Rhodamine-labeled phalloidin staining of morphologically differentiated 3T3L1 adipocytes demonstrated that F-actin predominantly exists juxtaposed to and lining the inner face of the plasma membrane (cortical actin) with a smaller amount of stress fiber and/or ruffling actin confined to the cell bottom in contact with the substratum. The extent of cortical actin disruption with various doses of either latrunculin B or Clostridium difficile toxin B (a Rho family small GTP-binding protein toxin) directly correlated with the inhibition of insulin-stimulated glucose uptake and GLUT4 translocation. The dissolution of the cortical actin network had no significant effect on proximal insulin receptor signaling events including insulin receptor autoprophosphorylation, tyrosine phosphorylation of insulin receptor substrate and Cbl, or serine/threonine phosphorylation of Akt. Surprisingly, however, stabilization of F-actin with jasplakinolide also resulted in a dose-dependent inhibition of insulin-stimulated glucose uptake and GLUT4 translocation. In vivo time-lapse confocal fluorescent microscopy of actin-yellow fluorescent protein demonstrated that insulin stimulation initially results in cortical actin remodeling followed by an increase in polymerized actin in the peri-nuclear region. Importantly, the insulin stimulation of cortical actin rearrangements was completely blocked by treatment of the cells with latrunculin B, C. difficile toxin B, and jasplakinolide. Furthermore, expression of the dominant-interfering TC10/T31N mutant completely disrupted cortical actin and prevents any insulin-stimulated actin remodeling. Together, these data demonstrate that cortical actin, but not stress fibers, lamellipodia, or filopodia, plays an important regulatory role in insulin-stimulated GLUT4 translocation. In addition, cortical F-actin does not function in a static manner (e.g. barrier or scaffold), but insulin-stimulated dynamic cortical actin remodeling is necessary for the GLUT4 translocation process.

Insulin stimulates glucose uptake in striated muscle and adipose tissue by inducing the translocation of the insulin-responsive glucose transporter isoform (GLUT4) from intracellular storage site(s) to the plasma membrane (1–5). In the basal state, GLUT4 cycles slowly between the plasma membrane and one or more intracellular compartments, with the vast majority of the transporter residing within the cell interior (6–10). Activation of the insulin receptor triggers a large increase in the rate of GLUT4 vesicle exocytosis, with a smaller decrease in the rate of internalization by endocytosis (8, 10–12). Thus, the overall insulin-dependent shift in the cellular dynamics of GLUT4 vesicle trafficking results in a net increase of GLUT4 protein levels on the cell surface, thereby increasing the rate of glucose uptake.

Over the past several years it has become increasingly apparent that the cell cytoskeleton can have substantial influence over vesicle trafficking events. For example, fast axonal transport of synaptic vesicles to the pre-synaptic membrane requires the microtubule cytoskeleton and motors (13–15). More recently, several studies (16–20) have also implicated microtubules in the translocation of GLUT4. The actin cytoskeleton has also been observed to have profound influence over regulated exocytosis; however, the functional role of actin in this process appears to be highly complex. For example, most secretory cells have a dense sheet of F-actin beneath and juxtaposed to the plasma membrane, referred to as cortical actin. Several studies (21–25) have suggested that this actin functions as a physical barrier to vesicle docking based upon its transient depolymerization during exocytosis and that secretion preferentially occurs at sites where the actin cortex is relatively thin. Furthermore, in some cases disruption of the actin cytoskeleton markedly potentiates agonist-stimulated secretion (26–29). In contrast, however, in many cell systems depletion of F-actin structures either by sequestering actin monomers or by stimulation of actin severing does not stimulate exocytosis but results in an inhibition of agonist-induced secretion (30–33).

Although it is unclear whether or not GLUT4 transport vesicles have properties more consistent with secretory granules or synaptic vesicles, several studies have suggested a role for F-actin in insulin-stimulated GLUT4 translocation. Treatment with the actin-depolymerizing agent cytochalasin D or the actin monomer-binding Red Sea Sponge toxins latrunculin A or B inhibited insulin-stimulated GLUT4 translocation (19, 34–37). Insulin has also been observed to induce membrane ruffling (lamellipodia) in a PI 3-kinase-dependent manner (38–42). Because PI 3-kinase function is also necessary for insulin-stimulated GLUT4 translocation, these data suggested that membrane ruffling might play an important regulatory role.

