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HAL Id: hal-02267464
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Submitted on 30 Jun 2020

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The GTP/GDP Cycling of Rho GTPase TCL Is an Essential Regulator of the Early Endocytic Pathway

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Submitted April 24, 2003; Revised August 1, 2003; Accepted August 20, 2003
Monitoring Editor: Anne Ridley

Rho GTPases are key regulators of actin dynamics. We report that the Rho GTPase TCL, which is closely related to Cdc42 and TC10, localizes to the plasma membrane and the early/sorting endosomes in HeLa cells, suggesting a role in the early endocytic pathway. Receptor-dependent internalization of transferrin (Tf) is unaffected by suppression of endogenous TCL by small interfering RNA treatment. However, Tf accumulates in Rab5-positive uncoated endocytic vesicles and fails to reach the early endosome antigen-1–positive early endosomal compartments and the pericentriolar recycling endosomes. Moreover, Tf release upon TCL knockdown is significantly slower. Conversely, in the presence of dominant active TCL, internalized Tf accumulates in early endosome antigen-1–positive early/sorting endosomes and not in perinuclear recycling endosomes. Tf recycles directly from the early/sorting endosomes and it is normally released by the cells. The same phenotype is generated by replacing the C terminus of dominant active Cdc42 and TC10 with that of TCL, indicating that all three proteins share downstream effector proteins. Thus, TCL is essential for clathrin-dependent endocytosed receptors to enter the early/sorting endosomes. Furthermore, the active GTPase favors direct recycling from early/sorting endosomes without accumulating in the perinuclear recycling endosomes.

INTRODUCTION

In addition to the prominent role of the GTPases of the Rab family as regulators of membrane traffic (Zerial and McBride, 2001), the actin and microtubule cytoskeleton plays a key role in vesicle movement and fusion through the action of the small GTPases from the Rho family (Etienne-Manneville and Hall, 2002). Rho proteins are molecular switches that are mostly known for their effects on actin cytoskeleton remodeling. The Rho family contains 21 members in mammals (Vignal et al., 2003), among which is TCL, the closest relative to TC10 and Cdc42 (Vignal et al., 2000). Like Cdc42, Rac1, and TC10, TCL can bind to proteins containing a Cdc42/Rac interactive binding (CRIB) domain. Overexpression of TCL induces cortical actin reorganization, as well as the formation of very dynamic ruffle-like structures associated with the formation of large cytoplasmic vesicles in REF52 fibroblast cells (Vignal et al., 2000). Although TCL and TC10 share common effectors, they have differential effects on actin cytoskeleton reorganization (Vignal et al., 2000). The divergence in their C-terminal domains suggests that the two proteins might be targeted to different intracellular membranes and thus have distinct functions.

Rho GTPases are involved in various cellular processes, including adhesion, cell polarization, motility and transformation, gene activation, and vesicular trafficking (Etienne-Manneville and Hall, 2002). Several Rho GTPases apparently regulate different aspects of intracellular membrane dynamics. On uptake of extracellular material, plasma membrane reorganization occurs. This involves the GTPases Rac1, RhoA, or Cdc42, during pinocytosis (Nobes and Marsh, 2000; Fiorentini et al., 2001) and phagocytosis (Ridley, 2001a,b). These Rho GTPases may also be involved in clathrin-mediated endocytosis (Lamaez et al., 1996; Ellis and Mellor, 2000; Rojas et al., 2001). Intracellular vesicle trafficking is also regulated by Rho GTPases. RhoG regulates lysosomal dynamics (Vignal et al., 2001), whereas Cdc42 controls transport from Golgi to endoplasmic reticulum (ER) (Luna et al., 2002). Several Rho GTPases also regulate endosomal vesicle trafficking: RhoD is involved in early/sorting endosome (EE/SE) movements (Murphy et al., 2001; Gasman et al., 2003), whereas RhoB seems to control transfer from the early to the late endosomal compartments (Gampel et al., 1999). In adipocytes, TC10 participates in the regulation of insulin-dependent transfer of the glucose transporter GLUT4 from its storage compartment to the plasma membrane. Notably, TC10 overexpression inhibits insulin-stimulated GLUT4 receptor translocation (Chiang et al., 2001). Transport of the GLUT4-containing vesicles involves actin cytoskeleton reorganization induced by TC10 activation (Kanzaki et al., 2001), possibly through N-WASP recruitment (Jiang et al., 2002). In contrast to TC10, TCL does not affect GLUT4 translocation.
from its storage compartment to the plasma membrane in 3T3L1 adipocytes (Chiang et al., 2002).

