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Skeletal Muscle Cells and Adipocytes Differ in Their Reliance on TC10 and Rac for Insulin-Induced Actin Remodeling

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Insulin causes distinct cortical actin remodeling in muscle and fat cells, and interfering with actin dynamics halts glucose transporter 4 (GLUT4) translocation to the membrane. Phosphatidylinositol 3-kinase (PI3-K) and the small G protein Rac govern myocyte actin remodeling, whereas TC10α contributes to adipocyte actin dynamics downstream of Cbl-associated protein (CAP) and Cbl, independently of PI3-K. Given the importance of insulin action in both cell types, it is paramount to determine whether signaling pathways and actin manifestations are cell type specific. We found CAP expression and insulin-mediated Cbl phosphorylation in differentiated myotubes but not in myoblasts. Unlike adipocytes, Cbl is phosphorylated on Y774 and Y731 in myotubes. TC10α and β-transcripts are amplified by RT-PCR in muscle cells, but the endogenous proteins are barely detectable using two unrelated antibodies. TC10α transfected into myoblasts is activated by insulin despite the lack of CAP expression and Cbl phosphorylation. Moreover, dominant-negative TC10α mutants do not prevent insulin-induced actin remodeling in either myoblasts or myotubes and do not interfere with insulin-mediated recruitment of c-*myc* epitope-tagged GLUT4 to the cell surface. In contrast to TC10α, endogenous Rac is readily detectable in both muscle cells and adipocytes and binds GTP after insulin in a PI3-K-dependent manner. These data suggest that whereas individual components of the CAP to TC10 pathway are regulated by insulin, a functional TC10-dependent signaling pathway leading to actin remodeling and GLUT4 translocation may not operate in myocytes, as it does in adipocytes. (*Molecular Endocrinology* 18: 359–372, 2004)

EMERGING EVIDENCE LINKS the actin cytoskeleton to intracellular traffic and the control of signal transduction. In particular, actin filaments participate in insulin signaling leading to glucose uptake in muscle and adipocyte cell lines. Thus, in both cell types actin filament-disrupting agents reduce insulin-stimulated glucose uptake and its underlying mechanism, glucose transporter translocation to the cell membrane (1–6). Actin remodeling occurs in response to insulin in both muscle and fat cells, albeit with different morphological manifestations. In L6 rat skeletal muscle cells, insulin causes actin reorganization into a subcortical criss-crossed mesh that promotes dorsal membrane protrusions resembling lamellipodia (3, 7). In contrast, in 3T3-L1 mouse adipocytes, actin bundles surround caveolae, membrane invaginations abundant in this cell type (8). Insulin increases actin polymerization within a subcortical band enclosing the cell as well as the dynamic organization of an actin pool in a perinuclear region (9). These different actin manifestations in the two cell types have been attributed to the activation of distinct members of the Rho family of GTPases: Rac in muscle cells (10) and TC10α in adipocytes (9, 11). Indeed, a dominant-negative mutant of Rac prevented actin remodeling and glucose transporter 4 (GLUT4) translocation in L6 muscle cells (10), whereas dominant-negative TC10α prevented actin dynamics (9, 11) and GLUT4 translocation (12) in 3T3-L1 adipocytes.

In both muscle and fat cells, GLUT4 translocation from intracellular membrane compartments to the plasma membrane requires the activation of phosphatidylinositol 3-kinase (PI3-K) downstream of the insulin receptor (13–17), which phosphorylates phosphatidylinositol-4,5-bisphosphate to generate phosphatidylinositol-3,4,5-trisphosphate. The latter lipid recruits phosphoinositide-dependent kinase to the plasma membrane, as well as its downstream substrates Akt/protein kinase B and the atypical protein kinase Cs λ and ζ. In muscle cells, actin remodeling depends on

Abbreviations: CA, Constitutionally active; CAP, Cbl-associated protein; CRIB, Cdc 42/Rac interactive binding; DN, dominant negative; GLUT4, glucose transporter 4; GLUT4myc, c-myc epitope-tagged GLUT4; GST, glutathione-*S*-transferase; HA, hemagglutinin; PI3-K, phosphatidylinositol 3-kinase; WT, wild type.

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PI3-K activation (2) and specifically on its primary product, phosphatidylinositol-3,4,5-trisphosphate (7). These insulin-induced actin structures locally concentrate PI3-K subunits p85α and p110α, as well as Akt and GLUT4-containing endo-membranes (7, 10), all of which are required for GLUT4 translocation in response to insulin. In contrast, in adipose cells, insulin-induced cortical actin remodeling implicated in GLUT4 translocation is independent of PI3-K activity, relying instead on a newly characterized signaling pathway. In these cells, insulin induces Cbl phosphorylation on tyrosine residues, resulting in its migration to caveolae via an adapter protein, CAP (Cbl-associated protein) (18–20). In such plasma membrane microdomains, Cbl binds a series of adaptor proteins including APS (adapter protein with a Pleckstrin homology and Src homology domain) (21), resulting in Cbl phosphorylation (22) and formation of a multiprotein complex that includes CrkII and the guanidine exchange factor C3G. This complex assembly ultimately results in the activation of TC10α (12, 23), which in turn governs actin remodeling that is required for GLUT4 translocation. Thus, it was proposed that the CAP to TC10 pathway acts in parallel to the PI3-K axis to induce GLUT4 translocation (12). How TC10-induced actin dynamics then promote GLUT4 translocation is not clear. Yet, in a semi-*in vitro* system, TC10α contributes to comet tail formation on GLUT4 vesicles (9, 24) via neuronal Wiskott-Aldrich syndrome protein (11). In intact adipocytes, dominant negative TC10α reduces the insulin-induced translocation of GLUT4 in parallel to inhibition of cortical actin dynamics (5).

The parallels and contrasts of the results obtained with muscle and fat cells have prompted us to test whether these are cell type-dependent idiosyncratic phenomena, or whether the CAP to TC10 pathway also operates in muscle cells. Similarly, it became important to explore whether insulin activates endogenous Rac in either cell type. Therefore, in this study we first examine the expression of elements of the CAP to TC10 pathway and whether insulin causes Cbl phosphorylation in muscle cells. We then compare Rac and TC10α activation in muscle and fat cells. We further explore whether PI3-K regulates the activation of such G proteins and how dominant negative mutant versions of TC10α affect actin remodeling and GLUT4 translocation in muscle cells. It is expected that these results will bring us closer to a more complete understanding of the particular role of small G proteins in insulin action and of the cell type-specific nature of the CAP to TC10 pathway.

RESULTS

Expression of CAP, Cbl, TC10, and Rac

Whereas Cbl is expressed in various cell types (25), CAP and TC10 expression patterns are more restricted and change during the course of cellular differentiation (26, 27). To begin to compare the role of the CAP to TC10 pathway in insulin signaling in adipose *vs.* skeletal muscle-derived cells, we first determined the expression level of CAP, Cbl, and TC10 in L6 myoblasts (d 2 cultures) and multinucleated L6 myotubes (d 5–7 cultures). Total cell lysates from L6 myoblasts and myotubes that stably express GLUT4myc (c-*myc* epitope-tagged GLUT4) were prepared and resolved by SDS-PAGE. As shown in Fig. 1A, CAP was detectable only in differentiated myotubes. In contrast, Cbl was expressed at similar levels in myoblasts and myotubes (Fig. 1A). A differentiation-dependent expression pattern of CAP was also observed in 3T3-L1 adipocytes, where increased expression of CAP was associated with differentiation from fibroblasts (preadipocytes) to adipocytes (28). We next determined the protein expression of TC10 in L6 muscle cells using two different polyclonal antibodies (Ab:A and Ab:B) raised against distinct TC10-derived peptides. Both antigenic peptides are identical in mouse and rat TC10 orthologs, allowing the detection of TC10 in cells from mouse or rat origin with the same efficiency (as detailed in *Materials and Methods*). Figure 1B shows immunoblot analysis of TC10 expression in L6 myoblasts and L6 myotubes using these two antibodies. When rodent wild-type (WT)-TC10α was overexpressed in myoblasts, a prominent band of approximately 25 kDa was readily observed with both antibodies (Fig. 1B, lane 1). Furthermore, both antibodies detected prominent bands in lysates of myoblasts overexpressing rodent HA-TC10α, migrating at 30 kDa (Fig. 1B, lane 2). However, endogenous levels of TC10 in nontransfected cells were difficult to discern in either myoblasts or myotubes with either antibody (Fig. 1B, lanes 3 and 4). In contrast, lysates from 3T3-L1 adipocytes showed a band that potentially represents endogenous TC10, when using Ab:B, which detects both TC10α and TC10β (Fig. 1B, lane 5). Using this antibody and serial dilutions of the 3T3-L1 lysates, we estimated that TC10 protein content in myotube lysates is at least 10-fold lower than in adipocytes. This was also reflected in immunoblotting for TC10 from total membrane fractions (data not shown). The nearly undetectable endogenous TC10 protein levels in L6 muscle cells prompted us to verify that TC10 is transcribed in these cells. TC10 mRNA has been detected in various tissues including muscle, heart, kidney, brain, and uterus (27), as well as in various cell lines including human 293 embryonic kidney, mouse NIH 3T3, 3T3-L1, and C2C12 myocytes, and rat PC12 and N1E-115 neuroblastoma (27, 29–32). TC10 transcripts of 2.3, 3.4, and 4.4 kb were detected in various cell types. Figure 1C shows that, similarly, L6 myoblasts and myotubes express both TC10α and TC10β transcripts as detected by RT-PCR. These data suggest that the TC10α and β genes are transcribed in muscle cells, but that the endogenous protein levels of both isoforms are markedly lower than in 3T3-L1 adipocytes. To facilitate analysis of whether TC10 can be activated by insulin in muscle

