23, 24), SCAMPs (25, 26), syntaxin 4 (27), and SNAP23 (28-30). Synip-1 in turn has been identified as a syntaxin 4-interacting protein and its interaction with syntaxin 4 is reported to be regulated by insulin (31). A unifying hypothesis has been derived from the work of many laboratories on these proteins which suggests that fusion of GLUT4-containing membranes with the plasma membrane involves VAMP2/syntaxin4 interaction regulated by SNAP23, MUNC 18, synip-1 and other proteins (4). The molecular mechanisms by which this process may be regulated by insulin is not yet understood.

It seems likely that insulin also regulates events in the GLUT4 translocation process that occur prior to plasma membrane docking or fusion. For example, in unstimulated 3T3-L1 adipocytes, an excellent model system for insulin action on GLUT4, most of the GLUT4 is present in perinuclear membranes and in other intracellular membranes that are not apparently connected to the plasma membrane (32, 33). Also, the time course of insulin action on GLUT4 translocation is rather slow (several minutes) compared to the stimulation of neurotransmitter release, which involves rapid fusion of synaptic vesicles near the cell surface. These characteristics of GLUT4 regulation are consistent with the hypothesis that insulin causes translocation of GLUT4-containing membranes over some distance prior to docking and fusion with the plasma membrane. However, no data are available on the mechanisms whereby GLUT4 membranes are sequestered and restrained in a perinuclear localization in 3T3-L1 adipocytes under basal conditions. Nor is there an understanding of the processes whereby GLUT4-containing membranes actually move to the plasma membrane.
In order to gain insight into these issues, we developed a method to purify GLUT4-containing membranes that does not involve anti-GLUT4 antibodies which can contaminate membrane preparations. Mass spectrometry analysis of proteins in the purified GLUT4–containing membranes identified the cytoskeletal proteins vimentin and alpha-tubulin, suggesting a role for intermediate filaments and microtubules in their localization. We provide strong evidence in favor of this concept. Importantly, our data support the hypothesis that dynein motor activity on microtubules is required to localize GLUT4 into perinuclear membranes that constitute the insulin-sensitive compartment. These results provide a new framework to test hypotheses on the movements of GLUT4 based on the regulation and coordination of cellular motors and cytoskeletal structures.
EXPERIMENTAL PROCEDURES

**Materials** - Rabbit polyclonal anti-GLUT4 antibody was raised against the C-terminal 12 amino acid sequence of GLUT4. Mouse anti-transferrin receptor was from Zymed. Rabbit polyclonal anti-VAMP2 antibody was from StressGen Biotechnologies Corp. Mouse monoclonal anti-vimentin antibody used in immunoblots and immuno-electron microscopy analysis was from Santa Cruz. Mouse monoclonal anti-α-tubulin antibody, used in immunoblot and immuno-electron microscopy analysis and the secondary antibodies conjugated to gold particles for immuno-electron microscopy were from Ammersham Pharmacia Biotech.

**Cell Culture** - 3T3-L1 fibroblasts were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 50 µg/ml streptomycin, 50 units/ml penicillin and 10% fetal bovine serum. 3T3-L1 fibroblasts (2-3 days postconfluent) were differentiated into adipocytes by incubating with DMEM supplemented with the same antibiotics, 10% fetal bovine serum, 0.5 mM isobutylmethylxantine, 0.25 µM dexamethasone and 2.5 µg/ml insulin for 3 days, grown in DMEM with 10% fetal bovine serum and 2.5 µg/ml insulin for 3 days and then grown in DMEM with 10% fetal bovine serum for an additional 5-8 days.

**Isolation and Fractionation of GLUT4-containing vesicles** - Adipocytes were isolated from epididymal fat pads of Male Sprague-Dawley Rats (125-150 g) by collagenase digestion using Krebs-Ringer/HEPES, pH 7.4, supplemented with 2% bovine serum albumin and 2 mM pyruvate. Cells were washed and incubated for a 30 min recovery period prior to initiation of experiments. Cells were then incubated at 37 °C with or without 100 nM insulin for 20 min. The cells were then washed with PBS and immediately homogenized in buffer A (50 mM HEPES, pH 7.4, 10 mM NaF, 1 mM NaPPi, 0.1 mM Na₃VO₄, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin and 10 µg/ml leupeptin), and then subjected to differential centrifugation as
described previously (6) to isolate the low density microsomal (LDM) subcellular fractions. Protein was quantified using the bicinchoninic acid protein determination kit (Pierce) with bovine serum albumin as standard. The GLUT4-enriched fractions were then isolated from LDM fractions utilizing the sedimentation sucrose velocity gradient centrifugation method exactly as previously described (34, 35). Briefly, 1.5 to 2 mg of LDM fractions were loaded onto a 10-35% sucrose velocity gradient (buffer B, 20 mM HEPES, pH 7.4, 100 mM NaCl, 1 mM EDTA, 2 mM dithiothreitol, 1 mM, 10 mM NaF, 1 mM NaPPi, 0.1 mM NaVO₄, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin and 10 µg/ml leupeptin) and centrifuged for 3.5 hrs at 24,000 rpm in a SW28 rotor (Beckman). Fractions containing GLUT4-membranes (Fractions 8 to 18) were pooled, pelleted by ultracentrifugation at 48,000 rpm for 1.5 hrs, resuspended in buffer B and then loaded onto an equilibrium density sucrose gradient (10-65% (w/v) in buffer B and centrifuged at 35,000 rpm for 18 hr in a SW 50.1 rotor (Beckman). After centrifugation, fractions were collected starting from the top of the gradient and analyzed for the total protein content determined by the Bradford assay (Bio-Rad).

