Characterization of TCL, a New GTPase of the Rho Family related to TC10 and Cdc42

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GTPases of the Rho family control a wide variety of cellular processes such as cell morphology, motility, proliferation, differentiation, and apoptosis. We report here the characterization of a new Rho member, which shares 85% and 78% amino acid similarity to TC10 and Cdc42, respectively. This GTPase, termed as TC10-like (TCL) is encoded by an unexpectedly large locus, made of five exons spanning over 85 kilobases on human chromosome 11. mRNA is 2.5 kilobases long and mainly expressed in heart. In vitro, TCL shows rapid GDP/GTP exchange and displays higher GTP dissociation and hydrolases than TC10. Using the yeast two-hybrid system and GST pull-down assays, we show that GTP-bound but not GDP-bound TCL protein directly interacts with Cdc42/Rac interacting binding domains, such as those found in PAK and WASP. Despite its overall similarity to TC10 and Cdc42, the constitutively active TCL mutant displays distinct morphogenic activity in REF-52 fibroblasts, producing large and dynamic F-actin-rich ruffles on the dorsal cell membrane. Interestingly, TCL morphogenic activity is blocked by dominant negative Rac1 and Cdc42 mutants, suggesting a cross-talk between these three Rho GTPases.

The Rho GTPases form a distinct family of the Ras superfamily of low molecular weight GTP-binding proteins. Rho proteins are key elements in the regulation of dynamic assembly of cytoskeletal components, that participate in physiological processes such as cell proliferation and motility, establishment of cell polarity (reviewed in 1), as well as in physiopathological processes such as cell transformation and metastasis (2, 3). The Rho family is made of two main groups. One comprises Rho-A(C), RhoD, Rnd1–3, and RhoL; the other comprises Rac1–3, RhoG, Cdc42, TC10, TTF/RhoH, and Chp (reviewed in Ref. 4). In fibroblastic cells, RhoA–C control the formation of focal adhesions and actin stress fiber bundling (5, 6), while RhoD causes rearrangements of the actin cytoskeleton and controls early endosome motility and distribution (7). Cdc42 and TC10 regulate the formation of filopodia (8, 9), while Rac proteins are required for growth factor-induced membrane ruffling and lamellipodia formation (5). In neurons, Rho proteins are required for axonal outgrowth (10), while in phagocytic cells, they play a role in the activation of NADPH oxidase (11), the polarization of helper T cells toward antigen-presenting cells (12), and apoptosis (13).

Like other Ras-related proteins, Rho GTPases adopt either an active GTP-bound or an inactive GDP-bound conformational state. Their activity is controlled positively by guanine nucleotide exchange factors, which catalyze the replacement of GDP with GTP, and negatively by GTPase-activating proteins, which accelerate the endogenous GTPase activity (14). Once loaded with GTP, the GTPase binds to and activates a set of downstream effector proteins directly or indirectly involved in the initiation of the cellular effects (reviewed in Ref. 15). Activation or inhibition of different Rho GTPases have been reported to control the formation of similar F-actin-containing structures, which might be a consequence of two distinct mechanisms. First, this might result from activation cascades involving several GTPases. This is the case for Cdc42, whose activation promotes the formation of filopodia as well as Rac-dependent lamellipodia (6); for RhoG, which promotes the formation of filopodia and lamellipodia through Rac1 and Cdc42 activities (16); and for RhoA, whose inactivation leads to the downstream activation of Rac1 and Cdc42 (17). Second, distinct GTPases might activate identical downstream effector proteins. This might be the case for Cdc42 and TC10, which share several effectors and both trigger the formation of filopodial extensions (9). It thus emerges that identifying all GTPases of the Rho family is an essential step toward understanding how these proteins act in concert in the control of cell physiology. Here we report the identification of a new Rho member in mammals, based on its homology with Cdc42 and TC10. Sequence analysis indicates that this new protein, termed as TCL, is encoded by a 2.5-kb mRNA preferentially expressed in heart. Like TC10 and Cdc42, TCL binds Cdc42/Rac interacting binding (CRIB)

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domains of PAK and WASP. However, expression of the constitutively active TCL and TC10 proteins elicits the formation of distinct cytoskeletal structures in fibroblasts.