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**Insulin-stimulated GLUT4 Translocation in Adipocytes Is Dependent upon Cortical Actin Remodeling**

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1 The abbreviations used are: GLUT4, insulin-responsive glucose transporter; F-actin, filamentous actin; IRS, insulin receptor substrate; PI 3-kinase, phosphatidylinositol 3-kinase; YFP, yellow fluorescent protein; GFP, green fluorescent protein; toxin B, Clostridium difficile toxin B; PKB, protein kinase B.
More recently, insulin-stimulated F-actin membrane ruffles in L6 myoblasts were found to accumulate at localized plasma membrane sites and to direct the localization of both the PI 3-kinase and GLUT4 protein (36, 43).

Although collectively these data suggest that the actin cytoskeleton plays a role in GLUT4 trafficking events, isolated primary rat adipocytes do not have significant amounts of stress fibers, lamellipodia, or filopodia but instead have a layer of cortical actin similar to that found in secretory cells (37). In this paper, we demonstrate that both disruption and stabilization of adipocyte cortical actin inhibit insulin-stimulated GLUT4 translocation. Furthermore, using time-lapse confocal fluorescent microscopy, we directly demonstrate that insulin induces cortical actin remodeling, and it is the dynamic actin rearrangement process that is necessary for insulin-stimulated GLUT4 translocation.

EXPERIMENTAL PROCEDURES

Materials—Clotostroidium difficile toxin B was obtained from Techlab Inc (Blacksburg, VA). Latrunculin B and jasplakinolide were purchased from Calbiochem and Molecular Probes (Eugene, OR), respectively. The monoclonal Cbl (7G10) and phosphotyrosine (PY20) antibodies were obtained from Upstate Biotechnology, Inc. (Lake Placid, NY) and Transduction Laboratories (Lexington, KY), respectively. Mouse monoclonal Phospha-K (Ser-473) and Akt (Thr-308) antibodies were purchased from Sigma and Santa Cruz Biotechnology, respectively. A rabbit polyclonal GLUT4 antibody (47) was obtained as described previously (44). Texas Red-conjugated donkey anti-rabbit IgG, Cy5-conjugated donkey anti-mouse IgG, and fluorescein isothiocyanate-conjugated donkey anti-sheep IgG were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Rhodamine-phalloidin and the pACTIN-enhanced yellow fluorescent protein were purchased from Sigma and CLONTECH (Palo Alto, CA), respectively. pKH3-TC10/T31N and Cde42/T17N were prepared as described previously (55).

Cell Culture and 2-Deoxyglucose Transport Assays—3T3L1 pre-adipocytes were cultured in Dulbecco’s modified Eagle’s medium containing 25 mM glucose, 10% calf serum at 37 °C in a 8% CO2 atmosphere and induced to differentiate into adipocytes as described previously (45). 3T3L1 adipocytes were washed with KP buffer (5 mM Na2HPO4, 20 mM HEPES, pH 7.4, 1 mM MgSO4, 1 mM CaCl2, 136 mM NaCl, 4.7 mM KCl, and 1% bovine serum albumin). Glucose transport was determined at 4 °C by incubation with 50 μM 2-deoxyglucose containing 0.5 μCi of 2-14Cdeoxyglucose in the absence or presence of 10 μM cytochalasin B. The reaction was stopped after 10 min by washing the cells 3 times with ice-cold phosphate-buffered saline. The cells were then solubilized in 1% Triton X-100 at room temperature for 30 min, and aliquots were subjected to scintillation counting. Protein concentration was determined by the method of Bradford.

Single Cell Microinjection—the microinjection and visualization of single 3T3L1 adipocytes was performed as described previously (46). Briefly, the cells were grown on coverslips, and prior to microinjection, the medium was changed to Lebovitz’s L-15 medium containing 0.1% bovine serum albumin. Differentiated 3T3-L1 adipocytes were impaled using Eppendorf model 5171 micromanipulator, and nuclei were injected with 50 μg/ml of the YFP-actin cDNA or 200 μg/ml of the TC10/T31N or Cde42/T17N cDNAs in 100 mM KCl, 5 mM Na3PO4, pH 7.2, with an Eppendorf model 5246 transjector. The cells were allowed to recover for 24 h and placed into a perfusion chamber maintained at 37 °C and visualized by time-lapse confocal fluorescent microscopy.

