Insulin stimulation results in the activation of cyclin-dependent kinase-5 (CDK5) in lipid raft domains via a Fyn-dependent phosphorylation on tyrosine residue 15. In turn, activated CDK5 phosphorylates the Rho family GTP-binding protein TC10α on threonine 197 that is sensitive to the CDK5 inhibitor olopatadine and blocked by small interfering RNA-mediated knockdown of CDK5. The phosphorylation deficient mutant T197A-TC10α was not phosphorylated and excluded from the lipid raft domain, whereas the phosphorylation mimetic mutant (T197D-TC10α) was lipid raft localized. Insulin resulted in the GTP loading of T197D-TC10α but not T197A-TC10α and in parallel, T197D-TC10α but not T197A-TC10α depolymerized cortical actin and inhibited insulin-stimulated GLUT4 translocation. These data demonstrate that CDK5-dependent phosphorylation maintains TC10α in lipid raft compartments thereby disrupting cortical actin, whereas subsequent dephosphorylation of TC10α through inactivation of CDK5 allows for the re-assembly of F-actin. Because cortical actin reorganization is required for insulin-stimulated GLUT4 translocation, these data are consistent with a CDK5-dependent TC10α cycling between lipid raft and non-lipid raft compartments.

Similar to fibroblasts, pre-differentiated adipocytes are elongated and relatively flat cells that contain well defined actin stress fibers. In contrast, following differentiation into mature adipocytes the cells become highly spherical with a relatively thick cortical actin lining the inner surface of the plasma membrane. This cortical actin structure in adipocytes is composed of patches of punctate F-actin that emanates from organized caveolae-rosettes containing the caveolin protein referred to as Cav-actin (1). This cortical organization of F-actin is dependent on caveola-rosette organization as disruption of caveola results in the dissolution of cortical actin, whereas depolymerization of cortical actin has no effect on caveola-rosette organization (2, 3). Although insulin induces the rapid breakdown of stress fibers and the appearance of lamellipodia in pre-adipocytes, in differentiated 3T3L1 adipocytes and primary rat adipocytes insulin primarily results in dynamic actin rearrangements in both the cortical and perinuclear regions (4, 5).

Caveolin-enriched domains in adipocytes have also been reported to associate with a variety of structural and functional proteins. For example, the structural protein flotillin is lipid raft localized and interacts with CAP, Cbl, and Fyn (6). In particular, the unusual Rho family member GTP-binding protein TC10 appears also to be lipid raft localized in adipocytes and has been implicated in the regulation of insulin-stimulated GLUT4 translocation through the assembly of membrane docking regulating proteins (7). In parallel, TC10 appears to differentially regulate two distinct compartmentalized actin populations. Overexpression of TC10 was found to disrupt cortical actin when lipid raft localized through its endogenous carboxyl-terminal domain that specifies both farnesylation and dual acylation, but not when mis-targeted through mutations that directed TC10 to non-lipid raft plasma membrane domains (8). Moreover, expression of a constitutive active (GTP-bound) TC10 mutant (TC10/Q75L) disrupted both Cav-actin and cortical actin structures, but induces massive actin polymerization in perinuclear regions (9). Recent studies using enhanced yellow fluorescent protein-tagged β-actin and real-time imaging also demonstrated insulin-regulated actin reassembly in both cortical and perinuclear regions that was prevented by the Rho-specific toxin Clostridium difficile toxin B (5). Thus, several lines of evidence exist for both a direct action of TC10 in mediating insulin-stimulated GLUT4 translocation and indirect actions through modification of actin organization.

In this regard, there are three TC10 isoforms, TC10α, TC10β, and long but only the constitutive over expression of TC10α inhibits insulin-stimulated GLUT4 translocation (10). These isoforms differ at their amino terminus and previous studies suggested that this domain is responsible for alterations of actin structure and the inhibitory effect on insulin-stimulated GLUT4 translocation (8). However, in addition to the differences in the amino-terminal extension, we have observed that there are other differences in the carboxyl-terminal regions, in particular the presence of a CDK5 consensus phosphorylation site in TC10α but not in TC10β. We have therefore...
examined the CDK5-dependent phosphorylation of TC10α and demonstrated that this event is necessary for the regulation of TC10α function, localization, and reorganization of cortical F-actin in adipocytes.

**MATERIALS AND METHODS**

*Reagents—* The HA2 polyclonal antibody was obtained from Sigma. CDK5, p35, and caveolin-1 antibodies were from Santa Cruz Biotechnology. Phospho-Thr MAPK/CDK substrate monoclonal antibody was from Cell Signaling. TC10 rabbit polyclonal antibody was prepared and affinity purified as previously described (1). F-actin antibody was from Abcam. Omnia™ Ser/Thr recombinant kit was from BioSource. ProteoExtract Subcellular Proteome Extraction Kit was from Calbiochem. EZ-Detect Cdc42 Activation Kit was from Pierce. Caveolea/Rafts Isolation Kit was from Sigma. CDK5 and Fyn RNAi were obtained from Invitrogen and Santa Cruz. ECL Plus Western Blotting Detection System was obtained from Amersham Biosciences. The horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG antibodies were obtained from Pierce. Cell culture media and reagents were from Invitrogen. All of other chemicals used in this study were purchased from Sigma.