**Immunoblotting** - Fractions from velocity gradients and equilibrium density gradient were prepared as described above and aliquots from these fractions were subjected to SDS-PAGE on resolving gels according to Laemmli (36). Separated proteins were electrophoretically transferred to nitrocellulose membrane, blocked with 3% nonfat milk and 1% BSA in TTBS (0.05% Tween 20 in Tris-buffered saline) and then incubated with primary antibody in TTBS containing 1% BSA. After incubation, membranes were washed with TTBS and incubated with horseradish peroxidase-labeled anti-mouse IgG for the detection of monoclonal antibodies or with horseradish peroxidase-labeled anti-rabbit IgG for detection of polyclonal antibodies. Proteins were visualized using an enhanced chemiluminescent substrate kit (Amershan Pharmacia Biotech) and immunoblot intensities were quantified by a scanning densitometer.
**Electron Microscopy** - GLUT4-containing membranes of the insulin sensitive fractions from the equilibrium density gradient were isolated as described above. Fractions were pooled, pelleted by centrifugation at 48,000 rpm for 2 h, resuspended in PBS and fixed in a final concentration of 2% paraformaldehyde in PBS. GLUT4-vesicles were then adsorbed to Formvar-coated gold grids and processed for double labeling as outlined in Martin et al. (24) and Sleeman et al. (37). Grids were incubated with 50µl of primary antibody diluted in 1% BSA and PBS as follows: anti-GLUT4, anti-IRAP, anti-vimentin, anti-α-tubulin or non-immune IgG, as a negative control. After incubation with each IgG fraction, grids were labeled with either 5 or 15 nm gold particles conjugated to the secondary antibody (goat anti-rabbit or goat anti-mouse). Grids were stained with 1% uranyl acetate, dried and viewed using a transmission electron microscope PHILLIPS CM.10.

**MALDI-TOF MS Analysis** - Proteins resolved by SDS-PAGE were visualized by silver staining (Bio-Rad) and the bands were excised from one single dimensional 5-15% gel. The silver stained proteins bands were destained and tryptically digested (trypsin) in gel according to Gharahdaghi et al. (38) with some slight modifications. The digested samples were further concentrated and desalted with Millipore Zip Tip C18 micro tips prior to MALDI-TOF analysis. MALDI-TOF analyses were performed on a Kratos Analytical Kompact SEQ Instrument, equipped with a curved field reflectron. Peptide masses were searched against the non-redundant protein database using MS-Fit of the Protein Prospector program developed by Clauser et al (39) at University of California, San Francisco. Fragmentation information obtained from individual peptides via Post-Source-Decay (PDS) analysis was searched against the non-redundant protein database using the protein prospector program MS-Tag.
Microinjection of 3T3-L1 adipocytes-- 3T3-L1 cells at 7-9 days post differentiation were released from the cell culture dishes using 0.5 mg/ml Collagenase D and 0.025% trypsin in PBS at 37°C. Cells were resuspended in DMEM containing 10% FBS, 50 units/ml and 50 µg/ml streptomycin, centrifuged at 1000 rpm in a Beckman GPK centrifuge, and resuspended in media once again. The cells were plated on grid coverslips (Eppendorf) in 12 well dishes at a density of approximately 1.5x10^5 cells per well. The next day approximately 300 cells were impaled using the Eppendorf model 5171 micromanipulator and injected using the Eppendorf model 5246 microinjector with approximately 0.1 pl of a 2 mg/ml solution of vimentin peptide Vm-1A (peptide sequence) or control peptide (Vimentin amino acids 1-18 mutated R10H) containing 20 µg/ml FITC conjugated Dextran. The cells were allowed to recover for 60 minutes and then fixed with 3.7% formaldehyde in PBS for 10 minutes. The coverslips were processed for immunofluorescence as described below.

Immunofluorescence--Formaldehyde fixed cells were permeabilized with methanol for 2 minutes and blocked in PBS+1% FBS +0.5% TritonX-100 for 15 minutes. GLUT4 antibody diluted 1:1000 was applied to the coverslips for 2 hours, washed and detected with rhodamine conjugated anti-rabbit secondary antibody. Microinjected cells, identified by FITC fluorescence were evaluated for GLUT4 dispersion.

Acetate Treatment and GLUT4 Translocation Assays - Coverslips containing 3T3-L1 adipocytes were incubated in Krebs Ringer's/HEPES, KRH (80 mM NaCl, 10 mM HEPES, 10 mM glucose, 5 mM KCl, 2 mM CaCl2, 2 mM NaPO4, 1 mM MgCl2) containing 70 mM sodium acetate, pH 6.4 for 15 min in a 37°C incubator. These conditions have previously been shown to selectively inhibit dynein activity (40). Acetate recovery was achieved by washing the coverslips 3 times with KRH without acetate and incubating at 37°C for 15 or 60 min, as indicated in the legends of Figures. Cells were then fixed in 3.7% formaldehyde as described.
above and GLUT4 visualized by immunofluorescence. For the effect of acetate treatment on insulin-stimulated GLUT4 translocation, cells were treated with KRH with or without acetate, allowed to recover for the indicated times and stimulated or not with insulin for 15 min.