Results

Adipocyte Differentiation Results in the Conversion of F-actin from Stress Fiber and Lamellipodia to Cortical Actin Structures—Fully differentiated adipocytes are relatively large round lipid-laden cells that are morphologically quite distinct from their precursor fibroblasts. Because the actin cytoskeleton is dynamically regulated and intimately involved in cell morphology and motility, we examined the organization of F-actin during the differentiation of 3T3L1 fibroblasts to morphologically mature adipocytes (Fig. 1). Prior to differentiation, the precursor adipocytes (fibroblasts) primarily display long organized stress fibers typical of F-actin found in other fibroblast cell types. Even though the fibroblasts are relatively flat cells, stress fibers can be detected throughout the cells but are more pronounced at the cell bottom in contact with the substratum (Fig. 1, panels a and e). However, as the cells differentiate into a large rounded morphological, the long stress fiber F-actin structures are converted into F-actin that lines the inner face of the plasma membrane (Fig. 1, panels b–d). In parallel, after 4 days of adipocyte differentiation the length of F-actin stress fibers visualized at the cell bottom were markedly decreased (Fig. 1, panel f). At longer times, the vast majority of stress fiber F-actin was reduced to small patches of punctate actin (Fig. 1, panels g and h). The distribution of F-actin in adipocytes is more readily apparent when visualized by stained z axis confocal images (Fig. 2, panels a–d). These data demonstrate that the major form of F-actin in fully differentiated 3T3L1 adipocyte is cortical actin underlying the plasma membrane but not stress fibers.

Previous studies (38–42, 47–49) have observed that insulin can acutely modulate membrane ruffling and stress fiber F-actin in a PI 3-kinase-dependent manner. Therefore, we next examined the relationship between stress fiber, membrane ruffles, cortical actin, and PI 3-kinase activity using the selective PI 3-kinase inhibitor wortmannin (Fig. 3). As expected, insulin conversion of F-actin from stress fiber and membrane ruffles to cortical actin structures occurs during adipocyte differentiation. 3T3L1 cells were fixed and labeled with rhodamine-labeled phallolidin during various stages of adipogenesis (0, 4, 8, and 12 days) as described under “Experimental Procedures.” F-actin was then visualized by confocal fluorescent microscopy in sections taken through the middle (panels a–d) and bottom (panels e–h) of the cells. This is a representative field of cells from three independent experiments.

Nonidet P-40, 100 mM NaCl, 2% glycerol, 1 mM EDTA, 1 mM sodium vanadate, 1 mM sodium pyrophosphate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 5 μg/ml leupeptin, 5 μg/ml pepstatin A. After 20 min of rotaiton at 4 °C, the extracts were centrifuged at 13,000 × g for 20 min to remove insoluble material, and 50 μg of the total protein were resolved by SDS-polyacrylamide gel electrophoresis. The gels were then subjected to immunoblotting with either a rabbit polyclonal Akt antibody, a β-actin antibody, the monoclonal phospho-Akt (Ser-473) antibody, or the phospho-Akt (Thr-308) antibody and visualized by the SuperSignal Chemiluminescence detection kit (Pierce). For immunoprecipitation, whole cell extracts were incubated for 2 h at 4 °C with 5 μg of the monoclonal Cbl antibody. The samples were then precipitated with protein G PLUS-Sepharose (Santa Cruz Biotechnology) and were immunoblotted as described above.
stained with rhodamine-labeled phalloidin, and visualized by confocal fluorescence microscopy. These data directly demonstrate that in adipocytes the regulation and cellular distribution of cortical F-actin was distinct from F-actin involved in the formation of stress fibers and membrane ruffling.