**Cell Culture—** 3T3L1 preadipocytes were cultured in Dulbecco’s modified Eagle’s medium containing 25 mM glucose, 10% calf serum at 37 °C with 8% CO2. Confluent cultures were induced to differentiate into adipocytes as previously described (11).

**Immunoprecipitation and Immunoblotting—** Scared frozen cells were rocked for 30 min at 4 °C with Nonidet P-40 lysis buffer (25 mM Hepes, pH 7.4, 10% glycerol, 50 mM sodium fluoride, 10 mM sodium pyrophosphate, 137 mM sodium chloride, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 mg/ml aprotinin, 1 mg/ml pepstatin, 5 mg/ml leupeptin). Insoluble material was separated from the soluble extract by centrifugation for 30 min at 4 °C, and the total protein amount in the supernatant was determined by the BCA method. After the addition of 4.5 μg of antibody to the whole cell lysates, samples (typically 2–3 mg of lysates) were incubated for 2 h at 4 °C. Then 50 μl of protein A/G-agarose was added and samples were consistently rocked for 1 h at 4 °C. After the incubation, samples were extensively washed three times with the Nonidet P-40 lysis buffer. The washed samples were resuspended in SDS sample buffer (125 mM Tris–HCl, pH 6.8, 20% (v/v) glycerol, 4% (w/v) SDS, 100 mM dithiothreitol, 0.1% (w/v) bromphenol blue), and heated at 100 °C for 5 min. Samples were separated by SDS-PAGE and electrophoretically transferred to polyvinylidene difluoride membranes. The samples were immunoblotted with monoclonal or polyclonal specific antibody as indicated in the figures and legends.

**Subcellular Extraction—** To analyze F-actin rearrangement, we first isolated cytoskeleton fractions by using the ProteoExtract Subcellular Proteome Extraction Kit. After cells were resuspended in Extraction Buffer I, cells were pelleted by centrifugation (300 × g, 10 min, 4 °C). The pellet was resuspended in Extraction Buffer II and rocked for 30 min at 4 °C and centrifuged for 10 min at 6,000 × g at 4 °C. The pellet was resuspended in Extraction Buffer III and rocked for 10 min at 4 °C. Then sample was centrifuged for 10 min at 7,000 × g at 4 °C. The pellet was resuspended in Extraction Buffer IV and this suspension was used as a cytoskeletal matrix protein extraction to analyze F-actin rearrangement condition. Namely, samples were applied on SDS-PAGE and transferred to polyvinylidene difluoride membrane and F-actin and G-actin amounts were estimated by Western blotting.

**TC10 Activity Assay—** EZ-Detect CDC42 activation kit was used. Cells were scraped with Lysis/Wash/Wash buffer and transferred into Eppendorf tubes. After rocking for 5 min at 4 °C, the sample was centrifuged for 15 min at 16,000 × g at 4 °C. 20 μg of GST-Pak1-p21-binding domain (PBD) was applied to the supplied spin cup and 700 μg of lysate added. The sample was mixed well and rocked for 60 min at 4 °C. Then samples were washed with Lysis/Wash/Wash buffer three times. After the TC10α bound to GST-Pak1-PBD was eluted, samples were applied on SDS-PAGE and transferred to a polyvinylidene difluoride membrane and immunoblotted with TC10 antibody.

**Transfection of 3T3L1 Adipocytes—** 3T3L1 adipocytes were transfected with mild trypsinization and electroporated with a total of 1 μg of plasmid under low-voltage conditions (0.16 kV, 950 microfarads) (12). The cells were then allowed to adhere to collagen-coated tissue culture dishes for 30–48 h, and the adipocytes were then serum starved for 4 h prior to incubation in the absence or presence of 100 nM insulin at 37 °C for the various time periods indicated in each figure.

