Cloning and Functional Characterization of Related TC10 Isoforms, a Subfamily of Rho Proteins Involved in Insulin-stimulated Glucose Transport*

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Insulin stimulates glucose transport via phosphatidylinositol 3-kinase-dependent and -independent pathways. The phosphatidylinositol 3-kinase-independent pathway involves activation of the G protein TC10. A cDNA encoding the mouse homolog of TC10 was cloned, and its gene was mapped at the distal end of chromosome 17. Additionally, a second gene was discovered with ~70% sequence identity to TC10. We refer to this gene as TC10β. Both isoforms of TC10 were activated by insulin upon transfection in 3T3L1 adipocytes. Cotransfection of cells with TC10α or β plus a dominant negative form of the c-cbl-associated protein CAP prevented the activation by insulin, implicating the CAP/Cbl pathway. Interestingly, both forms of TC10 were also localized in lipid raft fractions in transfected adipocytes. However, although overexpression of TC10α completely blocked glucose transport, TC10β only partially inhibited this process. Furthermore, TC10α overexpression disrupted adipocyte cortical actin, whereas TC10β had little if any effect. Thus, there are two isoforms of this key signaling intermediate, both of which are activated by insulin, but they may play different roles in initiating downstream effectors that influence glucose transport.

Insulin increases glucose uptake by stimulating the translocation of the GLUT4 glucose transporter isofrom from intracellular storage sites to the cell surface (1–3). Although it has been well established that the activation of phosphatidylinositol 3-kinase and the generation of phosphatidylinositol-3,4,5-trisphosphate is essential for this biological response, several lines of evidence indicate that it is not sufficient (4–9). Recent data suggest that the second requisite pathway might involve the insulin-stimulated tyrosine phosphorylation of Cbl (10–12). Cbl forms a complex with the adapter protein CAP,‡ a member of the SoHo family of proteins that contains a flotillin-binding SoHo domain in its amino terminus and three adjacent SH3 domains in its carboxyl terminus (11–13). Once phosphorylated, the Cbl/CAP complex is recruited to lipid raft plasma membrane subdomains through the interaction of CAP with flotillin. The expression of dominant-interfering CAP mutants that lack either the SH3 or SoHo domains prevented the localization of this complex to plasma membrane microdomains and inhibited the stimulation of glucose uptake and GLUT4 translocation by insulin (13).

Following insulin-stimulated tyrosine phosphorylation, Cbl recruits the SH2-containing adapter protein CrkII to lipid raft microdomains along with the guanine nucleotide exchange factor C3G (14). Upon its translocation, C3G appears to activate the Rho family protein TC10, a member of the family of small GTP-binding proteins expressed in muscle and adipose tissue (15). Upon overexpression in murine 3T3L1 adipocytes, insulin activates TC10 in a CAP-dependent but PI 3-kinase-independent manner (14). Although the physiologically relevant effectors that interact with TC10 are unknown, disruption of its activation blocks insulin-stimulated glucose transport and GLUT4 translocation. Moreover, the mistargeting of TC10 to a non-lipid raft domain by production of a TC10/K-Ras chimera or disruption of lipid raft microdomains via expression of a dominant-interfering mutant form of caveolin-3 also completely prevented the activation of TC10 by insulin (16).

Although these experiments suggest that TC10 is a critical player in the hormonal regulation of glucose transport, they relied almost exclusively on the overexpression of the human form of TC10 in mouse cells. To study the endogenous forms of TC10 in the highly insulin-responsive mouse 3T3L1 cell line, we cloned the mouse ortholog of TC10. Interestingly, these efforts led to the identification of a closely related gene, referred to as TC10β, and another variant termed TC10βLong (TC10/L). We describe here the characterization of this gene, the regulation of its gene product by insulin, and the evaluation of its role in glucose transport.

MATERIALS AND METHODS

Reagents—A mouse monoclonal anti-β2-adrenergic receptor antibody and a rabbit polyclonal anti-C3G antibody were purchased from Santa Cruz Biotechnology. A polyclonal anti-caveolin antibody was purchased from BD Transduction Laboratories. GST-PAK1 PBD-agarose was purchased from Cytoskeleton, Inc. A mouse muscle Marathon RACE library was purchased from CLONTECH.