Here, we investigate the specific cellular roles of TCL. We show that TCL localizes at the plasma membrane and in the EE/SE. Suppression of endogenous TCL by small interfering RNA (siRNA) perturbs the distribution of receptor-dependent internalized transferrin (Tf): Tf does not enter the early endosomes and remains in the decouated endocytic vesicles. Furthermore, Tf release is slower when TCL is knocked down. On the other hand, a constitutively active mutant of TCL (TCLda) provokes sequestration of Tf in EE/SE. If Tf cannot reach the perinuclear recycling endosomes (REs), it is still able to recycle directly from the early/sorting endosomes. Finally, TCLda and Cdc42a targeted to the TCl compartment have the same inhibitory effect as TCLda. We conclude that TCL is a new Rho GTPase involved in clathrin-dependent endocytosis: on one hand, TCL is necessary for the transport of endocytosed receptors from the decouated endocytic vesicles to the early/sorting endosomes and normal recycling, whereas on the other, TCL inactivation is required for endocytosed receptors to reach perinuclear recycling endosomes.

MATERIALS AND METHODS

Antibodies and Plasmids

Antibodies raised against GST-TCL (Vignal et al., 2000) were affinity purified on an MBP-TCL resin. Affinity-purified anti-Rab11 antibodies were described previously (Wilcke et al., 2000). Other primary antibodies: anti-transferrin receptor (TIR) and anti-green fluorescent protein (GFP) (Zymed Laboratories, South San Francisco, CA), anti-early endosome antigen-1 (EEA1) (Santa Cruz Biotechnology, Santa Cruz, CA), anti-histone H3 (Upstate Biotechnology, Lake Placid, NY), anti-p115 and anti-p230 (Transduction Laboratories, Lexington, KY), anti-calphespin D (Sigma-Aldrich, St. Louis, MO), and anti-Rab5 (BD Bioscience Pharmingen, San Diego, CA). Anti-Lamp1 antibodies were a gift from Bruno Cost (Institut Curie, Paris, France), and anti-glyceroldehyde-3-phosphate dehydrogenase (GAPDH) and anti-enriched green fluorescent protein (EGFR) were gifts from Urszula Hibner ( Institut de Genomique Moléculaire, Montpellier, France). Fluorescent Alexa 568- or Alexa 488-coupled proteins was visualized directly. Other proteins were visualized by double fluorescence. Cell extracts were centrifuged (20,000 × g for 5 min at 4°C) charge-coupled device camera 16-bit images, by using a MetaMorph (version 4.1i) control program (Princeton Scientific Instruments, Monmouth Junction, NJ) run by a PC-compatible microcomputer. Image stacks were deconvolved using Huygens2.3.5p2a program (Scientific Volume Imaging SVI, Hilversum, The Netherlands).

Antibodies and Plasmids

Subcellular Fractionation

Subcellular fractions were performed as described previously (Mary et al., 2002). Briefly, transfected HeLa cells were lysed in cold hypotonic buffer containing 10 mM Tris-HCl, pH 7.5, 0.5 mM MgCl2, 10 mM D-glucose, and 0.25 mM phenylmethylsulfonyl fluoride. Cell extracts were centrifuged (600 × g for 5 min at 4°C) to pellet nuclei and nuclei-associated structures, including the Golgi and endoplasmic reticulum membranes (N). Postnuclear supernatants were ultracentrifugated (100,000 × g for 45 min at 4°C) to pellet cytoplasmic membranes (M) and cytosolic proteins (C). Samples were fractionated on a 12% SDS-polyacrylamide gel and transferred to nitrocellulose membranes.

Percoll Gradients

Percoll gradients were performed as described previously (Mereses et al., 1995). Briefly, transfected HeLa cells were scrapped and pelleted for 5 min at 800 rpm, overlaid with 3 ml of homogenization buffer (250 mM sucrose, 10 mM triethanolamine, 10 mM acetic acid, 1 mM EDTA, pH 7.4, and 1 mM phenylmethylsulfonyl fluoride) and centrifuged for 5 min at 3000 rpm. Cells were resuspended in 1 ml of homogenization buffer and homogenized by 10 passages through a 22-gauge needle. After centrifugation for 10 min at 3000 rpm, the postnuclear supernatant was loaded at the top of a 9-mL cushion of 27% (w/v) sucrose in homogenization buffer. Gradients were centrifuged in a SW55 rotor for 65 min at 23,000 rpm, 4°C. Fractions of 0.5 ml were then collected from the top of the gradient. Each fraction was supplemented with 0.25% NP-40 and centrifuged for 25 min at 70,000 rpm in a TL100.2 rotor. Percoll-free supernatants were precipitated with 40% ammonium sulfate when appropriate and used for Western blots.