**In Vitro CDK5 Kinase Assay and TC10α Peptide Phosphorylation—** The Omni™ Ser/Thr Recombinant Kit (BioSource International) was used to assay CDK5 activity. CDK5 was immunoprecipitated from 2.0 mg of 3T3L1 adipocytes lysates in the absence or presence of insulin stimulation. Then, immunoprecipitated CDK5 or CDK5/p35 recombinant was mixed with either biotinylated S/T control peptide (250 μM) or biotinylated synthesized TC10α peptide (250 μM, SHIMAZU Biotech, Japan) in 1× kinase buffer containing ATP and dithiothreitol and the kinase reaction was performed at 30 °C for 60 min. The samples were transferred to the streptavidin-coated 96-well plate and incubated at room temperature for 20 min. The samples were extensively washed with TBST and incubated with phospho-Thr MAPK/CDK substrate monoclonal antibody at room temperature for 60 min. The samples were extensively washed again with TBST and incubated with mouse IgG horseradish peroxidase-conjugated antibody at room temperature for 60 min. Then samples were extensively washed with TBST and Tris-buffered saline and incubated with 1-Step Ultra TMB-ELISA (Pierce) for 20 min and the reaction was stopped with STOP solution (Cell Signaling). The absorbance was measured at 450 nm with a microplate reader (Molecular Devices).

**Caveoleae/Rafts Isolation—** One ml of Lysis buffer (Caveolea/Rafts Isolation Kit, Sigma) containing 1% Triton X-100 was

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2 The abbreviations used are: HA, hemagglutinin; CDK, cyclin-dependent kinase; MAPK, mitogen-activated protein kinase; RNAi, RNA interference; GST, glutathione S-transferase; PBD, p21-binding domain; eGFP, enhanced green fluorescent protein; siRNA, small interfering RNA; WT, wild type; TBST, Tris-buffered saline Tween-20.
added for each 10-cm dish. After cells were scraped and transferred to an Eppendorf tube, the sample was rocked for 30 min at 4 °C. The lysate was mixed with OptiPrep and the final OptiPrep concentration was adjusted to 35% concentration. Then the density gradient was made of 5 layers of OptiPrep with 35, 30, 25, 20, and 0%. The volume of each layer was 2 ml except for 1 ml of 0% OptiPrep. Samples were centrifuged at 200,000 × g for 4 h at 4 °C. After centrifugation, 1-ml fractions from the top to bottom of the ultracentrifuge tube were collected. As described in manufacturer’s instruction, caveolin-1 fractions were found in fractions 2–4 (25–35% OptiPrep) counting from the top and for the data presented the immunoblots are shown for fraction 2, which is the most abundant caveolin-1-enriched fraction.

**EGFP-GLUT4 Translocation Assay**—Fifty µg of eGFP-GLUT4 plasmid with 550 µg of interesting plasmids was electroporated to 3T3L1 adipocytes. The cells were allowed to adhere to collagen-coated tissue culture dishes for 30–48 h, and the adipocytes were serum starved for 2 h before incubation in the absence or presence of 100 nM insulin for 15 min at 37 °C. Transfected adipocytes were washed in phosphate-buffered saline and fixed for 10 min in phosphate-buffered saline containing 4% paraformaldehyde and 0.2% Triton X-100. The samples were mounted on glass slides with Fluorescent Mounting Medium (DakoCytomation). Cells were imaged using a confocal fluorescence microscope (model MRC-1024; Bio-Rad).

**Quantification of GLUT4 Translocation**—Quantification of transfected GLUT4 translocation was determined as described before (11, 12). Briefly, 3T3L1 adipocytes were co-transfected with 4 µg of eGFP-cMyc-GLUT4 plus 6 µg of various other cDNAs as indicated in each figure. Following basal or hormonal stimulation, the cells were cooled to 4 °C and incubated with a myc antibody followed by horseradish peroxidase-conjugated anti-mouse IgG antibody. The specific cell surface-bound horseradish peroxidase was then determined by incubation with the substrate, o-phenylenediamine dihydrochloride peroxidase (11, 12).

**Statistical Analysis**—All values are expressed as mean ± S.D. Data were evaluated for statistical significance using the one-way analysis of variance and Tukey-Kramer Multiple Comparison Test. The minimum level of significance was set at p < 0.05. The InStat 2 program was used for statistical analysis.

**RESULTS**

**Expression of CDK5 and p35 in 3T3L1 Differentiated Adipocytes**—CDK5 is a serine-threonine kinase that requires the presence of its co-activators p35 and p39 for functional enzymatic activity (13). Although CDK5 is ubiquitously expressed in mammalian tissues, it has been assumed that CDK5 activity is only present in neurons due to the selective expression of p35 and p39 (14–17). However, recent studies have observed the expression of p35 and p39 in pancreatic β cells (18, 19), suggesting the possible activation and potential role of CDK5 in other tissues. Therefore, we examined whether CDK5 and p35 are expressed in differentiated 3T3L1 adipocytes. As shown in Fig. 1, immunoprecipitation of CDK5 (Fig. 1A) and p35 (Fig. 1B) followed by immunoblotting demonstrated the presence of both of these proteins in differentiated adipocytes. Although the relative expression levels of CDK5 remain unchanged during adipogenesis, there was a significant increase in the expression of p35 in fully differentiated adipocytes compared with preadipocytes (Fig. 1C).

