Lipid Raft Targeting of the TC10 Amino Terminal Domain Is Responsible for Disruption of Adipocyte Cortical Actin

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Overexpression of the Rho family member TC10 α , disrupts adipocyte cortical actin structure and inhibits insulin-stimulated GLUT4 translocation when targeted to lipid raft microdomains. This appears to be independent of effector domain function because overexpression of the wild-type (TC10/WT), constitutively GTP-bound (TC10/Q75L), and constitutively GDP bound (TC10/T31N) all inhibit adipocyte cortical actin structure and GLUT4 translocation. To examine the structural determinants responsible for these effects, we generated a series of chimera proteins between TC10 with that of H-Ras and K-Ras. Chimera containing the 79 (TC10–79/H-Ras), 41 (TC10–41/H-Ras), or 16 (TC10–16/H-Ras) amino acids of the TC10 amino terminal extension fused to H-Ras disrupted cortical actin and inhibited insulin-stimulated GLUT4 translocation. In contrast, the same amino terminal TC10 extensions fused to K-Ras had no significant effect on either GLUT4 translocation or cortical actin structure. Similarly, expression of TC10 β was without effect, whereas fusion of the amino terminal 8 amino acid of TC10 α onto TC10 β resulted in an inhibition of insulin-stimulated GLUT4 translocation. Within the amino terminal extension point mutation analysis demonstrated that both a GAG and GPG sequences when lipid raft targeted was essential for these effects. Furthermore, expression of the amino terminal TC10 deletions Δ NT-TC10/WT or Δ NT-TC10/T31N had no detectable effect on cortical actin organization and did not perturb insulin-stimulated GLUT4 translocation. Surprisingly, however, expression of Δ NT-TC10/Q75L remained fully capable of inhibiting insulin-stimulated GLUT4 translocation without affecting cortical actin. These data demonstrate that inhibitory effect of TC10 overexpression on adipocyte cortical actin organization is due to the specific lipid raft targeting of the unusual TC10 amino terminal extension.

INTRODUCTION

The mechanisms and intracellular signaling pathways utilized by the insulin receptor to initiate biological responsiveness are quite complex. In the case of glucose uptake in adipose and striated muscle, the insulin-responsive glucose transporter protein (GLUT4) is predominantly localized to intracellular membrane compartments and undergoes a dramatic redistribution to the cell surface following insulin stimulation through a process termed translocation (Holman and Sandoval, 2001; Simpson *et al.*, 2001; Bryant *et al.*, 2002; Ducluzeau *et al.*, 2002; Ploug and Ralston, 2002). Although this translocation process increases the amount of cell surface GLUT4, recent data has also suggested that the transport activity itself is also activated by insulin (Sweeney *et al.*,

1999; Konrad *et al.*, 2001; Somwar *et al.*, 2001; Huang *et al.*, 2002). In addition to understanding the translocation process and the mechanisms activating glucose uptake, the intracellular signaling pathways mediating these processes is also poorly understood.

Currently, it is well established that activation of the insulin receptor tyrosine kinase results in the tyrosine phosphorylation of IRS proteins (White, 1998; Giovannone *et al.*, 2000). This results in the association and activation of the Type 1A phosphatidylinositol 3-kinase (PI 3-kinase) and the generation of phosphatidylinositol-3, 4,5-trisphosphate (PI3,4,5P3) at the plasma membrane (Alessi and Downes, 1998; Cantley, 2002; Saltiel and Pessin, 2002). PI3,4,5P3 serves to recruit the phosphoinositide-dependent protein kinase (PDK1) and protein kinase B (PKB) also known as Akt (Alessi *et al.*, 1997; Anderson *et al.*, 1998; Stephens *et al.*, 1998). PDK1 also phosphorylates PKB as well as atypical protein kinase C isoforms zeta and lambda (PKC ζ/λ) and

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evidence has been presented both for and against either PKB or PKC ζ/λ as the critical target necessary for insulin-stimulated glucose uptake (Kohn *et al.*, 1996; Alessi *et al.*, 1997; Bandyopadhyay *et al.*, 1997; Le Good *et al.*, 1998; Imamura *et al.*, 1999; Standaert *et al.*, 1999; Wang *et al.*, 1999; Kristiansen *et al.*, 2001; Matsumoto *et al.*, 2001; Yamada *et al.*, 2002). Nevertheless, a substantial amount of data have established that PI 3-kinase activation and generation of PI3,4,5P3 are essential for insulin-stimulated GLUT4 translocation (Cheatham *et al.*, 1994; Okada *et al.*, 1994; Martin *et al.*, 1996; Sharma *et al.*, 1998; Vollenweider *et al.*, 1999; Czech, 2000; Nakashima *et al.*, 2000). Although PI 3-kinase function is necessary, numerous studies have also suggested that this pathway is not sufficient to mediate the full extent of insulin-stimulated GLUT4 translocation (Isakoff *et al.*, 1995; Wiese *et al.*, 1995; Krook *et al.*, 1997; Guilherme and Czech, 1998; Egawa *et al.*, 2002). More recently, a second insulin signaling pathway has been proposed that utilizes the APS/CAP/Cbl complex to recruit and activate an unusual member of the Rho family of small GTP binding proteins, TC10 in a PI 3-kinase-independent manner (Ribon and Saltiel, 1997; Ribon *et al.*, 1998; Baumann *et al.*, 2000; Chiang *et al.*, 2001).

