

## Regulation of Cystic Fibrosis Transmembrane Regulator Trafficking and Protein Expression by a Rho Family Small GTPase TC10\*

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The cystic fibrosis transmembrane conductance regulator (CFTR)-interacting protein, CFTR-associated ligand (CAL) down-regulates total and cell surface CFTR by targeting CFTR for degradation in the lysosome. Here, we report that a Rho family small GTPase TC10 interacts with CAL. This interaction specifically up-regulates CFTR protein expression. Co-expression of the constitutively active form, TC10Q75L, increases total and cell surface CFTR in a dose-dependent fashion. Moreover, co-expression of the dominant-negative mutant TC10T31N causes a dose-dependent reduction in mature CFTR. The effect of TC10 is independent of the level of CFTR expression, because a similar effect was observed in a stable cell line that expresses one-tenth of CFTR. Co-expression of TC10Q75L did not have a similar effect on the expression of plasma membrane proteins such as Frizzled-3 and Pr-cadherin or cytosolic proteins such as tubulin and green fluorescent protein. TC10Q75L also did not have a similar effect on the vesicular stomatitis virus glycoprotein. Co-expression of constitutively active and dominant-negative forms of Cdc42 or RhoA did not affect CFTR expression in a manner similar to TC10, indicating that the effect of TC10 is unique within the Rho family. Metabolic pulse-chase experiments show that TC10 did not affect CFTR maturation, suggesting that it exerts its effects on the mature CFTR. Importantly, TC10Q75L reverses CAL-mediated CFTR degradation, suggesting that TC10Q75L inhibits CAL-mediated degradation of CFTR. TC10Q75L does not operate by reducing CAL protein expression or its ability to form dimers or interact with CFTR. Interestingly, the expression of TC10Q75L causes a dramatic redistribution of CAL from the juxtannuclear region to the plasma membrane where the two molecules overlap. These data suggest that TC10 regulates both total and plasma membrane CFTR expression by interacting with CAL. The GTP-bound form of TC10 directs the trafficking of CFTR from the juxtannuclear region to the secretory pathway toward the plasma membrane, away from CAL-mediated degradation of CFTR in the lysosome.

Rho family small GTPases are molecular switches in signal transduction pathways that link cell surface receptors to a variety of intracellular responses (1). They regulate cellular processes as diverse as vesicular trafficking, cytoskeleton or-

ganization, cell polarity, cell migration, and gene expression. Like other GTPases, members of the Rho family cycle between a GDP-bound (inactive) and a GTP-bound (active) state. In the GTP-bound state, the GTPase couples signals that are initiated by growth factors, adhesion factors, cytokines, and other molecules to a large number of downstream effectors.

Rho GTPases control multiple vesicular trafficking events that include macropinocytosis, phagocytosis, clathrin-mediated endocytosis, clathrin-independent endocytosis, mast cell degranulation, and exocytosis (2). TC10, a member of the Rho family GTPase, regulates the insulin-stimulated glucose transporter Glut4 trafficking in adipocytes (3). The TC10 pathway functions in parallel with phosphatidylinositol 3-kinase to stimulate GLUT4 translocation in response to insulin (4). TC10 interacts with Exo70, a component of the exocyst complex. The activated TC10 translocates Exo70 to the plasma membrane where it assembles into a multiprotein complex that may play a crucial role in targeting GLUT4-containing vesicles to the plasma membrane (5).

CFTR<sup>1</sup> trafficking in the Golgi compartments involves a number of processes that ultimately regulate the levels of CFTR in the cell and at the plasma membrane. After passing the ER quality control system, CFTR enters Golgi compartments via coat protein complex II (6, 7). CFTR follows a non-conventional pathway that is insensitive to dominant-negative Arf1, Rab1a/Rab2 GTPases, and syntaxin 5, eventually maturing from the core-glycosylated ER form to the complex-glycosylated form (7). Cycling through a late Golgi/endosomal system may be a prerequisite for CFTR maturation (7). The exocytosis of CFTR from the Golgi to the cell surface can be constitutive or regulated in a cell type-specific manner. From the cell surface, CFTR is endocytosed via the clathrin adaptor complex I (8–10). CFTR endocytosis requires the large GTPase dynamin activity and myosin VI (11, 12). Cell surface CFTR uses a Rab5-dependent step to enter early endosomes. Endosomal CFTR then either recycles back to the surface through a Rab11-dependent step or moves to late endosomes via Rab7 or to the trans-Golgi network via Rab9-mediated processes (13). CFTR in these post-Golgi compartments is degraded in the lysosomes and proteasomes. As an additional quality control system, any  $\Delta F508$  CFTR escaping the ER quality control machinery may be degraded in the cell periphery (14).

CAL (also known as PIST (PDZ domain protein interacting specifically with TC10), GOPC (Golgi-associated PDZ and coiled-coil motif-containing protein), and FIG (fused in glioblastoma)) is a Golgi-localized, CFTR-interacting protein that targets CFTR for degradation in lysosomal systems (11). CAL has one PDZ (PSD-95/DLG/ZO-1) domain and two coiled-coil do-

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<sup>1</sup> The abbreviations used are: The abbreviations used are: CFTR, cystic fibrosis transmembrane conductance regulator; GFP, green fluorescent protein; HA, hemagglutinin; ER, endoplasmic reticulum; DMEM, Dulbecco's modified Eagle's medium.

mains. The PDZ domain binds to the C terminus of CFTR (15). Yeast two-hybrid assays or *in vitro* binding assays indicate that the second coiled-coil domain binds to TC10 and the SNARE protein syntaxin 6 (16, 17). Overexpression of CAL retains CFTR in the cell and leads to enhanced lysosomal degradation of CFTR (11, 15).

In this report, we show that TC10 interacts with CAL in mammalian cells. The constitutively active form of TC10, TC10Q75L, redistributes CAL from the Golgi to the plasma membrane. TC10Q75L specifically increases the total cellular and the cell surface and CFTR. Thus, active TC10 regulates CFTR protein in a manner opposite to that of CFTR by CAL. TC10 may be a molecular switch that regulates CFTR levels at the plasma membrane and in the post-Golgi compartments in response to intracellular and extracellular stimuli.

#### EXPERIMENTAL PROCEDURES

**Cell Culture and Transfection**—African green monkey kidney cells (COS-7) (obtained from American Type Tissue Culture) were maintained in DMEM, 20 mM L-glutamine, 100 unit/ml penicillin, 100  $\mu$ g/ml streptomycin, and 10% fetal calf serum. Media and other components were purchased from Invitrogen. COS-7 cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

**Plasmids and Plasmid Constructions**—The HA-tagged TC10 (HA-TC10) was made by PCR amplification of human lung cDNA and subcloning into Sall/NotI sites of the HA-tagged pRK5 vector (pRK5-HA) (gift from Drs. A. A. Lanahan and P. F. Worley, The Johns Hopkins University). The constitutively active form of TC10 (HA-TC10Q75L) and the dominant-negative form of TC10 (HA-TC10T31N) were constructed with QuikChange<sup>TM</sup> site-directed mutagenesis kit (Stratagene, La Jolla, CA). All of the constructs were sequence-verified in both directions by automated fluorescent sequencing (The Johns Hopkins University Biosynthesis & Sequencing Facility).

Other plasmid constructs used were GFP-CFTR and GFP-CFTR $\Delta$ TRL (18), HA-NHE-RF (19), HA-CAP70 (20), Pr-cadherin-GFP (21), and Myc-CAL (15). Frizzled-3GFP is a gift from Dr. Y. Wang (The Johns Hopkins University). The HA-tagged, constitutively active, and dominant-negative forms of Cdc42 and RhoA are gifts from Dr. D. M. Raben (The Johns Hopkins University). Full-length vesicular stomatitis virus G protein (Indiana) cloned into pCDNA3.1 was a gift from Dr. C. E. Machamer (The Johns Hopkins University).