In neuronal cells, CDK5 is tyrosine phosphorylated (Tyr15) by the non-receptor tyrosine kinase Fyn (20). Insulin stimulation in 3T3L1 adipocytes resulted in an acute (5 min) CDK5 tyrosine phosphorylation as detected with a phospho-CDK5 antibody specific for Tyr15 (Fig. 1D). However, this phosphorylation was transient and returned to the basal level of Tyr15 phosphorylation by 15 min. Because insulin has been reported to activate Fyn (21), we reduced Fyn expression by siRNA interference that resulted in approximately 80% reduction in Fyn protein (Fig. 1E). Under these conditions, both the basal and insulin-stimulated Tyr15 phosphorylation of CDK5 was dramatically decreased without any significant effect on CDK5 protein expression.

To examine the physiological role of CDK5 on insulin-stimulated GLUT4 translocation, we took two different approaches. First, we overexpressed CDK5 and p35 in 3T3L1 adipocytes to increase CDK5 function (22). As shown in Fig. 1F, insulin-stimulated GLUT4 translocation was significantly reduced compared with vector-transfected adipocytes. In a complementary approach, we reduced CDK5 expression by siRNA interference that resulted in an approximate 70–80% reduction in CDK5 protein levels (Fig. 1C). Under these conditions, there was a significant enhancement of insulin-stimulated GLUT4 translocation (Fig. 1F). To control for off target effects, we examined several CDK5 siRNAs and each resulted in increased insulin-stimulated GLUT4 translocation, whereas overexpression of p35 with a CDK5 siRNA-resistant cDNA also resulted in an inhibition of insulin-stimulated GLUT4 translocation (data not shown).

**In vitro kinase activity measurement using CDK5 immunoprecipitated from basal and insulin-stimulated cells confirmed that insulin stimulation results in an increase in CDK5 kinase activity (Fig. 2D). Taken together these data demonstrate that insulin stimulation results in the kinase activation and tyrosine phosphorylation of CDK5 in a Fyn-dependent manner.**