Quantification of mRNA by Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

HeLa cells cotransfected with pSuper constructs and pEGFP were sorted 24 h after transfection by using a FACSAvant (BD Biosciences, San Jose, CA) measuring GFP fluorescence in FL-1. Total RNAs were then extracted from sorted transfected cells and oligoT-primed cDNAs were prepared using Superscript II reverse transcriptase (Invitrogen). The levels of the various cDNAs were determined by quantitative PCR by using the SYBR Green I technology on a Light Cycler (Roche Diagnostics, Sommerville, NJ) with the Taq Platinum DNA polymerase (Invitrogen). The primer pairs were used as follows: 5’-GTCATCCTACGACGCTTCC-3’ and 5’-GGATGACCCACACATCCC-3’ for TCL and 5’-CTTCGACCACTACGCAGTCA-3’ and 5’-GTTCCTTCATGCTGCTCTATGTC-3’ for TC10. The specificity of the primers was tested by DNA sequencing. All data are presented as a ratio to the GAPDH cDNA level (95% confidence limits).

Transferrin Internalization and Release

HeLa cells were depleted from endogenous Tf by incubation at 37°C for 1 h in internalization medium (DMEM, 10 mM HEPES, pH 7.4, and 0.1% BSA). For steady-state studies, cells were incubated for 45 min at 37°C with fluorescent transferrin (100 μg/ml) in internalization medium and then rinsed twice with ice-cold internalization medium before
fixation in paraformaldehyde. For Tf chase experiments, cells were first incubated for 20 min at 19°C with fluorescent-labeled Tf as described above, rinsed twice with ice-cold internalization medium, and then incubated for 20 min at 37°C in internalization medium, rinsed, and fixed as described above. Tf uptake and release were performed essentially as described previously (Peres et al., 2002). Briefly, for Tf uptake cells expressing GFP, GFP-TCLda or TCL siRNA was incubated in recycling medium (DMEM, 10% fetal calf serum, 10 mM HEPES, pH 7.4, 0.1% BSA, and 50 μg/ml unlabeled Tf). At desired time points, cells were fixed in 1% paraformaldehyde and fluorescence-activated cell sorting (FACS) analysis was performed using a FACScalibur (BD Biosciences), measuring GFP fluorescence in FL1 and Alexa 647 in FL-4. At last 10^6 cells were counted in each window.

RESULTS

TCL Is Inserted in Plasma and Intracellular Membranes of HeLa Cells

The expression and subcellular localization of TCL was studied using affinity-purified polyclonal antibodies to TCL (Vignal et al., 2000). On Western blots, the antibodies recognized myc-tagged wild-type TCL (mycTCLwt) expressed in HeLa cells, unlike the preimmune serum (Figure 1A, a). They immunoprecipitated endogenous TCL that migrated in SDS-PAGE as a 25-kDa band. This comigrated with the immunoprecipitated protein expressed from the full-length TCL cDNA inserted in a bicistronic vector (Figure 1A, b). Anti-TCL and anti-myc antibodies showed the same staining pattern in HeLa cells expressing myc-TCLwt. Thus, our anti-TCL antibodies detected overexpressed TCL by immunofluorescence (Figure 1B, a and b), whereas preimmune serum did not (Figure 1B, c and d). However, we were unable to detect the endogenous protein by immunofluorescence (our unpublished data), possibly due to the low amount of endogenous protein. Notably, the anti-TCL antibodies did not recognize overexpressed TCL in Western blot and immunofluorescence experiments (our unpublished data).