**Immunoblotting**—The cells were harvested and processed as described previously (15). Briefly, cells were solubilized in lysis buffer (50 mM NaCl, 150 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, and Complete protease inhibitor (Roche Applied Science). The cell lysates were spun at 14,000  $\times$  g for 15 min at 4  $^{\circ}$ C to pellet insoluble material. The protein concentrations of the supernatants were quantified with a BCA protein assay kit (Pierce). The normalized supernatants were subjected to SDS-PAGE and Western blotting, followed by ECL (Amersham Biosciences). The chemiluminescence signal on the polyvinylidene difluoride membrane was directly captured by a FujiFilm LAS-1000 plus system with 1,300,000-pixel cooled CCD camera having a linearity of 3.7 orders of magnitude. Quantification was carried out within the linear range using the Image Gauge version 3.2 software (FujiFilm). GFP-tagged proteins were detected with a rabbit polyclonal anti-GFP antibody (1:1000; BD Biosciences, Boston, MA). HA-tagged proteins were detected with a mouse monoclonal HA antibody (1:2000; Roche Applied Science) or a rabbit polyclonal HA antibody (1:500; Roche Applied Science). Myc-tagged proteins were detected with a mouse monoclonal Myc antibody (1:2000; Roche Applied Science). Vesicular stomatitis virus G was detected with a rabbit polyclonal antibody (gift of Dr. C. E. Machamer, The Johns Hopkins University). Tubulin was detected with a mouse monoclonal tubulin antibody (1:1000; Sigma).

**Surface Biotinylation**—Surface biotinylation of CFTR at the plasma membrane was described previously (15). Briefly, the cell surface proteins were labeled with cell-impermeable EZ-Link<sup>TM</sup> Sulfo-NHS-SS-Biotin (sulfo-succinimidyl-2-(biotinamido)ethyl-1,3-dithiopropionate; Pierce) at 4  $^{\circ}$ C for 15 min. The cell surface proteins were isolated from lysate by incubating with immobilized NeutrAvidin beads at 4  $^{\circ}$ C for 2 h (Pierce; catalog number 53151). The bound proteins were eluted with 2 $\times$  Laemmli sample buffer supplemented with 100 mM dithiothreitol at 42  $^{\circ}$ C for 30 min. The eluted proteins were subjected to SDS-PAGE and Western blotting followed by ECL (Amersham Biosciences). GFP-CFTR was detected with a rabbit polyclonal GFP antibody (1:1000; BD Biosciences).

**Pulse-Chase**—COS-7 cells were washed twice in methionine/cysteine-free DMEM and then incubated for 30 min in methionine/cysteine-free DMEM. The cells were incubated in methionine/cysteine-free DMEM containing TRAN-<sup>35</sup>S-label (250  $\mu$ Ci/ml) for 30 min. Subsequently, the cells were washed extensively with DMEM containing 10 mM of unlabeled methionine and 10 mM of unlabeled cysteine and chased in this solution for the indicated times. Radiolabeled CFTR was immunoprecipitated with M3A7 and L12B14 monoclonal antibodies (Upstate, Waltham, MA), separated on 7.5% SDS-PAGE, dried, and visualized by autoradiography. The radioactive signal on the dried SDS-PAGE was scanned with a FujiFilm BAS-100 plus system. Quantification was carried out within the linear range using the Image Gauge version 3.2 software (FujiFilm).

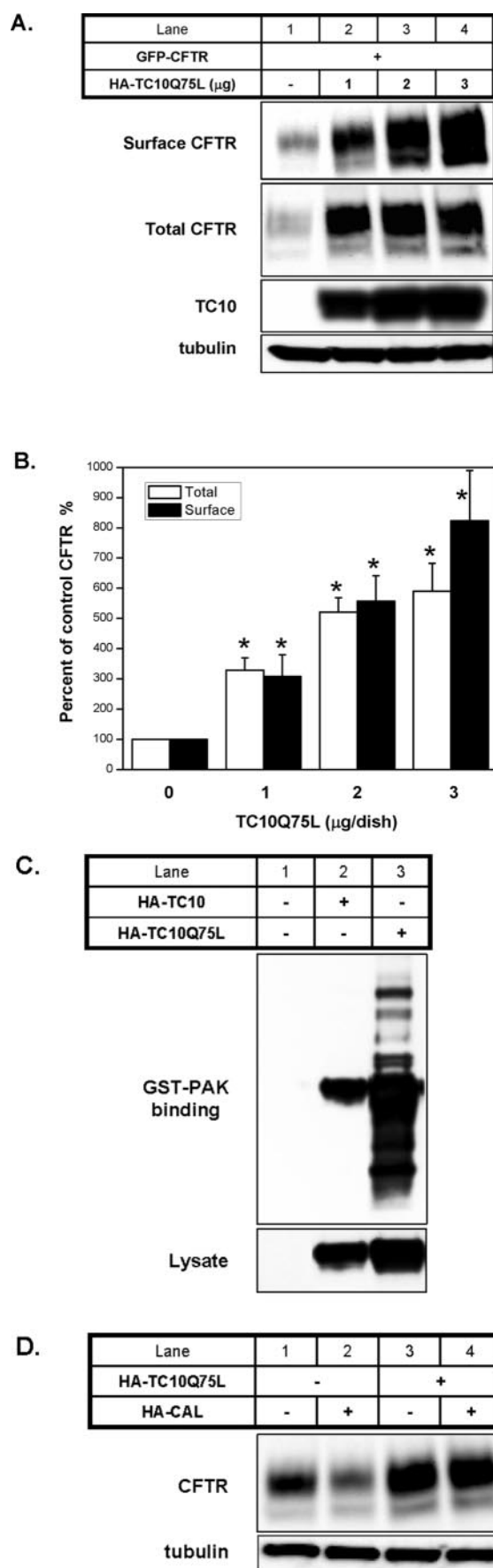
**Confocal Microscopy**—COS-7 cells were plated on glass coverslips 1 day before transfection. The cells were fixed in 4% paraformaldehyde and permeabilized in 0.2% Nonidet P-40 1 day post-transfection. Non-specific binding sites were blocked with 5% normal goat serum. The cells were stained with anti-HA monoclonal antibody (1:2000; Roche Applied Science) in 5% normal goat serum, washed with 1% bovine serum albumin, and incubated with goat anti-mouse Cy3 secondary antibodies in 1% normal goat serum (1:200; Jackson ImmunoResearch, West Grove, PA). The specimens were mounted and viewed on an UltraVIEW spinning disk confocal microscope (PerkinElmer Life Sciences).

**GST-PAK Binding Assay**—COS-7 cells transfected with HA-tagged constructs were lysed with magnesium-containing lysis buffer (25 mM HEPES, pH 7.5, 150 mM NaCl, 10 mM MgCl<sub>2</sub>, 1% Nonidet P-40, 10% glycerol, 25 mM NaF, and Complete protease inhibitor (Roche Applied Science)). GST-PAK-1 p21-binding domain (PBD)-agarose beads (Upstate, Lake Placid, NY) were added immediately to the cell lysate (10  $\mu$ g/ml). The lysates were gently mixed with the beads for 60 min at 4  $^{\circ}$ C. The agarose beads were washed three times in the magnesium-containing lysis buffer. The bound proteins were eluted with 2 $\times$  Laemmli sample buffer supplemented with 100 mM dithiothreitol at 42  $^{\circ}$ C for 30 min. The eluted proteins were subjected to SDS-PAGE and Western blotting, followed by ECL (Amersham Biosciences). HA-tagged proteins were detected with a rabbit polyclonal HA antibody (1:500; Roche Applied Science).