Expression Constructs—The coding sequence of mouse TC10α was PCR amplified from a mouse muscle cDNA library. The PCR product was cloned into the EcoRI site of a pH3 vector driven by the cytomegalovirus (CMV) promoter (a gift from Dr. Ian Macara) that has sequences encoding for three HA epitope tags at the 5′-end of the cloning sites. To construct constitutively active (Q67L) and dominant negative (T23N) mutants of TC10α, the wild type construct was mutated using the QuikChange site-directed mutagenesis kit (Stratagene). Comple-

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Cloning and Characterization of TC10β

**Results**

**Cloning of Murine TC10 Genes**—A mouse muscle RACE cDNA library was used to amplify a full-length cDNA encoding TC10. The first methionine was identified, although a stop codon was not found in the 180-bp 5′-untranslated region. The coding region was then amplified with mouse TC10-specific primers. The mouse TC10 gene contains an open reading frame of 205 amino acids that predicts a 23-kDa protein. The coding sequence of mouse TC10 is ~95% identical to the rat cDNA sequence (22). However, the encoded protein sequence is completely identical to rat TC10 and exhibits only two amino acid changes (Val to Ile at 143 and Ile to Val at 172) from its human homolog (Fig. 1A).

We performed an NCBI BLAST search to identify mouse EST sequences similar to the TC10 cDNA. Mouse ESTs were found that showed ~70% identity to the rat TC10 cDNA sequence (22). PCR primers were designed to amplify the coding region of this mouse mRNA with the 3T3L1 adipocyte cDNA library. A full-length mRNA was cloned that encodes a 204-amino acid protein with 83% identity to TC10. After this analysis was completed, the cloning of TCL (TC10-like) was re-attempted (23), which is a human ortholog of this mouse gene. Among the mouse ESTs, we identified other clones with sequences at the 5′-end different from the cDNA that was amplified. These clones contain a 10-amino acid extension of the coding sequence. Thus, we speculate that this TC10-like gene utilizes a different promoter or splicing process to generate two isoforms that vary at the amino terminus. A reverse transcription PCR confirmed that the longer form is also expressed in 3T3L1 adipocytes (data not shown).

The two mouse TC10 genes share identical sequences throughout the five Ras canonical boxes (G1–G5) as well as the effector domain. The main differences between the two genes are clustered in the amino- and carboxyl-terminal ends (Fig. 1B). Despite these differences, they both have a unique CXXCXXAAX box, a presumed signal for palmitoylation and prenylation. We refer to these two genes as TC10α and TC10β (Fig. 1B).

**Chromosomal Mapping of Mouse TC10α**—We performed a mouse-hamster radiation hybrid panel analysis to determine the chromosomal location of TC10α (ResGen). A human 180-kb bacterial artificial cosmid clone (GenBank™ accession no. AC018662) covering human TC10α was available in the GenBank™. There are two clustered genes arranged tail-to-tail in this bacterial artificial cosmid clone. One gene represents TC10α, and the other phosphatidylinositol glycan class F (PIGF) PIGF was mapped on human chromosome 2p16-21. The human TC10α gene contains five exons spanning 38 kb (Fig. 2A). Based on the human TC10 genomic organization, we designed a PCR forward primer in intron 1 (5′-GGGCAATTCGTCCTACTTCT-3′) and a reverse primer (5′-GGGTCTAGGATCATACATT-3′) in intron 2 to amplify the mouse-specific genomic fragment by PCR for RH mapping. This primer set specifically amplified a 400-bp product using mouse 129 genomic DNA and no product using hamster A29 DNA as control. By analyzing 100 RH cell lines by PCR, mouse TC10α was localized on the distal end of chromosome 17, 20.09 cR from D17Mit129 (lod >3.0) (Fig. 2B). These mapping results re-
revealed that mouse TC10 is also close to the PIGF gene, suggesting that the genomic organization of this region is evolutionarily conserved.