**EXPERIMENTAL PROCEDURES**

**Data Base Search—**Searches in human and murine data bases for sequences homologous to TC10 cDNA were done using BLAST (18) and ENTREZ NCBI web facilities. One human genomic sequence was identified (AL048971.2) as well as human ESTs (AA020825, AA020848, AA244648, AA434636, AA461242, AA923054, AA066890, AA091480, AA136146, AA368275, AA370786, AA375815, AS085092, NA40545, and W59948), and murine ESTs (AA051640, AA537565, AA126285, AI322287, AI462525, AI510273, AI920345, AI391399, AW060455, AW121127, W12115, and W16055). Human- and mouse-derived TCL protein sequences were aligned with other Rho GTPases using MULATLIN (19). Distance matrices were computed and used to draw a similarity tree using ClustalW (20; Treeview (21).

Construe—**IMAGE clones 269952 and 368723 (human) and 1383415 and 316263 (mouse) containing TCL full-length cDNAs were obtained from the United Kingdom (UK) Human Genome Mapping Project Resource Center (Cambridge, UK). The human TCL open reading frame (ORF) was amplified by PCR and cloned in pBlueScript KS. Q79L and T355 mutations were introduced using the Gene Editor kit (Pall Biotechnology). The PCR box was deleted by XhoI digestion. The original clones as well as wild-type and mutated PCR fragments were fully sequenced. Wild-type and mutated ORFs were subcloned in prokaryotic pGEX-4T-2 vector (Life Science), yeast pBTM116, and mammalian pEGFP-C2 (CLONTECH). Pak-1 ORF was obtained by PCR amplification and subcloned in pGAD1318. Wasp fragment (amino acids 201–321) containing the CRIB domain was swapped from pGEX-KG to pGAD1318. Pak-1-Kinectin (coding amino acids 677–913) was isolated from an interaction screen with RhoG (16). Gex-PAK expressing GST protein fused to human PAK1B CRIB domain (amino acids 5–131) was kindly provided by Anja Bathoorn (Netherlands Cancer Institute, Amsterdam, Netherlands). For in vitro interaction purposes, the insert containing PAK1B CRIB domain was swapped from pGEX-KG to pGAD1318. Pak-1-Kinectin (coding amino acids 677–913) as well as human 293 (Bosc) cells were cultured at 37 °C in the presence of 5% CO2 in Dulbecco’s modified Eagle medium supplemented with 1% fetal calf serum. Cells were plated on 18-mm diameter glass coverslips 16–24 h before transfection. Cells were transfected using the LipofectAMINE method, as described by the supplier (Life Technologies, Inc.). Four hours after the transfection, the medium was replaced by Dulbecco’s modified Eagle medium supplemented with 10% fetal calf serum. Expression levels were observed under fluorescence microscopy 8–24 h after transfection.

**GST-CRIB Pull-down Assay—**Interactions were performed as described by Sander et al. (23). Boc cells were transfected with constructs expressing enhanced green fluorescent protein (GFP) alone (CLONTECH) or fused to the constitutive active mutants of Cdc42, RhoG, TC10, and TCL. 24 h after transfection, cells were rapidly washed in ice-cold PBS and lysed on ice in 50 mM Tris-HCl (pH 7.4), 2 mM MgCl2, 1% Triton X-100, 10% glycerol, and 100 mM NaCl, and protein inhibitor mixture. Lysates were centrifuged for 5 min at 17,000 × g at 4 °C, and samples were taken from the supernatant to estimate total protein concentration. 20 μg of GST-CRIB fusion bound to Sepharose beads were added to cell lysate and incubated for 30 min at 4 °C. Beads were washed three times in PBS, re-suspended in lysis buffer, and bound proteins were eluted in Laemmli sample buffer. Total proteins and CRIB affinity-purified proteins were analyzed by Western blotting using a monoclonal antibody directed against GFP (CLONTECH).