One surprising and unexplained aspect of these data was that overexpression of the wild-type (TC10/WT), a constitutively GTP-bound active mutant (TC10/Q75L) and a constitutively GDP-inactive mutant (TC10/T31N), all inhibited insulin-stimulated GLUT4 translocation (Chiang *et al.*, 2001). Moreover, unlike other Rho family GTP binding proteins, TC10 contains a carboxyl terminal CAAX domain that targets TC10 to plasma membrane lipid raft microdomains (Murphy *et al.*, 2001; Watson *et al.*, 2001). The inhibitory effects of overexpressed TC10 were dependent on lipid raft microdomain compartmentalization, whereas targeting of TC10 to nonlipid raft domains of the plasma membrane had no effect (Watson *et al.*, 2001, 2003). Because the inhibitory actions of TC10 are independent of effector domain activation but require appropriate intracellular compartmentalization strongly suggests that a specific protein interacting structural domain of TC10 is responsible. In this regard, overexpression of lipid raft microdomain targeted TC10 was recently observed to disrupt adipocyte cortical actin and several studies have demonstrated that actin structure is necessary for insulin-stimulated GLUT4 translocation (Tsakiridis *et al.*, 1998; Wang *et al.*, 1998; Omata *et al.*, 2000; Kanzaki *et al.*, 2002). In this study, we have identified and characterized a novel amino terminal structural motif in TC10 that is responsible for the disruption of adipocyte cortical actin and inhibition of insulin-stimulated GLUT4 translocation when specifically targeted to plasma membrane lipid raft microdomains.

MATERIALS AND METHODS

Materials

The mammalian expression plasmid pKH3-TC10/WT encoding for the human TC10, mouse TC10 α and TC10 β isoforms were prepared as described previously (Chiang *et al.*, 2001, 2002). R-Ras1 and R-Ras2 were subcloned into pKH3 vector from pCGN vector and pZip vector, respectively. Murine 3T3L1 preadipocytes were purchased from the American Type Tissue Culture repository and differentiated as described by Min *et al.* (1999). Monoclonal anti-HA and anti-Myc antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Fluorescent secondary antibodies were

purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Rhodamine-phalloidin was purchased from Sigma (St. Louis, MO).

Cell Culture and Transient Transfection of 3T3L1 Adipocytes

Murine 3T3L1 preadipocytes were cultured in DMEM supplemented with 25 mM glucose and 10% calf serum at 37°C with 8% CO₂. Cells were then differentiated into adipocytes as previously described (Olson *et al.*, 1997). Differentiated adipocytes were electroporated using the Gene Pulsar II (Bio-Rad, Hercules, CA) with settings of 0.16 kV and 950 μ F (Min *et al.*, 1999). After electroporation, cells were plated on 6-well plates and allowed to recover in adipocytes medium for ~16–18 h. The next day the cells were starved with serum-free DMEM for 2–3 h and then stimulated with 100 nM insulin for 30 min.

Immunofluorescence and Image Analysis

Transfected adipocytes were washed in phosphate-buffered saline (PBS) and fixed for 15 min in 4% paraformaldehyde containing 0.2% Triton X-100. Cells were washed briefly in PBS and then blocked in 5% donkey serum (Sigma) plus 1% bovine serum albumin (BSA; Sigma) for 1 h. Primary and secondary antibodies were used at 1:100 dilutions in blocking solution, and samples were mounted on glass slides with Vectashield (Vector Labs, Burlingame, CA). Cells were imaged using confocal fluorescence microscopy (LSM 510; Zeiss, Thornwood, NY). Images were then imported into Adobe Photoshop (Adobe Systems, Inc., San Jose, CA) for processing and composite files were generated.

Preparation of TC10 Amino Terminal Deletion, Substitutions, and Chimeras

Amino terminal deletions PGAGRSSMAHGPALML (Δ NT-TC10/WT) and substitutions for amino acids GAG at positions 3–5 to ASA (TC10/GAG-ASA) and GPG at position 12–14 to ASA (TC10/GPG-ASA) were generated from the human TC10/WT cDNA using the PCR with oligonucleotide primers containing the appropriate base-substitutions. The amino terminal extension of TC10 (amino acids 1–16, 1–41, and 1–79), R-Ras1 and R-Ras2 (amino acids 1–16) were fused to both H-Ras and K-Ras as described previously (Horton *et al.*, 1993).

Single-Cell Microinjection

The microinjection and visualization of single 3T3L1 adipocytes were performed as described previously (Baumann *et al.*, 2000). Briefly, the cells were grown on coverslips, and the medium was changed to Leibovitz's L-15 medium containing 0.1% BSA before microinjection. Differentiated 3T3L1 adipocyte nuclei were injected with 200 μ g/ml cDNAs in microinjection buffer containing 100 mM KCl, 5 mM Na₂PO₄, pH 7.2, with Eppendorf model 5171 micromanipulator. The cells were allowed to recover for 16–18 h and cells were fixed for 15 min in 4% paraformaldehyde containing 0.2% Triton X-100. Cells were washed briefly in PBS and then blocked in 5% donkey serum (Sigma) plus 1% BSA (Sigma) for 1 h. Primary antibodies was used at 1:100 dilutions in blocking solution and rhodamine-phalloidin staining for actin structure at 1:2500 dilutions in blocking solution. Samples were mounted on glass slides with Vectashield (Vector Labs). Cells were imaged using confocal fluorescence microscopy (Zeiss LSM 510). Images were then imported into Adobe Photoshop (Adobe Systems, Inc.) for processing and composite files were generated.

Figure 1. Schematic and sequence relationship between TC10, H-Ras, K-Ras, R-Ras1, and R-Ras2. (A) Chimera between the TC10 α amino terminal 79-, 41-, and 16-amino acid residues were fused to either H-Ras or K-Ras. (B) The amino terminal amino acid sequences of TC10 α are compared with that of R-Ras1 and R-Ras2, all of which contain similar carboxyl terminal targeting motifs. (C) The amino terminal amino acid sequences of TC10 α , TC10 β , and the TC10 α/β chimera are shown.