GLUT4 translocation was assayed in plasma membrane lawns, generated by using a technique similar to the one previously described (41, 42). Briefly, membrane lawns were fixed on the coverslips with 3.7% formaldehyde for 10 min, washed twice with PBS and incubated for 45 min with 2% BSA in PBS. To quantify GLUT4 on lawns, the coverslips were incubated with a 1:1000 dilution of rabbit anti-GLUT4 polyclonal antibody in PBS and 0.05% Tween-20. Coverslips were washed 5 times for 3 min each and they were incubated with a 1:1000 dilution of FITC conjugated goat anti-rabbit mixed with 10 μg/ml rhodamine conjugated wheat germ agglutinin (to quantitate plasma membrane). The coverslips were washed as before and postfixed for 10 min with 3.7% formaldehyde followed by a final wash with PBS. They were then mounted on slides with 90% glycerol in PBS (DABCO) and viewed with x 60 objective on a Nikon Diaphot 200 inverted microscope coupled to a Bio-Rad MRC1024 processing unit. Images were analyzed by the Lasersharp processing software.
RESULTS

Purification of insulin-responsive GLUT4-containing membranes. In order to purify GLUT4-containing membranes, low density microsomes were first prepared from primary, unstimulated rat adipocytes by standard techniques. This crude membrane fraction contains most of the GLUT4 present in unstimulated adipocytes and is composed primarily of intracellular membranes (6). These membranes were then subjected to sucrose velocity gradient centrifugation (Fig. 1), which reportedly yields a more purified GLUT4-enriched membrane preparation (34, 35). Consistent with previous results, this technique separates about 90% of the total membrane protein (fractions 1-7) away from the GLUT4-enriched membranes (fractions 8-18). Insulin treatment of rat adipocytes prior to disruption of the cells and preparation of these membranes causes a marked decrease in the yield of GLUT4 present in the latter fractions (Fig. 1A and upper panel of Fig. 1B). However, no such insulin effect is observed when total membrane protein is measured (lower panel of Fig 1B) because these membranes are still highly contaminated with membranes that do not contain GLUT4 and are not insulin-responsive.

Fractions 8-18 containing most of the GLUT4 from the sucrose velocity gradient were therefore subjected to equilibrium gradient centrifugation to further resolve the membrane species present (Fig. 2). Most of the membrane protein was distributed over fractions 5-20 after this procedure, whereas most of the GLUT4 was distributed within fractions 7-14. Importantly, this GLUT4 was separated into two types of membranes that could be distinguished based on their sensitivity to insulin. The amount of GLUT4 in fractions 7-9 was decreased when the cells were treated with insulin before homogenization and preparation of membranes, whereas the GLUT4 in fractions 10-20 was not effected by insulin treatment of the adipocytes (Fig. 1A and upper panel of Fig 1B). Strikingly, measurement of total membrane protein in the fractions of this gradient revealed a similar profile: about a 50% reduction in fractions 7-9 due to insulin
action, with no insulin effect observed in fractions 10-20 (lower panel of Fig. 1B). This observed insulin-mediated decrease in total membranes recovered in fractions 7-9 indicates the successful partial purification of membranes of the insulin-responsive compartment or compartments in primary adipocytes. Similar data has been obtained using 3T3-L1 adipocytes (not shown).

Two additional approaches were used to characterize the membranes resolved by equilibrium gradient centrifugation. First, each of the fractions from the gradient were analyzed by SDS-PAGE and silver staining of the constituent proteins (Fig. 3). This analysis revealed that most of the membrane proteins in fractions 7 and 8 were dramatically reduced when membranes were derived from insulin-treated adipocytes. Certain proteins in fractions 6 and 9 showed the same effect, whereas many did not (Fig. 3). These results suggest that membranes resolved in fractions 7 and 8 are highly purified insulin-responsive membranes, while those in fractions 6 and 9 are only partially purified. Membranes in higher density fractions show no detectable insulin-sensitivity in spite of the presence of significant GLUT4 protein. Of note is the finding that many of the protein bands in the insulin-sensitive membranes are also present in the membranes that are not responsive to the hormone (compare lanes 7-9 to lanes 10-13 in Fig. 3). These data are consistent with the hypothesis that the insulin-sensitive membranes containing GLUT4 contain many of the same constituent proteins as other cell membranes that function in a hormone-insensitive mode.

A second set of experiments was conducted on the membranes fractionated by equilibrium gradient centrifugation to determine the distribution of transferrin receptors, thought to be present in endosomal membranes, and VAMP2, thought to be associated with insulin-insensitive GLUT4-containing membranes (18, 19). Surprisingly, both these proteins were present in the fractions that were responsive to insulin and their distributions were more restricted to
these fractions than was GLUT4 itself (Fig 4). These data suggest the insulin-sensitive membranes in these fractions are contaminated by recycling endosomes or that transferrin receptor is present in the insulin-sensitive membranes or both. The restriction of VAMP2 to the insulin-sensitive fractions is consistent with data showing that VAMP2 function is necessary for GLUT4 translocation to the plasma membrane in response to insulin (23, 24).