**Immunohistochemistry—**Cells were fixed for 5 min in 3.7% formalin (in PBS) followed by a 2-min permeabilization with 0.1% Triton X-100 (in PBS) and incubation in PBS containing 0.1% bovine serum albumin. Primary antibody incubations were performed in GB-1 (1:250 dilution) for 2 h at room temperature. Cells were then washed in PBS, containing 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 100 μM EDTA, and 0.25 mg/ml -1 incubated on ice for 1 h, and sonicated. Unsoluble material was removed by centrifugation, 300 μl of glutathione-S-Sepharose 4B beads (Amersham Pharmacia Biotech) were added to the cleared lysate, incubated for 30 min at 4 °C, and washed three times in PBS. GST fusion proteins were eluted twice in 50 mM Hepes-NaOH (pH 7.5) containing 10 mM dithiothreitol, 1 mM MgCl2, 1 μM GTP, and 20 mM reduced glutathione (Sigma). Recombinant GST-TCL was further used for biochemical studies and for raising antibodies. Rabbits were submitted to three rounds of injection of 0.1 mg of GST-TCL. Immunogens were depleted on GST-bound Sepharose, then affinity-purified on GST-TCL-bound Sepharose.

**Expression and GTP Hydrolysis Assays—**For loading assays, 1 μM GTPase was incubated with 10 μM nucleotide ([32p]GTP-S or [3H]GDP) in Load Buffer (50 mM Hepes-NaOH (pH 7.4), 100 mM KCl, 2 mM MgCl2, and 1 mM dithiothreitol). For off-rate measurements, 1 μM GST-GTPases was incubated for 2 min at 37 °C in the presence of 50 μM nucleotide (ppGpp or GDP) in MgCl2-free Load Buffer. Nucleotide exchange was started by adding 2 mM MgCl2 and 1 mM unlabeled GTP. For loading and exchange kinetics, 25-μl aliquots (25 pmol) were taken at different time points, added to 55 μl of cold “Stop Buffer” (50 mM Tris-HCl (pH 7.5), 10 mM MgCl2,) and filtered on 0.45-μm membranes (Schleicher & Schuell). kcat values were calculated assuming single-exponential kinetics. For GTP hydrolysis assays, GST-GTPases were loaded for 2 min at 37 °C with 10 μM [32p]GTP in MgCl2-free Load Buffer supplemented with 5 mM Mg2+. GTP hydrolysis was started by adding 2 mM MgCl2, 25-μl aliquots (25 pmol) at different time points, mixed with 975 μl of charcoal in 50 mM NaH2PO4, and centrifuged. [32p]P, release was measured by counting the supernatant.

**RNA Hybridization—**Mouse MTN blot (CLONTECH) was pre-hybridized for 30 min. at 65 °C in 5 ml of ExpressHyb buffer (CLONTECH), then hybridized for 1 h at 65 °C in the same buffer containing denatured [α-32P]UTP-labeled murine probe (1.2 × 108 cpm/ml).
using a 63× (numeric aperture, 1.32) objective mounted on a piezoelectric stepping motor. Stacks were first restored with Huygens (Scientific Volume Imaging b.v, Hilversum, The Netherlands). Briefly, Huygens is an iterative program that encodes light as 32-bit gray levels and reassigns it at high probability to specific voxels in the stack using a point spread function. This results in removing fuzziness contained in the stack. In the present study, the maximum likelihood estimation algorithm was used throughout (24, 25). Restored stacks were then further processed with Imaris (BitPlane, Zurich, Switzerland) for visualization and volume rendering. Huygens and Imaris programs were run on a four-processor Origin 2000 and a two-processor Octane (SGI), respectively.

Time-lapse Video Microscopy—For living cell studies, a laboratory made device maintained cells in a 37 °C, 5% CO2, 80% relative humidity atmosphere. Epifluorescence illumination was ensured by a halogen light bulb (100 watts). GFP positive cells grown on 0.17-mm-thick glass coverslip were studied 18 h after transfection with an inverted DMRBE using a 63× (numeric aperture, 1.32). Cell images were captured (exposure time, 400 ms) every 10 s for 10 min as time series of 16-bit files. For video presentation, selected images were cropped, assembled, and compressed using Quicktime (version 4.0).