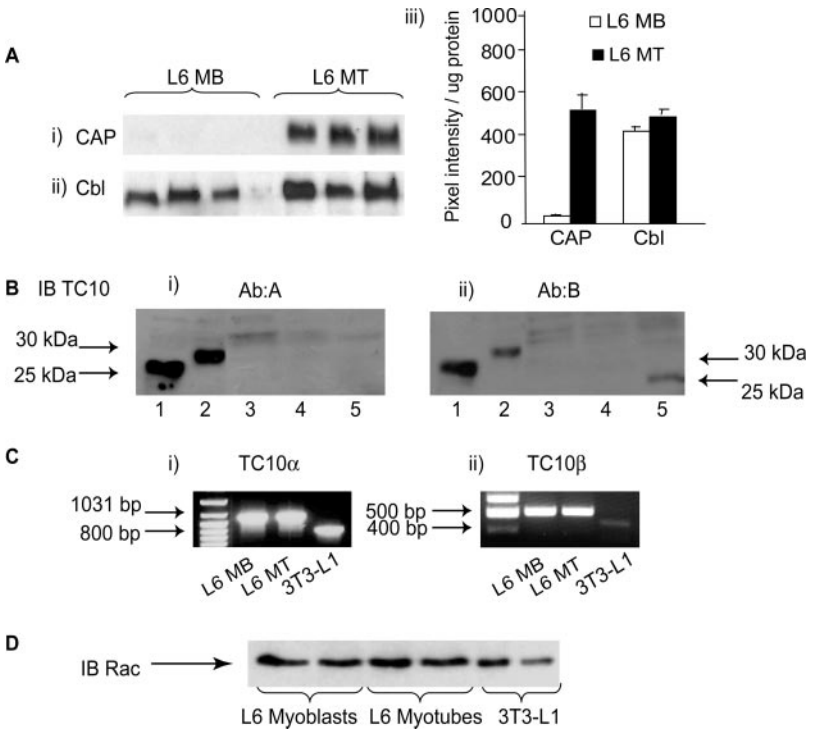

Fig. 1. Protein Expression of CAP, Cbl, TC10, and Rac

A, Representative triplicate sample immunoblot analysis of endogenous CAP (i) and Cbl (ii) protein in total cell lysates prepared from L6 myoblasts (MB) and differentiated myotubes (MT). Four to five independent experiments were quantified as described in *Materials and Methods*. The pixel intensity to μg protein ratio is presented as the mean ± SE. B, TC10 protein expression from total cell lysates of L6 myoblasts, L6 myotubes, and 3T3-L1 adipocytes using two different anti-TC10 antibodies (see *Materials and Methods*). Total cell lysates (40 μg) were prepared from L6 myoblasts overexpressing untagged (lane 1) and HA-tagged TC10 (lane 2). Lanes 3–5 represent 80 μg of total cell lysates from L6 myoblasts, L6 myotubes, and 3T3-L1 adipocytes, respectively. C, RT-PCR amplification of TC10α (i) and TC10β (ii) from L6 MB, MT, and 3T3-L1 adipocytes. The primers were designed according to specific species sequences to amplify a 1100- and 855-bp fragment of TC10α from muscle and adipocytes, respectively, or 488- and 425-bp fragments of TC10β from muscle and adipocytes, respectively. D, Endogenous Rac expression in 40 μg total cell lysates from L6 myoblasts, L6 myotubes, and 3T3-L1 adipocytes. Blots shown in panels B–D are representative of two additional experiments.

cells, subsequent studies were conducted with myoblasts overexpressing epitope-tagged TC10α, the same experimental approach used to characterize the CAP to TC10 pathway in 3T3-L1 adipocytes (12). Furthermore, although in adipocytes both TC10α and β have been shown to be activated by insulin and dependent on CAP, only TC10α significantly participated in insulin-mediated actin remodeling and GLUT4 translocation (31). Therefore, cells were transfected with only TC10α, and will be referred to as TC10 from here on.

In contrast to TC10 expression, Rac was readily detected as a 22-kDa protein in lysates from L6 myoblasts, L6 myotubes, as well as 3T3-L1 adipocytes (Fig. 1D). The Rac protein expression level remained unchanged during muscle cell differentiation.

Cbl Phosphorylation by Insulin

We next assessed whether insulin stimulates tyrosine phosphorylation of Cbl in muscle cells as it does in 3T3-L1 adipocytes (18, 28). Cbl was immunoprecipitated from L6 myoblasts and L6 myotubes with polyclonal anti-Cbl antibodies conjugated to Sepharose beads and assayed for phosphorylation using antiphosphotyrosine antibodies (Fig. 2). In L6 myoblasts, insulin did not stimulate tyrosine phosphorylation of Cbl, whereas differentiated L6 myotubes exhibited a 2- to 2.5-fold ($P < 0.01$) increase in its phosphotyrosine content by 3 min of insulin stimulation (Fig. 2D). Although readily observed, this degree of tyrosine phosphorylation of Cbl in myotubes was significantly lower than that in 3T3-L1 adipocytes, where a 3.5- to 4.5-fold ($P < 0.01$) increase was detected (Fig. 2E). Immunoblotting of the same membranes for total Cbl was used to verify equal loading of the gels (Fig. 2, A–C, *lower panels*). These results suggest that in muscle cells Cbl is tyrosine phosphorylated only after differentiation into myotubes, correlating with the increase in CAP expression (Fig. 1A).

Cbl can be phosphorylated on several distinct tyrosine residues, each leading to the recruitment of specific Cbl effector proteins (33). To further compare

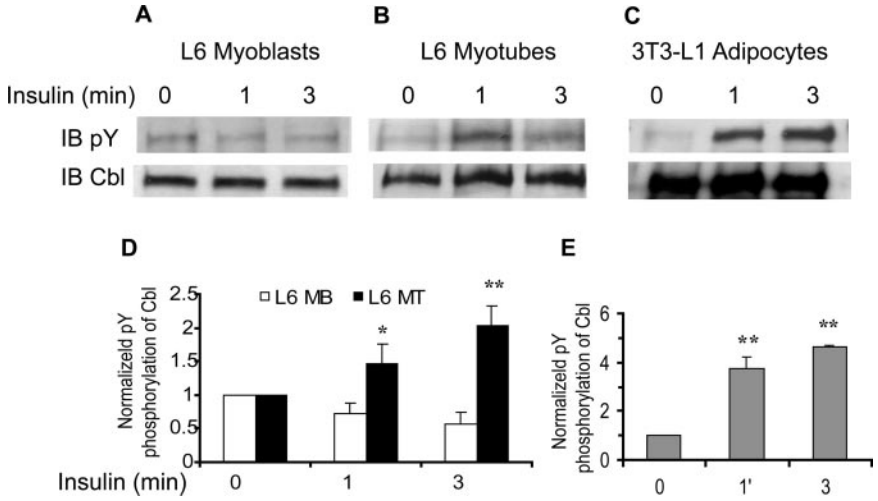

Fig. 2. Insulin-Induced Tyrosine Phosphorylation of Cbl

Cbl was immunoprecipitated from 500 μg total cell lysate protein prepared from L6 myoblasts (A), L6 myotubes (B), or 3T3-L1 adipocytes (C) and immunoblotted for phosphotyrosine (pY). The same membranes were stripped and reblotted for Cbl. Insulin stimulated tyrosine phosphorylation of Cbl in L6 myoblasts (MB), L6 myotubes (MT) (D), or 3T3-L1 adipocytes (E). Pixel intensity was normalized to total Cbl, and a value of 1 was assigned to the basal state. Results of four to six independent experiments are presented as the mean ± SE. *, $P < 0.05$; and **, $P < 0.01$ compared with basal state.