**Identification of cytoskeletal proteins in GLUT4-containing membranes.** In order to identify proteins present in the insulin-sensitive membranes containing GLUT4, in separate experiments the equivalent of fractions 7 and 8 shown in the experiment of Figure 3 were pooled, analyzed by SDS-PAGE and the gels silver stained (Fig. 5). These results confirmed that many of the resident proteins in the membranes derived from insulin-treated cells were present at lower abundance compared to controls (arrowheads in Fig. 5). Many of the protein bands, combined from both lanes, were subjected to tryptic hydrolysis and the peptides analyzed by mass spectrometry. Of the proteins identified by this procedure, peptides derived from GLUT4 itself appeared in two closely spaced bands. Remarkably, the lower of these bands also contained a peptide corresponding to the phosphorylated form of the COOH-terminus of GLUT4 (Fig. 5), indicating significant amounts of phosphorylated GLUT4 are present in insulin-sensitive membranes. In addition, peptides corresponding to several proteins previously reported to be present in these membranes were identified, including the IGF-II/mannose-6-phosphate receptor, IRAP, amine oxidase, long chain acyl-CoA synthetase and SCAMPs (Fig. 5). Two proteins not previously known to be present in insulin-sensitive GLUT4-containing membranes were also identified—vimentin, an intermediate filament subunit, and α-tubulin, the microtubule protein.

Two approaches were taken to determine if vimentin and α-tubulin are actually directly associated with membrane vesicles that also contain GLUT4 and are insulin-sensitive. First, the membrane preparations obtained from the equilibrium gradient centrifugation were analyzed by
immunoelectron microscopy using anti-GLUT4, anti-vimentin and anti-tubulin antibodies. As shown in Figure 6, most of the vesicles in our preparations are reactive with anti-GLUT4 (large colloidal gold particles), indicating relatively low contamination with membranes that do not contain the transporter. A fraction of these GLUT4-positive membrane vesicles also directly react with anti-vimentin (Fig. 6C and 6D, small colloidal gold particles) and anti-tubulin (Fig. 6E and 6F, small colloidal gold particles). Non-immune antibodies showed no detectable staining of these membranes under the conditions of these experiments, while the anti-GLUT4 was readily detected (Fig. 6G and H). These results indicate that some GLUT4-containing membrane vesicles are associated with the cytoskeletal proteins vimentin, α-tubulin or both.

To further assess association of vimentin and α-tubulin with insulin-sensitive membranes, the abundance of these cytoskeletal proteins was estimated using Western analysis in each of the membrane fractions obtained by equilibrium gradient centrifugation. Figure 7 shows a comparison of the relative abundance of GLUT4 protein versus vimentin and α-tubulin throughout these fractions. Both vimentin and alpha-tubulin are present in all of the membrane fractions of the gradient except for the top few fractions. Strikingly, both these proteins are greatly reduced in abundance in the same gradient fractions in which GLUT4 is also reduced in response to the action of insulin. In membrane fractions of higher density, the concentrations of GLUT4, vimentin, and α-tubulin are all unaffected by prior treatment of cells with insulin. Taken together, the experiments depicted in Figures 5-7 demonstrate that two cytoskeletal proteins, vimentin and α-tubulin, are bound to subpopulations of the GLUT4-containing membranes that are insulin-responsive in rat adipocytes.

**Perinuclear localization of GLUT4 depends on cytoskeletal integrity.** The intermediate filament protein vimentin has been previously implicated in playing a role in intracellular trafficking of proteins and membranes (43-45), and appears also to function in localization of
intracellular structures (44-45). It is also well established that microtubules and their associated motor proteins dynein and kinesins direct the cellular localization of cellular organelles such as mitochondria, the endoplasmic reticulum and golgi membranes (for review, see Ref. 46). Based on the findings presented in Figures 5-7 indicating that some GLUT4-containing membranes prepared from 3T3-L1 adipocytes are associated with tubulin as well as vimentin, we tested whether the intact cytoskeleton is required for perinuclear GLUT4 localization. To address this question, experiments were conducted to disrupt intermediate filaments in 3T3-L1 adipocytes by microinjection of peptide Vm-1A (RVTMQNLNDRLASYLDKV), derived from the helix initiation 1A domain of vimentin, which has been previously shown to cause disassembly of intermediate filaments in other cell types (47). Figure 8A confirms previous findings that cultured adipocytes do contain an extensive network of intermediate filaments (48, 49) and documents that introduction of this peptide into 3T3-L1 adipocytes disrupts this filamentous network (47).

Consistent with the known integration of intermediate filaments with microtubules via interconnecting proteins in mammalian cells (50), microinjection of peptide Vm-1A into cultured adipocytes was also found to disassemble microtubules (Fig. 8B). This observation is similar to published data in other cell systems (47). Microinjection into 3T3-L1 adipocytes of a peptide identical to Vm-1A except for a single residue change (H substituted for R10), which has been shown to be ineffective in disrupting vimentin dimerization (47), failed to alter cytoskeletal morphology (not shown).