Scanning Electron Microscopy (SE)—Transfected REF-52 cells were grown on glass coverslips, fixed in 0.1 M sodium cacodylate (pH7.2) containing 2% glutaraldehyde and 0.1 M sucrose for at least 1 h, and processed for SE as described (26). Samples were observed using a Hitachi S4000 scanning microscope at 15 kV. For all experiments, at least 50 cells were examined. Images were processed as above. To estimate the fraction of transfected cells, one coverslip was processed for F-actin, TCLQ79L, and TC10Q75L detection by immunofluorescence. In all experiments, the same proportion of cells with altered morphology was observed either by SE or immunohistochemistry.

RESULTS

Characterization of TCL, a TC10-like Protein—A general survey of nucleotide sequences encoding new GTPases of the Rho family was performed in human and murine expressed sequence tag (EST) data bases. We isolated a set of 16 human and 14 murine EST sequences with 70% identity to human or murine TC10 ORF (27). These sequences showed lower levels of identity with Cdc42, RhoG, or Rac1. Merging overlapping ESTs produced a 2,195-bp human and a 2,252-bp murine consensus cDNA sequences, whose ORFs were 87% identical. ORFs from both species were PCR-amplified from IMAGE clones 269952 and 316263, respectively, and fully sequenced (accession numbers AJ276567 and AJ276568). Human and murine ORFs potentially encode 214 and 204 amino acids proteins, respectively, that share 95% sequence similarity (93% identity), all changes being clustered within the amino-terminal and carboxy-terminal ends. These proteins contain the canonical G1–3 boxes involved in the nucleotide binding and conserved among Ras-like GTPases, as well as a two-cysteine “CC AAX” box, a substrate for geranylgeranyl- and farnesyltransferases. Protein sequence alignment with other GTPases of the Rac/Cdc42 subfamily showed that, like TC10, these proteins contain an amino-terminal extension compared with Cdc42, Rac, or RhoG, which differs in length between man and mouse (Fig. 1A). Sequence alignment of human Rho proteins was next used to compute pairwise similarity matrices and to draw a similarity dendrogram, which clearly showed that the new protein shares a significant stem with TC10 (Fig. 1B). For these reasons, this protein will be referred to as TCL (TC10-like).

TCL Gene Structure—Data base search also revealed significant matches within a 169,645-bp genomic fragment from human chromosome 14 (AL049871.2, Genoscope, France). Further analysis showed that this fragment contains all genomic counterparts of whole of TCL cDNA sequence (Fig. 2). The human TCL gene is made of 5 exons, the first one (39,379–40,011) encoding the 5′-untranslated region and the first 59 amino acids. The second exon (102,071–102,132) encodes amino acids 60–79 and is separated by an unusually long intron (62,061 bp) from the first exon. Exon 3 (113,934–

![Fig. 1. Comparison of TCL protein with other Rho proteins.](image-url)
114,099) encodes amino acids 80–134, exon 4 (116,082–116,178) encodes amino acids 135–165, and exon 5 (123,839–125,067) amino acids 166–214. Thus, the human TCL gene represents the largest gene encoding a Rho GTPase characterized so far, spanning over 86 kb.