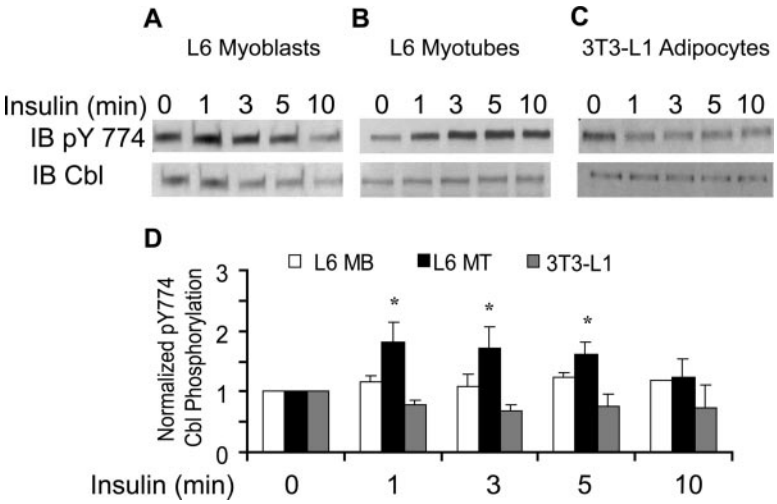

Fig. 3. Insulin-Stimulated Cbl Phosphorylation on Y774

Representative immunoblots of Cbl phosphorylation on Y774 were determined using 40 μg total cell lysates prepared from L6 myoblasts (A), L6 myotubes (B), and 3T3-L1 adipocytes (C). The same membranes were stripped and reblotted for Cbl. D, Pixel intensity was normalized to total Cbl, and a value of 1 was assigned to the basal state. Results of four to five independent experiments are presented as the mean ± SE. *, $P < 0.05$ compared with the basal state, as determined using Student's *t* test assuming unequal variance.

insulin-induced Cbl phosphorylation in L6 muscle cells and 3T3-L1 adipocytes, we made use of specific antibodies raised against phosphorylated tyrosine residues Y774 and Y731 on Cbl, serving as binding sites for CrkII and the p85 regulatory subunit of PI 3-K, respectively. Insulin induced a 2-fold increase in phosphorylation of Cbl on tyrosine 774 in L6 myotubes (Fig. 3, B and D). As expected from the data obtained with antiphosphotyrosine antibodies (Fig. 2), insulin did not stimulate phosphorylation of this tyrosine residue in L6 myoblasts. Surprisingly, however, insulin did not induce Cbl phosphorylation on tyrosine 774 in 3T3-L1 adipocytes (Fig. 3, C and D), suggesting that the reported Cbl-CrkII interaction induced in these cells by insulin (12) occurs through the phosphorylation of an alternative CrkII binding site (25). Similar to the insulin-induced tyrosine phosphorylation of residue 744, phosphorylation on tyrosine 731 was observed in L6 myotubes (2- to 3-fold increase by 1 min) (Fig. 4, B and D) but not in L6 myoblasts or 3T3-L1 adipocytes.

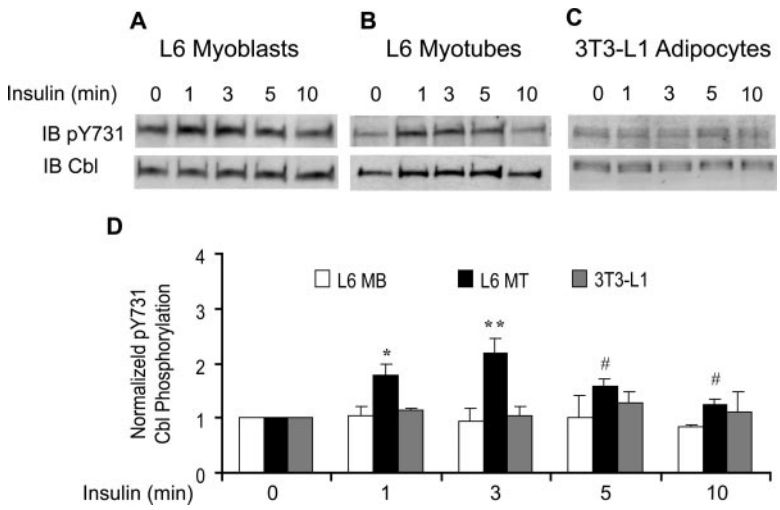

Fig. 4. Insulin-Stimulated Cbl Phosphorylation on Y731

Representative immunoblots of Cbl phosphorylation on Y731 were determined using 40 μg total cell prepared lysates from L6 myoblasts (A), L6 myotubes (B), and 3T3-L1 adipocytes (C). The same membranes were stripped and reblotted for Cbl. D, Pixel intensity was normalized to total Cbl, and a value of 1 was assigned to the basal state. Results of three to five independent experiments are presented as the mean ± SE. *, $P < 0.05$; and **, $P < 0.01$ compared with basal state using ANOVA. #, $P < 0.05$ compared with the basal state, as determined using Student's *t* test assuming unequal variance.

Hence, regardless of the identity of the Cbl sites phosphorylated in response to insulin in 3T3-L1 adipocytes, they are different from those involved in the insulin response in muscle cells.

Rac and TC10 Activation by Insulin

Chiang *et al.* (12) have shown activation of TC10 downstream of CAP and Cbl within 5 min of insulin stimulation in 3T3-L1 adipocytes transfected with plasmids encoding a tagged version of this small GTPase. The extent of GTP loading of TC10 after insulin stimulation was not altered by wortmannin. Interestingly, in those experiments endogenous TC10 activity was never measured, nor was the effect of wortmannin on basal levels of activated (GTP-bound) TC10. To assess whether insulin-stimulated activation of TC10 could similarly occur in muscle cells, L6 myoblasts were transfected with a plasmid encoding hemagglutinin (HA)-tagged WT TC10. Upon GST-CRIB (glutathione-*S*-transferase-Cdc 42/Rac interactive binding) pull down, immunoblotting for the HA-epitope allowed for the assessment of TC10 activation. Negative and positive controls were obtained by treating nonstimulated lysates with excess GDPβ and GTPγS, respectively. After 1 min, insulin caused a 3-fold increase in the amount of GTP-bound (activated) HA-TC10 in myoblasts. The level of activation of TC10 remained stable for 10 min after insulin stimulation (Fig. 5A). To determine whether TC10 activation in muscle cells was PI3-K dependent, L6 myoblasts transfected with HA-TC10 were treated with 100 nM wortmannin for 20 min before and during insulin stimulation. The level of insulin-stimulated TC10 activation was not affected by wortmannin (Fig. 5B), similar to findings in adipocytes.

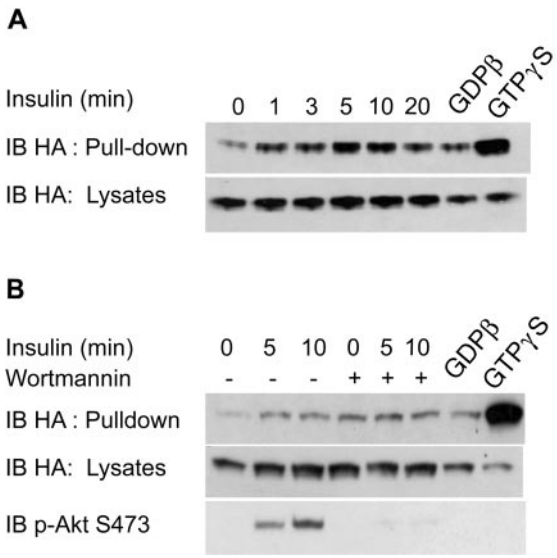

Fig. 5. TC10 Activation by Insulin

Representative immunoblots of L6 myoblasts transiently expressing HA-tagged TC10α were treated with insulin at the indicated time points and subjected to GST-CRIB pull-down assay. Total cell lysates prepared from each condition were immunoblotted for HA-epitope to confirm level of expression. A, Insulin-stimulated activation of TC10α. B, Cells were pretreated with or without 100 nM wortmannin for 20 min, followed by 5 and 10 min stimulation with 100 nM insulin. Controls with nonhydrolysable GDP or GTP are shown as indicated. Phospho-Akt immunoblots are shown as an index of insulin and wortmannin action.