RESULTS

Lipid Raft Compartmentalization of the TC10 Amino Terminal Domain Inhibits Insulin-stimulated GLUT4 Translocation

Recently, we have observed that overexpression of wild-type TC10 isoform (TC10/WT), a constitutively active GTP-bound mutant (TC10/Q75L) and constitutively inactive GDP-bound mutant (TC10/T31N), all inhibited insulin-stimulated GLUT4 translocation (Chiang *et al.*, 2001). These data suggest that neither GTP binding nor effective domain function is responsible for this effect. Because overexpression of H-Ras or K-Ras does not have any significant effect on insulin-stimulated GLUT4 translocation in adipocytes (Watson *et al.*, 2001), we utilized both H-Ras and K-Ras backbone sequences to identify the structural domain in TC10 responsible for this inhibitory action.

Sequence alignment of Rho family members revealed that TC10 is most divergent from most of these family members at the amino and carboxyl termini. The TC10 carboxy terminus is related to the H-Ras carboxy terminus in that they both have an appropriate context of cysteine residues necessary for both farnesylation and dual palmitoylation (Hancock *et al.*, 1989; Seabra, 1998; Choy *et al.*, 1999; Resh, 1999; Apolloni *et al.*, 2000; Watson *et al.*, 2001). These posttranslational modifications result in the lipid raft microdomain compartmentalization of both H-Ras and TC10 (Dupree *et al.*, 1993; Li *et al.*, 1996; Song *et al.*, 1996; Watson *et al.*, 2001). Therefore, we initially prepared three amino terminal TC10/H-Ras fusion proteins that contain the TC10 amino terminal 79 residues (TC10-79/H-Ras), 41 residues (TC10-41/H-Ras), and 16 residues (TC10-16/H-Ras) fused to H-Ras (Figure 1). After expression by electroporation, confocal immunofluorescence localization demonstrated that all three TC10/H-Ras chimera distributed in a manner indistinguishable from that of TC10/WT or H-Ras (Figure 2A, panels 1-5). These proteins were localized to the peri-nuclear secre-

tory membrane compartments and the plasma membrane. In contrast, the K-Ras carboxyl terminal domain is not palmitoylated and only contains a single cysteine residue in the appropriate context for farnesylation (Hancock *et al.*, 1990; Choy *et al.*, 1999; Resh, 1999). This results in the localization of K-Ras to nonlipid raft microdomains of the plasma membrane and exclusion from the endomembrane system (Apolloni *et al.*, 2000). Consistent with these data, overexpression of both K-Ras and TC10-16/K-Ras chimera was exclusively plasma membrane associated with little if any protein localized to the secretory membrane compartments (Figure 2A, panels 6 and 7).

As typically observed in cells expressing the GLUT4-EGFP fusion protein, insulin stimulation resulted in a marked translocation of GLUT4 from intracellular storage sites to the plasma membrane (Figure 2B, panels 1 and 2). This is readily detected by the appearance of a strong plasma membrane rim of fluorescence (Figure 2B, panel 2). As previously reported (Watson *et al.*, 2001), expression of TC10/WT but not H-Ras inhibited insulin-stimulated GLUT4-EGFP translocation (Figure 2B, panels 3-6). Similar to TC10/WT, expression of TC10-79/H-Ras, TC10-41/H-Ras, and TC10-16/H-Ras all inhibited insulin-stimulated GLUT4 translocation (Figure 2B, panels 9-14). In contrast, neither K-Ras (Figure 2B, panels 7 and 8) nor the TC10-16/K-Ras chimera (Figure 2B, panels 15 and 16) had any significant effect on insulin-stimulated GLUT4 translocation.

These data were quantified by determining the number of cells displaying a continuous cell surface GLUT4-EGFP fluorescence (Figure 2C). In control vector-transfected cells, insulin stimulated GLUT4 translocation in $65 \pm 2.4\%$ of the adipocyte population and was not significantly different in cells coexpressing H-Ras/WT ($58 \pm 3.0\%$) or K-Ras/WT ($62 \pm 3.0\%$). Similarly, coexpression with TC10-16/K-Ras resulted in a comparable degree of insulin-stimulated GLUT4 translocation ($58 \pm 1.5\%$). In contrast, there was a marked inhibition of insulin-stimulated GLUT4 translocation in adipocytes expressing TC10/WT ($23 \pm 3.1\%$), TC10-79/H-Ras ($29 \pm 1.3\%$), TC10-41/H-Ras ($30 \pm 2.1\%$), and TC10-16/H-Ras ($23 \pm 1.3\%$). Together, these data demonstrated that the amino terminal 16 amino acids of TC10 contain the necessary information to inhibit insulin-stimulated GLUT4 translocation independent of effector binding function. Importantly, this property of TC10 is only manifested when the amino terminal domain is targeted to lipid raft microdomains.