**Biochemical Properties of TCL—** We next examined the kinetics of [35S]GTPγS and [3H]GDP loading of TCL. Since the recombinant protein was purified in the presence of GDP, GDP dissociation represents the first and the rate-limiting step. The increase in TCL bound to labeled nucleotides therefore reflects the dissociation of the original bound GDP. At 37 °C and in a physiological buffer containing 2 mM MgCl2, TCL bound [35S]GTPγS and [3H]GDP at the same rate (t1/2 = 2 min) (Fig. 4A). Assumming single-exponential kinetics, TCL dissociates from GDP at an apparent rate of 0.34 ± 0.05 min⁻¹. Fig. 4B shows GTP–GDP dissociation kinetics of TCL and TC10. GTPases were first loaded with [35S]GTP under conditions of low magnesium buffer and then allowed to dissociate from the labeled nucleotide by incubation at 37 °C with 2 mM MgCl2 and 1 mM cold GTP. Under these conditions, TCL dissociated from GTP–S with a koff approximating 0.085 ± 0.01 min⁻¹, while TC10 showed a slower dissociation rate (koff = 0.055 ± 0.008 min⁻¹). These koff values are higher than values previously determined at 30 °C for TC10 (0.006–0.018 min⁻¹) (9, 28). Fig. 4C shows the basal GTPase activity of TCL and TC10. GTPases were loaded with 32P–GTP in low magnesium, and the release of 32Pi, was measured at 37 °C after 2 mM MgCl2 addition.

Under these conditions, TCL hydrolyzed 7% of its bound GTP per minute, while TC10 hydrolyzed 5% of it. These values are in the range of those reported for TC10 hydrolysis at 37 °C (0.06 min⁻¹) (28).

**Active TCL Interacts with PAK and WASP—** As mentioned above, TCL and TC10 protein sequences are highly similar in their amino-terminal region, and are strictly conserved in their effector loop (HsTCL amino acids 48–59, Fig. 4A). To evaluate whether TCL can bind targets containing a CRIB domain as TC10 does, we first generated TCL mutants expected to be preferentially loaded with GDP or GTP. To produce a constitutively active TCL protein, glutamine 79 was replaced by leucine (Q79L). This substitution is equivalent to the classical G61L mutation previously shown to reduce the overall GTP hydrolysis rate in other Rho GTPases (reviewed in Ref. 27). We also replaced threonine 35 by an asparagine (T35N), whose equivalent substitution in Ras (T17N) has been reported to lock the GTPase in the GDP-bound form (30). Both mutants were expressed as LexA fusions and assayed for interaction with PAK-1 and with the CRIB domain of WASP fused to Gal4. As shown in Fig. 5A, a high β-galactosidase activity was detected when LexA-TCLQ79L was co-expressed with Gal4-DPAK-1 or Gal4-PAK-1 while RhoG12V did not show any interaction. Yeasts co-expressing LexA-TCLQ79L failed to produce any β-galactosidase activity, indicating that only the GTP-bound form of TCL binds PAK-1 and WASP. Similar levels of β-galactosidase activity were observed when LexA-TC10Q75L or LexA-Cdc42G12V were expressed, while RhoG12V did not show any interaction. Yeasts co-expressing LexA-TCLQ79L or LexA-Cdc42G12V failed to produce any β-galactosidase activity, indicating that only the GTP-bound form of TCL binds PAK-1 and WASP. Similar levels of expression were detected for Cdc42, TC10, and TCL constructs in yeast extracts (Fig. 5A, lower panel). As a negative control, we co-expressed Gal4 fused to a kinase fragment, which specifically binds to RhoG12V and Rac1G12V (16). This fragment failed to produce any interaction with TCLQ79L, TC10Q75L, and Cdc42G12V fusions. The interaction of GTP-bound TCL was confirmed in mammalian cells using a CRIB affinity assay (23) (Fig. 5B). Lysates from Bosc cells expressing GFP or GFP fused to Cdc42G12V, RhoG12V, TC10Q75L, or TCLQ79L were incubated with GST-CRIBPAK bound to Sepharose beads. Western blotting analysis of bound proteins using an anti-GFP antibody shows that GFP-Cdc42G12V, GFP-TC10Q75L, and GFP-TCLQ79L were efficiently bound, whereas GFP or GFP-RhoG12V proteins were not detected (Fig. 5B, upper panel). As a control, lysates from GFP-TCLQ79L-expressing cells were incubated in the presence of GST (Fig. 5B, last lane), which produced no interaction. The presence of wild-type and fusion GFP in total lysates was checked.
by Western blotting, which showed that all constructs had been expressed at comparable levels (Fig. 5B, lower panel). To establish that TCL directly interacts with CRIB domains, affinity-purified MBP fused to CRIBPAK was incubated with glutathione beads bound to GST-TCLwt loaded with GDP or GTP. Fig. 5C shows that TCL.GTP efficiently interacted with MBP-CRIBPAK, while barely detectable levels were observed with TCL.GDP (upper and lower right panels). MBP alone produced no interaction with either form of TCL. The amount of input and pulled-down purified proteins was controlled by Western blotting using anti-MBP and anti-GST antibodies (upper panels), anti-TCL antibodies (middle panels), and Ponceau staining (lower panels). These experiments therefore establish that TCL efficiently and directly binds CRIB domains.