**TC10α Is Phosphorylated by CDK5**—Because TC10α has a predicted CDK5 phosphorylation site at Thr197 (ILTPKHKHT197 VKKRIGS), we next transfected 3T3L1 differentiated adipocytes with a cDNA encoding for a HA epitope-tagged either wild type TC10α (WT-TC10α) or the alanine for threonine substitution mutant (T197A-TC10α) that is predicted to prevent CDK5-dependent phosphorylation (Fig. 2A). In the basal state, WT-TC10α was phosphorylated as detected by HA immunoprecipitation followed by immunoblotting with a phospho-Thr MAPK/CDK substrate monoclonal antibody. Insulin stimulation for 5 min resulted in increased TC10α phosphorylation that declined to half of the maximum phosphorylation following 15 min of insulin stimulation. On the other hand, the expressed T197A-TC10α mutant protein was a poor substrate compared with WT-TC10α. Under these conditions, the total expressed amount of TC10α protein remained relatively unchanged. Consistent with a CDK5-dependent phosphorylation of TC10α, pretreatment of the cells with a
FIGURE 1. Expression of CDK5 and p35 in 3T3L1 differentiated adipocytes and regulation by Fyn-dependent phosphorylation on Tyr15. A, CDK5 was immunoprecipitated (IP) with either a rabbit IgG (left lane) or a specific rabbit polyclonal CDK5 antibody (right lane) from whole cell lysates of 3T3L1 differentiated adipocytes. CDK5 was immunoblotted (IB) with a specific mouse monoclonal CDK5 antibody as described under “Materials and Methods.” Representative experiments independently performed three times are shown. B, p35 was immunoprecipitated with either a rabbit IgG (left lane) or a specific rabbit polyclonal p35 antibody (right lane) from whole cell lysates of 3T3L1 adipocytes. p35 was immunoblotted with a specific goat polyclonal p35 antibody as described under “Materials and Methods.” Representative experiments independently performed three times are shown. C, CDK5 was immunoprecipitated with a specific rabbit polyclonal CDK5 antibody from undifferentiated cells (Day 0), immature adipocytes (Day 4), and mature adipocytes (Day 8). CDK5 was immunoblotted with a specific mouse monoclonal CDK5 antibody as described under “Materials and Methods.” Similarly, p35 was immunoprecipitated with a specific rabbit polyclonal p35 antibody from undifferentiated cells (Day 0), immature adipocytes (Day 4), and mature adipocytes (Day 8). p35 was immunoblotted with a specific goat polyclonal p35 antibody as described under “Materials and Methods.” Representative experiments independently performed three times are shown. D, differentiated 3T3L1 adipocytes were either untreated or stimulated with 100 nM insulin for 5 and 15 min at 37 °C. CDK5 was immunoprecipitated with polyclonal CDK5 antibody and one-fifth of the immunoprecipitate was subjected to CDK5 immunoblotting with monoclonal CDK5 antibody and four-fifths of the immunoprecipitate was immunoblotted with a phospho-CDK Tyr15 antibody. These are representative immunoblots independently performed three times. Phosphorylated CDK5 band intensity was compared with the unstimulated sample and expressed as the mean ± S.D. (*, *p < 0.05). E, a Fyn-specific RNAi or scrambled RNAi was introduced to differentiated adipocytes by electroporation. After 48 h, the adipocytes were either untreated or stimulated with 100 nM insulin for 5 and 15 min at 37 °C. Total cell lysates were immunoblotted for expression levels of the Fyn protein. One-fifth of the CDK5 immunoprecipitate was immunoblotted with a polyclonal CDK5 antibody and four-fifths of the immunoprecipitate was immunoblotted with a phospho-CDK Tyr15 antibody. Representative experiments independently performed three times are shown. F, CDK5 and p35 were overexpressed in differentiated adipocytes by electroporation as described under “Materials and Methods.” After 48 h, the adipocytes were either untreated or stimulated with 100 nM insulin for 30 min at 37 °C. Similarly, a CDK5-specific RNAi or scrambled RNAi was introduced to differentiated adipocytes and 48 h later the cells were either untreated or stimulated with 100 nM insulin for 30 min at 37 °C. The extent of GLUT4 translocation was determined by the quantitative colorimetric assay as described above. These data were obtained from the average of four independent experiments and expressed as the mean ± S.D. (**, **p < 0.01).
FIGURE 2. Insulin-stimulated phosphorylation of TC10 on threonine 197 is CDK5 dependent. A, HA epitope-tagged WT-TC10 or T197A-TC10 mutant protein was expressed in differentiated 3T3L1 adipocytes as described under “Materials and Methods.” Forty-eight h later, the cells were either left untreated or stimulated with 100 nM insulin for 5 and 15 min at 37 °C. The expressed HA-TC10 was immunoprecipitated with a HA antibody and phosphorylated TC10 was detected by immunoblotting with phospho-Thr MAPK/CDK substrate monoclonal antibody and phosphorylated TC10 band intensity was compared with unstimulated sample expressing HA-WT-TC10 (**, *p < 0.01; *, p < 0.05). The immunoprecipitated HA-TC10 amount was confirmed by HA immunoblot. These are representative immunoblots independently performed three times and expressed as the mean ± S.D. B, differentiated 3T3L1 adipocytes were transfected with HA epitope-tagged WT-TC10 and incubated in the absence or presence of the CDK5 inhibitor olomoucine (10 mM) for 60 min. The cells were then either left untreated or stimulated with 100 nM insulin for 5 min, followed by HA immunoprecipitation (IP) and immunoblotting (IB) with the phospho-Thr MAPK/CDK substrate monoclonal antibody and HA antibody. These are representative immunoblots independently performed three times and expressed as the mean ± S.D. Phosphorylated TC10 band intensity was compared with unstimulated sample without olomoucine (**, *p < 0.01). C, differentiated adipocytes were electroporated with CDK5 RNAi or scrambled RNAi and incubated for 48 h. The adipocytes were then either untreated or stimulated with 100 nM insulin for 5 min at 37 °C as described under “Materials and Methods.” TC10 was immunoprecipitated with polyclonal TC10 antibody and one-fifth of the immunoprecipitated TC10 protein was subjected to TC10 immunoblotting with a polyclonal TC10 antibody and four-fifths of the immunoprecipitated TC10 protein was immunoblotted with a phospho-Thr MAPK/CDK substrate monoclonal antibody. These are representative experiments independently performed three times. D, either biotinylated Ser/Thr control peptide or biotinylated synthesized TC10 peptide was phosphorylated by CDK5/p35 recombinant or CDK5 immunoprecipitated from 2.0 mg of 3T3L1 adipocytes lysates in the absence or presence of insulin stimulation (100 nM, 5 min) as described under “Materials and Methods.” These results are independently performed four times and expressed as the mean ± S.D.
CDK5 and GLUT4

CDK5-specific inhibitor (olomoucine) reduced both the basal and acute insulin-stimulated WT-TC10α phosphorylation with no change in the amount of TC10α protein expression (Fig. 2B). To confirm that olomoucine inhibition of TC10α phosphorylation was due to a blockade of CDK5, we reduced CDK5 expression using a CDK5-specific siRNA. Although there was no change in TC10α expression, the knockdown of CDK5 markedly inhibited insulin-stimulated TC10α phosphorylation (Fig. 2C). Using both a S/T control peptide designed as a substrate for CDK5 and the specific TC10α peptide, both peptides displayed similar extents of phosphorylation by recombinant CDK5/p55 (Fig. 2D, first two bars). Under basal conditions S/T control peptide and TC10α peptide were also phosphorylated by CDK5 immunoprecipitated from 3T3L1 adipocytes extracts. As expected, extracts isolated from insulin-stimulated 3T3L1 adipocytes displayed increased phosphorylation of both peptides (last two bars). These data demonstrated that insulin not only activates the kinase activity of CDK5 but that TC10α is a direct substrate of CDK5 kinase.