Figure 9 depicts the results of microinjection of Vm-1A peptide or its biologically inactive R10H homolog (control peptide) on the intracellular GLUT4 distribution in 3T3-L1 adipocytes after 60 min. Uninjected adipocytes or those microinjected with control peptide mostly display a normal, perinuclear localization of GLUT4, with some of the glucose
transporter also distributed in vesicular structures throughout the cytoplasm. In contrast, GLUT4 in many of the cultured adipocytes microinjected with the Vm-1A peptide appears dispersed away from the juxtanuclear regions of the cells (Fig. 9). In some cases the GLUT4 in these latter cells apparently remains associated with several large membrane clusters, whereas in other cells the dispersion appears more complete. The Vm-1A peptide-induced movement of GLUT4 away from the juxtanuclear regions correlates with the disassembly of both intermediate filaments and microtubules (Figs. 8 and 9).

Images similar to those obtained with the Vm-1A peptide above were also observed when 3T3-L1 adipocytes are treated with the microtubule-disrupting agent nocodazole (not shown). The microtubule motor dynein is known to be required for the perinuclear localization of endoplasmic reticulum and golgi membranes, as well as the recycling endosome that is transited by the transferrin receptor (51, 52). Heuser (40) showed that dynein motor activity can be reversibly disrupted without inhibition of kinesin function by slight acidification of the cytoplasm, causing perinuclear lysosomes to disperse to the cell periphery. Using this method, we monitored the disposition of GLUT4 in 3T3-L1 adipocytes immediately after a 15 min treatment with acetate buffer, pH 6.4, or following a recovery period of 3 hours in physiological buffer. Figure 10 shows that cytoplasmic acidification of 3T3-L1 adipocytes causes dispersion of much of the perinuclear GLUT4 to vesicular structures throughout the cytoplasm. Subsequent incubation of the acetate-treated adipocytes for 3 hours at physiological pH restored normal perinuclear GLUT4 localization. Thus, the data in Figure 10 show in cultured adipocytes a tight correlation between a treatment known to disrupt the microtubule-based motor dynein and the perinuclear disposition of GLUT4.

The reversible dispersion of GLUT4 from the perinuclear region of 3T3-L1 adipocytes in response to cytoplasmic acidification offers the opportunity to test whether perinuclear
localization of GLUT4 is necessary for the responsiveness of GLUT4 to insulin-mediated translocation to the cell surface. Figure 11 shows that a 15 min acetate treatment of 3T3-L1 adipocytes, which causes dispersion of GLUT4, prior to washing at physiological pH and incubation with insulin for 10 min blocks the hormone’s effect on recruitment of GLUT4 to plasma membranes. In these experiments, cell surface GLUT4 is estimated by the binding of anti-GLUT4 antibody to plasma membrane sheets that are adsorbed to class cover slips following light sonication of attached cells. When acetate-treated cells are allowed to recover for 60 min at physiological pH, GLUT4 returns to a perinuclear localization (not shown for these experiments, see Fig. 10), and insulin responsiveness of GLUT4 measured by its translocation to the cell surface membrane is also restored (Fig.11). These data document a correlation between normal localization of GLUT4 in the juxtanuclear region of cultured adipocytes and sensitivity of intracellular GLUT4 to insulin action.
DISCUSSION

The major conclusion of the studies presented here is that the characteristic perinuclear localization of GLUT4 in 3T3-L1 adipocytes requires an intact cytoskeleton, and that this requirement may reflect the action of the microtubule-based motor dynein. Disruption of the intermediate filament and microtubule systems in these cells by microinjection of the vimentin-derived peptide Vm-1A (Fig. 8) or by the microtubule-disrupting agent nocodazole (not shown) leads to dispersion of the perinuclear GLUT4 into the cell periphery (Fig. 9). It is well established that intermediate filaments and microtubules are highly integrated in mammalian cells, and disruption of either of these cytoskeletal elements has deleterious effects on the other (47, 50). Our data are consistent with this concept in that introduction of the vimentin-derived peptide not only disperses intermediate filaments in the cultured adipocytes but also disrupts microtubules (Fig. 8). Interestingly, vimentin-containing intermediate filaments have been reported to surround small lipid droplets in cultured adipocytes during differentiation, and may have a function in lipogenesis and lipid storage (48). Also, reorganization of intermediate filaments is apparently mediated by cdc2-directed vimentin phosphorylation during mitosis and may play a role in vesicle orientation during cell division (43). However, ablation of the vimentin gene does not cause major phenotypic abnormalities in mice (53). Thus, while it is not possible to determine whether intermediate filament disruption or microtubule disruption or both are required to disperse perinuclear GLUT4 to the cell periphery, it seems likely that microtubules play the major role.

Our findings that vimentin-derived peptide or nocodazole causes dispersion of perinuclear-localized GLUT4 are consistent with a large body of literature strongly supporting a direct role of the microtubule-based cytoskeleton in localizing cellular organelles (54, 55).
Membranes of the endoplasmic reticulum and golgi complex that are retained in juxtanuclear regions of cells are dispersed to peripheral regions upon disruption of microtubules (56, 57), similar to our findings with GLUT4. Thus the perinuclear membrane system that sequesters GLUT4 in unstimulated 3T3-L1 adipocytes appears to be localized within cells by the same mechanisms that regulate the localization of other major cellular organelles.