Amino Terminal Extension of TC10 Disrupts Cortical Actin Structure

Several recent studies have reported that the cortical actin structures in muscle and adipocytes play an essential role in the insulin-stimulated GLUT4 translocation process (Tsakiridis *et al.*, 1998; Wang *et al.*, 1998; Omata *et al.*, 2000; Kanzaki *et al.*, 2001; Tong *et al.*, 2001; Jiang *et al.*, 2002). Because TC10 has been observed to alter actin polymerization both in vitro and in vivo (Kanzaki *et al.*, 2002), we next assessed the effect of the various chimeras on cortical actin organization by single cell microinjection (Figure 3). Phalloidin staining of adipocytes demonstrated the presence of a thick filamentous actin network surrounding the plasma membrane as well as polymerized actin in the peri-nuclear region (Figure 3A, panels 2, 5, 8, and 11). In cells expressing

Figure 2. Targeting of the amino terminal TC10 extension to lipid raft microdomains inhibits insulin-stimulated GLUT4 translocation. (A) Differentiated 3T3L1 adipocytes were electroporated with 50 μ g of the HA epitope-tagged TC10/WT (panel 1), H-Ras/WT (panel 2), TC10-79/H-Ras (panel 3), TC10-41/H-Ras (panel 4), TC10-16/H-Ras (panel 5), K-Ras/WT (panel 6), and TC10-16/K-Ras (panel 7) chimera cDNAs as described in MATERIALS AND METHODS. Eighteen hours later, the cells were fixed and the subcellular localization was determined by confocal fluorescent microscopy. (B) Differentiated 3T3L1 adipocytes were coelectroporated with 50 μ g of GLUT4-EGFP plus 200 μ g of the empty vector (panels 1 and 2), TC10/WT (panels 3 and 4), H-Ras/WT (panels 5 and 6), K-Ras/WT (panels 7 and 8), TC10-79/H-Ras (panels 9 and 10), TC10-41/H-Ras (panels 11 and 12), TC10-16/H-Ras (panels 13 and 14), and TC10-16/K-Ras (panels 15 and 16) cDNAs. Eighteen hours later, the cells were then treated without (Basal, panels 1, 3, 5, 7, 9, 11, 13, and 15) or with 100 nM insulin (Insulin, panels 2, 4, 6, 8, 10, 12, 14, and 16) for 30 min. The cells were then fixed and the subcellular localization of GLUT4-EGFP was assessed by confocal fluorescent microscopy. (C) GLUT4 translocation was quantified by determining the number of cells displaying a continuous plasma membrane GLUT4-EGFP fluorescent signal from the counting of 50 cells per experiment. These data were obtained from the average of four independent experiments (200 cells total).

Figure 3. The amino terminal TC10 extension disrupts adipocyte cortical actin when targeted to lipid raft microdomains. (A) Differentiated 3T3L1 adipocyte nuclei were micro-injected with 200 μ g/ml HA-tagged TC10/WT (panels 1–3), TC10-79/H-Ras (panels 4–6), TC10-41/H-Ras (panels 7–9), and TC10-16/H-Ras (panels 10–12) cDNAs. Eighteen hours later, the cells were fixed and the localization of the expressed proteins was determined by confocal fluorescent microscopy using an FITC-labeled anti-HA antibody (panels 1, 4, 7, and 10). The cells were also colabeled with rhodamine-phalloidin to observe the distribution of filamentous actin (panels 2, 5, 8, and 11). The merged images are presented in panels 3, 6, 9, and 12. (B) Quantification of the number of cells displaying a disrupted cortical actin structure was determined from the counting of more than 50 cells per experiment were that expressed TC10/WT, TC10-79/H-Ras, TC10-41/H-Ras, TC10-16/H-Ras, and TC10-16/K-Ras proteins. These data were obtained from the average of four independent experiments (>200 cells total).

TC10/WT (Figure 3A, panels 1–3), there was a near complete disruption of the cortical network with little effect on peri-nuclear actin structure. This is in marked contrast to cells expressing H-Ras, which had little, if any effect on cortical actin labeling (our unpublished results). Similar to TC10/WT, adipocytes expressing TC10-79/H-Ras had a complete loss of cortical actin organization (Figure 3A, panels 4–6). Expression of TC10-41/H-Ras and TC10-16/H-Ras also resulted in a loss of cortical actin structure (Figure 3A, panels 7–12). Although the loss of cortical actin structure was readily apparent, TC10-41/H-Ras and TC10-16/H-Ras were somewhat less effective than TC10/WT and TC10-79/H-Ras (Figure 3B). In any case, expression of neither K-Ras nor TC10-16/K-Ras had any significant effect on cortical actin organization (our unpublished results). Quantification of the number of cells displaying a disruption of cortical actin is presented in Figure 3B. Cortical actin was disorganized in $51 \pm 0.5\%$, $51 \pm 1.7\%$, $31.8 \pm 3.3\%$, and $34.5 \pm 7.2\%$ of cells expressing TC10/WT, TC10-79/H-Ras, TC10-41/H-Ras, and TC10-16/H-Ras, respectively. In contrast cells expressing H-Ras, K-Ras, or TC10-16/K-Ras had $9.5 \pm 0.9\%$, $9.8 \pm 0.6\%$, and $9.8 \pm 1.0\%$ disrupted cortical actin, respectively. These data demonstrate that the amino terminal TC10 domain disrupts adipocyte cortical actin organization when targeted to lipid raft microdomains.

Amino Terminus Mutations Inactivate the Inhibitory Properties of TC10

Comparison of the amino terminal domains of all small GTP binding proteins that are predicted to undergo carboxyl terminal farnesylation and palmitoylation indicated that R-Ras1 has an amino terminal extension that exhibits some similarity with TC10 (Figure 1B). As observed for TC10, coexpression of R-Ras1 with GLUT4-EGFP resulted in an inhibition of insulin-stimulated GLUT4 translocation compared with empty vector-transfected cells (Figure 4, panels 1–4). As additional controls, R-Ras2 has an identical effector and carboxyl terminal targeting domains as R-Ras1 but with an unrelated amino terminal extension. Coexpression of R-Ras2 with GLUT4-EGFP had no significant effect on insulin-stimulated GLUT4 translocation (Figure 4, panels 5 and 6). Furthermore, replacement of the R-Ras1 carboxyl terminal targeting domain with the K-Ras carboxy terminus resulted in a chimera that was unable to inhibit insulin-stimulated GLUT4 translocation (Figure 4, panels 7 and 8). These data demonstrate that both the R-Ras1 and TC10 amino terminal extensions have similar functions when targeted to lipid raft microdomains.