To localize the protein intracellularly, HeLa cell extracts were separated in three fractions: nucleus and associated membranes (N), cytoplasm (C), and high-speed sedimenting membranes, corresponding to plasma membrane and microsomes (M) (Figure 1C). Endogenous TCL, as well as overexpressed wild-type, dominant active Q79L (TCLda) or inactive T35N (TCLdn) mutant proteins was enriched in the two membrane fractions (N and M) and absent from the cytosol (C) (Figure 1C). Deletion of the carboxy-terminal polybasic and CAAX box motives of TCL, which are required for membrane anchoring, resulted the protein relocalizing in the cytosolic fraction (Figure 1C, mycTCLΔCAAX). Postnuclear supernatants, corresponding to the C and M fractions described above were then fractionated on a 27% Percoll gradient to separate lysosomes from the plasma membrane and the other vesicular compartments (Meresse et al., 1995). Endogenous TCL protein predominantly frac-
tionated in the medium fractions of the gradient, corresponding to the vesicular compartments that contain TIR and Rab5 and in the upper fractions of the gradient corresponding to the plasma membrane. TCL was absent from the bottom lysosomal fractions containing cathepsin D (Figure 1D, a). Importantly, myc-TCLwt and myc-TCLda proteins showed similar fractionation patterns (Figure 1D, b and c, respectively), as did the overexpressed untagged protein (our unpublished data). These results show that endogenous TCL and expressed TCL mutants are inserted in cellular membranes through the C-terminal polybasic and CAAX box sequences. TCL is present at the plasma membrane and in various intracellular membrane compartments but not in lysosomes.

**TCL Localizes at the Plasma Membrane and in Early Endocytic Compartments**

TCL was localized more precisely using immunofluorescence and colocalization with endogenous makers or GFP-tagged Rab proteins. In HeLa cells expressing mycTCLwt, TCL immunoreactivity was detected at the plasma membrane (Figure 1B, a and b) and as a punctate staining throughout the cytoplasm (Figure 2, b, d, f, h, and j). TCL significantly colocalized with EEA1 (Figure 2, a and b), a
marker of the EE/SE compartment, and TIR (Figure 2, c and d), which cycles between the plasma membrane, EE/SE, and RE compartments. No colocalization was observed with the cis- and median-Golgi marker p115 (Figure 2, e–f), with the lysosomal protein Lamp1 (Figure 2, g and h) or with the trans-Golgi network marker p230 (Figure 2, i and j). The morphology of these compartments was not affected by mycTCLwt expression (our unpublished data). These results corroborate and expand the fractionation experiments, indicating that TCL localizes to the plasma membrane and compartments of the endocytic pathway.

Different Rab GTPases define distinct compartments within the endocytic pathway. Rab4 and Rab5 are in early/sorting endosomes, whereas Rab11 is found in recycling endosomes and Rab7 locates in the late endosomes (Zerial and McBride, 2001). GFP-tagged wild-type Rab GTPases have been widely used to define the various compartments of the endosomal pathway (Meresse et al., 1995; Bottger et al., 1996; Sonnichsen et al., 2000). When coexpressed with GFP-tagged Rab4, 5, and 7, myc-TCLwt shows substantial colocalization with Rab4 and Rab5 (Figure 3, a–d), but not with Rab7 (Figure 3, e and f). GFP-TCLwt only shows very limited colocalization with endogenous Rab11 (Figure 3, g and h). Dominant active TCL presented a similar distribution (our unpublished data). In HeLa cells, mycTCLdn occurred in very bright and scattered aggregates, suggesting the protein is degraded (our unpublished data). Therefore, we did not use this mutant for further functional analyses. In summary, TCL- and the Q79L-activated mutant protein associate predominantly with membranes of the EE/SE and the plasma membrane. It is absent from recycling and late endosomes, from the Golgi apparatus, and secretion vesicles.

Suppression of Endogenous TCL Expression Impairs Normal Functioning of the Early Endocytic Pathway

We next examined the effect of suppressing TCL expression on the endosomal pathway, by using an siRNA delivered by a pSuper polymerase III expression vector (Brummelkamp et al., 2002). TCL siRNA specifically inhibited the expression of GFP-tagged wild-type TCL, whereas the control siRNA had no effect (Figure 4A). To examine the effect of siRNA expression on endogenous TCL mRNA, HeLa cells were transfected with the pSuper constructs, sorted by FACS by using a GFP marker, and the amount of TCL mRNA was determined by quantitative RT-PCR. In cells expressing TCL siRNA, the level of TCL mRNA was ~40% the amount in control pSuper transfected cells, whereas TC10 mRNA was
not affected (Figure 4B). Trace amounts of TCL mRNA were detected 72 h after transfection, and the level was too low for reliable quantification (our unpublished data). The siRNA thus specifically interferes with endogenous TCL expression.