CDK5 Localizes to Lipid Rafts Microdomains—Because TC10α is known to be lipid raft associated, we next investigated the localization of CDK5 by comparing its plasma membrane distribution with an established lipid raft marker caveolin-1 (23). To do this, the caveolae/raft fraction was isolated by gradient fractionation from 3T3L1 adipocytes as previously described (24). CDK5 co-fractionated with caveolin-1 in adipocytes expressing WT-TC10α, T197A-TC10α, as well as cells expressing T197D-TC10α that mimics TC10 phosphorylation (Fig. 3A).

Because CDK5 is lipid raft associated, we next examined the possible role of CDK5-dependent phosphorylation of TC10α to regulate TC10α plasma membrane localization. As shown in Fig. 3B, WT-TC10α was colocalized with caveolin-1, whereas the amount of T197A-TC10α colocalized with caveolin-1 was substantially reduced. In contrast, expression of an aspartic acid substitution mutant expected to mimic phosphorylation (T197D-TC10α) display an equivalent amount of colocalization with caveolin-1. The reduced amount of T197A-TC10α that co-fractionated with caveolin-1 was not due to differences in protein expression as the level of each TC10α construct was essentially identical (Fig. 3B). These data are consistent with Thr197 phosphorylation being a regulatory event controlling the partitioning of TC10α between non-lipid raft and lipid domains.

TC10α Phosphorylation and GTP/GDP Exchange—Previous studies have demonstrated that insulin stimulates the activation of TC10α by increasing GTP binding through the exchange of GTP for GDP (25). To assess the role of TC10α phosphorylation on GTP loading, we took advantage of the PBD domain that was not further changed following insulin stimulation. Thus, these data indicated that Thr197 phosphorylation functionally regulates the ability of TC10α to become activated through increased GTP/GDP exchange.

TC10α Phosphorylation Regulates F-actin Polymerization State—Several studies have observed that the lipid raft targeting of constitutively overexpressed TC10α results in a disruption of adipocyte cortical actin organization with a concomitant inhibition of insulin-stimulated GLUT4 translocation (1, 2, 5–10, 25). As the data presented in Fig. 3 are consistent with

FIGURE 3. CDK5 and phosphorylated TC10α colocalizes with caveolin 1 and phosphorylation of TC10α regulates GTP/GDP conversion. A, the caveolin-enriched lipid raft fraction was isolated from 3T3L1 adipocytes as described under “Materials and Methods” and caveolin-1 and CDK5 were immunoblotted (IB) with specific antibodies. These are representative experiments independently performed three times. B, adipocytes were transfected with HA-tagged WT-TC10α, T197A-TC10α, or T197D-TC10α and 48 h later the caveolin-enriched lipid raft fraction was isolated and quantitated as described under “Materials and Methods.” The total cell lysates were immunoblotted for the expressed TC10α protein (top panel) and the caveolin-enriched lipid raft fraction was immunoblotted for the expressed TC10α proteins (top panel) and caveolin-1 (middle panel). These are representative experiments independently performed three times. C, adipocytes were transfected with WT-TC10α or T197A-TC10α, or T197D-TC10α and either left untreated or stimulated for 5 min with 100 nM insulin. The relative amount of GTP-loaded TC10α protein was determined by the GST-Pak1-PBD pull-down assay as described under “Materials and Methods.” Whole cell lysate samples were immunoblotted for the total amount of TC10α protein expression. These are representative results independently performed three times. These are representative experiments independently performed three times.
Thr^{197} phosphorylation resulting in both GTP loading and lipid raft localization, cytoskeletal and cytosolic fractions were isolated and immunoblotted for actin content (Fig. 4). Expression of WT-TC10α and T197D-TC10α resulted in a marked disruption of F-actin in adipocytes compared with cells transfected with the empty vector. In contrast, adipocytes transfected with the CDK5 phosphorylation defective mutant (T197A-TC10α) had no significant change in F-actin assembly. Moreover, the total amount of F-actin present was not affected by insulin stimulation and paralleled the changes observed with the TC10α phosphorylation mutants in the basal state. Thus, these data are consistent with the disruption of cortical F-actin related to the localization of TC10α to the lipid raft domains that are in turn regulated by the Thr^{197} phosphorylation state.