This was further confirmed by using both anti-TC10 antibodies (Ab:A and Ab:B) instead of the anti-HA to detect activated TC10 in the pull-down assay (data not

shown). Effective inhibition of PI3-K by wortmannin was confirmed by demonstrating the nearly complete prevention of insulin-stimulated phosphorylation of Akt in the same cell lysates used for the pull-down assay (Fig. 5). Surprisingly, however, inhibition of PI3-K enhanced the basal level of activated TC10 above which there was no further stimulation by insulin. This suggests that there is a tonic inhibitory input by PI3-K on the TC10 signaling pathway in nonstimulated myoblasts. Furthermore, when overexpressed in myoblasts, TC10 can be activated by insulin through a signaling mechanism that does not involve CAP or Cbl.

We next assessed whether insulin stimulation results in the activation of endogenous Rac in adipocytes and muscle cells using the GST-CRIB pull-down assay as described in *Materials and Methods*. It is worth stressing that the detection of Rac activation required a different concentration of Mg^{2+} in the pull-down assay, compared with the concentration used to measure TC10 activation. In contrast to TC10, endogenous levels of Rac were readily detectable in both muscle and adipose cells (Fig. 1). Accordingly, activation assays of Rac were done with untransfected cells. Using anti-Rac antibodies to identify pulled-down activated (GTP-bound) Rac, insulin stimulation of this GTPase was observed within 5 min and was sustained for 10 min both in L6 myoblasts and L6 myotubes (Fig. 6, A–D). Insulin-induced Rac activation at 5 and 10 min was prevented by a 20-min pretreatment with 100 nM wortmannin, an effect that was more apparent at the 10-min time point. Importantly, wortmannin pretreatment did not affect the basal level of Rac activation. Effective inhibition of PI3-K was confirmed, as before, by immunoblotting the same lysates for phosphorylated Akt. Similar results were also obtained using LY 294002, a structurally unrelated inhibitor of PI3-K (data not shown). Rac activation was also assessed in 3T3-L1 adipocytes and interestingly, insulin induced endogenous Rac activation with a similar time course as in muscle cells. Rac activation in 3T3-L1 adipocytes was also PI3-K dependent, and this effect was also more apparent at the 10-min time point (Fig. 6, E and F). Thus, Rac activation in response to insulin was seen in L6 muscle cells and 3T3-L1 adipocytes, and in both cell types this process was PI3-K dependent.

TC10 Is Not Involved in Insulin-Induced Actin Remodeling in Muscle Cells

The small GTPases Rac and TC10 have been implicated in insulin-induced actin remodeling in L6 muscle cells and 3T3-L1 adipocytes, respectively. Although different manifestations of actin remodeling were reported, possible cell type specificities of the GTPases were not addressed. Accordingly, myoblasts were transfected with WT, dominant negative (DN), or con-

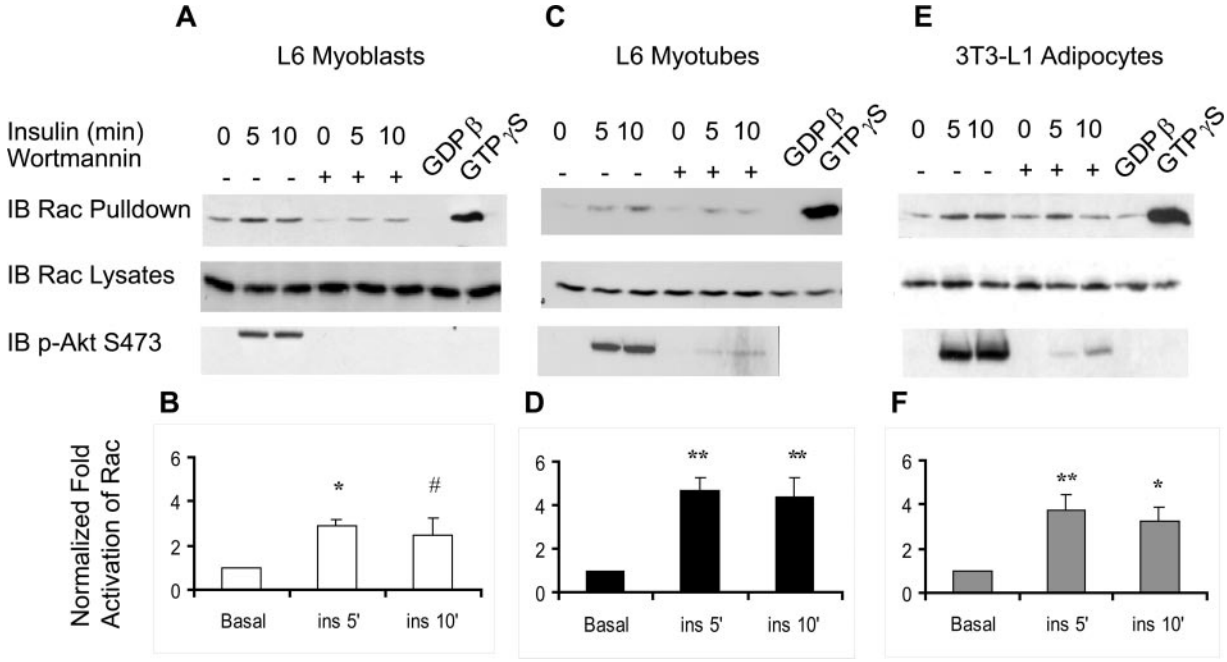

Fig. 6. Rac Activation by Insulin

Endogenous Rac activation by insulin and the effect of wortmannin were determined in L6 myoblasts (A and B), L6 myotubes (C and D), and 3T3-L1 adipocytes (E and F). Pixel intensity was normalized to total Rac in cell lysates, and a value of 1 was assigned to the basal state. Results of three to six independent experiments are presented as the mean ± SE. *, $P < 0.05$; and **, $P < 0.01$ compared with the basal state using ANOVA. #, $P < 0.05$ compared with basal state, as determined using Student's *t* test assuming unequal variance.

stitutively active (CA) HA-TC10. Twenty-four hours after transfection, cells were stimulated with insulin, fixed, and immunostained for HA to allow identification of the transfected cells. Filamentous actin was decorated with rhodamine-conjugated phalloidin. As previously reported, insulin-stimulated L6 myoblasts exhibited actin remodeling into mesh-like structures resembling lamellipodia protruding above the dorsal surface of the cell (Fig. 7, H, J, and L), whereas no significant change could be detected in stress fiber structure. In the nonstimulated basal state, the stress fibers were indistinguishable between untransfected cells and cells expressing moderate levels of WT or DN TC10. Interestingly, expression of CA TC10 in nonstimulated L6 myoblasts cells caused actin to disorganize into punctate clusters, associated with thinning or loss of stress fibers when compared with the untransfected cells. These cells also contained a rim of actin about the periphery of the transfected cell that did not protrude above the dorsal plane (Fig. 7F, *arrowhead*). Irrespective of these alterations in actin morphology in the basal state, insulin caused the formation of characteristic filamentous actin structures that elevated and caused peripheral membrane folds resembling lamellipodia (Fig. 7, *asterisks*), which were indistinguishable in cells expressing WT, CA, or DN TC10 from those in the neighboring untransfected cells. The above alterations in actin morphology in the basal and insulin-stimulated states were documented in 50–130 untransfected, WT, DN, or CA TC10 expressing cells. The percent of cells in which each actin manifestation was observed is summarized in Table 1. Thus, although overexpressed TC10 is activated by insulin in L6 myoblasts, this small GTPase has no noticeable effect on actin morphology in the insulin-stimulated state. Furthermore, normal insulin-stimulated actin remodeling was also observed when DN TC10 was introduced into myotube (supplemental Fig. 1, published as supplemental data on The Endocrine Society's Journals Online web site at http://mend.en-