We then performed steady-state Tf internalization for 45 min at 37°C in control and siRNA-treated cells. In nontransfected cells and in pSuper or in control siRNA transfected cells (Figure 4C, stars, a and c), Tf accumulated normally in the juxtanuclear recycling endosomes, with little punctate staining throughout the cells. The latter corresponds to endocytic vesicles, early/sorting, and peripheral recycling endosomes. In contrast, cells expressing TCL siRNA presented a scattered distribution of Tf (Figure 4C, stars, b). This effect was due to the suppression of endogenous TCL, because coexpression of TCL siRNA and GFP-TCL bearing three silent mutations in the siRNA target sequence resulted in normal distribution of Tf (our unpublished data). Tf-containing vesicles did not colocalize with AP2, EEA1, or Rab11 (Figure 4D, a–d). Interestingly, in TCL siRNA-expressing cells, internalized Tf is predominantly in Rab5-positive vesicles (Figure 4D, d–f). This suggests that when TCL is absent, internalized Tf is sequestered in AP2-negative decoated endocytic vesicles without further transport to the EEA1-positive early/sorting and Rab11-positive recycling endosomes.

To analyze the effect of TCL suppression on transferrin recycling, we used FACS. HeLa cells were cotransfected with pEGFP and either pSuper expressing TCL siRNA (TCLsiRNA) or the empty pSuper vector (pSuper) were incubated with Tf coupled to Alexa 647 at 37°C. After 60 min, the mean Alexa647-Tf was measured. The inset shows the fluorescence in TCL siRNA-transfected cells and in nontransfected cells (NT). Labeled Tf was then chased with unlabeled Tf. The mean Alexa 647-Tf fluorescence remaining in the cells (percentage of remaining Tf) was measured after 0, 2.5, 5, 15, 30, and 90 min of chase in pSuper (circles) and TCL siRNA (closed diamonds)-transfected cells and in nontransfected cells (open diamonds); 100% corresponds to the amount of fluorescence measured after 60 min of internalization at 37°C and before the chase. The plots represent means ± SEM of three independent experiments of Tf release that counted at least 10^4 cells per time point.
with pEGFP and with either pSuper alone or pSuper expressing TCLsiRNA. Cells were depleted of endogenous Tf by serum starvation and then put in suspension and incubated with Alexa 647-conjugated Tf. After 60 min, Tf uptake was comparable in the TCL siRNA-expressing cells and in nontransfected (NT) cells (Figure 4E, inset). Labeled Tf was then removed and chased by unlabeled Tf for 90 min. Interestingly, during the first 20 min of chase, Tf release was significantly slower in TCLsiRNA-expressing cells compared with the nontransfected and to the control pSuper-transfected cells (Figure 5, closed diamonds, open diamonds, and circles, respectively). After 10 min, about one-half of the internalized Tf had recycled out of the nontransfected and the pSuper-transfected cells, whereas at that time >70% was still in TCLsiRNA-expressing cells. This did not reflect partial trapping of Tf in TCLsiRNA-expressing cells as after the 90 min of chase the amount of labeled Tf remaining was comparable in all cases. Thus, TCL is a new Rho GTPase essential for the normal functioning of the early endocytic pathway. Suppression of the endogenous protein leads to accumulation of receptor-bound internalized Tf in Rab5-positive decorated endocytic vesicles. TCL knock down also modifies the dynamics of Tf release.

**Expression of Dominant Active TCL Favors the Recycling of Tf Directly from Early/Sorting Endosomes**

Rho GTPases cycle between an inactive GDP-bound and an active GTP-bound form. We thus examined the effect of expressing a GTP-bound dominant active mutant (Q79L) of TCL on receptor-mediated Tf endocytosis. In most cells expressing GFP-TCLwt, Tf distribution seemed unaltered relative to control cells (Figure 5A, a and b). However, in cells expressing GFP-TCLda, Rhod-Tf presented a scattered distribution and did not accumulate in the juxtanuclear recycling endosomes (Figure 5A, c). Its distribution was normal in cells expressing dominant active Q75L TC10 mutant (TC10da; Figure 6A, a) or G12V Cdc42 mutant (Cdc42da; unpublished data). Thus, TCLda specifically perturbs the distribution of endocytosed Tf.
active and inactive form is essential for normal trafficking of clathrin-dependent endocytosed receptors.

The accumulation of internalized Tf in early/sorting endosomes observed upon dominant active TCL expression may reflect preferential recycling of Tf directly from EE/SE to the plasma membrane without prior distribution in the juxtanuclear positive recycling endosomes, or a block of Tf in EE/SE without further recycling. To examine whether Tf was still able to recycle, we used FACS to analyze nontransfected cells and cells expressing GFP alone or GFP-TCLda that were loaded with Alexa 647-labeled transferrin for 60 min at 37°C. Tf uptake was comparable in all cases (Figure 5C, inset). Labeled Tf was then removed from the medium and chased by unlabeled Tf for 90 min to allow labeled Tf release. Tf release was similar in cells expressing either GFP-TCLda or GFP and in nontransfected cells (Figure 5C). Together, these results suggest that dominant active TCL expression does not affect Tf uptake and release from the cells, but it favors the preferential recycling of Tf from early/sorting endosomes to the plasma membrane without prior transit through the pericentriolar recycling endosomes.