TC10α Phosphorylation and Insulin-stimulated GLUT4 Translocation—Because the ability of overexpressed TC10α to inhibit insulin-stimulated GLUT4 translocation correlates with cortical F-actin depolymerization, we determined the effect of the TC10α mutants. Adipocytes were co-transfected with a cMyc-GLUT4-eGFP reporter and either WT-TC10α or the T197D-TC10α or T197A-TC10α mutants (Fig. 5). Overexpression of WT-TC10α and T197D-TC10α had no effect on the basal state of GLUT4 distribution but markedly inhibited insulin-stimulated GLUT4 translocation to the plasma membrane, whereas T197A-TC10α was without any significant inhibitory effect. These data are quantified in Fig. 5B using a quantitative colorimetric assay that detects the amount of GLUT4 at the cell surface (11, 12). Thus, these data are consistent with Thr^{197} phosphorylation of TC10α as providing an important signal for the depolymerization of adipocyte F-actin and inhibition of insulin-induced GLUT4 translocation.

To examine whether the TC10α inhibition of insulin-stimulated GLUT4 translocation was CDK5-dependent, CDK5 expression was reduced by RNAi gene silencing. As previously observed (Fig. 1F), the knockdown of CDK5 resulted in enhanced insulin-stimulated GLUT4 translocation (Fig. 5C). As expected, overexpression of WT-TC10α had no effect on the basal state of GLUT4 translocation but inhibited insulin-stimulated GLUT4 translocation (Fig. 5C). However, CDK5 knockdown prevented the inhibitory effects of WT-TC10α and resulted in a normal extent of insulin-stimulated GLUT4 translocation (*, p < 0.05).

On the other hand, overexpression of T197A-TC10α in these experiments resulted in a small reduction of insulin-stimulated GLUT4 translocation that was fully reversed by siRNA-mediated knockdown of CDK5 (Fig. 5C). More importantly, overexpression of T197D-TC10α had no effect on the basal state of GLUT4 translocation but markedly inhibited insulin-stimulated GLUT4 translocation (Fig. 5C). Under these conditions, the CDK5 was completely unable to restore insulin-stimulated GLUT4 translocation (Fig. 5C). These data are consistent with CDK5-dependent phosphorylation of Thr^{197} being responsible for the inhibitory function of TC10α on insulin-stimulated GLUT4 translocation.

DISCUSSION

Previous studies have observed that overexpression of WT-TC10α, a constitutively GTP-bound active mutant (Q75L-TC10α) and a constitutively GDP-inactive mutant (T31N-TC10α), all inhibited insulin-stimulated GLUT4 translocation (25, 27). Moreover, unlike other Rho family GTP-binding proteins, TC10α contains a carboxyl-terminal CAAX domain that targets TC10α to plasma membrane lipid raft microdomains (8). Recently, the inhibitory effect of overexpressed TC10α was reported to depend on lipid raft microdomain compartmentalization, whereas targeting of TC10α to non-lipid raft domains of the plasma membrane were without effect (8). Thus, the fact that the inhibitory action of TC10α is independent of effector domain activation but requires appropriate intracellular compartmentalization strongly suggests that a specific protein interacting with the structural domain of TC10α is responsible.

Nevertheless, TC10α regulation appears to play an important role in the insulin regulation of GLUT4 translocation as insulin stimulation results in GTP loading and subsequent recruitment of the exocyst complex required for GLUT4 transporter vesicle docking (27). In addition, TC10α appears to have marked effects on adipocyte cortical F-actin organization and remodeling (1, 2, 5–10, 25, 27). Thus, the question of how insulin regulates TC10α signals mediating actin function remains as an open question.

As previous studies have also demonstrated that overexpression of wild type, constitutively active, or constitutively inactive TC10α mutants, but not TC10β, inhibited insulin-stimulated GLUT4 translocation, glucose uptake, and disrupted cortical
CDK5 and GLUT4

**FIGURE 5.** TC10 phosphorylation state regulates GLUT4 translocation. A, differentiated 3T3L1 adipocytes were co-transfected with the myc-GLUT4-GFP reporter plus WT-TC10α, T197A-TC10α, or T197D-TC10α as described under “Materials and Methods.” Forty-eight h later, the cells were treated with or without 100 nM insulin for 30 min and the cells fixed and subjected to confocal fluorescence microscopy for the localization of the Myc-GLUT4-GFP. These are representative confocal microscopic images independently performed four times. B, the data obtained in A was confirmed using a quantitative colorimetric assay that detects the amount of exofacial exposed Myc epitope as described under “Materials and Methods.” These data were obtained from the average of four independent experiments and expressed as the mean ± S.D. (*, p < 0.05). C, differentiated 3T3L1 adipocytes were co-transfected with Myc-GLUT4-GFP plus WT-TC10α, T197A-TC10α, or T197D-TC10α and either scrambled or CDK5 RNAi. Forty-eight h later, the cells were incubated in the absence or presence of 100 nM insulin for 30 min. The cells were fixed and the extent of Myc-GLUT4-GFP translocation was quantified by the quantitative colorimetric assay as described above. These data were obtained from the average of six independent experiments and expressed as the mean ± S.D. (*, p < 0.05; **, p < 0.01; ***, p < 0.001). C, control; I, insulin.