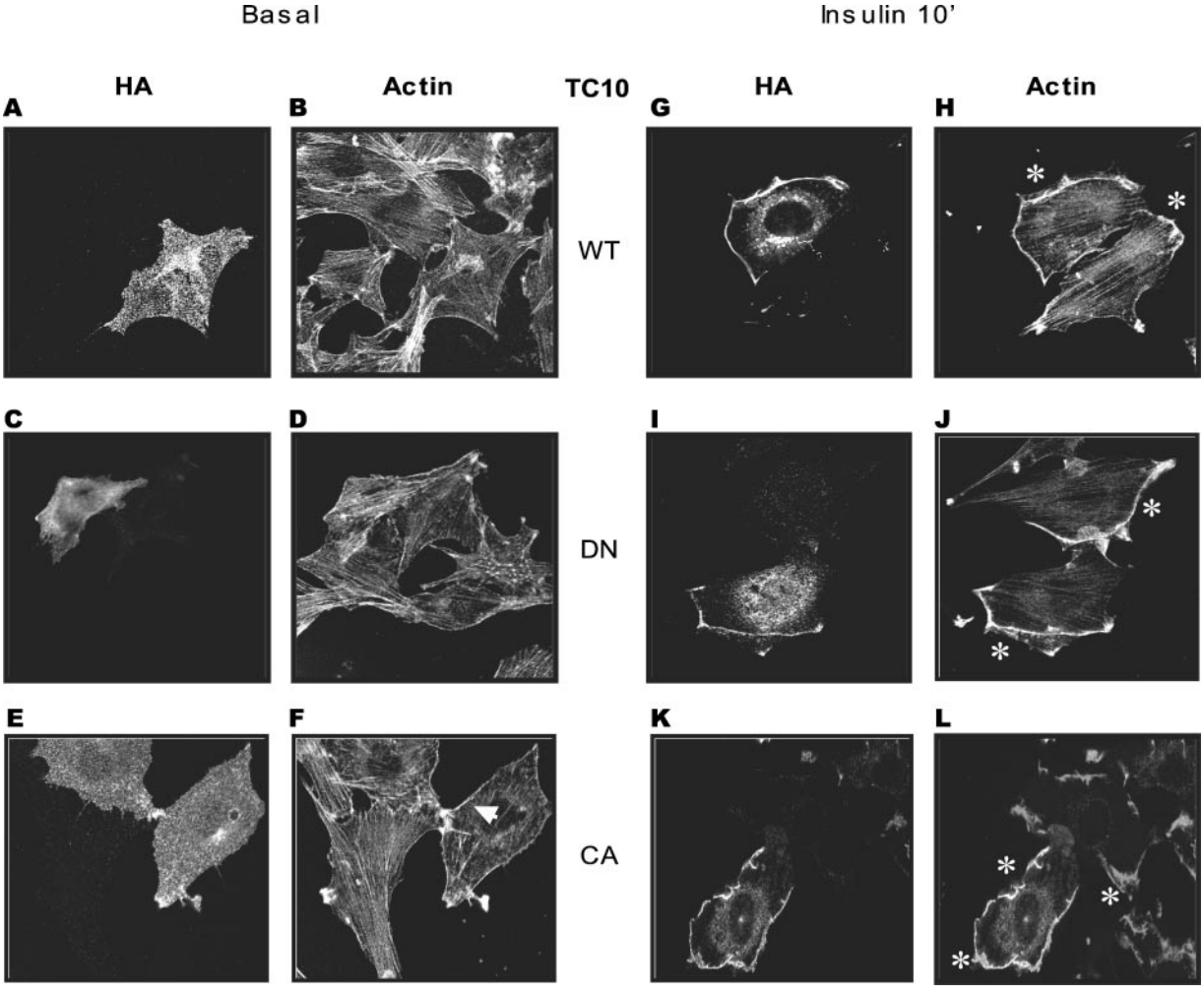

Fig. 7. Effect of TC10 on Insulin-Stimulated Actin Remodeling in Myoblasts

Shown are images of attached L6 myoblasts overexpressing WT (A, B, G, and H), DN (C, D, I, and J), or CA (E, F, K, and L) HA-TC10α. Serum-deprived (4 h) L6 myoblasts were untreated or stimulated with 100 nM insulin for 10 min at 37 C as indicated. Cells were permeabilized, and the HA-epitope and filamentous actin were detected as described in *Materials and Methods*.

Table 1. Effect of TC10 on Actin Morphology in Unstimulated and Insulin-treated L6 Muscle Cells

Actin Morphology Manifestation		% of Cells Showing the Indicated Actin Manifestations			
		Untransfected	Transfected with TC10		
			WT	CA	DN
Stress fiber disruption	− Insulin	0	0	75	0
Rim	− Insulin	3	13	75	4
Rim	+ Insulin	2	16	16	5
Insulin-induced lamellipodia	+ Insulin	98	100	100	98

Confocal images were acquired of rhodamine-phalloidin-stained actin in untransfected L6 myoblasts or myoblasts transfected with HA-tagged WT, CA, or DN TC10α before and after insulin treatment for 10 min. Transfected cells were identified using an anti-HA antibody followed by Alexa488-conjugated secondary antibody. The different actin manifestations were categorized as general stress fiber disruption or rim or ruffle formation. The results shown are the percentage of cells displaying each manifestation. The total number of cells analyzed ranged from 50–132 from four independent experiments.

dojournals.org) cells in which CAP is expressed and Cbl is tyrosine phosphorylated in response to insulin. These finding suggest that even in differentiated muscle cells, TC10 is not involved in actin remodeling, as shown in adipocytes (5, 8, 9).

The above findings are in contrast to the reported action of TC10 to increase the dynamics of transfected, fluorescently tagged actin in 3T3-L1 adipocytes (5, 8). The two cell types differ substantially in their morphology: the fully differentiated 3T3-L1 adipocyte is a rather round globular cell devoid of elongated stress fibers, rising occasionally more than 20 μm above the substratum. The L6 muscle cell is rather spread out and flat, mounting vertically to no more than 7–8 μm. To assess the dependency of insulin-induced actin remodeling on cell shape, L6 myoblasts were detached from the substratum and allowed to reattach to glass coverslips for 10 min in the absence or presence of insulin (Fig. 8, A–D). Insulin-induced treatment of these cells caused dramatic protrusions of actin structures about the periphery of the cell (Fig. 8D). Unlike the case of the flat, attached cells, these actin structures are not confined to the dorsal membrane surface and can be seen in the ventral aspect of the cell. Pretreatment of the cells with wortmannin for 20 min before detachment, followed by insulin treatment during reattachment, nearly abolished the insulin-induced actin remodeling observed in rounded-up myoblasts (Fig. 8F). A similar observation was made in the flat, adherent myoblasts (Fig. 8, A, C, and E). This finding confirms that even when muscle cells are forced to adopt a rounded morphology devoid of stress fibers, insulin-induced actin remodeling is largely, if not completely, PI3-K dependent, correlating with the activation of Rac rather than TC10.

TC10 Is Not Involved in Insulin-Induced GLUT4myc Translocation in Muscle Cells

It is thought that TC10 activation in 3T3-L1 adipocytes complements the signaling input downstream of PI3-K, resulting in the full insulin-mediated recruitment of GLUT4 at the cell surface. We therefore assessed whether TC10 was indeed involved in insulin-mediated GLUT4 translocation in L6 myoblasts. Surface GLUT4 was assayed using immunofluorescent detection of a myc epitope introduced on the first exofacial loop of the GLUT4 molecule (34), whereas myoblasts overexpressing HA-tagged WT, DN, or CA TC10 were identified by permeabilizing the cells after surface staining, followed by immunofluorescent detection of HA. Neither HA-TC10 protein affected the intensity of the surface GLUT4myc signal in the basal state (Table 2). The increase in surface GLUT4myc content induced by insulin is typically 1.5- to 2-fold of that observed in unstimulated cells (34, 35). Consistently, we found, in nontransfected myoblasts (devoid of HA staining), that insulin caused a 1.5- to 1.6-fold increase in the intensity of the signal arising from GLUT4myc at the cell surface. Myoblasts overexpressing DN, CA, or WT TC10 showed the same increase in surface GLUT4myc signal (Table 2). These data suggest that TC10 does not play a role in insulin-stimulated GLUT4 translocation in muscle cells.

DISCUSSION

Functionality of the CAP to TC10 Pathway in Skeletal Muscle Cells

It was recently proposed that activation of the small GTPase TC10 is a central signaling event in insulin regulation of GLUT4 translocation, acting in parallel to the well-characterized signaling pathway involving activation of PI3-K. Further, it was postulated that insulin-mediated tyrosine phosphorylation of Cbl, complexed to CAP, leads to the corecruitment of CrkII and C3G and to the consequent activation of TC10 (12, 18). The model further states that TC10 promotes various types of actin remodeling through neuronal Wiskott-Aldrich syndrome protein, ultimately contributing to GLUT4 mobilization (8, 11). Until now, this novel insulin signaling pathway has only been reported in