**TCL Function Requires Effector Proteins That Can Also Be Activated by TCL10 and Cdc42**

TCL10, Cdc42, and TCL are able to bind the same effector proteins, in particular CRIB-domain containing proteins (Joberty et al., 1999; Vignal et al., 2000). We compared the effect of the expression of dominant active TCL, TCL10, and Cdc42 on the localization of receptor-dependent internalized Tf. We analyzed steady-state Tf internalization in HeLa cells expressing the different mutant proteins. After fixation, we scored the number of transfected cells where Tf accumulated normally in the juxtanuclear recycling endosomes (see control and TCLwt, Figure 5A, a and b) and of transfected cells in which Rhod-Tf presented a scattered distribution as defined above in the case of TCLda expression (see TCLda, Figure 5A, c). Overexpression of dominant active TCL10 (TCL10da) and Cdc42 (Cdc42da) resulted in normal Tf accumulation in the juxtanuclear recycling compartment (shown for TCL10da, Figure 6A, a) in ~70% of the cells (Figure 6B). It was reported previously that the hypervariable region determines to which membrane compartment Rho proteins are targeted (Michaelson et al., 2001). To target TCL10da and Cdc42da to the same compartments as TCL, we replaced the C-terminal basic regions and CAAX boxes of TCL10da and Cdc42da with that of TCL. Remarkably, the resulting chimeric proteins (TCL10daCterTCL and Cdc42daCterTCL) affected the distribution of internalized Tf (shown for TCL10da, Figure 6A, compare a and b) in ~70% of the cells, similarly to TCLda (Figure 6B). Rhog, a more divergent GTPase, is unable to bind CRIB domain-containing proteins (Gauthier-Rouviere et al., 1998). Interestingly, targeting dominant active Rhog (Rhogda) to the TCL compartments as described above (RhogdaCterTCL) had little effect on Tf distribution (Figure 6B). Thus, TCL activity in the early endocytic pathway relies on its insertion in specific membrane compartments, as defined by its C-terminal domain, and involves binding to effector proteins that can also be activated by TCL10 and Cdc42.

**DISCUSSION**

Several studies have highlighted the role of Rho GTPases in the control of intracellular membrane traffic. We identified TCL, a new member of the Rho family that is similar to TCL10 and Cdc42 (Vignal et al., 2000). Here, we report that TCL function in the early endocytic pathway requires activation of effectors that can also bind Cdc42 and TCL10. (A) HeLa cells expressing GFP-TCL10da (a) or TCL10daCterTCL (b) were incubated with rhodamine-labeled Tf, which was then visualized microscopically. The insets show the GFP fluorescence in the transfected cells. Images shown are representative of >200 transfected cells. Bar, 10 μm. (B) HeLa cells expressing the indicated GFP-tagged chimeric proteins were incubated with rhodamine-labeled Tf for 45 min at 37°C. After fixation, internalized Tf was visualized by immunofluorescence. Bar graphs show the percentage of cells expressing the indicated GFP fusion proteins that have scattered transferrin distribution (percentage of scattered Tf). The points represent the average and SEM of four independent experiments involving >600 cells. The frequencies of cells with scattered Tf did not differ between RhoGda and RhoGdaCterTCL (stars, Mann-Whitney U test, P > 0.05 in all pairwise comparisons). All other differences were statistically significant (Mann-Whitney, P = 0.0286 for each comparison).