A principal role for microtubules in membrane trafficking and organization reflects the balance of molecular motor activity directed towards the plus ends (cell periphery) versus minus ends (perinuclear region) of microtubules (for reviews see refs 46, 54). The microtubule-based motor dynein has been shown to direct many membranes to the juxtanuclear regions of cells, including endosomes (52), melanosomes (58), golgi (51, 59), and lysosomes (60). Upon inhibition of dynein activity, these membranes disperse out to the cell periphery. Kinesins are microtubule-based molecular motors that in general direct protein and organelle movement towards the plus ends of microtubules (for review see ref 46). For example, the dispersion of mitochondria throughout the cytoplasm is dependent upon kinesin function (61). Heuser (40) showed that dynein activity as opposed to kinesin activity could be selectively inhibited by a cytoplasmic acidification procedure, which was subsequently confirmed by the demonstration that the dynein motor complex is disrupted under these conditions (62). As has been shown for other perinuclear organelles in response to brief treatment of cells at pH 6.4, perinuclear GLUT4 in cultured adipocytes was found in the present study to disperse into the cell periphery upon acidification of the cytoplasm (Fig. 10). This response is apparently completely reversible upon incubation of the cells at physiological pH for 3 hr. These data are consistent with the hypothesis that the microtubule-based motor dynein functions to localize GLUT4-containing membranes into the perinuclear regions of cultured adipocytes.
The endosomal membrane system in which the transferrin receptor and other proteins continuously recycle between the cell surface membrane and intracellular membranes is also partially localized in a perinuclear disposition, which is disrupted by nocodazole (63, 64). However, exocytosis of transferrin receptor from this compartment is not impaired by this treatment, apparently dissociating the cellular position of this organelle from its function (65). In the case of regulated GLUT4 exocytosis there may be such a relationship. Disruption of dynein by incubation of 3T3-L1 adipocytes at low pH for 15 minutes followed by return to pH 7.4 did significantly inhibit the ability of insulin to cause translocation of GLUT4 to the plasma membrane (Fig. 11). Restoration of GLUT4 to its perinuclear disposition following a 1 hr recovery period at physiological pH occurred in conjunction with a return of normal insulin responsiveness. While these data will require confirmation with alternative techniques to disrupt dynein function, they suggest the possibility that dynein motor activity is necessary to confer insulin sensitivity to GLUT4-containing membranes in the juxtanuclear region of adipocytes. Further more detailed experiments are underway to test this hypothesis.

One of the fundamental questions about the mechanism by which insulin regulates GLUT4 is whether insulin causes intracellular membranes that contain GLUT4 to actually move and fuse with the plasma membrane or whether insulin causes only GLUT4 itself to move more rapidly through constitutively recycling membrane compartments (3, 4, 66). The former possibility is currently favored because proteins such as VAMP2 that are involved in membrane docking and fusion, and are present in GLUT4-containing membranes, are also translocated to the plasma membrane in response to insulin (23, 24). However, this data is indirect. Here we provide strong evidence in favor of this hypothesis by directly monitoring the mass of intracellular membranes enriched in GLUT4. A new method to prepare GLUT4-containing membranes without the use of anti-GLUT4 antibodies was devised for this purpose (Fig. 2).
This method is simple in that it employs a single sucrose equilibrium gradient centrifugation step to further purify GLUT4-containing membranes obtained by a previously published velocity gradient centrifugation procedure (34, 35). Following both centrifugation steps, the method yields about 10μg of purified GLUT4-containing membranes from a starting preparation of about 1mg of primary rat adipocyte low density microsomes. Importantly, insulin treatment of adipocytes prior to homogenization causes a lower yield of these intracellular membranes (Figs. 2-4), consistent with the concept that insulin action directs membranes contained in this fraction to the plasma membrane where they fuse.

It is likely that the purified membrane preparations we obtain that exhibit maximum insulin sensitivity (e.g., fractions 7 and 8 of Fig. 3) contain various other cell membranes in addition to the GLUT4 containing membranes that represent the insulin-sensitive compartment. Thus, much of the transferrin receptor is present in these fractions (Fig. 4), and this receptor is thought to be present mostly in recycling endosomes that apparently show only modest sensitivity to insulin (12, 16). However, this problem also applies to GLUT4-containing membrane preparations obtained by immuno-adsorption with anti-GLUT4 antibodies because GLUT4 is also present in the recycling endosome compartment (12, 15-17). Furthermore, immuno-electron microscopy shows that virtually all the membrane vesicles obtained by the method described here do actually contain GLUT4 (Fig 6). This indicates that there is little contamination of the insulin-sensitive membranes by membranes in which GLUT4 does not transit. The great advantage of the present technique is that it allows for the first time the purification of membranes that can be observed to be insulin-sensitive without the use of reagents that can contaminate the preparations such as immunoglobulin polypeptides. This contrasts with other methods where membranes enriched with GLUT4 are obtained, but do not seem to be reduced in abundance when cells are treated with insulin (16, 67).
containing membranes purified by the method described here thus appear to be particularly excellent for the application of microsequencing techniques to identify resident proteins.

It is interesting to note that significant amounts of the GLUT4 present in the membrane fractions obtained by velocity gradient centrifugation of low density microsomes is not apparently regulated by insulin, as revealed by further separation of these membranes by sucrose equilibrium gradient centrifugation (compare Figs 1 and 2). Membranes in fractions 10 and greater in the latter gradient show no detectable decrease when obtained from insulin treated cells. These membranes are unlikely to be derived from the plasma membrane because there is little contamination of low density microsomes by plasma membranes (6) and there is little GLUT4 present at the cell surface in unstimulated adipocytes. Thus there appears to be GLUT4 in intracellular membrane compartments that are exclusive of the recycling endosomal system and the insulin-sensitive compartment. Further work will be required to determine the cellular origin of these membranes that contain GLUT4.