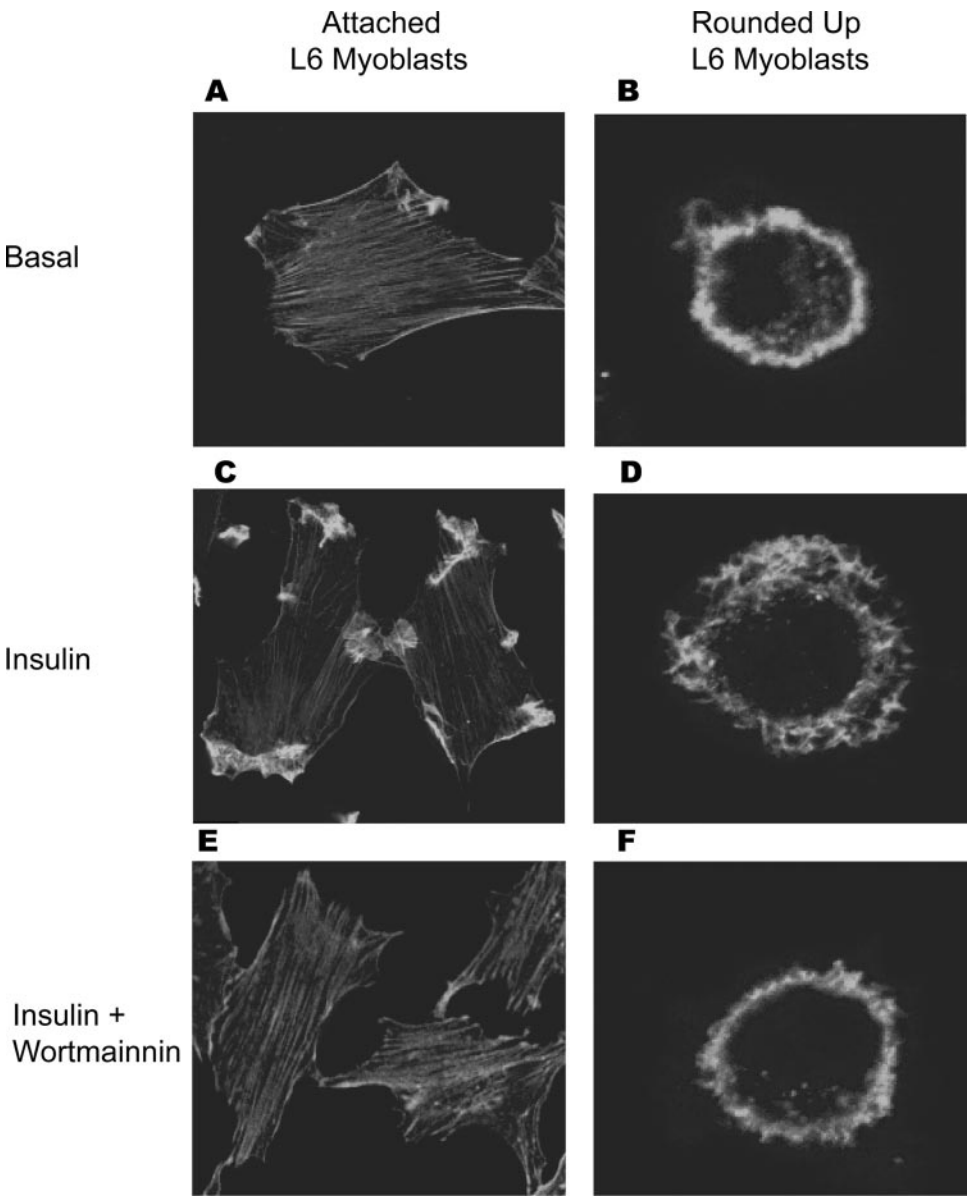

Fig. 8. Immunofluorescence of Actin in L6 Myoblasts

Shown are images of attached (A, C, and E) and semiattached rounded-up L6 myoblasts (B, D, and F). Serum-deprived (4 h) L6 myoblasts were untreated (A and B) or stimulated with 100 nM insulin for 10 min at 37 C (C and D), or with 100 nM wortmannin for 20 min followed by 10 min stimulation with 100 nM insulin (E and F). Cells were permeabilized, and actin was stained with rhodamine-conjugated phalloidin as described in *Materials and Methods*.

3T3-L1 adipocytes into which WT or mutant forms of TC10 were introduced. Curiously, there are no reports of activation of endogenous TC10 in 3T3-L1 adipocytes. The first aim of the present study was to assess the functionality of such a signaling cascade in skeletal muscle cells.

Several of the key elements in this signaling sequence (*e.g*. CAP, Cbl) were found to be expressed in the L6 skeletal muscle cell line. Moreover, CAP expression increased upon muscle cell differentiation, as reported in 3T3-L1 cells (28). However, endogenous levels of TC10 protein were very low in the muscle cells as shown using two different antibodies, one of which readily detected endogenous TC10 proteins in 3T3-L1 adipocytes. Moreover, we were unable to detect activation of endogenous TC10 by insulin in L6 muscle cells using a pull-down assay. In contrast, insulin caused activation of this GTPase when L6 myoblasts overexpressed the protein. Yet, these nondifferentiated myoblasts express very little CAP compared with myotubes, and insulin-mediated Cbl phosphorylation in myoblasts is much weaker than in differentiated cells. These findings suggest that insulin can activate exogenous TC10 in muscle cells without the participation of CAP and p-Cbl. Hence, CAP does not define the TC10 pathway in muscle cells as it does in adipocytes.

Table 2. Effect of TC10 Mutants on Insulin-Mediated Recruitment of Surface GLUT4myc in L6 Myoblasts

	Average Fold GLUT4myc Translocation			
	Basal		Insulin	
	Nontransfected Neighboring Cells	Transfected Cells	Nontransfected Neighboring Cells	Transfected Cells
TC10 Mutant				
WT TC10	1	1.03 ± 0.03[a]	1.56 ± 0.08[a]	1.58 ± 0.12[a]
CA TC10	1	0.99 ± 0.04[a]	1.64 ± 0.19[a]	1.60 ± 0.20[a]
DN TC10	1	1.03 ± 0.01[a]	1.61 ± 0.12[a]	1.64 ± 0.15[a]

L6 myoblasts were transfected and treated with insulin as detailed in *Materials and Methods*. Surface myc staining was first performed in unpermeabilized cells, followed by permeabilization and immunofluorescent detection of HA-tagged TC10 to allow identification of transfected and untransfected cells. Images were obtained by laser confocal microscope as detailed in *Materials and Methods*. The results shown are the means (± SE) of the fold increase in surface GLUT4myc signal over nonstimulated, untransfected cells in the same experiment. For each group 75–125 cells were analyzed from three independent experiments. Significant difference between the values obtained in basal transfected, insulin-stimulated nontransfected, and transfected *vs.* basal nontransfected states was assessed as described in *Materials and Methods* and using ANOVA. [a] $P < 0.05$.

The above differences between 3T3-L1 adipocytes and muscle cells in the functionality of the CAP to TC10 pathway are further highlighted by the analysis of insulin-stimulated phosphorylation of Cbl on specific tyrosine residues. Although in the differentiated state of both cell types, insulin causes overall tyrosine phosphorylation of Cbl, different tyrosine residues are involved. Our results show that in L6 myotubes, but not in 3T3-L1 adipocytes, insulin induces tyrosine phosphorylation of Y731 and Y774. These phosphotyrosine residues interact, respectively, with the p85 regulatory subunit of PI3-K and with CrkII. Because in 3T3-L1 adipocytes Cbl binds CrkII in response to insulin, it is somewhat surprising that Y774 was not phosphorylated when these cells were stimulated by the hormone. Yet, the Cbl-CrkII interaction in 3T3-L1 adipocytes may also be mediated by Y700, a second CrkII binding site (33). Intriguingly, the finding that Y731 was phosphorylated in insulin-stimulated L6 myotubes suggests a possible mechanism for cross-talk between the Cbl-dependent signaling pathway(s) and the PI 3-K pathway. This notion is further supported by the recent work of Farese *et al.* (36), whereby expression of Cbl-encoding mutations at Y731 and Y371, two tyrosine phosphorylation sites within a YXXM (p85 binding) motif, prevented glucose uptake and PI3-K-mediated insulin signaling. Furthermore, APS (adapter protein with a Pleckstrin homology and Src homology domain) has been recently documented to regulate insulin receptor phosphorylation and subsequent PI3-K signaling (37). Similarly, rosiglitazone caused Cbl phosphorylation in a PI3-K-dependent manner (38), and the PI3-K and Cbl pathways were suggested to converge at the level of atypical PKC complexed with Par 6 and Par3 (39). In the present study, we demonstrate that, after inhibition of PI3-K with wortmannin, insulin-induced TC10 activity was not reduced, but the basal level of GTP-loaded TC10 was increased. This tonic increase in basal activity suggests that PI3-K exerts a negative regulatory input on the GTP-loading of TC10 at the basal state. Possible targets for PI3-K input could be at the guanine activation protein level (*i.e*. hyperactivating GTP hydrolysis), or at the guanine exchange factor level (*i.e*. inhibiting GDP to GTP exchange). Importantly, the effect of wortmannin on TC10 in the absence of insulin was not examined in the previous studies with 3T3-L1 adipocytes. It therefore appears that cross-talk mechanisms do exist between the PI3-K and the CAP to TC10 pathways in L6 muscle cells.