Disruption of Polymerized Actin Inhibits Insulin-stimulated Glucose Uptake and GLUT4 Translocation—Previous studies (19, 34, 36, 37, 50) have reported that the actin-depolymerizing agent cytochalasin D and the actin monomer sequestering agents latrunculin A or latrunculin B can inhibit insulin-stimulated GLUT4 translocation in 3T3L1 adipocytes and in primary adipocytes. Consistent with these findings, latrunculin B inhibited insulin-stimulated glucose uptake in a dose-dependent manner (Fig. 4A). As observed previously, rhodamine-labeled phalloidin staining demonstrated that the differentiated adipocytes contain polymerized actin underneath and juxtaposed to the inner face of the plasma membrane (Fig. 4B, panel a). In parallel to the reduction in insulin-stimulated glucose transport activity, latrunculin B treatment resulted in a dose-dependent disruption of this cortical actin structure (Fig. 4B, panels b–e). In the absence of latrunculin B only 2% of the cells displayed a discontinuous cortical actin rim, whereas the cortical actin structure was disrupted in 13% of the cells at 1 μM, 31% at 5 μM, 71% at 20 μM, and 91% at 60 μM latrunculin B. It is well established that Rho family members of small GTP-binding proteins play important regulatory roles in the control of actin polymerization (51–54). In addition, we have recently observed that the Rho family member protein TC10 plays a critical function in the insulin-stimulated GLUT4 translocation process (55). We therefore took advantage of C. difficile toxin B, which inactivates Rho family small GTP-binding proteins (56, 57). Incubation of adipocytes with toxin B resulted in a slight increase in glucose uptake in the basal state and also resulted in a biphasic response, with a slight enhancement of insulin-stimulated glucose uptake at 10 ng/ml toxin B followed by a dose-dependent inhibition (Fig. 5A). In the basal state, toxin B treatment caused a slight increase in not only glucose uptake but also 125I-transferrin cell surface binding (data not shown), indicating that toxin B slightly increases general membrane exocytosis and/or inhibits endocytosis. In any case, higher concentrations of toxin B also resulted in a disruption of cortical actin structure (Fig. 5B, panels a–e), suggesting that the Rho family of small GTP-binding proteins primarily maintain the actin cytoskeleton organization in 3T3L1 adipocytes. Similar to the effect of latrunculin B, treatment with 0.01, 0.1, 0.5, and 1.0 μg/ml toxin B resulted in 14, 38, 74, and 85% of the cells displaying disrupted cortical actin structures.

To confirm that the inhibition of glucose uptake by the disruption of cortical actin was, in fact, because of a block in GLUT4 translocation, the isolated plasma membrane sheets were examined for the presence of the GLUT4 protein (Fig. 6). As expected, insulin-stimulated a robust translocation of GLUT4 to the plasma membrane as detected by the appearance of a strong GLUT4 immunofluorescent signal (Fig. 6, panels a...
However, treatment with either latrunculin B or toxin B markedly inhibited the insulin-stimulated formation of plasma membrane GLUT4 immunofluorescence (Fig. 6, panels c and d).

**Toxin B and Latrunculin B Do Not Affect Proximal Insulin Signaling Events**—Previous studies (43, 58, 59) have suggested that insulin receptor downstream signaling effectors (IRS and PI 3-kinase) may interact with the cytoskeleton. Thus, the effect of toxin B and latrunculin B to disrupt cortical actin could potentially result in an inhibition of insulin signaling rather than a direct requirement for actin in the GLUT4 translocation process. To address this issue, we next examined the effect of toxin B and latrunculin B on insulin receptor autophosphorylation and IRS tyrosine phosphorylation (Fig. 7A). Although toxin B treatment resulted in a small decrease in insulin-stimulated IRS tyrosine phosphorylation, there was no significant effect on insulin receptor autophosphorylation (Fig. 7A, lanes 1–4). In addition, latrunculin B had no effect on either insulin receptor autophosphorylation or IRS tyrosine phosphorylation (Fig. 7A, lanes 5 and 6). Because IRS phosphorylation leads to the activation of PI 3-kinase, we also examined the phosphorylation of the downstream serine kinase Akt/PKB that also has been implicated in the regulation of GLUT4 translocation (60–64). Importantly, neither toxin B nor latrunculin B had any significant effect on insulin-stimulated Akt phosphorylation at the threeine 308 or serine 473 activation sites (Fig. 7B, lanes 1–6).

Recently, we have reported (46, 55) that the insulin activation of the PI 3-kinase pathway is not sufficient to mediate...
the stimulation of GLUT4 translocation by insulin appears to be dependent upon cortical actin, it is possible that either stable cortical actin structures and/or dynamic cortical actin rearrangements are functionally required. To address this issue, we took advantage of jasplakinolide, a cell-permeable agent that promotes the formation and/or stabilization of actin filaments (65-67). Similar to latrunculin B and toxin B, jasplakinolide also inhibited insulin-stimulated glucose uptake in a dose-dependent manner (Fig. 8A). Jasplakinolide was also found to prevent insulin-stimulated GLUT4 translocation as assessed by the plasma membrane sheet assay (Fig. 8B, panels a-c). Because jasplakinolide binds to the same site and competes for phalloidin binding to F-actin, we were unable to examine the effect of jasplakinolide on cortical actin structure using rhodamine-labeled phalloidin. However, we were able to examine the effect of jasplakinolide using time-lapse confocal fluorescent microscopy of adipocytes expressing YFP-actin (see Fig. 10). In any case, these data indicate that insulin-induced actin rearrangements but not static actin structures are necessary for GLUT4 translocation.