F-actin (10), it is likely that specific sequence differences were responsible. Inspection of the amino acid sequence indicated the presence of a CDK5 phosphorylation site in TC10α (Thr197) that is not present in TC10β. Thus, we predicted that CDK5-dependent phosphorylation of TC10α may provide an important control mechanism for the differential function of TC10α versus TC10β.

In this regard, we have confirmed the presence of the CDK5 in differentiated 3T3L1 adipocytes as well as the presence of the CDK5 regulatory protein, p35, which is sufficient for CDK5 kinase activity (11, 28). Although, the Abl non-receptor tyrosine kinase was reported to phosphorylate CDK5 on Tyr15 that resulted in increased CDK5 kinase activity (29), we have also observed that the Src family kinase Fyn also phosphorylates CDK5 at Tyr15 in 3T3L1 adipocytes, similar to that found neuronal cells (18). In fact, we have observed a CDK5 basal phosphorylation at Tyr15 that also underwent a rapid increase but transient insulin-dependent phosphorylation in adipocytes. The fact that CDK5 displayed a relatively low basal level of Tyr15 phosphorylation was consistent with a relatively low level of CDK5 kinase activity. The insulin-stimulated increase in CDK5-Tyr15 phosphorylation correlated with an increase in CDK5 substrate kinase activity. Thus, our data strongly suggest that CDK5 is activated in the basal state as well as further activated following brief insulin stimulation and that Fyn is the upstream tyrosine kinase that mediates CDK5 activation.

In addition to its role in CDK5 protein kinase activation, p35 has also been reported to directly associate with the GTP-bound isoforms of the Rac, Rho family GTPases that are master regulators of actin polymerization (30). TC10α is a member of the Rho family that is lipid raft localized and we have observed that CDK5 colocalizes to caveolin-enriched domains, similar to that observed for TC10α. TC10α also appears to be a CDK5 substrate as mutation of the consensus phosphorylation site and pretreatment with either the CDK5 inhibitor olomoucine or CDK5 RNAi reduced TC10α phosphorylation on Thr197.

Having established evidence that TC10α is a CDK5 substrate and undergoes Thr197 phosphorylation, expression of a phosphorylation-deficient mutant T197A as well as phosphorylation mimic mutant T197D demonstrated that phosphorylation at this site controls not only lipid raft localization and GTP/GDP conversion but also F-actin organization and insulin-stimulated GLUT4 translocation. Based upon these data, we can postulate the following model for the control of GLUT4 translocation by TC10α. In the basal state, TC10α is phosphorylated and lipid raft localized, which defines the resting state of adipocyte cortical F-actin. Insulin stimulation transiently increases TC10α phosphorylation thereby altering its positioning from non-lipid raft to lipid raft plasma membrane domains. Because TC10α has intrinsic actin depolymerization activity, this results in a reduction in the amount of cortical F-actin. However, subse-
quent dephosphorylation results in redistribution back toward non-lipid raft domains, thereby increasing cortical F-actin organization. As dynamic actin remodeling (polymerization/depolymerization) is necessary for GLUT4 translocation (5, 25), this provides access of the GLUT4 transporter vesicles to the plasma membrane localized exocyt docking sites. This model also accounts for the effect of overexpression of TC10α to inhibit GLUT4 translocation and depolymerize cortical actin. As CDK5 is basally active, overexpression of TC10α results in net accumulation of TC10α actin. This regulation of cortical F-actin organization. As dynamic actin remodeling (polymerization/depolymerization) is necessary for GLUT4 translocation (5, 25), this provides access of the GLUT4 transporter vesicles to their plasma membrane docking sites. This model also accounts for the effect of overexpression of TC10α to inhibit GLUT4 translocation and depolymerize cortical actin. As CDK5 is basally active, overexpression of TC10α results in net accumulation of TC10α actin. As CDK5 is basally active, overexpression of TC10α results in net accumulation of TC10α actin. As CDK5 is basally active, overexpression of TC10α results in net accumulation of TC10α actin.

In conclusion, our data indicate that CDK5 plays an important role in the basal and insulin-stimulated phosphorylation of a key regulatory site (Thr197) in the TC10α isoform. This phosphorylation event establishes the localization of TC10α to either lipid raft or non-lipid raft domains and thereby modulates the intrinsic cortical actin polymerization and depolymerization properties of TC10α. This regulation of cortical F-actin dynamics thereby allows or prevents access of GLUT4 transporter vesicles to their plasma membrane docking sites, an event essential for membrane fusion and glucose uptake in adipocytes.

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