**Statistical Analysis**—The data are presented as the means  $\pm$  standard deviations. Statistical significance was determined by Student's *t* test. We assigned significance at *p* < 0.05.

#### RESULTS

**Is the Expression of Mature CFTR Protein Influenced by TC10?**—The CFTR-interacting protein, CAL, was shown to reduce the total cellular and the cell surface GFP-CFTR when co-expressed in COS-7 cells (11, 15). Previous studies have shown that GFP-CFTR exhibits single channel properties and intracellular trafficking patterns similar to that of wild type CFTR (18). In co-transfection studies, the HA-tagged, constitutively active form of TC10, HA-TC10Q75L, was used to study the effects of TC10 on CFTR (Fig. 1). TC10Q75L lacks GTPase activity and thus locks TC10 in the GTP-bound, constitutively active form (22). As expected, HA-TC10Q75L is enriched with GTP (Fig. 1C). As shown in Fig. 1 (A and B), co-expression of HA-TC10Q75L dramatically increases both the total cellular and the cell surface GFP-CFTR. For example, when 1  $\mu$ g/dish HA-TC10Q75L was transfected, the total CFTR was 323  $\pm$  40% (*p* < 0.05, *n* = 3), and the cell surface GFP-CFTR was 302  $\pm$  71% (*p* < 0.05, *n* = 3) higher than that of the controls. Meanwhile, the abundance of tubulin, a cytoskeleton protein, was not affected (Fig. 1A). Co-expression of wild type TC10 had no effect on CFTR expression (data not shown). To verify these results further, the effect of TC10 on CFTR was investigated in a COS-7-derived cell line expressing a low level of CFTR, 4F2 (11). As shown in Fig. 1D, TC10Q75L also significantly increased the steady state levels of CFTR. We previously demonstrated that binding of CAL to the PDZ-binding motif at the C terminus of CFTR reduced the total cellular CFTR by enhancing its lysosomal degradation. The effect of CAL is shown in Fig. 1D. Lane 2 is 60.9  $\pm$  5.2% (*p* < 0.05, *n* = 3) of that of lane 1 (*p* < 0.05, *n* = 3). Surprisingly, TC10Q75L reversed CAL-mediated CFTR degradation. Lane 4 is 101.5  $\pm$  6.8% (*p* < 0.05,



**FIG. 1. Dose-dependent increases of cell surface and total CFTR by constitutively active TC10.** A, COS-7 cells were co-transfected with GFP-CFTR (3  $\mu\text{g}/\text{dish}$ ) and HA-TC10Q75L as indicated. The cells were lysed 48 h post-transfection. The cell surface proteins were

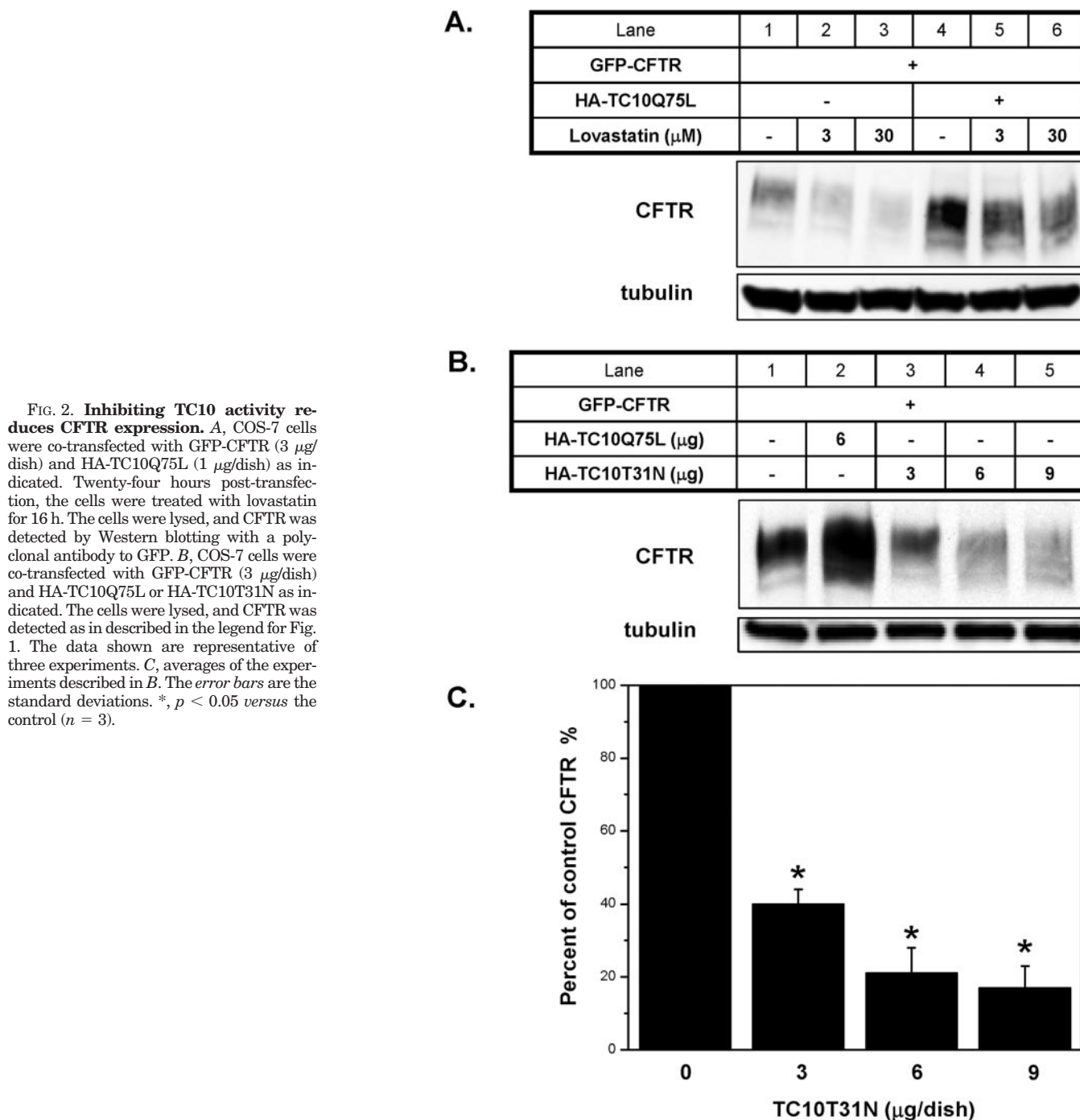
$n = 3$ ) of that of lane 3. These results suggest that TC10Q75L may suppress the inhibitory effect of CAL through a direct interaction with CAL.

**Inhibiting TC10 Reduces the Steady-state Level of CFTR**—TC10 is modified by lipids at the C-terminal cysteine residues for farnesylation and dual palmitoylation (23–25). The CAAX farnesylation motif is required for TC10 to enter the exocytic pathway from ER to Golgi. Upstream of CAAX sequence, a CXXC motif is found that functions in dual palmitoylation and plasma membrane targeting (26, 27). Lovastatin, a 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor, blocks farnesylation (28, 29). Lovastatin treatment reduced the steady-state level of GFP-CFTR (Fig. 2A). Thirty  $\mu\text{M}$  lovastatin treatment reduces GFP-CFTR to  $18.9 \pm 3.1\%$  ( $p < 0.05$ ,  $n = 3$ ) of untreated control cells (compare lane 3 with lane 1). Lovastatin also inhibits the effects of TC10Q75L on CFTR (lane 6 is  $45.1 \pm 7.8\%$  of that of lane 4 ( $p < 0.05$ ,  $n = 3$ )), further confirming that its effect on CFTR is through TC10 (Fig. 2A). To test whether lovastatin specifically inhibited the TC10 pathway, the HA-tagged, GDP-bound, dominant-negative form of TC10, TC10T31N, was co-transfected with GFP-CFTR into COS-7 cells. HA-TC10T31N decreased the level of GFP-CFTR (Fig. 2, B and C). When 3  $\mu\text{g}/\text{dish}$  HA-TC10T31N was transfected, the total CFTR is reduced to  $40 \pm 4\%$  of the control ( $p < 0.05$ ,  $n = 3$ ).