TC10 and R-Ras both have a conserved GPG sequence located within their respective amino terminal extensions

Figure 4. Lipid raft microdomain targeting of R-Ras1 inhibits insulin-stimulated GLUT4 translocation. Differentiated 3T3L1 adipocytes were coelectroporated with 50 μ g of GLUT4-EGFP plus 200 μ g of the empty vector (panels 1 and 2), HA-tagged R-Ras1 (panels 3 and 4), R-Ras2 (panels 5 and 6), and R-Ras1/K-Ras (panels 7 and 8) cDNAs. Eighteen hours later, the cells were then treated without (Basal, panels 1, 3, 5, and 7) or with 100 nM insulin (Insulin, panels 2, 4, 6, and 8) for 30 min. The cells were then fixed and the subcellular localization of GLUT4-EGFP was assessed by confocal fluorescent microscopy. These are representative images of cells obtained from three to four independent determinations.

(Figure 1B). To evaluate the potential role of this sequence within TC10, we substituted the GPG sequence at position 12–14 with ASA (TC10/GPG-ASA). We also replaced the GAG sequence at residues 3–5 with ASA (TC10/GAG-ASA). Although expression of TC10/WT (Figure 5, panels 3 and 4) effectively inhibited insulin-stimulated GLUT4 translocation, expression of these mutants TC10/GPG-ASA and TC10/GAG-ASA were unable to block the insulin stimulation of GLUT4 translocation (Figure 5, panels 5–8). In parallel, TC10/WT (Figure 6, panels 1–3) disrupted cortical actin, whereas TC10/GPG-ASA and TC10/GAG-ASA had no significant effect (Figure 6, panels 4–9). The finding that inactivating point mutations occur at the very amino terminal (amino acids 1–3) and carboxyl end of this extension (amino acids 12–14) suggests that the entire structure (residues 1–16) of this extension is required.

To further examine the properties of the TC10 amino terminal domain, we deleted the amino terminal 16 amino acids of TC10 (Δ NT) in the context of the wild-type TC10 (Δ NT-TC10/WT), constitutively inactive GDP-bound TC10 mutant (Δ NT-TC10/T31N), and constitutively active GTP-bound TC10 mutant (Δ NT-TC10/Q75L). Because the carboxyl terminal posttranslational modification and targeting domain was unaffected in these amino terminal TC10 deletions, overexpression of these constructs resulted in an identical intracellular distribution as TC10/WT with localization to both the plasma membrane and the peri-nuclear endo-

membrane system (Figure 7A, panels 1–4). Coexpression of GLUT4-EGFP with TC10/WT resulted in the typical inhibition of insulin-stimulated GLUT4 translocation compared with control empty vector-transfected adipocytes (Figure 7B, panels 1–4). As expected, coexpression of Δ NT-TC10/WT and Δ NT-TC10/T31N had no significant effect on insulin-stimulated GLUT4 translocation (Figure 7B, panels 5–8). Surprisingly, however, coexpression of Δ NT-TC10/Q75L resulted in a marked inhibition of insulin-stimulated GLUT4 translocation (Figure 7B, panels 9 and 10). Quantification of the above results showed that insulin-stimulated GLUT4-EGFP translocation in $69 \pm 2.0\%$ in empty vector control-transfected cells, $71 \pm 2.1\%$ in of Δ NT-TC10/WT-transfected, and $68 \pm 2.9\%$ in Δ NT-TC10/T31N-transfected cells. In contrast, insulin-stimulated GLUT4 translocation was only $24 \pm 2.6\%$ and $21 \pm 1.5\%$ in cells expressing TC10/WT and Δ NT-TC10/Q75L (Figure 7C).

Analysis of cortical actin organization again demonstrated that the expression of TC10/WT resulted in the disruption of adipocyte cortical actin compared with the surrounding nonmicroinjected control cells (Figure 8, panels 1–3). Consistent with the lack of inhibition of insulin-stimulated GLUT4 translocation, expression of Δ NT-TC10/WT and Δ NT-TC10/T31N did not affect cortical actin organization (Figure 8, panels 4–9). It should be noted, however, that expression of Δ NT-TC10/T31N did appear to reduce the extent of perinuclear actin (Figure 8, panels 7–9). In any case,

Figure 5. Point mutations in the TC10 amino terminal extension neutralize the inhibitory effects on insulin-stimulated GLUT4 translocation. Differentiated 3T3L1 adipocytes were coelectroporated with 50 μ g of GLUT4-EGFP plus 200 μ g of the empty vector (panels 1 and 2), TC10/WT (panels 3 and 4), TC10/GPG-ASA (panels 5 and 6), or TC10/GAG-ASA (panels 7 and 8) cDNAs. Eighteen hours later, the cells were then treated without (Basal, panels 1, 3, 5, and 7) or with 100 nM insulin (Insulin, panels 2, 4, 6, and 8) for 30 min. The cells were then fixed and the subcellular localization of GLUT4-EGFP was assessed by confocal fluorescent microscopy. These are representative images of cells obtained from three to four independent determinations.