The sucrose equilibrium density gradient step of the procedure described here reveals interesting protein profiles of the various membrane fractions that are separated. Surprisingly, many of the major protein bands that are visualized in the gel lanes after SDS-PAGE and silver staining are common throughout the gradient (Fig. 3). This indicates that the membranes containing GLUT4 that are translocated to the cell surface in response to insulin contain many proteins that are also present in membranes not responsive to insulin. These results imply that relatively few major proteins may be unique to the insulin-sensitive membrane compartment or compartments. Perhaps a relatively small number of proteins are required to confer insulin sensitivity to this compartment. Alternatively, many additional proteins may be present in the GLUT4-containing membranes that are insulin-responsive, but at much lower abundance than these major proteins. Further work will be required to sort out this question. The methodology
developed here should greatly facilitate the identification of low abundance proteins present in the insulin-sensitive GLUT4-containing membranes. Current experiments are directed to this end.
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ACKNOWLEDGEMENTS

We thank Drew Freilich for technical assistance during a preliminary stage of this study, Dr. Andrew Cherniack for helpful discussion and Jane Erickson for excellent assistance with preparation of the manuscript.
FOOTNOTES

1The Abbreviations used are:

IRAP, Insulin-regulated aminopeptidase
VAMP, Vesicle-associated membrane protein
SCAMP, Secretory carrier-associated membrane proteins
SNAP, Soluble NSF (N-ethylmaleimide-sensitive factor) attachment protein
Synip, Syntaxin 4-interacting protein
LEGENDS

Figure 1 - Isolation of GLUT4-containing membranes from rat adipocytes by sucrose velocity gradient centrifugation. Rat adipocytes were treated or not with insulin for 20 min and low density microsomes (LDM) were prepared as described in Experimental Procedures. LDM subcellular fractions were centrifuged in a 28 ml 10-35% sucrose gradient and 1 ml fractions were collected from the top to the bottom. A. Immunoblot analysis of fractions using anti-GLUT4. B. The data shown in panel A for GLUT4 protein from control (open circles) or insulin stimulated (closed circles) cells were quantified by scanning densitometry. C. Total
protein from control (open square) or insulin-stimulated (closed square) fractions were measured as described in Experimental Procedures.

**Figure 2 - Fractionation of GLUT4-containing membranes by equilibrium density gradient centrifugation.** Fractions containing GLUT4-membranes from Figure 1 (fractions 8 to 18) were pooled, pelleted and then loaded on the top of an 5 ml equilibrium density sucrose gradient (10-65%) as described in Experimental Procedures. **A.** Immunoblot analysis of fractions using anti-GLUT4. **B.** The data shown in panel A for GLUT4 protein from control (closed square) or insulin stimulated (open square) cells were quantified using scanning densitometry. **C.** Total protein from control (closed circles) or insulin-stimulated (open circles) fractions were measured as described in Experimental Procedures.

**Figure 3 - Silver staining of the GLUT4-containing membranes purified by equilibrium density gradient centrifugation.** Aliquots from fractions of the equilibrium gradients from Figure 2 were subjected to gradient SDS-PAGE (5-15% acrylamide) and proteins visualized by silver stain. Shown are fractions close to the top of gradient that contain proteins sensitive (fractions 6 to 9) or not (fractions 10 to 13) to insulin treatment. No bands were detected by silver stain in fractions 1 to 4. Depicted is a gel representative of 4 different experiments.

**Figure 4 - Distribution of VAMP2, transferrin receptor and GLUT4 in equilibrium density gradient fractions.** Aliquots from fractions of the equilibrium gradients from Figure 2 were subjected to SDS-PAGE and Western blot analysis as described in Experimental Procedures, using anti-GLUT4, anti-transferrin receptor (TfR) and anti-VAMP2 antibodies as indicated. Data shown are representative of 3 different experiments.

**Figure 5 - Sequences of protein peptides obtained from insulin-sensitive GLUT4-containing membrane.** Fractionation of GLUT4-containing membranes by equilibrium density
gradient was as described in Figure 2 was performed and the insulin-sensitive fractions, from control (CON) or insulin-stimulated cells (INS), pooled, pelleted, resolved by a 5-15% gradient SDS-PAGE and visualized with silver stain. Bands were excised and subjected to proteolytic digestion and peptides identified by mass spectrometry. The sequences of peptides obtained from some insulin-sensitive bands are indicated. The arrows indicate the insulin sensitive bands.

**Figure 6 - Vimentin and α-tubulin associate with insulin-responsive GLUT4-containing membranes isolated by equilibrium density gradient.** Aliquots of fractions from equilibrium gradient centrifugations performed as shown in Figure 2 were subjected to SDS-PAGE and Western blot analysis as described in Experimental Procedures. A. Anti-vimentin and anti-GLUT4 immunoblots of fractions from an experiment using primary rat adipocytes treated (+) or not (-) with insulin. B. Anti-α-tubulin and anti-GLUT4 immunoblots from another representative experiment using primary rat adipocytes treated (+) or not (-) with insulin.