**Stabilization of Polymerized Actin Also Inhibits Insulin-stimulated Glucose Uptake and GLUT4 Translocation**—Because the stimulation of GLUT4 translocation by insulin appears to be dependent upon cortical actin, it is possible that either stable cortical actin structures and/or dynamic cortical actin rearrangements are functionally required. To address this issue, we took advantage of jasplakinolide, a cell-permeable agent that promotes the formation and/or stabilization of actin filaments (65-67). Similar to latrunculin B and toxin B, jasplakinolide also inhibited insulin-stimulated glucose uptake in a dose-dependent manner (Fig. 8A). Jasplakinolide was also found to prevent insulin-stimulated GLUT4 translocation as assessed by the plasma membrane sheet assay (Fig. 8B, panels a-c). Because jasplakinolide binds to the same site and competes for phalloidin binding to F-actin, we were unable to examine the effect of jasplakinolide on cortical actin structure using rhodamine-labeled phalloidin. However, we were able to examine the effect of jasplakinolide using time-lapse confocal fluorescent microscopy of adipocytes expressing YFP-actin (see Fig. 10). In any case, these data indicate that insulin-induced actin rearrangements but not static actin structures are necessary for GLUT4 translocation.

**Stabilization of Polymerized Actin Also Inhibits Insulin-stimulated Glucose Uptake and GLUT4 Translocation**—Because the stimulation of GLUT4 translocation by insulin appears to be dependent upon cortical actin, it is possible that either stable cortical actin structures and/or dynamic cortical actin rearrangements are functionally required. To address this issue, we took advantage of jasplakinolide, a cell-permeable agent that promotes the formation and/or stabilization of actin filaments (65-67). Similar to latrunculin B and toxin B, jasplakinolide also inhibited insulin-stimulated glucose uptake in a dose-dependent manner (Fig. 8A). Jasplakinolide was also found to prevent insulin-stimulated GLUT4 translocation as assessed by the plasma membrane sheet assay (Fig. 8B, panels a-c). Because jasplakinolide binds to the same site and competes for phalloidin binding to F-actin, we were unable to examine the effect of jasplakinolide on cortical actin structure using rhodamine-labeled phalloidin. However, we were able to examine the effect of jasplakinolide using time-lapse confocal fluorescent microscopy of adipocytes expressing YFP-actin (see Fig. 10). In any case, these data indicate that insulin-induced actin rearrangements but not static actin structures are necessary for GLUT4 translocation.
nuclei with a relatively low concentration of YFP-actin cDNA (50 μg/ml in the microinjection pipette). The functional properties of the expressed YFP-actin were confirmed under these conditions, as insulin characteristically stimulated membrane ruffling of the YFP-actin in pre-differentiated 3T3L1 fibroblasts (data not shown). In the absence of TC10/T31N, expression of YFP-actin displayed the typical polymerization pattern being distributed between cortical and peri-nuclear regions. As observed previously (Fig. 9), insulin stimulation increased the amount of both polymerized cortical and peri-nuclear actin. In contrast, expression of TC10/T31N completely disrupted the cortical actin localization of YFP-actin consistent with the rhodamine-labeled phalloidin staining of fixed cells (Fig. 11A, panels b and c). As a control, we also expressed a dominant-interfering mutant of another Rho family member established to regulate actin dynamics in fibroblasts, Cdc42/T17N (51, 70, 71). In contrast to TC10/T31N expression of Cdc42/T17N had no significant effect on adiocyte cortical actin with only 7% of the cells having any morphological changes in phalloidin labeling (Fig. 11A, panels d–f). This is consistent with the inability of insulin to activate Cdc42 in adipocytes (55).