To examine the effect of TCLda expression on Tf distribution within the endocytic pathway, HeLa cells transfected with GFP, GFP-TCLwt, or GFP-TCLda were allowed to internalize Alexa 647-labeled Tf at 19°C for 20 min to specifically load EE/SE and prevent its further transport to REs. After uptake at 19°C, internalized Tf accumulated normally in EE/SE, labeled with EEA1, in GFP-TCLda- and GFP-TCLwt-expressing cells as in GFP-expressing control cells. As in the case of TCL suppression, TCLda expression did not affect Tf internalization (Figure 5C, inset). Labeled Tf was then removed from the medium and replaced by unlabeled Tf for 20 min at 37°C to allow Tf to be transported to the perinuclear recycling endosomes and to recycle. Cells were then fixed, stained with anti-EEA1 or anti-Rab11 antibodies, and observed by confocal microscopy. After transfer into a microscope, the transfected cells were then fixed, stained with anti-EEA1 or anti-Rab11 antibodies, and observed by confocal microscopy. After transfer into a microscope, the transfected cells were then fixed, stained with anti-EEA1 or anti-Rab11 antibodies, and observed by confocal microscopy.
localizes to the plasma membrane and early/sorting endosomes. When endogenous TCL is suppressed by siRNA, receptor-dependent internalized transferrin remains in coated vesicles and fails to enter the early/sorting and recycling endosomes. Moreover, Tf release is significantly slower upon TCL knockdown. On the other hand, TCLda causes internalized Tf to accumulate in early/sorting endosomes, from which Tf recycles directly instead of being transported to the recycling endosomes. The effect of TCLda requires its insertion in membranes of the early endocytic pathway and relies on downstream effector proteins that can also be activated by TC10 and Cdc42.

**TCL Controls the Early Endocytic Pathway**

Actin dynamics plays a key role in early steps of endocytosis in *Saccharomyces cerevisiae* (Fujimoto et al., 2000), and increasing evidence is in favor of the involvement of actin filaments in these processes in mammalian cells (Schafer, 2002). Rho GTPases are major regulators of actin cytoskeleton dynamics and thus participate in the control of endocytosis (Ridley, 2001b; Qualmann and Mellor, 2003). Both actin- and microtubule-dependent events govern the distribution of endocytosed proteins throughout the cell. The early steps of endocytosis involve actin reorganization, and actin polymerization would participate in short-range endocytic vesicle movements, whereas long-range endosome mobility toward perinuclear recycling endosomes would be mediated by microtubules (van Deurs et al., 1995; Merrifield et al., 1999; Leung et al., 2000; Murray et al., 2000; Taunton et al., 2000; Schafer, 2002).

Our present results show that the Rho GTPase TCL is localized to the plasma membrane and the early/sorting endosomes. TCL is involved in the control of the early endocytic pathway. Its activity is not required for endocytic vesicle formation: suppression of TCL expression by siRNA did not prevent receptor-dependent Tf internalization. Nevertheless, TCL is required for subsequent steps of endocytosis. In cells expressing TCL siRNA, internalized transferrin was sequestered in Rab5-positive decoupled endocytic vesicles and it was not further distributed into the early/sorting and the recycling endosomes. Moreover, suppression of TCL delayed transferrin recycling to the plasma membrane. These results suggest that TCL is involved in endocytic vesicle movements, and that it is essential for clathrin-dependent endocytosed receptors to be transported to the early/sorting endosomes (Figure 7).

Expression of TCLda also allowed normal Tf internalization in HeLa cells, contrary to what was reported for Rac1 and RhoA (Lamaze et al., 1996). Nevertheless, TCLda prevented Tf transport to the juxtanuclear recycling endosomes. Instead, Tf remained scattered in the cells and accumulated in early/sorting endosomes. TCLda did not perturb subsequent Tf release, indicating that the early/sorting endosomes are still able to move within the cell cortex. After Tf internalization, the Tf receptor can recycle through two pathways: it can go back to the plasma membrane directly from the early/sorting endosomes, or it can initially be transported to the recycling endosomes (Sheff et al., 1999).

Internalized Tf can recycle back to the plasma membrane even if the juxtanuclear recycling compartment is removed from the cells (Sheff et al., 2002). Consistent with the former, our results indicate that in intact cells, TCL activation would favor direct recycling of receptor bound Tf from the early/sorting endosomes. Recycling receptors clearly fail to enter a Rab11-positive compartment when active TCL is expressed. Membrane homeostasis between EE/SE and REs should be affected, because physiologically, the two membrane domains are actively exchanging material (Sonnichsen et al., 2000). This may explain why, although cargo molecules like Tf/TfR complexes are not reaching the Rab11-positive membranes, we still see an effect on RE cellular distribution in TCLda-expressing cells (Figure 5). The present results suggest that TCLda favors Tf direct recycling from EE/SE endosomes to the plasma membrane. The levels of active TCL may thereby influence the kinetics of individual receptor return to the plasma membrane after ligand internalization, by regulating the balance between the two recycling pathways. One is actin-dependent short range recycling from peripheral cytoplasmic compartments directly to the plasma membrane, and the other is microtubule-dependent long range recycling through the perinuclear recycling endosomes (Figure 7).