**Figure 7 - GLUT4, vimentin and α-tubulin colocalization in GLUT4-containing membranes.** Immuno-electron microscopy of GLUT4-vesicles from insulin-sensitive fractions, isolated by an equilibrium density gradient as described in Figure 2 was performed. GLUT4-containing membranes were fixed in 2% paraformaldehyde and adsorbed to Formvarcoated gold grids. The grids were labeled with specific primary antibodies and were detected using secondary antibodies conjugated to different sized gold particles. A, B. colocalization of GLUT4 (15 nm, large arrowheads) and IRAP (5 nm, small arrows). C, D. colocalization of GLUT4 (15 nm, large arrowheads) and vimentin (5 nm, small arrows). E, F. colocalization of GLUT4 (15 nm, large arrowheads) and α-tubulin (5 nm, small arrows). G, H - Grids were labeled with anti-GLUT4 antibody (15 nm, large arrowheads) and non-immune mouse IgG antibody.

**Figure 8 - Effect of microinjection of Vm-1A peptide on integrity of 3T3-L1 adipocyte microtubules and intermediate filaments.** 3T3-L1 adipocytes were microinjected
with a solution containing 20 µg/ml fluorescein isothiocyanate (FITC)-coupled dextran and 2 mg/ml Vm-1A peptide. Following microinjection, the cells were allowed to recover for 60 min, fixed and stained with monoclonal anti-α-tubulin (A, top panel) or anti-vimentin (B, lower panel) antibodies. Primary antibodies were detected using Cy5-conjuged anti-mouse and Cy3-conjuged anti-goat antibodies. Microinjected cells were identified via FITC-coupled dextran.

**Figure 9** - Microinjection of Vm-1A peptide into 3T3-L1 adipocytes disperses perinuclear GLUT4 compartments. 3T3-L1 adipocytes were microinjected with a solution containing 20 µg/ml fluorescein isothiocyanate (FITC)-coupled dextran and 2 mg/ml control peptide (middle panel) or 2 mg/ml of Vm-1A peptide (lower panel). Following microinjection, the cells were allowed to recover for 60 min, then they were fixed and stained with rabbit polyclonal anti-GLUT4 antibody. Primary antibody was detected using Cy3-conjuged anti-rabbit antibody. Microinjected cells were identified by FITC-coupled dextran.

**Figure 10** - Inhibition of dynein-motor activity by cytoplasmic acidification disperses perinuclear GLUT4. 3T3-L1 adipocytes were treated or not with 70 mM sodium acetate, pH 7.5, for 15 min, washed with KHR and then cells were fixed (middle panel) or allowed to recover for 3 hr and then fixed (right panel). Cells were then stained with rabbit anti-GLUT4 antibody followed by a FITC-coupled secondary anti-rabbit antibody. Stained cells were observed as described in Experimental Procedures. The data shown are representative from 3 different experiments.

**Figure 11** - Effect of inhibition of dynein activity by cytoplasmic acidification on insulin-stimulated GLUT4 translocation. A. 3T3-L1 adipocytes were treated or not with 70 mM sodium acetate for 15 min, washed with KHR and treated or not with 100 nM insulin for 10 min. Another group of cells were allowed to recover for 60 min and then were treated with 100 nM insulin for 10 min. Lawns of plasma membrane were then generated as described in
Experimental Procedures. The lawns were incubated with rabbit anti-GLUT4 antibody followed
by FITC-coupled secondary anti-rabbit antibody and GLUT4 stain visualized as described in
Experimental Procedures.  B. GLUT4 protein in the lawns shown in A were quantified by
measuring the fluorescence intensity using Photoshop analysis software. Data correspond to the
average of 4 experiments ± S.E.
Figure 1

A

GLUT4

Basal

Insulin

B

250

200

150

100

50

0

GLUT4 Protein

Fractions

C

Protein

Fractions
Figure 4

Fractions

GLUT4 →

TfR →

VAMP-2 →

Insulin-Sensitive Fractions
Figure 6

A

Top

Insulin: + + + + + + + + + + + + + + + + + + + + + + + + + +

Vimentin

GLUT4

Fractions

Insulin-sensitive

Bottom

B

Insulin: + + + + + + + + + + + + + + + + + + + + + + + + + +

α-Tubulin

GLUT4

Insulin-sensitive
Figure 9

Uninjected

Control Peptide

Vm-1A
Figure 10

**Control**

**Acetate 15 min**

**KRH wash**

**Acetate 15 min**

**KRH recovery**

**120 min**
Figure 11

A  Treatment

KRH  Control

Acetate 15 min prior to KRH  Control

KRH  insulin

Acetate 15 min prior to KRH

Acetate 15 min prior to KRH recovery for 60 min  insulin

B  

Fluorescence intensity

Insulin  -  -  +  +  +

Treatment  - Acetate (15 min)  - Acetate (15 min)  Recovery (60 min)
Perinuclear localization and insulin-responsiveness of GLUT4 requires cytoskeletal integrity in 3T3-L1 adipocytes
Adilson Guilherme, Masahiro Emoto, Joanne M. Buxton, Sahana Bose, Rosanna Sabini, William E. Theurkauf, John Leszyk and Michael P. Czech

*J. Biol. Chem.* published online August 18, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M003432200

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