To determine whether the expressed TC10/T31N protein also prevented insulin-stimulated cortical actin rearrangements, we co-microinjected adipocytes with TC10/T31N and YFP-actin (Fig. 11B). In the absence of TC10/T31N, expression of YFP-actin displayed the typical polymerization pattern being distributed between cortical and peri-nuclear regions. As observed previously (Fig. 9), insulin stimulation increased the amount of both polymerized cortical and peri-nuclear actin. In contrast, expression of TC10/T31N completely disrupted the cortical actin localization of YFP-actin consistent with the rhodamine-labeled phalloidin staining of fixed cells (Fig. 11B, panel a). Furthermore, in the presence of TC10/T31N insulin was unable to induce the appearance of or change in cortical actin structure (Fig. 11B, panels b–d). The dynamics of YFP-actin in adipocytes expressing TC10/T31N can be more readily observed in the time-lapse imaging presented in the Supplementary Material. In any case, the disruption of cortical actin remodeling by expression of TC10/T31N is consistent with the inhibition of insulin-stimulated GLUT4 translocation (55).

DISCUSSION

One of the most intensively studied actions of insulin is its ability to enhance glucose uptake through the translocation of the insulin-responsive GLUT4 glucose transporter from intracellular storage sites to the plasma membrane. This is a highly complex and dynamic process that appears to require at least two independent but cooperative signal transduction pathways (46, 55). The GLUT4 protein continuously cycles through various endomembrane compartments in both the basal and insulin states (1–3, 5). Although it is well established that insulin increases the rate of exocytosis, whether this occurs from a direct trafficking of a specialized GLUT4 compartment, the
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**Fig. 10.** Latrunculin B, C. difficile toxin B, and jasplakinolide inhibit insulin-stimulated cortical actin rearrangements and peri-nuclear actin polymerization in vivo. 3T3L1 adipocyte nuclei were microinjected with an expression plasmid encoding for YFP-actin as described under “Experimental Procedures.” The YFP-actin-expressing cells were then pretreated with 20 μM latrunculin B (LatB; panels a–d), 0.5 μg/ml C. difficile toxin B (Toxin B; panels e–h), or 10 μM jasplakinolide (Jasp; panels i–l) for 2 h followed by time-lapse confocal fluorescent microscopy. The complete time-lapse image is provided in the Supplementary Material. These are representations of insulin-stimulated time courses of YFP-actin observed in 4–8 individual cells examined.

**Fig. 11.** Expression of TC10/T31N disrupts cortical actin and blocks insulin-stimulated remodeling. A, 3T3L1 adipocytes were transfected with a plasmid encoding for Myc-TC10/T31N (panels a–c) or Myc-Cdc42/T17N (panels d–f) as described under “Experimental Procedures.” The cells were then fixed and dual labeled with rhodamine-labeled phalloidin, and a monoclonal Myc antibody was examined by confocal fluorescent microscopy. These are representative fields of cells from three independent determinations. B, 3T3L1 adipocyte nuclei were co-microinjected with an expression plasmids encoding for YFP-actin and TC10/T31N as described under “Experimental Procedures.” The YFP-actin and TC10/T31N-expressing cells were then incubated in the absence or presence of insulin for the times indicated. The complete time-lapse image is provided in the Supplementary Material. These are representations of insulin-stimulated time courses of YFP-actin observed in four independent cells.

Recently, several studies have suggested that both the microtubule and actin-based cytoskeleton networks may play important roles by providing an organized network for the movement or trafficking of the insulin-stimulated vesicle compartments to the cell surface (16–20, 34–37, 43, 58, 59). These findings are primarily based on the observation that disruption of F-actin and/or microtubules inhibits GLUT4 translocation and that a portion of the GLUT4 compartments appears to co-localize with these cytoskeleton structures. These data are consistent with the cytoskeleton providing a molecular scaffold allowing for the organized trafficking of insulin-stimulated GLUT4-containing compartments to the plasma membrane.

However, the actin cytoskeleton is quite complex and different cell types display multiple distinct organization patterns of F-actin with each undergoing different modes of regulation and extents of polymerization/depolymerization. This problem has been further complicated by studies examining the role of F-actin in different secretory and membrane transport processes. In many cases, a negative or barrier function for F-actin in membrane transport has been proposed as actin-depolymerizing agents can markedly potentiate agonist-stimulated secretory events (21–25). In other contexts, F-actin appears to provide a critical scaffolding function, and in some cases the rapid polymerization/depolymerization on membrane compartments can act as molecular motors (72–74). In the case of GLUT4 translocation, this problem is further exacerbated by the observation that different model cell systems appear to display different cellular organizations of F-actin. Furthermore, insulin has been observed to markedly induce membrane ruffling in a PI 3-kinase- and Rac1-dependent manner (38–42, 75–77). However, membrane ruffling is a characteristic of cells undergoing active motility, whereas insulin-responsive adipocytes and skeletal muscle are typically non-motile cells.