Collectively, our study suggests that differentiated muscle cells express various components of the CAP to TC10 pathway, and that insulin induces tyrosine phosphorylation of Cbl. Yet, it is uncertain whether these proteins participate in a functional signaling pathway from the insulin receptor through CAP and Cbl to TC10, as was reported in 3T3-L1 adipocytes (also see below). This conclusion is supported by the findings of Thirone *et al.* (40), where CAP expression and Cbl phosphorylation were observed in mature rat adipocytes but not in muscle tissue. Furthermore, activation of exogenously expressed TC10 may receive regulatory input from PI3-K in muscle cells.

The Role of Rac and TC10 in Insulin Action

Previous findings indicated that the small GTPase Rac is involved in insulin-mediated actin remodeling in skeletal muscle cells (10) but not in adipocytes (41). Here we compared in parallel the expression and activation of Rac in both cell lines. Unlike TC10, endogenous levels of Rac were readily detected in both muscle cells and 3T3-L1 adipocytes. Moreover, after insulin stimulation, both cell types exhibited rapid activation of endogenous Rac in a PI3-K-dependent manner.

The following observations correlate Rac activation with actin remodeling in muscle cells in response to insulin stimulation: 1) Insulin-mediated actin reorganization in muscle cells is largely PI3-K dependent, based on pharmacological and molecular approaches (1, 3). Here we show that this also holds true when the

cells are semidetached and rounded up. This PI3-K-dependent actin remodeling correlates with insulin-induced Rac activation that is wortmannin inhibitable. 2) A dominant negative Rac mutant prevented insulin-mediated actin remodeling and the gain in surface GLUT4 in muscle cells (10). In contrast to muscle cells, in 3T3-L1 adipocytes, even though Rac is activated by insulin (Fig. 6), dominant negative Rac had no effect on hexose transport (41, 42). Thus, despite the fact that insulin can activate endogenous Rac both in adipocytes and muscle cells, it is possible that the role of Rac in insulin-mediated actin remodeling differs between the two cell types. Furthermore, it is possible that other GTPases may also participate in insulin signaling. Indeed, a recent study shows that Cdc 42, a GTPase with similarities to TC10, is required for the stimulation of glucose uptake into 3T3-L1 adipocytes (43).

As mentioned above, in L6 myoblasts, activation of exogenous TC10 by insulin occurred in the absence of CAP expression and Cbl phosphorylation. This renders this cell system ideal for assessing the role of TC10 in actin remodeling, independent from additional signals arising from the CAP-Cbl pathway. The following observations dissociate TC10 from insulin-mediated actin remodeling and GLUT4 translocation in muscle cells: 1) Wortmannin abolished insulin-mediated actin remodeling in this cell type but did not reduce TC10 activation. 2) A dominant negative TC10 mutant did not prevent insulin-mediated actin remodeling in myoblasts. Similar results were obtained in myotubes, in which the CAP and Cbl components are assembled and regulated by insulin. 3) Actin remodeling occurred in myoblasts that do not express CAP nor respond to insulin by phosphorylating Cbl. It is noteworthy, however, that upon constitutively active TC10 overexpression, the stress fiber architecture was altered and a prominent peripheral actin band was evident. This suggests a potential capacity of TC10 to regulate actin morphology in muscle cells. Alternatively, it is plausible that such action results from competition of the highly expressed TC10 mutant with guanine exchange factors and guanine activation proteins needed for the function of other small GTPase such as RhoA, which is known to control stress fiber morphology (44). Regardless of the changes in actin morphology observed in cells exogenously expressing constitutively active TC10, insulin still induced the characteristic formation of actin structures in muscle cells. 4) Finally, unlike in the adipocytes, TC10 had no effect on GLUT4 translocation in response to insulin in L6 myoblasts. When this manuscript was under review, a paper appeared dissociating TC10 activation from its downstream actions on actin remodeling and GLUT4 translocation in adipocytes (45), suggesting that the link between the upstream CAP and Cbl leading to TC10 activation may not be linearly linked to actin and GLUT4 via TC10.

In conclusion, muscle cells and adipocytes appear to differ in their reliance on TC10 and Rac for insulin-mediated actin remodeling and GLUT4 translocation. In skeletal muscle cells, a functional CAP to TC10 signaling pathway toward actin remodeling in response to insulin is deemed unlikely, although individual components of this pathway are assembled and regulated by insulin. TC10 protein was barely detectable, and actin remodeled in the absence of CAP or Cbl phosphorylation. Insulin-dependent actin remodeling was prevented by wortmannin as was Rac activation. Moreover, dominant negative Rac, but not dominant negative TC10, prevents insulin-dependent actin dynamics and GLUT4 translocation. Rac is therefore the likely candidate small GTPase involved in insulin-stimulated actin reorganization that is necessary for GLUT4 translocation in muscle cells, downstream of PI3-K.

MATERIALS AND METHODS

Plasmids, Reagents, and Antibodies

Wild type (WT) TC10α was kindly provided by Dr. Mark Rush (New York University, New York, NY). Three-X-HA-tagged versions of TC10α (HA TC10) were gifts provided by Dr. Ian Macara (University of Virginia, Charlottesville, VA). Plasmid encoding enhanced green fluorescent protein was purchased from CLONTECH (Palo Alto, CA). Effectene transfection kits and plasmid DNA purification columns were purchased from Qiagen (Mississauga, Ontario, Canada). Lipofectamine 2000 transfection kits were purchased from Invitrogen (Carlsbad, CA). Human insulin was purchased from Eli Lilly (Mississauga, Ontario, Canada). PI3-K inhibitors, wortmannin and LY 294002, were purchased from BIOMOL Research Laboratories, Inc. (Plymouth Meeting, PA). Mouse monoclonal antibodies against Rac, Cbl, phosphotyrosine (4G10), and rabbit polyclonal antibodies for CAP were purchased form Upstate Biotechnology, Inc. (Lake Placid, NY). Rabbit polyclonal antibodies for Cbl and myc (A-14) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Phospho-Cbl antibodies against Y731 and Y774, phospho-Akt against S473, and anti-HA antibodies were purchased from Cell Signaling (Beverly, MA). Two different affinity-purified polyclonal TC10 antibodies recognizing rodent TC10 were used. One antibody (Ab:A) was a gift from Dr. Mark Rush (New York University) and was raised against amino acids 139–152 of human TC10α. This peptide sequence shares 99% identity with the corresponding sequences in the mouse and rat TC10α (which are 100% identical within themselves), but not with TC10β or TC10-like (TCL, the human ortholog), which is globally 77% identical to TC10α in amino acid sequence. A second antibody (Ab:B) was a gift from Dr. Alan Saltiel (University of Michigan, Ann Arbor, MI) and was raised against the peptide PASYYHNVQEEWVPELKDCMP (20). This sequence shares 100% identity to sequences from the human, mouse, and rat TC10β/TCL, and 85% amino acid identity with sequences in the human, mouse, and rat TC10α (which are 100% identical between rat and mouse TC10α). Yet, this antibody cross-reacts with both proteins (20). Rhodamine-conjugated phalloidin and Alexa 488-conjugated goat antimouse antibodies were purchased from Molecular Probes (Eugene, OR).

Cell Culture and Transfections

L6 muscle cells expressing c-*myc* epitope-tagged GLUT4 (GLUT4myc) were maintained in myoblast monolayer culture, or differentiated into multinucleated myotubes as described

previously (2). Myotubes in six-well plates or 10-cm dishes were ready for experimentation 5–7 d post seeding. Transfection of L6 GLUT4myc myoblasts (24 h post seeding) and myotubes (on d 6) was performed using Effectene and Lipofectamine 2000 reagent, respectively, essentially as specified by the manufacturer. Experiments with transfected cells were conducted 20–24 h after transfection.

3T3-L1 adipocytes were cultured to confluency in DMEM supplemented with 20% calf serum, 1% glutamine, and penicillin-streptomycin cocktail. Forty-eight hours after confluence, differentiation was induced in DMEM containing 10% fetal bovine serum, 0.11 mg/ml 3-isobutyl-1-methylxanthine, 1 μM dexamethasone, and 1 μg/ml insulin. Forty-eight hours later, the medium was changed to 10% fetal bovine serum DMEM containing 1μg/ml insulin for an additional 2 d. Experiments were performed on cells 11–12 d after induction of differentiation, when exhibiting greater than 90% adipocyte morphology (46).