Figure 6. Point mutations in the TC10 amino terminal extension neutralize the inhibitory effects on cortical actin organization. Differentiated 3T3L1 adipocyte nuclei were microinjected with 200 μ g/ml HA-tagged TC10/WT (panels 1–3), TC10/GPG-ASA (panels 4–6), or TC10/GAG-ASA (panels 7–9) cDNAs. Eighteen hours later, the cells were fixed and the localization of the expressed proteins was determined by confocal fluorescent microscopy using a FITC-labeled anti-HA antibody (panels 1, 4, and 7). The cells were also colabeled with rhodamine-phalloidin to observe the distribution of filamentous actin (panels 2, 5, and 8). The merged images are presented in panels 3, 6, and 9. These are representative fields of cells observed in four independent experiments.

although expression of in Δ NT-TC10/Q75L inhibited insulin-stimulated GLUT4 translocation, this construct also did not appear to alter cortical actin organization (Figure 8, panels 10–12). Instead, this mutant markedly increased the amount of polymerized actin localized to the peri-nuclear endomembrane compartments. In any case, data further demonstrate that the amino terminus of TC10 is responsible for the disruption of cortical actin.

Because the visualization of actin structure was performed in microinjected cells whereas GLUT4 translocation was assessed in electroporated cells, we wanted to ensure that the effects observed were directly comparable by the same technique. We therefore microinjected 3T3L1 adipocytes with GLUT4-EGFP plus several of the germane TC10 constructs (Figure 9). As previously observed by electroporation, microinjection of TC10/WT resulted in a marked inhibition of insulin-stimulated GLUT4 translocation (Figure 9A, panels 1–4). In contrast, adipocytes microinjected with GLUT4-EGFP plus either Δ NT-TC10 or TC10/ASA had no significant effect on insulin-stimulated GLUT4 translocation (Figure 9A, panels 5–12). Quantification of these data are presented in Figure 9B. These data confirm that the disruption of cortical actin by the amino terminal domain of TC10 directly correlates with the loss of insulin-stimulated GLUT4 translocation in adipocytes.

The Amino Terminus of Mouse TC10 α in the Context of Mouse TC10 β Confers Inhibition of GLUT4 Translocation and Disruption of Cortical Actin

In addition to human TC10, we and others have identified three related mouse TC10 isoforms, termed TC10 α , TC10 β , and TC10 β -long and demonstrated that only expression of mouse TC10 α inhibited insulin-stimulated GLUT4 translocation and disrupted cortical actin organization (Chiang *et al.*, 2002). Mouse TC10 α is highly related to human TC10 and shares the identical amino terminal domain with the exception of an 8-amino acid extension in the human TC10 (Figure 1). Because both mouse TC10 α and TC10 β containing the same carboxyl terminal targeting sequences but primarily differ at the amino terminus, we next prepared a chimera replacing the mouse TC10 β amino termini with that of mouse TC10 α (Figure 1C). As expected, microinjection of TC10 α inhibited insulin-stimulated GLUT4 translocation, whereas expression of TC10 β had no significant effect (Figure 10A, panels 1–8). However, the TC10 α / β chimera was as effective as TC10 α in inhibiting insulin-stimulated GLUT4 translocation (Figure 10A, panels 9–12). These data are quantified and presented in Figure 10B. Thus, these data further demonstrate that the mouse TC10 α amino terminal domain when overexpressed in the context of heterologous proteins containing the appropriate targeting domains (H-Ras and TC10 β) functions as a potent inhibitor of insulin-stimulated GLUT4 translocation.

DISCUSSION

We have recently observed that insulin can stimulate a signaling pathway leading to the activation of TC10 α in adipocytes (Baumann *et al.*, 2000; Chiang *et al.*, 2001). This apparently results from the insulin-dependent tyrosine phosphorylation of the Cbl proto-oncogene product and its recruitment to a lipid raft plasma membrane microdomain via the adapter proteins APS and CAP (Ribon *et al.*, 1998; Ahmed *et al.*, 2000; Baumann *et al.*, 2000). Importantly, overexpression of TC10 α was found to specifically inhibit insulin-stimulated GLUT4 translocation. The inhibitory function of TC10 occurred not only when an inactive constitutively GDP-bound mutant form of TC10 was expressed but also for an active constitutively GTP-bound mutant and more surprisingly when the wild-type TC10 was expressed. Furthermore, expression of mouse TC10 β had no significant effect on insulin-stimulated GLUT4 translocation despite sharing an identical effector and GTP binding domain with mouse TC10 α (Chiang *et al.*, 2002). These data suggest that a structural domain independent of GTP or effector binding was responsible for this inhibition of insulin signaling. Moreover, the ability of TC10 to inhibit insulin-stimulated GLUT4 translocation was also dependent on the intracellular targeting information provided by posttranslational modification of the carboxy terminus (Watson *et al.*, 2001).

A specific role for the carboxyl-terminal domains of the small GTP binding proteins of the Ras family in directing different intracellular trafficking routes and plasma membrane subdomain compartmentalization have been well established (Choy *et al.*, 1999; Roy *et al.*, 1999; Apolloni *et al.*, 2000; Michaelson *et al.*, 2001). For example, farnesylation and