Thus, to examine further the relationship between actin structure, insulin action, and GLUT4 translocation, we initially compared the F-actin structures during adipocyte differentiation. As expected, the pre-adipocytes have a fibroblast morphology and displayed typical actin stress fibers and membrane ruffles. Insulin stimulation in these cells resulted in increased membrane ruffling that was inhibited by the selective PI 3-kinase inhibitor wortmannin. In contrast, morphologically differentiated adipocytes primarily express F-actin around the cell cortex (cortical actin) that lines the inner face of the plasma membrane. This cortical actin did not display insulin-stimulated membrane ruffling but appeared to undergo dynamic remodeling (polymerization/depolymerization). These results are in excellent agreement with the analysis of F-actin in primary rat adipocytes, which were also found to predominantly display a cortical actin network (37).
In addition, the morphologically fully differentiated adipocytes also contained a smaller amount of punctate F-actin localized to the cell bottom in contact with the substratum. Similar to fibroblasts, the adipocyte cell bottom also underwent an insulin-stimulated membrane ruffling in a wortmannin-sensitive manner. However, this actin does not appear to be responsible for insulin-stimulated GLUT4 translocation, as this is only a minor fraction of the adipocyte F-actin localized to a small portion of the cell surface membrane. In addition, expression of constitutively active and dominant-interfering Rac1 mutants markedly modulates actin at the cell bottom but does not have any significant effect on insulin-stimulated GLUT4 translocation. These findings are also consistent with previous reports (78–80) indicating that Rac-mediated changes in the actin cytoskeleton do not affect insulin-stimulated glucose uptake or GLUT4 translocation in adipocytes. Nevertheless, other members of the Rho family of small GTP-binding proteins have also been implicated in various aspects of F-actin dynamics in adipocytes, consistent with the inability of insulin to activate Cdc42 to or to regulate GLUT4 translocation (55). Thus together, these data specifically implicate adipocyte actin remodeling through TC10 function as an essential process in GLUT4 translocation.

In summary, fully differentiated adipocytes primarily express cortical actin that must undergo active insulin-stimulated remodeling to allow for insulin-stimulated GLUT4 translocation. This cortical actin network appears to be regulated by TC10, a member of the Rho family of small GTP-binding protein. Although the insulin stimulation of membrane ruffling in fibroblasts and at the cell bottom of adipocytes is clearly PI 3-kinase-dependent, insulin regulation of cortical actin rearrangements appears to be a necessary event in GLUT4 translocation. More importantly, our data are inconsistent with a model of cortical actin remodeling as a passive molecular network allowing for the trafficking of the GLUT4-containing compartments to the plasma membrane. Instead, these data demonstrate that cortical actin remodeling plays an integral role in the trafficking of GLUT4 vesicles. Further studies will be necessary to determine whether cortical actin rearrangements in vivo reflect actin-based motility of GLUT4 vesicles or some other dynamic change in actin-based trafficking function.

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REFERENCES

1. Pessin, J. E., Thurmond, D. C., Elmendorf, J. S., Coker, K. J., and Okada, S. (1999) J. Biol. Chem. 274, 2593–2596.

2. Rea, S., and James, D. E. (1997) Diabetes 46, 1667–1677.

3. Sardaru, K. V., and Plitch, P. F. (1996) Am. J. Physiol. 371, E1–E14.

4. Goodyear, L. J., and Kahn, B. B. (1998) Annu. Rev. Med. 49, 235–261.

5. Simpson, F., Whitehead, J. P., and James, D. E. (2001) Traffic 2, 2–11.

6. Slot, J. W., Geuze, H. J., Gigengack, S., James, D. E., and Lienhard, G. E. (1991) Proc. Natl. Acad. Sci. U.S.A. 88, 7815–7819.

7. Slot, J. W., Geuze, H. J., Gigengack, S., Lienhard, G. E., and James, D. E. (1991) J. Cell Biol. 113, 123–135.

8. Jhun, B. H., Rampal, A. L., Lin, H., Lachaal, M., and Jung, C. Y. (1992) J. Biol. Chem. 267, 17110–17115.

9. Rodnick, K. J., Slot, J. W., Stuuldska, D. R., Hanpete, D. E., Robinson, J. L., Geuze, H. J., and James, D. E. (1992) J. Biol. Chem. 267, 6278–6285.