**Is the Effect of TC10 Specific for CFTR?**—To test the specificity of TC10 on CFTR, we studied the effects of co-transfecting TC10 with other proteins, both cytosolic protein, GFP, and membrane-associated proteins VSV-G, GFP-tagged Frizzled-3, and PrCad. Neither GFP nor Frizzled-3 binds to CAL (15). All of the GFP-tagged proteins used have the same cytomegalovirus promoters, eliminating a nonspecific effect on promoter activity. Co-transfection of HA-TC10Q75L did not dramatically alter the abundance of these proteins (Fig. 3, A and B). In addition, HA-TC10Q75L had no effect on the cell surface of VSV-G (Fig. 3B). These data suggest that TC10 increases GFP-CFTR by mechanisms that do not involve transcription, acting on the GFP moiety of the fusion proteins or the exocytosis of plasma membrane proteins in general.

TC10 is a member of the Rho family GTPase and is closely related to Cdc42 and RhoA. They share over 90% amino acid identity. Rho family GTPase are involved in vesicular trafficking, cytoskeleton organization, cell polarity, cell migration, and gene expression. As shown in Fig. 3C, neither the constitutively active (HA-Cdc42-QL) nor the dominant-negative (HA-Cdc42-N17) form of Cdc42 affects the abundance of CFTR. Co-expression of the constitutively active and dominant-negative forms of RhoA also has no effect on the abundance of CFTR (data not shown). Thus, not only is the effect of TC10 specific for CFTR, but other

labeled with membrane-impermeable Sulfo-NHS-SS-Biotin at 4  $^{\circ}\text{C}$ . The cell surface proteins were isolated by NeutrAvidin beads. The cell surface proteins and lysate were separated on SDS-PAGE. GFP-CFTR was detected by Western blotting using an anti-GFP polyclonal antibody. HA-TC10Q75L was detected using anti-HA polyclonal antibody. Tubulin was detected using an anti-tubulin monoclonal antibody to demonstrate equal loading of total cell lysates. B, averages of the experiments described in A. The *open columns* are values from total cellular CFTR. The *solid columns* are values from cell surface CFTR. The *error bars* are the standard deviations. \*,  $p < 0.05$  versus the control ( $n = 3$ ). C, COS-7 cells were transfected with HA-TC10 (3  $\mu\text{g}/\text{dish}$ ) or HA-TC10Q75L (1  $\mu\text{g}/\text{dish}$ ) as indicated. The GTP-bound forms were isolated with GST-PAK-agarose, subjected to SDS-PAGE, and detected by Western blotting using anti-HA polyclonal antibody. D, the COS-7 cell line, 4F2, stably expressing GFP-CFTR in low abundance (15) was transfected with HA-CAL (6  $\mu\text{g}/\text{dish}$ ) or HA-TC10Q75L (1  $\mu\text{g}/\text{dish}$ ) as indicated. The cells were lysed, and the proteins were detected as described above. The data shown in A, C, and D are representative of three experiments.



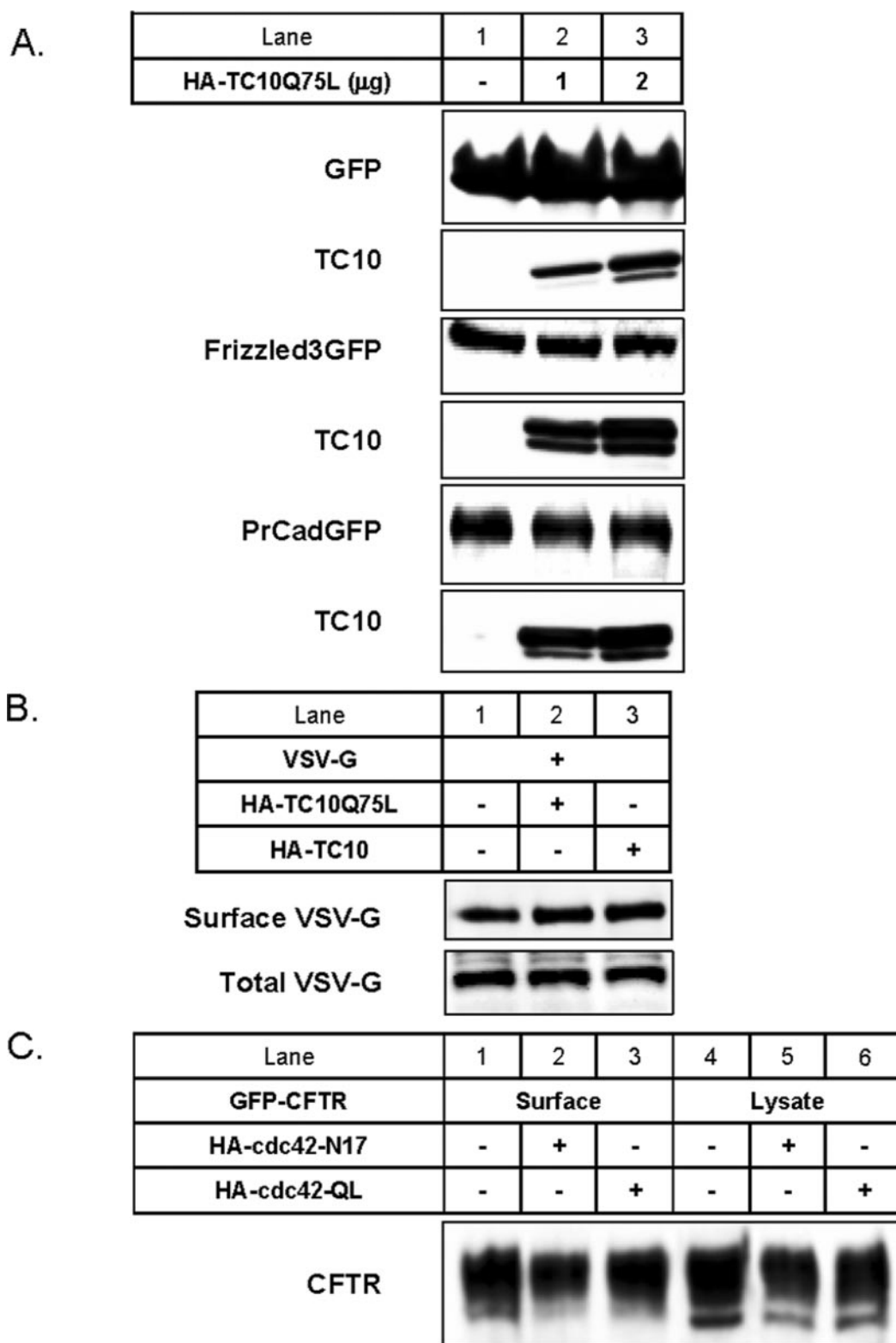
**FIG. 2. Inhibiting TC10 activity reduces CFTR expression.** *A.* COS-7 cells were co-transfected with GFP-CFTR (3  $\mu\text{g}/\text{dish}$ ) and HA-TC10Q75L (1  $\mu\text{g}/\text{dish}$ ) as indicated. Twenty-four hours post-transfection, the cells were treated with lovastatin for 16 h. The cells were lysed, and CFTR was detected by Western blotting with a polyclonal antibody to GFP. *B.* COS-7 cells were co-transfected with GFP-CFTR (3  $\mu\text{g}/\text{dish}$ ) and HA-TC10Q75L or HA-TC10T31N as indicated. The cells were lysed, and CFTR was detected as in described in the legend for Fig. 1. The data shown are representative of three experiments. *C.* averages of the experiments described in *B.* The error bars are the standard deviations. \*,  $p < 0.05$  versus the control ( $n = 3$ ).