**Specific Downstream Targets or Specific Localization of GTPase and Regulators?**

TCL, TC10, and Cdc42 share extensive sequence similarities in their effector binding domains and they all bind to CRIB domain-containing proteins, such as PAK, WASP, BORG, and SPEC families, unlike the more divergent GTPase RhoG.
Rho GTPases as Regulators of Vesicle Trafficking

Several Rho family proteins localize to vesicular structures and affect their intracellular transport. This was shown for RhoB and RhoD (Ellis and Mellor, 2000; Murphy et al., 2001; Gasman et al., 2003) and more recently for Cdc42 (Luna et al., 2002, TC10 (Chiang et al., 2001; Watson et al., 2001), RhoG (Vignal et al., 2001), and TCL (this report). The involvement of Rho GTPases in the regulation of intracellular membrane traffic now seems a general property of this family of proteins (Qualmann and Mellor, 2003). Recent studies suggest that their common function may be to regulate the transport of vesicles from one compartment to another, most likely through their action on cytoskeleton dynamics (Schafer, 2002). For instance, RhOD localizes to early/sorting endosomes and is involved in controlling the movement of early and late endosomes (Murphy et al., 2001; Gasman et al., 2003), whereas RhoG regulates microtubule-dependent lysosomal transport through its interaction with kinetin (Vignal et al., 2001). RhoB is associated with multivesicular bodies and is thought to be involved in regulating their trafficking toward the late endosomes (Ellis and Mellor, 2000). Although the precise molecular mechanisms linking Rho GTPases and vesicle dynamics are still unknown, actin polymerization, driven by the Arp2/3 complex and the WASP family of proteins, seems to play an essential role in intracellular organelle movements (Taunton et al., 2000; Eizet al., 2002). This can be activated in vitro by the three Rho GTPases TCL, TC10, and Cdc42 (Abe et al., 2003). N-WASP and the Arp2/3 complex are involved both in Cdc42-regulated transport from the endoplasmic reticulum to the Golgi apparatus (Luna et al., 2002), and in TCL-dependent secretion vesicle transport (Jiang et al., 2002). N-WASP can also drive actin-dependent endosome movements (Taunton et al., 2000; Schafer, 2002). Our results suggest that TCL may have a similar function in driving actin-dependent endocytic vesicle movements toward early/sorting endosomes and to the plasma membrane. TCL suppression in HeLa cells affects transport of endocytic vesicles to EEA1-labeled EE/SE. This could reflect from impaired dynamics of the vesicles, due to the lack of actin polymerization at their surface in the absence of N-WASP recruitment by active TCL. Conversely, increasing the level of active TCL, which can recruit N-WASP at the vesicle surface (our personal observations), would maintain sustained actin polymerization at the surface of the vesicles. This would prevent their interaction with microtubules, thereby precluding their transport toward the juxtanuclear recycling compartment.

Exchange factors and/or GTPase-activating proteins most likely control the regulation of TCL activity, rather than GDP-dissociation inhibitors, which can extract GTPases from their target membranes. Fractionation analyses showed that the endogenous TCL is associated with membranes, and we could not detect the protein in the cytoplasm. Moreover, the potential GDI-RhoGTPase interaction site, as defined for Cdc42, contains identical substitutions in TCL and TC10, namely, Glu-Asp-Cys-Ser instead of Arg66-His103-His104-Arg186 in Cdc42. These substitutions rendered TCL insensitive to the action of RhoGDI, unlike Cdc42, whose membrane association is affected by RhoGDI expression (Hoffman et al., 2000). An interesting potential activator of TCL is Intersectin, an important scaffolding protein of the early endocytic pathway. Intersectin has a DbI homology domain able to activate Cdc42, and it regulates actin assembly via N-WASP and Arp2/3 (Hussain et al., 2001; Qualmann and Mellor, 2003). Given the role of Intersectin in the early endocytic pathway and the high degree of homology between TCL and Cdc42, it is tempting to speculate that Intersectin may activate TCL in vivo. In a cellular context, the effective binding of a particular target to a given Rho GTPase is difficult to estimate. Furthermore, it will be essential to know the subcellular distribution of the different partners, in particular GEFs and GAPs, to decipher the signaling cascades downstream of closely related GTPases such as TCL, TC10, and Cdc42.
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(PIP2)-induced vesicle movement depends on N-WASP and involves Nck, WIP, and Grb2. J. Biol. Chem. 277, 57771–57776.