Mouse TC10α and β Expression and Localization in 3T3L1 Adipocytes—Because the primary event in the CAP/CrkII/C3G pathway appeared to be the translocation of these proteins to lipid rafts in response to insulin, we suspected that TC10, as the target of these proteins, might reside in this plasma membrane microdomain. Following the transfection of 3T3L1 adipocytes with HA-tagged TC10α or β, cells were fractionated by
a continuous sucrose density gradient and immunoblotted with HA, flotillin, or caveolin antibodies. As shown in Fig. 3A, both TC10α/H9251 and /H9252 colocalized with caveolin-enriched fractions. To further confirm the localization of TC10α/H9251 and β, we electroporated HA-tagged TC10 constructs and immunostained with anti-caveolin and anti-HA antibodies. The majority of TC10α/H9251 and /H9252 were co-immunostained with caveolin on the plasma membrane and were found in rosette structures at higher magnification on the lower section of the cells, as described previously for hTC10 (Fig. 3B). Merging of the images revealed that every rosette structure identified by anti-caveolin staining exhibited staining for the two forms of TC10. These data are consistent with previous results demonstrating co-staining of the two forms of mTC10 with caveolin in rosette structures on plasma membrane sheets (16), again indicating that the localization of endogenous TC10 is restricted to lipid raft microdomains in differentiated adipocytes.

Mouse TC10α and β Are Activated in Response to Insulin—The detection of the two isoforms of TC10 in lipid rafts suggests that they might both be similarly activated by insulin. To examine whether mouse TC10α and β can be activated in 3T3L1 adipocytes like human TC10α, cells were electroporated with 400 µg of HA-TC10α and β. Cells were plated on coverslips and recovered for 4–5 days. Cells were then immunostained with anti-caveolin and anti-HA antibodies as described under “Materials and Methods.”
downstream of the CAP/Cbl pathway, we coexpressed the HA-tagged forms of the proteins with a mutant form of CAP. CAPΔSH3 lacks the three SH3 domains and fails to interact with Cbl. Overexpression of this mutant blocks the translocation of Cbl to lipid raft microdomains (12). Therefore, 3T3L1 adipocytes that were transfected with mTC10α or β plus wild type CAP or the dominant-interfering CAP mutant (CAPΔSH3) were assessed for TC10 activation (Fig. 5). As expected, cotransfection with wild type CAP did not influence activation of either of the TC10 isoforms. However, activation of both forms of TC10 by insulin was inhibited by the overexpression of CAPΔSH3, indicating that TC10α and β are both downstream of the CAP/Cbl pathway.

**TC10α but Not β Blocks the Stimulation of Glucose Transport by Insulin**—Our previous studies revealed that overexpression of wild type, dominant negative, and constitutively active mutants of human TC10α significantly inhibited insulin-stimulated glucose uptake and GLUT4 translocation in 3T3L1 adipocytes (14). To investigate the function of mouse TC10α, β, and βL, wild type, constitutively active (Q67L for TC10α, Q69L for TC10β and Q79L for TC10βL), and dominant negative (T23N for TC10α, T25N for TC10β, and T35N for TC10βL) mutants were generated by site-directed mutagenesis (Fig. 1C). 3T3L1 adipocytes were then cotransfected with the GLUT4-EGFP fusion proteins plus the various mTC10 cDNAs, and cells were treated with or without insulin. The representative confocal immunofluorescent images are presented for the dominant-interfering mutants in Fig. 6. As established previously, insulin stimulated the translocation of an intracellular sequestered GLUT4-EGFP fusion protein to the plasma membrane, characterized by the appearance of a plasma membrane rim fluorescence (Fig. 6, panels a and e). The expression of mTC10α/T23N potently inhibited insulin-stimulated GLUT4-EGFP translocation (Fig. 6, panels b and f), mTC10β/T25N also prevented insulin-stimulated GLUT4-EGFP translocation, but was not nearly as effective as mTC10α/T23N (Fig. 6, panels c and g). Furthermore, mTC10βL/T35N had no significant effect on GLUT4-EGFP translocation (Fig. 6A, panels d and h).

Quantitation of the effects of wild type, constitutively active, and dominant-interfering mutants of mTC10α, mTC10β and mTC10βL on GLUT4 translocation is presented in Fig. 6B. Insulin stimulated the translocation of the fusion protein to the cell surface in ~75% of the transfected cells, whereas only 15% of the cells exhibited rim fluorescence in the basal state. Cotransfection with each of the three isoforms of mTC10α produced an 80% reduction in the number of cells responding to insulin. Cotransfection of the adipocytes with the corresponding forms of TC10β resulted in partial inhibition with ~50% of the cells displaying insulin-stimulated GLUT4-EGFP translocation. Although there was a very slight reduction in the extent

![Fig. 4](http://www.jbc.org/content/13071/1/13067/F4.large.jpg)  
**Fig. 4. Insulin activates both TC10α and β.** Differentiated 3T3L1 adipocytes were electroporated with 50–60 μg of HA-tagged human TC10, mouse TC10α, or mouse TC10β. Cells were starved for 3 h before treatment with 100 nM insulin. 50 μg of lysates from different time points were immunoblotted with an anti-HA antibody and are shown in the lower panel. 240 μg of lysate were incubated with GST-PAK1 for 1 h followed by four washes. An HA immunoblot is shown in the upper panel.