Rho-GTPases do not have similar effects on CFTR levels.

*What Is the Effect of TC10 on CFTR Maturation?*—To test the possibility that TC10 increases CFTR synthesis and/or maturation, we examined the synthesis and maturation of CFTR using metabolic labeling and pulse-chase experiments. In Fig. 4A, COS-7 cells were transfected with GFP-CFTR with or without HA-TC10Q75L and, after 16 h, metabolically pulse-labeled with [ $^{35}\text{S}$ ]methionine and cysteine for 30 min. The maturation of CFTR was followed by chasing for 1–4 h. Maturation can be gauged by the initial appearance and subsequent decrease of newly synthesized core-glycosylated CFTR (band B), with a concomitant increase in the mature C band. Fig. 4A shows that GFP-CFTR matures similarly whether transfected or not transfected with TC10Q75L. There is no significant difference in fractional conversion between the B band at 0 h and the C band at 4 h in the absence or presence of HA-TC10Q75L ( $26.6 \pm 3.9\%$  versus

$28.4 \pm 2.6\%$ , respectively,  $p > 0.05$ ,  $n = 3$ ). Therefore, TC10 exerts its effects on mature CFTR and does not affect the synthesis and trafficking to Golgi. To test this hypothesis directly, the effect of TC10Q75L on mature CFTR was examined. GFP-CFTR becomes fully mature after chasing for 4 h (Fig. 4A). There is no significant difference in the amount of CFTR in TC10Q75L-transfected and untransfected cells. The mature CFTR was then followed as cells were chased for an additional 4–20 h. As shown in Fig. 4B, at 20 and 26 h, more  $^{35}\text{S}$ -labeled mature CFTR in cells transfected compared with those not transfected with HA-TC10Q75L (lane 4 is  $171.7 \pm 17.8\%$  of lane 2,  $p < 0.05$ ,  $n = 3$ ). Taken together, these data suggest that TC10 does not affect early CFTR biosynthesis in the ER.

*TC10 Interacts with CAL but Does Not Reduce CAL Protein Level or Reduce Its Dimerization or Association with CFTR*—TC10 binds to CAL in a yeast-two hybrid assay and *in vitro*

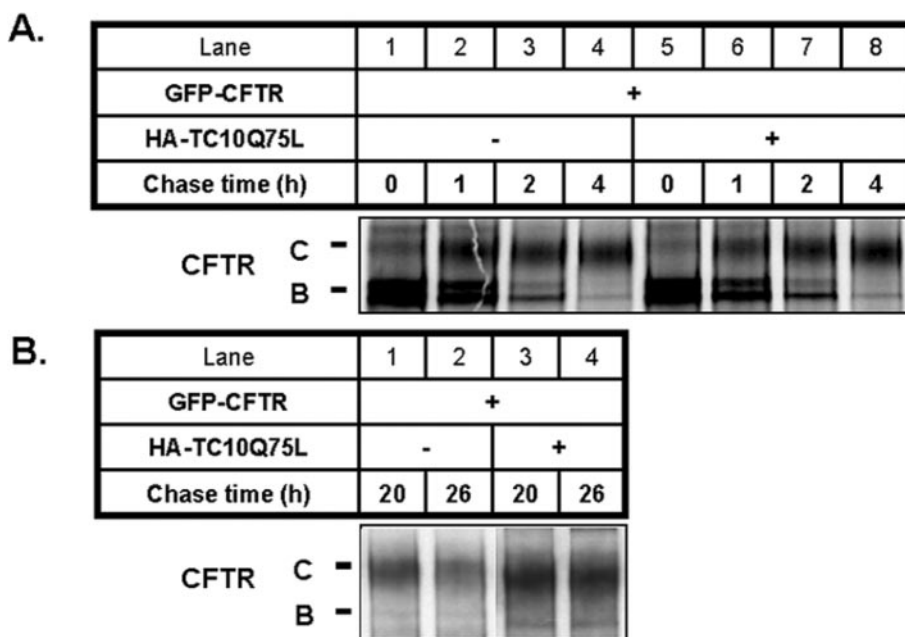


**FIG. 3. The effect of TC10 is specific for CFTR.** *A*, COS-7 cells were co-transfected with HA-TC10Q75L (1  $\mu\text{g}/\text{dish}$ ) and GFP, Frizzled-3GFP, or Pr-cadherin-GFP (*PrCadGFP*) (3  $\mu\text{g}/\text{dish}$ ) as indicated. The cells were lysed, and the proteins were detected as described in the legend for Fig. 1. *B*, COS-7 cells were co-transfected with VSV-G (3  $\mu\text{g}/\text{dish}$ ) and HA-TC10Q75L (1  $\mu\text{g}/\text{dish}$ ) as indicated. The cell surface proteins and total cell lysates were detected as in Fig. 1, except a rabbit polyclonal VSV-G antibody was used. *C*, COS-7 cells were co-transfected with GFP-CFTR (3  $\mu\text{g}/\text{dish}$ ) and either HA-Cdc42N17 (3  $\mu\text{g}/\text{dish}$ ) or HA-Cdc42N17 (3  $\mu\text{g}/\text{dish}$ ) as indicated. The cell surface proteins and total cell lysates were detected as described in the legend for Fig. 1. The data shown are representative of three experiments. Tubulin controls were performed for these experiments. There were no differences in protein loading (data not shown). VSV-G, vesicular stomatitis virus G.

(17). To test its binding in mammalian cells, we co-transfected Myc-tagged CAL (Myc-CAL) and HA-TC10Q75L into COS-7 cells and performed immunoprecipitation studies. As shown in Fig. 5A, immunoprecipitation of Myc-CAL brings down HA-

TC10Q75L. We have previously shown that CAL reduces cell surface and total CFTR (11, 15). We now show that TC10Q75L binds to CAL, increases the abundance of CFTR, and reverses the inhibitory effect of CAL. To shed more light on the mech-

**FIG. 4. Metabolic pulse-chase experiments.** *A*, COS-7 cells were transfected with GFP-CFTR and HA-TC10Q75L. The cells were metabolically labeled for 30 min with [<sup>35</sup>S]methionine 16 h post-transfection. The cells were then chased in normal medium supplemented with unlabeled cysteine and methionine for 1–4 h as indicated. *B*, after the initial 4-h chase, CFTR maturation was complete. The cells were chased for an additional 20–26 h. Longer exposure times were used to allow visualization. The data shown are representative of three experiments.



anisms by which TC10 and CAL might affect CFTR, we next examined CAL protein expression, the interaction of CFTR and CAL, and the homodimerization of CAL.

One possibility is that CAL protein expression might be down-regulated by TC10Q75L. To test this, HA-TC10Q75L was co-transfected with HA-tagged CAL, NHE-RF, or CAP70 (Fig. 5B). All three proteins have been shown to bind to CFTR through PDZ domain interaction. In the presence of TC10Q75L, the protein level of HA-tagged CAL is increased to  $328 \pm 34\%$  of the control ( $p < 0.05$ ,  $n = 3$ ). As controls, NHERF and CAP70 are unchanged.