Figure 7. Effects of TC10 amino terminal deletions on GLUT4 translocation. (A) Differentiated 3T3L1 adipocytes were electroporated with 50 μ g of the HA epitope-tagged TC10/WT (panel 1), HA epitope-tagged Δ NT-TC10/WT (panel 2), Myc-tagged Δ NT-TC10/Q75L (panel 3), and Myc-tagged Δ NT-TC10/T31N (panel 4) cDNAs as described in MATERIALS AND METHODS. Eighteen hours later, the cells were fixed and the subcellular localization was determined by confocal fluorescent microscopy. (B) Differentiated 3T3L1 adipocytes were coelectroporated with 50 μ g of GLUT4-EGFP plus 200 μ g of the empty vector (panels 1 and 2), TC10/WT (panels 3 and 4), Δ NT-TC10/WT (panels 5 and 6), Δ NT-TC10/T31N (panels 7 and 8), and Δ NT-TC10/Q75L (panels 9 and 10) cDNAs. Eighteen hours later, the cells were then treated without (Basal, panels 1, 3, 5, 7, and 9) or with 100 nM insulin (Insulin, panels 2, 4, 6, 8, and 10) for 30 min. The cells were then fixed and the subcellular localization of GLUT4-EGFP was assessed by confocal fluorescent microscopy. These are representative images of cells obtained from three to four independent determinations. (C) GLUT4 translocation was quantified by determining the number of cells displaying a continuous plasma membrane GLUT4-EGFP fluorescent signal from the counting of 50 cells per experiment. These data were obtained from the average of four independent experiments (200 cells total).

palmitoylation of the H-Ras carboxyl terminal CxCxxCaax sequence results in the biosynthetic trafficking and localization of H-Ras to caveolin-enriched plasma membrane microdomains (Dupree *et al.*, 1993; Li *et al.*, 1996; Song *et al.*, 1996). In contrast, the K-Ras carboxyl terminal sequence KKKKKKxxCaax is only subjected to farnesylation and is excluded from the secretory membrane system, resulting in its localization to nonlipid raft domains of the plasma membrane (Hancock *et al.*, 1990; Choy *et al.*, 1999; Apolloni *et al.*, 2000). Using these trafficking domains, we previously demonstrated that the inhibitory function of TC10 only occurred when localized to lipid raft microdomains but not to non-

lipid raft domains of the plasma membrane (Watson *et al.*, 2001).

On the basis of this information, we speculated that unusual amino terminal extension of TC10 α , when coupled with a lipid raft targeting carboxyl terminal domain, was responsible for the inhibition of insulin-stimulated GLUT4 translocation. We specifically addressed this hypothesis by expressing chimera proteins containing the amino terminal domain of TC10 fused to full-length H-Ras and K-Ras. The amino terminal TC10-16/H-Ras but not the TC10-16/K-Ras fusion protein inhibited insulin-stimulated GLUT4 translocation. In addition, TC10 β had no effect on insulin-

Figure 8. Effects of TC10 amino terminal deletions on adipocyte cortical actin organization. Differentiated 3T3L1 adipocyte nuclei were microinjected with 200 $\mu\text{g}/\text{ml}$ HA-tagged TC10/WT (panels 1–3), HA-tagged $\Delta\text{NT-TC10/WT}$ (panels 4–6), Myc-tagged $\Delta\text{NT-TC10/T31N}$ (panels 7–9), and Myc-tagged $\Delta\text{NT-TC10/Q75L}$ (panels 10–12) cDNAs. Eighteen hours later, the cells were fixed and the localization of the expressed proteins was determined by confocal fluorescent microscopy using a FITC-labeled anti-HA or anti-Myc antibody (panels 1, 4, 7, and 10). The cells were also colabeled with rhodamine-phalloidin to observe the distribution of filamentous actin (panels 2, 5, 8, and 11). The merged images are presented in panels 3, 6, 9, and 12. These are representative fields of cells observed in four independent experiments.

stimulated GLUT4 translocation, whereas substitution of the TC10 α amino termini on TC10 β resulted in a marked inhibition of GLUT4 translocation. Similarly, R-Ras1 also contains a related amino terminal extension but has a completely distinct effector domain. Nevertheless, expression of R-Ras1 also inhibited insulin-stimulated GLUT4 translocation, whereas R-Ras2, which has an unrelated amino terminus, was unable to alter insulin action. Furthermore, TC10 inhibitory function was completely abrogated by selective point mutations within the amino terminal extension and deletion of this domain in the context of TC10/WT or TC10/T31N. These data clearly demonstrate that the amino terminal domain provides an inhibitory function but only when localized to lipid raft microdomains by the appropriate carboxyl terminal targeting motif.

Rho-family GTPases play critical roles in cytoskeleton organization, proliferation, differentiation, and apoptosis (Neudauer *et al.*, 1998). Although the members of this family, such as RhoA, Cdc42, and Rac share many regulators and effectors, they apparently produce different phenotypes when expressed as gain-of-function mutants in cells (Neudauer *et al.*, 1998). It has been recently documented that insulin stimulates dynamic actin remodeling at both the inner surface of the plasma membrane and in the perinuclear region that is sensitive to *Clostridium difficile* toxin B, a Rho family-specific toxin (Kanzaki and Pessin, 2001; Kanzaki *et al.*, 2001). In parallel, TC10 overexpression has been observed to disrupt adipocyte cortical actin and inhibition of dynamic actin remodeling in vivo prevents insulin-stimulated GLUT4 translocation (Kanzaki *et al.*, 2001). Similarly, TC10 amino terminal/H-Ras chimeras that inhibited insulin-stimulated GLUT4 translocation also resulted in a disruption of adipocyte cortical actin. Furthermore, mutations that prevent the inhibition of insulin-stimulated GLUT4

translocation had no effect on cortical actin integrity. As with the case of GLUT4 translocation, only those TC10 amino termini containing constructs that were targeted to lipid raft microdomains disrupted cortical actin. In this regard, recent studies have observed that caveolin forms large organized caveolae/lipid raft structures in the plasma membrane (Brown and London, 1998; Fujimoto *et al.*, 1998; Baumann *et al.*, 2000; Watson *et al.*, 2001; Kanzaki and Pessin, 2002; Parton *et al.*, 2002). These large organized caveolin rosette structures appear to be the sites of cortical actin anchoring and are consistent with only lipid raft targeted TC10 displaying cortical actin-depolymerizing properties.