![Fig. 5](http://www.jbc.org/content/13071/1/13067/F5.large.jpg)  
**Fig. 5. Inhibition of TC10 activation with CAPΔSH3.** 3T3L1 adipocytes were co-electroporated with 60 μg of HA-TC10α (A) or β (B) plus 500 μg of FLAG-tagged CAP or CAPΔSH3. Cells were starved for 3 h before harvest. Cell extracts were incubated with 7 μg of GST-PAK1 for 1 h followed by three washes. Panel A shows an HA immunoblot of pull-down samples from different time points. Panel B shows the HA and FLAG immunoblots with 50 μg of lysate.
forms that perturb adipocyte cortical actin are capable of inhibiting insulin-stimulated GLUT4 translocation.

**DISCUSSION**

The stimulation of glucose transport by insulin requires both phosphatidylinositol 3-kinase-dependent and -independent pathways (9, 19). We recently described a novel signaling pathway that is segregated into a lipid raft subdomain of the plasma membrane. The insulin receptor catalyzes the tyrosine phosphorylation of the protooncogene Cbl, which is recruited to the receptor with the adaptor protein CAP (10, 11, 26). Upon Cbl phosphorylation, the CAP/Cbl complex is translocated to lipid rafts via the interaction of the SoHo domain of CAP with the hydrophobic protein flotillin (12, 13). Phospho-Cbl can in turn recruit the SH2/SH3 adapter protein CrkII to these microdomains along with the guanyl nucleotide exchange protein C3G. The insulin-stimulated recruitment of C3G to lipid rafts brings the exchange factor into proximity with the Rho family protein TC10, which appears to reside in lipid rafts because of its unique carboxyl-terminal sequences (14, 16). TC10 undergoes activation via the C3G-catalyzed exchange of GTP for GDP, and the blockade of this pathway by overexpression of dominant negative forms of CAP prevents the stimulation of glucose transport by insulin (13, 14).

A key determinant in the activation of TC10 by insulin lies in its localization in lipid raft microdomains. This property of the protein appears to be defined by its carboxyl terminal sequences. A TC10/K-Ras chimera was not activated by insulin.
and did not target to lipid rafts, whereas a TC10/H-ras chimera was insulin sensitive and localized into lipid rafts (16). Interestingly, the second isoform of TC10 described here (TC10β) is also activated by insulin treatment and is also localized in lipid rafts, providing further support for the functional importance of spatial targeting of this protein. Indeed, TC10α and β are similarly activated by insulin in a CAP-dependent manner and localized in identical fractions by sucrose density gradients as well as by immunolocalization by confocal microscopy.

Despite similarities in function, the three isoforms of mTC10 differ in their impact on insulin-stimulated GLUT4 translocation. The overexpression of mTC10α profoundly inhibits GLUT4 translocation in response to insulin, as was observed for the human form of this protein. This inhibitory effect was produced with the wild type, constitutively active, and inactive forms of the protein. In contrast, TC10β only slightly inhibited insulin-stimulated GLUT4 translocation, whereas TC10βL was essentially without effect. Although the underlying reasons for these differences in biological effect remain unknown, the ability to inhibit GLUT4 translocation appears to correlate with the disruption in cortical actin structure. This may also account for the surprising observation that overexpression of the wild type, constitutively active, and dominant-interfering mutants of the TC10α isoforms are all inhibitory. Furthermore, these findings suggest that there are important determinants of effector binding in the amino-terminal sequences, where the TC10 isoforms are the most divergent. Alternatively, the active form of the protein might prevent the interaction of endogenous TC10 with its effectors, perhaps targeting the protein to an incorrect location or dramatically changing the kinetics of the interaction. Indeed, preliminary results suggest that candidate effector molecules show different affinities for TC10α and β in pull-down and immunoprecipitation experiments. In any case, TC10 isoforms appear to serve as signaling intermediates in the CAP/Cbl pathway, and the identification of multiple TC10 proteins in lipid raft microdomains will provide important clues toward understanding how TC10 isoforms mediate the effects of insulin on its target cells.
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