The PDZ domain of CAL binds to the C terminus of CFTR and enhances the lysosomal degradation of CFTR (11). TC10Q75L may bind to CAL and dissociate it from CFTR. To test this possibility, we performed experiments shown in Fig. 5C. The data show that co-expression of TC10Q75L does not prevent the binding of CAL to CFTR.

Another possibility is that TC10Q75L may disrupt CAL function by disrupting homodimerization. CAL is a homodimer through a coiled-coil interaction (15). CFTR is reportedly a homodimer (30). It is possible that the dimeric form of CAL is required for CFTR binding and that its disruption of dimer may render it nonfunctional. To test this possibility, we examined the dimerization of CAL by co-immunoprecipitation experiments using the HA- and Myc-tagged CAL. Fig. 5D shows that co-expression of TC10Q75L does not disrupt the dimerization of CAL.

*TC10Q75L Redistributes the Subcellular Localization of CAL*—TC10 plays a key role in the insulin-stimulated translocation of Glut4 to the plasma membrane in adipocytes (3). To test the effect of TC10 on the intracellular trafficking of CAL, we used confocal microscopy to examine the intracellular localization and distribution of CAL and TC10. Similar to H-ras, TC10 is synthesized by free ribosomes in the cytosol and then predominantly enters the conventional exocytic pathway from ER to Golgi en route to the plasma membrane (26, 27). In transfected COS-7 cells, wild type HA-tagged TC10 is localized to the perinuclear secretory membrane compartments and the plasma membrane (Fig. 6, *a* and *d*). HA-TC10 overlaps with Myc-CAL in the perinuclear region (Fig. 6, *b*, *e*, and *f*). Co-expression of wild type HA-TC10 does not change the subcellular distribution of Myc-CAL. Interestingly, co-expression of the constitutively active HA-TC10Q75L dramatically changes

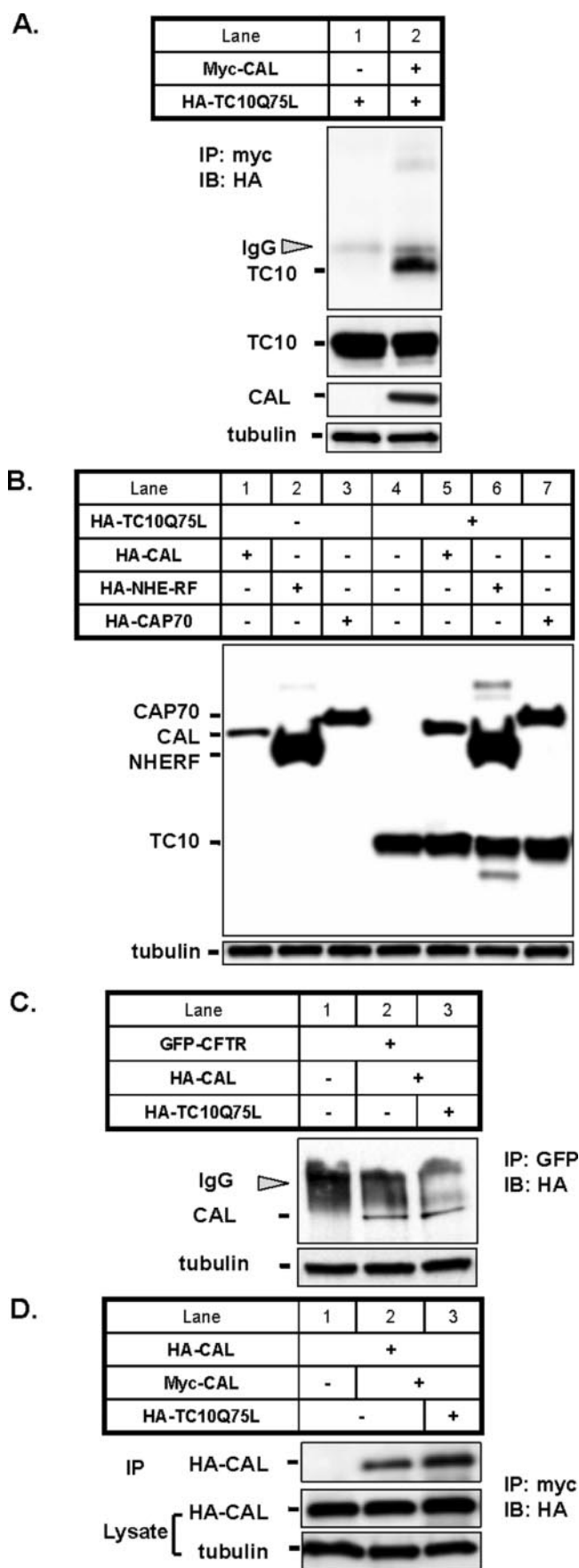
the localization of Myc-CAL from the Golgi to the plasma membrane, where it overlaps with HA-TC10Q75L (Fig. 6, *g–i*). These data suggest that the GTP-bound active form of TC10 redistributes CAL from the Golgi to the plasma membrane, removing the CAL from the Golgi where it may direct the trafficking of CFTR to lysosomes.

#### DISCUSSION

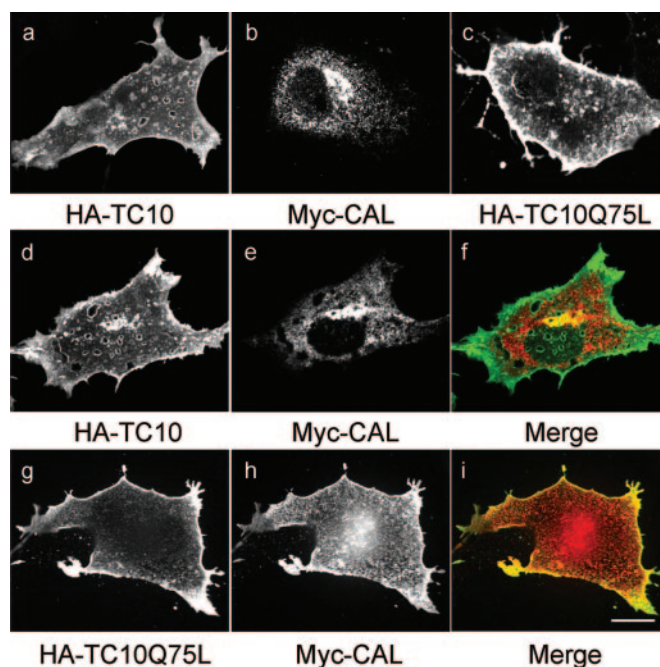
In this paper, we report that a Rho family small GTPase TC10 interacts with CAL in the COS-7 cells and up-regulates CFTR protein expression. We previously identified CAL as a novel CFTR-interacting protein and showed that CAL retains CFTR in the intracellular compartment, thus decreasing the cell surface expression of CFTR (15). CAL down-regulates the protein level of mature CFTR by targeting the retained CFTR to lysosomal degradation (11, 31). Here, we show that the constitutively active form of TC10, TC10Q75L, interacts with CAL and increases in cell surface and total CFTR (Fig. 1A). The effect of TC10 is independent of the CFTR expression level, because the same effect was observed in a stable COS-7-derived cell line expressing CFTR at about one-tenth the level seen in transiently transfected cells (Fig. 1D).