To further gain insight into the function of the TC10 amino terminus to disrupt cortical actin and inhibit insulin-stimulated GLUT4 translocation, we examined the properties of amino terminal deletion mutants. As expected, expression of $\Delta\text{NT-TC10/WT}$ and $\Delta\text{NT-TC10/T31N}$ lost their ability to inhibit insulin-stimulated GLUT4 translocation. In parallel these mutants were unable to disrupt cortical actin. Surprisingly however, expression of $\Delta\text{NT-TC10/Q75L}$ appeared to uncouple cortical actin from GLUT4 translocation. That is, expression of $\Delta\text{NT-TC10/Q75L}$ retained the ability to inhibit insulin-stimulated GLUT4 translocation but had no effect on cortical actin structure. There are several possible explanations for this observation. First, although cortical actin structure may remain intact, cortical actin remodeling may be prevented by expression of $\Delta\text{NT-TC10/Q75L}$. It is also possible that inhibitory effect of TC10/Q75L may be unrelated to changes in cortical actin and reflects a requirement for TC10 GTP/GDP cycling. In this regard, the lack of effect of $\Delta\text{NT-TC10/T31N}$ may be that this mutant does not function as a dominant-interfering mutant. Alternatively, the inhibitory action of $\Delta\text{NT-TC10/Q75L}$ could be related to the large increase in peri-nuclear actin polymerization because this is

Figure 9. Examination of GLUT4 translocation by microinjection of TC10 mutants. (A) Differentiated 3T3L1 adipocyte nuclei were microinjected with GLUT4-EGFP plus HA-tagged TC10/WT (panels 1–4), Δ NT-TC10 (panels 5–8), or TC10/GAG-ASA (panels 9–12) cDNAs. Eighteen hours later, the cells were either left untreated or incubated with 100 nM insulin for 30 min. The cells were then fixed and the subcellular localization of GLUT4-EGFP (panels 1, 2, 5, 6, 9, and 10) and HA-TC10 (panels 3, 4, 7, 8, 11, and 12) was assessed by confocal fluorescent microscopy. These are representative images of cells obtained from three to four independent determinations. (B) GLUT4 translocation was quantified by determining the number of cells displaying a continuous plasma membrane GLUT4-EGFP fluorescent signal from the counting of 30–40 cells per experiment. These data were obtained from the average of three to four independent experiments (90–120 cells total).

the apparent storage site for the insulin-responsive GLUT4 storage compartment. Clearly, further studies are necessary to resolve these issues and to further elucidate the function of the endogenous TC10 protein and the mechanisms by which the amino terminal domain inhibits cortical F-actin.

In any case, we have previously postulated that TC10 plays an important regulatory role in the insulin signaling pathway involved in GLUT4 translocation (Chiang *et al.*, 2001). This was based on the ability of insulin to activate TC10 and that expression of upstream TC10 regulators had dramatic effects on insulin-stimulated GLUT4 translocation. Furthermore, overexpression of TC10 was found to inhibit insulin-stimulated GLUT4 translocation. Based on our current data, the TC10 inhibitory function is a direct structural property of its unusual amino terminal extension and is apparently independent of GTP binding or effector function. One interpretation of these data is that TC10 is not directly

involved in an insulin-signaling pathway regulating GLUT4 translocation but reflects the requirement for cortical actin. Alternatively, it also remains possible that TC10 has both positive and negative regulator functions that balance the rate of actin polymerization/depolymerization. However, when overexpressed and targeted to lipid raft domains, the negative activity (disruption of cortical actin) becomes predominant, thereby masking the function of the endogenous TC10. Further studies will be necessary to resolve these issues and to determine the specific role that TC10 may be playing in insulin signaling.

In summary, we have identified an unusual amino terminal extension present in TC10 and R-Ras1 that interacts with a component(s) necessary for the maintenance of adipocyte cortical actin structure. This domain functions in a dominant-negative manner disrupting cortical actin but only when targeted to lipid raft microdomains of the plasma

Figure 10. Comparison of mouse TC10 α and TC10 β on insulin-stimulated GLUT4 translocation. (A) Differentiated 3T3L1 adipocyte nuclei were microinjected with GLUT4-EGFP plus HA-tagged mouse TC10 α (panels 1–4), TC10 β (panels 5–8), or TC10 α/β (panels 9–12) cDNAs. Eighteen hours later, the cells were either left untreated or incubated with 100 nM insulin for 30 min. The cells were then fixed and the subcellular localization of GLUT4-EGFP (panels 1, 2, 5, 6, 9, and 10) and HA-TC10 (panels 3, 4, 7, 8, 11, and 12) was assessed by confocal fluorescent microscopy. These are representative images of cells obtained from three to four independent determinations. (B) GLUT4 translocation was quantified by determining the number of cells displaying a continuous plasma membrane GLUT4-EGFP fluorescent signal from the counting of 30–40 cells per experiment. These data were obtained from the average of three to four independent experiments (90–120 cells total).

membrane. The targeting of this motif corresponds to precise localization of the F-actin attachment sites, termed Cavactin. Importantly, the ability of overexpressed TC10/WT and TC10/T31N to inhibit insulin-stimulated GLUT4 translocation appears to be independent of GTP binding and effector domain function but is dependent on the disruption of cortical actin. In contrast, overexpression of a constitutively active TC10 mutant (TC10/Q75L) inhibited of insulin-stimulated GLUT4 translocation and enhanced peri-nuclear actin polymerization but without any significant affect on cortical actin. This latter finding directly demonstrates that the TC10 activation state has dramatic effects on actin polymerization and insulin-stimulated GLUT4 translocation.

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