Active TCL Modifies the Actin Cytoskeleton of REF-52 Cells—Previous studies have established that expression of TC10Q75L produces numerous peripheral filopodial extensions and a reduction in the level of stress fibers, a phenotype similar but exaggerated compared with that of Cdc42G12V (9). We thus looked for differential morphogenic activity of TCL Q79L in REF-52 fibroblastic cells. To this aim, TCL Q79L and TC10Q75L were expressed as carboxyl-terminal fusions with the GFP and the distribution of F-actin was examined. 100% of cells expressing GFP-TCLQ79L (Fig. 6A, panel a) displayed strong and localized F-actin accumulation (panel d), as well as an overall reduction in actin stress fibers. In 40% transfected cells, a low number of thin F-actin-rich peripheral extensions were observed (Fig. 6C), in comparison, about 80% of cells expressing GFP-TC10Q75L (panel b) produced numerous

Fig. 4. Biochemical properties of E. coli expressed GST-TCL protein. A, kinetics of GDP and GTP-S binding on TCL (25 pmol/point) at 37 °C in 2 mM Mg²⁺. B, kinetics of GTP-S dissociation from TCL and TC10 at 37 °C in 2 mM Mg²⁺ with 1 mM GTP as competitor. C, GTP hydrolysis by TCL and TC10 proteins at 37 °C in 2 mM Mg²⁺.
filopodial extensions (>30/cell) associated with a reduction in actin stress fibers and the presence of F-actin dots, which might correspond to small protrusions on the dorsal membrane (panel e). We also examined the effects of GFP-TCL35N expression (panel c). All expressing cells maintained a morphology similar to control cells but showed a slight but significant increase in actin stress fibers (panel f), suggesting that this mutant indeed behaves as a dominant inhibitor. The phenotypic differences observed between TCLQ79L and TC10Q75L were not a consequence of differential expression, since comparable efficiencies of transfection and expression were obtained for all constructs (Fig. 6B). To further characterize F-actin structures elicited by TCLQ79L expression, images of a TCL-expressing cell region were acquired at different focusing planes and processed for deconvolution and volume rendering. A deconvolved section at 4.7 μm above the stress fiber plane clearly showed that the strong F-actin signal observed on the cell shown in Fig. 6C (boxed in panel b) is distributed according to a ruffle pattern (Fig. 6D, panel a). This was better evidenced by volume rendering (Fig. 6D, panel b), which showed that the overall structure adopts a ruffle morphology.

Effects of Active TCL on Cell Morphology—To compare the effects of TCLQ79L and TC10Q75L expression on cell morphology, we next analyzed transfected cells by scanning electron microscopy (Fig. 7). More than 40% of cells (which would correspond to at least 80% of TCLQ79L expressing cells) displayed an elongated morphology, with a low number of long and thin extensions at their periphery (panel a). Most cells exhibited one to three large protrusions on the dorsal membrane (boxed in panel a and 8-fold enlarged in panel c). These dorsal structures show a shape very similar to the one obtained after volume rendering of F-actin structures (see Fig. 6D, panel b). By comparison, cells expressing TC10Q75L (Fig. 7, panel b) displayed a motile cell morphology, associated with the formation of numerous filopodia. A reduced number of wormlike structures was observed on the dorsal membrane, in consistency with the