RNA Isolation and RT-PCR

Total RNA was isolated from 10-cm dishes of rat L6 myoblasts, myotubes, or mouse 3T3-L1 adipocytes using the RNAqueous Midi Kit from Ambion (Austin, TX). RNA was purified and extracted as directed by the manufacturer (Ambion). RNA (5 μg) was reverse transcribed in a 40-μl reaction as described by the manufacturer (Invitrogen).

TC10 fragments from L6 muscle cells (rat origin) were amplified from the cDNA by PCR with the following primers

TC10α 5′→3′ primer: AAAGGTCTAAGCGGCAGCGAACAG-

3′→ 5′ primer: GAGCAACCGAAAGAAGAAGGCAGA;

TC10β 5′→3′ primer: GCGTGAAACTGGCGAAAGCG-ATAG

3′→ 5′ primer: TGAGAGACAGGACTTGGTTACCGG

These primers amplified 1100- and 488-bp fragments of rat TC10α and β, respectively. TC10 from 3T3-L1 adipocyte cells (mouse origin) was amplified with the following primers:

TC10α 5′→3′ primer: AAAGGTCTAAGCGGCAGCGA-ACAG

3′→ 5′ primer: GTGTAGGTGTCGTCTTCCGTGTTG;

TC10β 5′→3′ primer: GACAGACCCTTTTCATCCCTCCTC

3′→ 5′ primer: GTCCTGTCCTCCTAATGTTGGTCG

These primers amplified 855- and 425-bp fragments of mouse TC10α and β, respectively. PCR was carried out in 50-μl reactions as described by the manufacturer (Invitrogen).

Immunoblot Analysis and Immunoprecipitation

Expression patterns of various proteins from total cell lysates were analyzed by immunoblotting. Cells were treated with or without 100 nM insulin for the indicated time points. After stimulation, cells were washed twice with ice-cold PBS containing 1 mM Na_3VO_4. Total cell lysates were made with 2× Laemmli sample buffer containing freshly added inhibitors [1 mM Na_3VO_4, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, protease inhibitor cocktail (PharMingen, San Diego, CA), and okadaic acid]. Lysates were then heated for 5 min at 65 C and passed through a 27-gauge needle. Forty Protein (40 μg) was resolved with 10% SDS-PAGE and transferred to polyvinylidene difluoride membrane for 2 h. Membranes were blocked with 3% BSA in Tris-buffered saline (500 mM Tris-base, 150 mM NaCl, pH 7.5) and then immunoblotted overnight at 4 C with primary antibodies as specified in the text. Horseradish peroxidase-conjugated secondary antibodies were applied for 1 h at room temperature, and detection by chemiluminescence was performed as specified by the manufacturer (PerkinElmer Corp., Norwalk, CT). Immunoblots were quantified by scanning and analysis of pixel intensity of individual bands using NIH Image (version 1.61) graphics.

For immunoprecipitation, cells were treated with insulin as above, and lysates were prepared in immunoprecipitation buffer containing 135 mM NaCl, 50 mM Tris-HCl, pH 8, 10 mM NaF, 1% Triton X-100 and 1 mM each of EDTA, Na tetrapyrophosphate, Na_3VO_4, and freshly added protease inhibitor cocktail. After 15 min of rotation and a quick 12,000 × *g* centrifugation at 4 C, lysates were incubated with 4 μg polyclonal anti-Cbl antibody, rotating at 4 C overnight, after which 70 μl protein beads (mix 50:50 of Protein A and G) were added for an additional 2 h at 4 C. Complexes were obtained by short centrifugation at 12,000 × *g*, washed on ice four to five times with immunoprecipitation buffer and eluted with 2× Laemmli sample buffer. The proteins were resolved on a 5% SDS-PAGE, followed by immunoblotting with phosphotyrosine (4G10) or monoclonal anti-Cbl antibodies.

Rac and TC10 Activation by Insulin

To determine whether TC10α or Rac was activated by insulin, cell lysates were subjected to pull-down experiments with a GST-fusion protein of the CRIB domain of p21 kinase conjugated to glutathione beads that specifically bind activated forms of Rho GTPases. Cells were grown as described above in 10-cm dishes (for TC10 activation, myoblasts were transfected with 5 μg HA-TC10α cDNA) and serum starved for 3–5 h before stimulation with 100 nM insulin for the indicated time periods. Cells were then washed twice with ice-cold PBS supplemented with 1 mM Na_3VO_4 and lysed in buffer containing 150 mM NaCl, 10 mM $MgCl_2$ (or 30 mM for TC10 activation), 5 mM HEPES, pH 7.5, 2% glycerol, 1% Igapal, 1 mM $NaVO_4$, 1 mM EDTA, 20 μl/ml protease inhibitor cocktail (provided by BD Biosciences, Oakville, Ontario, Canada) and 1 mM phenylmethylsulfonyl fluoride. Lysates were immediately centrifuged at 12,000 × *g* for 1 min at 4 C, an aliquot was removed for total lysate analysis, and the remainder was incubated with previously prepared GST-CRIB conjugated to glutathione Sepharose beads as described previously (47, 48). After 30 min rotation at 4 C, the active (GTP-bound) GTPases that bind the CRIB domain were pulled down by brief centrifugation at 12,000 × *g*, washed three times with lysis buffer, eluted with 2× Laemmli sample buffer, and heated for 3–5 min at 100 C. Pulled down proteins were resolved by 10–12% SDS-PAGE gels, followed by immunoblotting using anti-Rac, anti-TC10, or anti-HA antibodies.

Fluorescence Microscopy

L6 GLUT4myc myoblasts grown on 25-mm diameter glass coverslips were transfected with 0.5 μg of cDNA encoding WT, DN, or CA HA-TC10α, whereas myotubes were transfected with 4 μg DN HA-TC10. Cells were deprived of serum for 4 h and then treated with 100 nM insulin for up to 10 min at 37 C. Cells were fixed with 3% (vol/vol) paraformaldehyde in PBS for 5 min at 4 C and 25 min at room temperature, briefly permeabilized with 0.1% (vol/vol) Triton X-100 in PBS for 3 min, and blocked for 15 min with 5% milk in PBS. It was crucial to fix the cells immediately at 4 C after insulin stimulation, and to permeabilize in 0.1% (vol/vol) Triton X-100 for exactly 3 min to preserve actin morphology. To identify transfected cells, monoclonal anti-HA (1: 2000) antibody was used, followed by a secondary antibody conjugated to the fluorophore Alexa 488. Labeling of actin filaments with rhodamine-coupled phalloidin was carried out as described previously (7).

Rounded-up myoblasts were prepared as described previously (49). Briefly, L6-GLUT4myc myoblasts were detached from the substratum using Ca^{2+}- and Mg^{2+}-free PBS. Trypsin was avoided to better preserve cell surface proteins, such as the insulin receptor. After resuspending in HEPES-buffered RPMI, myoblasts were allowed to reattach onto glass coverslips for 10 min in the absence or presence of 100 nM insulin at 37 C. Where indicated, 100 nM wortmannin was added to the medium 20 min before cell detachment and during the 10-min incubation with insulin. Cells were then

immediately fixed in ice-cold 4% formaldehyde in PBS containing Ca^{2+} and Mg^{2+} for 20 min and further processed to allow actin decoration with rhodamine-conjugated phalloidin exactly as described above. Images were obtained by laser confocal microscopy (Zeiss LSM 510, Carl Zeiss, Thornwood, NY). Acquisition parameters were adjusted to exclude saturation of the fluorescent pixels. The focal plane was chosen to best observe the actin structures in the basal and insulin-stimulated states, as described previously (7).

Surface GLUT4myc detection was performed as described previously (34). In short, after treatment, cells were processed on ice at 4 C to prevent internalization of GLUT4. Cells were then briefly fixed with 3% (vol/vol) paraformaldehyde in PBS for 5 min, blocked with 5% milk in PBS, and allowed to react with a polyclonal anti-myc antibody (A-14, 1:250) for 1 h. After surface myc labeling, cells were permeabilized, blocked (15 min, 5% milk in PBS), and reacted with a monoclonal anti-HA. Secondary antirat and antimouse antibodies conjugated to the fluorophore Cy3 and Alexa 488, respectively, were used, followed by a final fixing with paraformaldehyde for 5 min at 4 C followed by 25 min at room temperature. Confocal microscopy was used as above, by choosing a focal plane immediately above the glass coverslip, and quantification of the intensity of the signal observed was performed as described previously (7). The results obtained from the ventral cell surface matched those in which a complete Z-stack was acquired, and a projection image was used for quantitation (data not shown).

Statistical Analysis

Unless otherwise specified in the figure legend, different groups were compared using ANOVA with Tukey's multiple comparison *post hoc* analysis using Prism version 3 (GraphPad Software, Inc., San Diego, CA).

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