The effect of TC10 depends on its GTP-bound active state. Only the GTP-bound, constitutively active TC10Q75L up-regulates CFTR (Fig. 1A). Wild type TC10 has no effect (data not shown). The GDP-bound, dominant-negative TC10T31N down-regulates CFTR (Fig. 2B). TC10, therefore, can serve as a molecular switch in regulating CFTR protein levels. Depending on the state of GTP or GDP occupancy, TC10 may direct the trafficking of CFTR to the liable lysosomal pathway or to a more stable exocytosis pathway en route to the plasma membrane. In addition, farnesylation is also required for the effect of TC10Q75L on CFTR (Fig. 2A). This is consistent with the earlier observation that lovastatin treatment reduces total cellular CFTR in human tracheal epithelial Calu-3 cells (32).

We used two approaches to address the specificity of the TC10 effect. First, our finding shows that co-expression of TC10Q75L has no effect on the expression of the other plasma membrane proteins such as VSV-G, Frizzled-3, and Pr-cadherin, or a cytosolic protein, GFP (Fig. 3, *A* and *B*). Second, the co-expression of the constitutively active and dominant-negative forms of Cdc42 or RhoA, the most closely related proteins



**FIG. 5. Interactions of TC10, CAL, and CFTR.** *A*, COS-7 cells were transfected as indicated. *Top panel*, cell lysates were immunoprecipitated (IP) with anti-Myc rabbit polyclonal antibody. TC10 was detected with an anti-HA mouse monoclonal antibody. *Bottom two panels*, cell



**FIG. 6. Constitutively active TC10 changes the subcellular distribution of CAL.** COS7 cells were transfected with either HA-TC10 (*a*), Myc-CAL (*b*), or HA-TC10Q75L (*c*) (*top row*), doubly transfected with HA-TC10 and Myc-CAL (*d-f*; *middle row*) or HA-TC10Q75L and Myc-CAL (*g-i*; *bottom row*). TC10 was detected by a monoclonal antibody to the HA epitope (*green* in *overlay panels f* and *i*). CAL was detected by a monoclonal antibody to the Myc epitope (*red* in *overlay panels f* and *i*) and visualized by confocal microscopy. CAL is typically expressed in the Golgi apparatus and vesicles. Note from *d-f* that wild type TC10 has no effect on its intracellular localization. In contrast, *g-i* show that the constitutively active TC10 (HA-TC10Q75L) redistributes and overlaps with CAL. Scale bar, 10  $\mu$ m. The data shown are representative of three experiments.

with amino acid identities of 90% to TC10, do not have a similar effect on the expression of CFTR (Fig. 3C).

Metabolic pulse-chase experiments indicate that TC10 does not affect CFTR maturation, suggesting that it exerts its effects on the mature CFTR (Fig. 4A). Pulse-chase experiments confirmed that TC10Q75L indeed stabilizes the newly synthesized mature CFTR (Fig. 4B).

The interaction of TC10 with CAL was demonstrated by co-immunoprecipitation experiments (Fig. 5A). TC10Q75L does not reduce the CAL protein level or the ability of CAL to form dimers or to interact with CFTR (Fig. 5, B and C). Importantly, TC10Q75L reverses CAL-mediated CFTR degradation (Fig. 1D). The co-expression of TC10Q75L causes a dramatic redistribution of CAL from the juxtannuclear region to the plasma membrane and causes CAL to overlap with TC10Q75L (Fig. 6). These data suggest a model in which the small GTPase TC10 regulates both the plasma membrane and total CFTR expression by interacting with the CFTR-binding partner CAL. The GTP-bound TC10 directs the trafficking of CFTR from the juxtannuclear region to the secretory pathway toward the

lysates were loaded onto SDS-PAGE directly, and TC10 and CAL were detected with HA and Myc antibodies, respectively. *B*, COS-7 cells were co-transfected with HA-TC10-Q75L and HA-CAL, HA-NHERF, and HA-CAP70 as indicated. Total cell lysates were detected as described in the legend for Fig. 1. *C*, COS-7 cells were co-transfected with GFP-CFTR, HA-CAL, and HA-TC10Q75L as indicated. The cell lysates were immunoprecipitated with polyclonal GFP antibody. CAL was detected with anti-HA antibodies. *D*, COS-7 cells were co-transfected with HA-CAL, Myc-CAL and Myc-TC10Q75L as indicated. Cell lysates were immunoprecipitated with a rabbit polyclonal anti-Myc antibody. CAL was detected with rabbit polyclonal anti-HA antibodies. The data shown are representative of three experiments. *IB*, immunoblot.

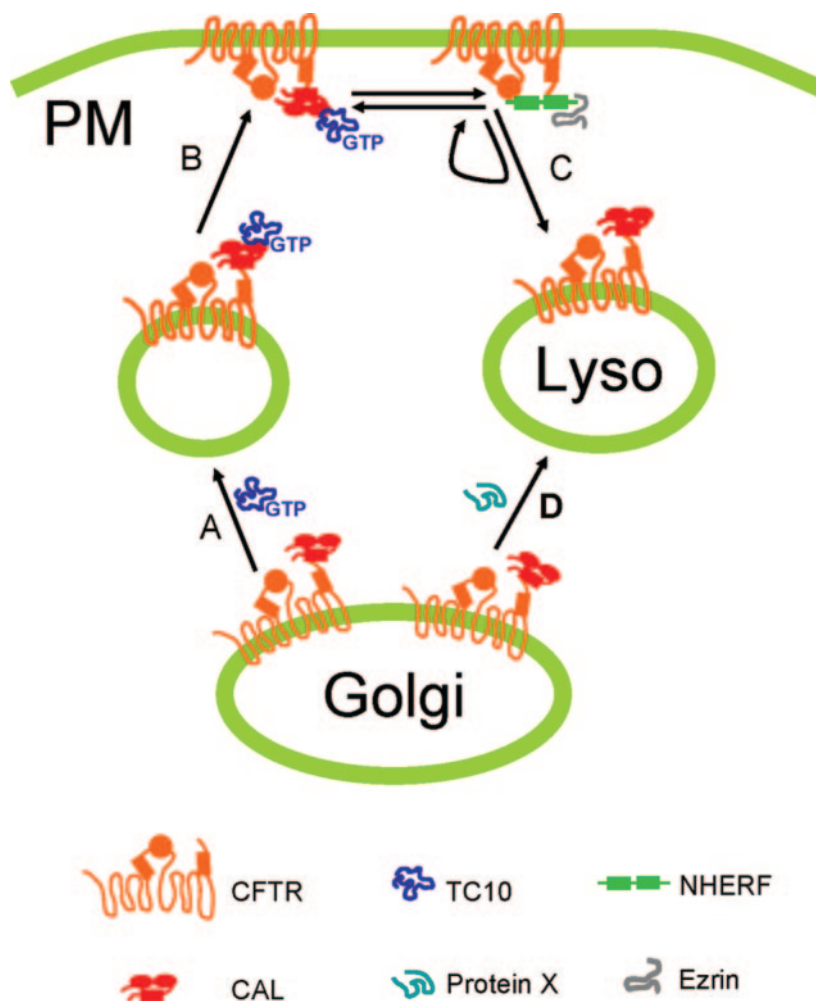


FIG. 7. Model demonstrating regulation of CFTR trafficking by TC10 and CAL. A, TC10 binds to CAL-CFTR complex at the Golgi and promotes CFTR trafficking to the cell surface. B, CFTR traffics to the plasma membrane, where it is stabilized by interaction with NHERF. C, CFTR is endocytosed from the cell surface. CFTR is either recycled back to the cell surface or traffics to the lysosome. D, CAL promotes CFTR trafficking to lysosome. PM, plasma membrane; Lyso, lysosome.

plasma membrane, away from the CAL-mediated degradation of CFTR in the lysosome (Fig. 7).