Fig. 6. Cytoskeletal effects of active TCL. A, rat REF-52 fibroblasts were transfected with plasmids allowing the expression of GFP fused to TCLQ79L (panels a and d), TC10Q75L (panels b and e), and TCL35N (panels c and f). 18 h after transfection, cells were fixed and examined for GFP fluorescence (panels a–c) and F-actin distribution (panels d–f). For each panel set, at least 100 independent expressing cells were analyzed. Bar, 10 μm. B, transfection and relative expression efficiency of GFP constructs. 18 h after transfection, the fraction of GFP-positive cells was measured from three independent experiments by examining a 100-cell population (shaded bars). GFP intensities were quantified from a 400-ms exposure time images on at least 50 positive cells using the MetaMorph program (open bars). Values correspond to the average of 12-bit encoded integrated fluorescence levels of expressing cells. Error bars represent S.E. values. C, TCLQ79L expressing REF-52 used for image deconvolution and volume rendering. The cell was visualized for GFP (panel a) and stained for F-actin (panel b). The actin structure used for image processing is boxed. Bar, 10 μm. D, detailed analysis of TCL-dependent actin structures. Panel a, optical section of the actin structure boxed in C). A stack of 80 optical sections (z step = 0.088 μm) was deconvolved using the Huygens system. Shown is a processed optical section located at 4.7 μm above the stress fibers plane. Bar, 1 μm. Panel b, volume rendering of the actin structure boxed in C). Deconvolved images were processed with Imaris 3.0 (BitPlane). (x, y, z) represents original coordinate axes of the image stack. Arrowheads indicate stress fibers.
F-actin staining (Fig. 6A, panel e). Identical overall level of expression were observed for both constructs, as monitored by Western blotting (Fig. 7, panel d) and epifluorescence analysis (data not shown). To get an insight in the way TCL- and TC10-dependent structures are dynamically regulated, we analyzed expressing cells by time-lapse videomicroscopy (see Supplemental Material). TCLQ79L-expressing cells exhibited a limited number (average 2.2 ± 0.5, n = 27) of very dynamic dorsal ruffles whose activity appeared associated with the formation of large cytoplasmic vesicles. In contrast, TC10 produced an average of 6.5 (± 1.2, n = 15) dynamic peripheral flat protrusions in which 5–10 filopodia showed back and forth elongation. Although small lamellipodia were occasionally observed, dorsal structures or cytoplasmic vesicles were never detected in TC10Q75L-expressing cells. These data confirm that, although sharing a high similarity in their primary structures, TCLQ79L and TC10Q75L elicit different effects on cell morphology.

Dominant Negative Rac1 and Cdc42 Inhibit TCL Activity—
The presence of dorsal structures in TCLQ79L-expressing cells prompted us to examine whether Rac1 and Cdc42 might participate to the establishment of TCL phenotype (Fig. 8). To this aim, we coexpressed in REF-52 cells GFP-TCLQ79L (panels a and d) with Myc-tagged Rac1T17N (panel b) or Cdc42T17N (panel e). These mutants have been previously shown to act as inhibitors of endogenous Rac1 and Cdc42 activities (reviewed in Ref. 1). As observed on panel c, cells coexpressing TCLQ79L and Rac1T17N still displayed an elongated shape, but maintained a high level of actin stress fibers and were devoid of ruffles on the dorsal membrane. Cells coexpressing TCLQ79L and Cdc42T17N (panel f) were also devoid of ruffles and maintained a high stress fiber content. As a control, cells coexpressing GFP-TCLQ79L and Myc-tagged Rac1wt (panels g and h) still showed a reduced stress fiber content and elicited the formation of dorsal ruffles. Expression of Myc-tagged Rac1 and Cdc42 alone had no effect on F-actin distribution (data not shown), as reported previously (16, 22). This indicates that coexpression of the dominant negative versions of Rac1 and Cdc42 inhibit the establishment of TCLQ79L phenotype.