10. Yang, J., and Holman, G. D. (1993) J. Biol. Chem. 268, 4600–4605.

11. Czech, M. P., and Buxton, J. M. (1993) J. Biol. Chem. 268, 9187–9190.

12. Sato, S., Nishimura, H., Clark, A. E., Kozuka, I. J., Vranic, M., Simpson, S. J., Pessin, J. E., Quan, M. J., Cushman, S. W., and Holman, G. D. (1993) J. Biol. Chem. 268, 17520–17529.

13. Schroer, T. A. (1992) Curr. Opin. Neurobiol. 2, 618–621.

14. Terada, S., and Hirokawa, N. (2000) Curr. Opin. Neurobiol. 10, 566–573.

15. Martin, M. A., Hurd, D. D., and Saxton, W. M. (1999) Cell Mol. Life Sci. 56, 200–216.

16. Fletcher, L. M., Welsh, G. I., Oatsey, P. B., and Tavare, J. M. (2000) Biochem. J. 352, 267–276.

17. Guillemere, A., Emoto, M., Buxton, J. M., Bose, S., Sabini, R., Theerkauf, W. E., Lelkes, P. I., Friedman, J. E., and Oplatka, A. (1986) J. Cell Biol. 107, 113, 113, 113, 113.

18. Vadas, E., Langille, S. E., and Cushman, S. W. (2001) J. Biol. Chem. 276, 10677–10683.

19. Olson, A. L., Trumbly, A. R., and Gibson, G. V. (2001) J. Biol. Chem. 276, 10706–10714.

20. Vitale, M. L., Rodriguez Del Castillo, A., Tekahar0, L., and Trifaro, J. M. (1991) J. Cell Biol. 113, 1057–1066.

21. Carballaj, M. E., and Vitale, M. L. (1997) Endocrinology 138, 5374–5384.

22. Vitale, M. L., Seward, E. P., and Trifaro, J. M. (1995) Neuron 14, 353–363.

23. Neuman, J. C., Price, L. S., Ridley, A. J., and Koffler, A. (1996) Mol. Biol. Cell 7, 1429–1442.

24. Bernstein, B. W., and Bamburg, J. R. (1989) Neuroscience 33, 579–584.

25. Muallem, S., Kwiatkowska, K., Xu, X., and Yin, H. L. (1995) J. Cell Biol. 129, 589–598.

26. Sontag, J. M., Aunis, D., and Bader, M. F. (1988) Eur. J. Cell Biol. 46, 316–326.

27. Kelkes, P. L., Friedman, J. E., Rosenheck, K., and Oplatka, A. (1986) FERES 90, 308, 357–363.

28. Matter, K., Dreyer, F., and Akters, K. (1989) J. Neurochem. 52, 370–376.

29. Li, G., Rographer-Brandel, E., Just, I., Jonas, J. C., Akters, K., and Wollheim, C. B. (1994) Mol. Cell. Biol. 5, 1199–1213.

30. Merida, K., Oka, M., and Hamano, S. (1988) Biochem. Pharmacol. 37, 3357–3359.

31. O’Konski, M. S., and Pandol, S. J. (1993) Pannears 8, 638–646.

32. O’Konski, M. S., and Pandol, S. J. (1993) J. Clin. Invest. 91, 649–657.

33. Tsakiridis, T., Vranic, M., and Klip, A. (1994) J. Biol. Chem. 269, 29934–29942.

34. Tsakiridis, T., Bergman, A., Somwar, R., Taha, C., Akters, K., Cruz, T. F., Klip, A., and Downey, G. P. (1998) J. Biol. Chem. 273, 28228–28331.

35. Wang, Q., Bilan, P. J., Tsakiridis, T., Hinke, A., and Klip, A. (1998) Biochem. J. 331, 917–928.

36. Omata, W., Shibata, H., Li, L., Takata, K., and Kojima, I. (2000) Biochem. J. 346, 321–328.

37. Kadowaki, T., Koyasu, S., Nishida, E., Sakai, H., Tukaku, F., Yahara, I., and Kasuga, M. (1986) J. Biol. Chem. 261, 16141–16147.

38. Martin, S. S., Harata, T., Morris, A. J., Klippel, A., Williams, L. T., and Pessin, J. E. (1997) Manuscript in preparation.

M. Kanazi, R. T. Watson, D. C. Thurmond, A. S. Saltiel, and J. E. Pessin, manuscript in preparation.