As shown previously, CAL is localized at Golgi and Golgi-derived vesicles at steady state (15, 33). It may transiently associate with the plasma membrane (11). Expression of a dominant-negative dynamin 2 mutant inhibits CFTR endocytosis and arrests CAL at the cell periphery, where dynamin regulates vesicle budding (11). Fig. 6 provides evidence further supporting this notion. In this case, the constitutively active TC10, but not the wild type TC10, translocated CAL to the plasma membrane. This is also consistent with the finding that co-expression of TC10Q75L increases the protein level of CAL (Fig. 5B).

Alternative splice variants of CAL bind to CFTR, the chloride channel, CIC-3B, the wnt receptors, Frizzled-5 and -8, the  $\delta 2$  subunit of glutamate receptor, a neural member of the epidermal growth factor, CALEB/NGC, and the  $\beta 1$ -adrenergic receptor through PDZ domain interactions (15, 31, 34–37). Overexpression of CAL reduces the CIC-3B protein expression level (31) and inhibits  $\beta 1$ -adrenergic receptor surface expression (37). Thus, regulation of CAL by TC10 will also likely be important for the trafficking of other membrane proteins that interact with CAL.

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#### REFERENCES

- Takai, Y., Sasaki, T., and Matozaki, T. (2001) *Physiol. Rev.* **81**, 153–208
- Symons, M., and Rusk, N. (2003) *Curr. Biol.* **13**, R409–R418
- Saltiel, A. R., and Pessin, J. E. (2003) *Traffic* **4**, 711–716
- Chiang, S. H., Baumann, C. A., Kanzaki, M., Thurmond, D. C., Watson, R. T., Neudauer, C. L., Macara, I. G., Pessin, J. E., and Saltiel, A. R. (2001) *Nature* **410**, 944–948
- Inoue, M., Chang, L., Hwang, J., Chiang, S. H., and Saltiel, A. R. (2003) *Nature* **422**, 629–633
- Kopito, R. R. (1999) *Physiol. Rev.* **79**, S167–S173
- Yoo, J. S., Moyer, B. D., Bannykh, S., Yoo, H. M., Riordan, J. R., and Balch, W. E. (2002) *J. Biol. Chem.* **277**, 11401–11409
- Weixel, K. M., and Bradbury, N. A. (2000) *J. Biol. Chem.* **275**, 3655–3660
- Prince, L. S., Peter, K., Hatton, S. R., Zaliauskiene, L., Cotlin, L. F., Clancy, J. P., Marchase, R. B., and Collawn, J. F. (1999) *J. Biol. Chem.* **274**, 3602–3609
- Lukacs, G. L., Segal, G., Kartner, N., Grinstein, S., and Zhang, F. (1997) *Biochem. J.* **328**, 353–361
- Cheng, J., Wang, H., and Guggino, W. B. (2004) *J. Biol. Chem.* **279**, 1892–1898
- Swiatecka-Urban, A., Boyd, C., Coutermarsh, B., Karlson, K. H., Barnaby, R., Aschenbrenner, L., Langford, G. M., Hasson, T., and Stanton, B. A. (2004) *J. Biol. Chem.* **279**, 38025–38031
- Gentzsch, M., Chang, X. B., Cui, L., Wu, Y., Ozols, V. V., Choudhury, A., Pagano, R. E., and Riordan, J. R. (2004) *Mol. Biol. Cell* **15**, 2684–2696
- Benharouga, M., Haardt, M., Kartner, N., and Lukacs, G. L. (2001) *J. Cell Biol.* **153**, 957–970
- Cheng, J., Moyer, B. D., Milewski, M., Loffing, J., Ikeda, M., Mickle, J. E., Cutting, G. R., Li, M., Stanton, B. A., and Guggino, W. B. (2002) *J. Biol. Chem.* **277**, 3520–3529
- Charest, A., Lane, K., McMahon, K., and Housman, D. E. (2001) *J. Biol. Chem.* **276**, 29456–29465
- Neudauer, C. L., Joberty, G., and Macara, I. G. (2001) *Biochem. Biophys. Res. Commun.* **280**, 541–547
- Moyer, B. D., Loffing, J., Schwiebert, E. M., Loffing-Cueni, D., Halpin, P. A., Karlson, K. H., Ismailov, I. I., Guggino, W. B., Langford, G. M., and Stanton, B. A. (1998) *J. Biol. Chem.* **273**, 21759–21768
- Wang, S., Raab, R. W., Schatz, P. J., Guggino, W. B., and Li, M. (1998) *FEBS Lett.* **427**, 103–108
- Wang, S., Yue, H., Derin, R. B., Guggino, W. B., and Li, M. (2000) *Cell* **103**, 169–179
- Rattner, A., Smallwood, P. M., Williams, J., Cooke, C., Savchenko, A., Lyubarsky, A., Pugh, E. N., and Nathans, J. (2001) *Neuron* **32**, 775–786
- Neudauer, C. L., Joberty, G., Tatsis, N., and Macara, I. G. (1998) *Curr. Biol.* **8**, 1151–1160
- Michaelson, D., Silletti, J., Murphy, G., D'Eustachio, P., Rush, M., and Philips, M. R. (2001) *J. Cell Biol.* **152**, 111–126
- Murphy, G. A., Solski, P. A., Jillian, S. A., Perez, O., D'Eustachio, P., Der, C. J.,

- and Rush, M. G. (1999) *Oncogene* **18**, 3831–3845
25. Neudauer, C. L., and Macara, I. G. (2000) *Methods Enzymol.* **325**, 3–14
26. Apolloni, A., Prior, I. A., Lindsay, M., Parton, R. G., and Hancock, J. F. (2000) *Mol. Cell. Biol.* **20**, 2475–2487
27. Chunqiu, H. J., and Pessin, J. E. (2003) *Mol. Biol. Cell* **14**, 3578–3591
28. Alberts, A. W., Chen, J., Kuron, G., Hunt, V., Huff, J., Hoffman, C., Rothrock, J., Lopez, M., Joshua, H., Harris, E., Patchett, A., Monaghan, R., Currie, S., Stapley, E., Albers-Schonberg, G., Hensens, O., Hirshfield, J., Hoogsteen, K., Liesch, J., and Springer, J. (1980) *Proc. Natl. Acad. Sci. U. S. A.* **77**, 3957–3961
29. Maltese, W. A., Sheridan, K. M., Repko, E. M., and Erdman, R. A. (1990) *J. Biol. Chem.* **265**, 2148–2155
30. Ramjeesingh, M., Kidd, J. F., Huan, L. J., Wang, Y., and Bear, C. E. (2003) *Biochem. J.* **374**, 793–797
31. Gentzsch, M., Cui, L., Mengos, A., Chang, X. B., Chen, J. H., and Riordan, J. R. (2003) *J. Biol. Chem.* **278**, 6440–6449
32. Shen, B. Q., Widdicombe, J. H., and Mrsny, R. J. (1995) *J. Biol. Chem.* **270**, 25102–25106
33. Yao, R., Ito, C., Natsume, Y., Sugitani, Y., Yamanaka, H., Kuretake, S., Yanagida, K., Sato, A., Toshimori, K., and Noda, T. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99**, 11211–11216
34. Hassel, B., Schreff, M., Stuebe, E. M., Blaich, U., and Schumacher, S. (2003) *J. Biol. Chem.* **278**, 40136–40143
35. Yao, R., Maeda, T., Takada, S., and Noda, T. (2001) *Biochem. Biophys. Res. Commun.* **286**, 771–778
36. Yue, Z., Horton, A., Bravin, M., DeJager, P. L., Selimi, F., and Heintz, N. (2002) *Neuron* **35**, 921–933
37. He, J., Bellini, M., Xu, J., Castleberry, A. M., and Hall, R. A. (2004) *J. Biol. Chem.* **279**, 50190–50196