**DISCUSSION**

The Rho family of GTPases represents a group of more than fifteen proteins sharing at least 50% identity in their amino acid sequence. These proteins have been shown to control many aspects of cell physiology, including the formation of dynamic F-actin rich structures, cell proliferation, and apoptosis, as well as cell transformation. The initiation of these cellular events is mediated by guanine nucleotide exchange factors, acting upstream of the GTPases (31), and by protein kinases, lipid kinases, coiled-coil proteins, and transcription factors, acting downstream of the GTPases (15). Rho proteins have been shown to display different patterns of tissue expression, and to exert their effects through a coordinated network of regulation. Here we report the identification of TCL, a new member of the Rho family more closely related to TC10 and Cdc42. We found that TCL is encoded by an unusually large gene located on human chromosome 14 and shows high levels of mRNA expression in heart. As far as the protein is concerned, the dominant active TCL protein binds to the same CRIB-containing effectors as TC10 and Cdc42. Despite this similarity, expression of the dominant active TCL in fibroblastic cells exhibits unique features, eliciting the formation of F-actin-rich structures on the dorsal membrane, sensitive to Rac1 and Cdc42 inhibition.
The identification of TCL originated from a global search in nucleic acids data bases for expressed cDNA sequence tags encoding new GTPases of the Rho family. Although we also searched for sequences similar to human Rac1, Cdc42, RhoG, TC10, Chp, and RhoA, only TCL sequence was recovered as a new Rho GTPase. This might reflect a situation in which nearly all sequences encoding Rho GTPases have been now determined. However, a limited number of Rho-encoding mRNAs might still remain unknown that are either expressed at very low levels or in tissues not used for constructing cDNA libraries. Indeed, although we found many ESTs for other Rho proteins, only two sequences homologous to Chp (32) were found in the human EST data base and none in the murine data base. Human genomic database search revealed that TCL gene is located on chromosome 14 and is made of five exons, spanning over 86 kb. This represents an unusually large locus for a Ras-like GTPase. Indeed, Ras-like genes identified so far span from 4 kb (Ha-, Ki-, N-, and R-Ras) to 25 kb (RhoG) (33). The human TCL gene might be even larger, since most Ras-like genes contain a 5'-non-coding exon (34). Nevertheless, analysis of the genomic sequences upstream of the first exon did not identify transcription regulatory elements such as TATA or CAAT boxes nor polypyrimidine tracts usually found in the 3' end of intronic sequences. Instead, it revealed the presence of three ESTs unrelated to TCL (AL039470, AL039471, and AL698602) corresponding to a distinct transcription unit located 5 kb upstream of TCL. These data suggest that the promoting sequences of TCL gene should not lie farther than 5 kb upstream of the first exon.

The characterization of TCL as a new protein closely related to TC10 and Cdc42 raises the question as to whether these members control distinct or redundant pathways. We observed that TCL is preferentially expressed in heart, while TC10 is expressed at high levels in heart and skeletal muscle, Cdc42 being ubiquitously distributed (9). Our data also demonstrate that TCL has the ability to bind the same CRIB-containing effectors as TC10 and Cdc42. Thus, the comparison of their respective tissue expression and downstream effectors would rather call for a functional redundancy between TCL and TC10. As far as biochemical activities are concerned, our data show that TCL exhibits dissociation and hydrolysis rates both 40% higher than TC10. In addition, TCL rapidly exchanges GDP for GTP. This suggests that wild-type TCL might be predominantly bound to GTP in vivo, unless down-regulated by a GTPase-activating protein. This supports our observations that overexpression of wild-type TCL induces the same phenotypic changes as TCLQ79L, although at a lower efficiency (data not shown).

Despite the similarities between TCL and TC10, we observed striking differences in their cellular effects. Whereas TC10Q79L expression produces numerous filopodial extensions and microvilli, TCLQ79L expressing cells show a limited number of ruffled structures on their dorsal membrane associated with large intracytoplasmic vesicles (see videos in Supplemental Materials). How so closely related proteins might produce so distinct effects as well as downstream targets should help elucidating the mechanisms by which TCL exerts its morphogenic activity.

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