THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 279, No. 3, Issue of January 16, pp. 1892–1898, 2004
Printed in U.S.A.
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Modulation of Mature Cystic Fibrosis Transmembrane Regulator Protein by the PDZ Domain Protein CAL*

Received for publication, August 5, 2003, and in revised form, October 6, 2003
Published, JBC Papers in Press, October 21, 2003, DOI 10.1074/jbc.M308640200

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We have previously identified the cystic fibrosis transmembrane regulator (CFTR)-interacting protein CAL and demonstrated that CAL modulates CFTR plasma membrane expression by retaining CFTR within the cell. Here, we report that in addition to regulating membrane expression, CAL also regulates the expression of mature CFTR. The co-expression of hemagglutinin-tagged or Myc-tagged CAL with green fluorescent protein (GFP)-CFTR in COS-7 cells causes a dose-dependent reduction in mature GFP-CFTR, independent of its tags. Bafilomycin A1, a lysosomal proton pump inhibitor, increases mature GFP-CFTR, confirming previous reports of lysosomal degradation of mature CFTR. Importantly, bafilomycin A1 reverses CAL-mediated CFTR degradation. The proteasome inhibitor, MG132, on the other hand, does not reverse the effect of CAL. CAL has no effect on CFTR maturation, suggesting that it exerts its effects on mature CFTR. Co-expression of CAL enhances the degradation of CFTR. We showed previously that CAL reduces the half-life of CFTR at the cell surface. Here we show that expression of dominant-negative dynamin 2 K44A, a large GTPase inhibitor that is known to inhibit clathrin-mediated endocytosis and vesicle formation in the Golgi, increases cell surface CFTR as measured by surface biotinylation. More importantly, dynamin 2 K44A also restores cell surface CFTR in CAL-overexpressing cells and partially blocks the CAL-mediated degradation of mature CFTR. These data suggest a model in which CAL retains CFTR in the cell and targets CFTR for degradation.

Cystic fibrosis is a common lethal genetic disease caused by autosomal recessive mutations of cystic fibrosis transmembrane regulator (CFTR)[1] (1, 2). The best-known and probably the primary defect in cystic fibrosis is salt and water transport in plasma membranes of epithelial cells lining the organs of lung, pancreas, liver, intestines, sweat duct, and reproductive systems (3). CFTR is a membrane protein that belongs to the ATP-binding cassette transport family. It functions as a cAMP-dependent protein kinase- and ATP-regulated Cl^- channel (4), as well as a regulatory protein for the epithelial sodium channel (5) and other channels (6), and as a putative receptor for *Pseudomonas aeruginosa* (7). These well characterized functions of CFTR require its correct synthesis, maturation, and trafficking from endoplasmic reticulum (ER) and Golgi to the cell surface.

Misfolded proteins are recognized by ER quality control system and are targeted for degradation in ER-associated degradation (8). Immature, wild type CFTR is folded inefficiently in the ER with a large fraction degraded before reaching the plasma membrane (9). That which is folded efficiently traffics to the Golgi and acquires complex glycosylation characteristic of the mature CFTR. The most common mutation in CF, ΔF508CFTR, is a misfolded protein and is recognized as such by the ER, translocated to the cytosol, and degraded by the 26 S proteasome (9). Thus, the predominant majority of the ΔF508 never matures and traffics to the plasma membrane.

The degradation of mature CFTR is less well understood. Recently, Benharouga *et al.* (10) found that both the lysosome and proteasome are involved. They found that lysosomal inhibitors increase the half-life of mature wild type CFTR in baby hamster kidney cells transfected with CFTR and in the endogenous CFTR in Caco-2 and T84 cells. Interestingly, C-terminal truncation mutants do not traffic to the lysosome. Instead, they are degraded in the proteasome (10). Thus, CFTR appears to enter different degradative pathways depending on signals within its C terminus.

CFTR possesses a type I, C-terminal, PDZ (PSD-95/DLG/ZO-1)-binding motif. Several PDZ domain proteins known to interact with CFTR include NHE-RF/EBP-50, CAP70, and CAL (CFTR-associated ligand) (11–15). The C-terminal tail of CFTR plays a potential regulatory role in modulating its Cl^- channel activity. Adding recombinant NHE-RF or CAP70 fusion proteins to the cytoplasmic side of CFTR *in vitro* increases the activity of CFTR (15, 16). In addition, NHE-RF anchors CFTR to cytoskeleton stabilizing the cell surface CFTR (13, 17). Both NHE-RF and its related protein, E3KARP, link cAMP-dependent protein kinase to CFTR (13, 18). Moreover, NHE-RF was shown to link β_2-adrenergic receptor to CFTR (19). Formation of such macromolecular complexes facilitates the efficiency of activation CFTR.

CAL (also known as PIST (PDZ domain protein interacting specifically with TC10), GOPC (Golgi-associated PDZ and coiled-coil motif containing), and FIG (fused in glioblastoma)) (11, 20–22) is a CFTR-interacting PDZ domain protein that associates predominantly with the Golgi apparatus. Overexpression of CAL reduces CFTR chloride currents in mammalian cells by decreasing the expression of CFTR in the plasma membrane (11). This effect of CAL can be overcome by NHE-RF, which restores the plasma membrane expression of CFTR. CAL favors the retention of CFTR within the cell, whereas NHE-RF favors surface expression by competing with CAL for

* This work was supported by Cystic Fibrosis Foundation Research Development Programs (to W. B. G.) and National Institutes of Health Grants DK-48977 (to W. B. G.) and HL-47122 (to W. B. G.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: CFTR, cystic fibrosis transmembrane regulator; NHE-RF, Na^+/H^+ exchanger regulatory factor; Dyn2, dynamin 2; GFP, green fluorescent protein; HA, hemagglutinin; ER, endoplasmic reticulum; DMEM, Dulbecco's modified Eagle's medium; TGN, trans-Golgi network; SNARE, soluble NSF attachment protein receptors.

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the binding of CFTR (11). Interestingly, overexpression of CAL also reduces the amount of CFTR (23). These findings point to a role for CAL in the intracellular trafficking of CFTR. Consistent with this notion, CAL interacts with proteins involved in membrane vesicle trafficking such as the SNARE protein, syntaxin 6, and the small GTPase, TC10 (20, 22). Furthermore, CAL also interacts with and potentially regulates the intracellular trafficking of the Wnt receptors, frizzled 5 and 8, neural members of the epidermal growth factor receptor family, CALEB/NGC (21, 24), and the chloride channel, CLC-3B (23).

Recently, PDZ domain interactions were found to be involved in trafficking of other plasma membrane proteins both to and from the plasma membrane. For example, synapse-associated protein 102 is involved in the delivery of *N*-methyl-D-aspartate receptors to the cell surface (25). In addition, NHE-RF regulates β_2-adrenergic receptor recycling to plasma membrane (26).

In addition to the PDZ domain, the C-tail of CFTR contains motifs for endocytosis. A tyrosine motif at the C terminus of CFTR binds directly with the μ light chain of the AP2 complex involved in sorting CFTR for endocytosis into clathrin-coated pits (27). Surface CFTR is endocytosed rapidly. For example, within 5 min, 50% CFTR at the plasma membrane is endocytosed via AP2 clathrin-coated pits. CFTR contained within endosomes can be either recycled back to the plasma membrane or degraded in the lysosome. Because surface CFTR has a long half-life (24–48h), the majority of CFTR must be recycled back to the cell surface instead of targeted to the lysosome for degradation. It is not clear how this process is regulated. In this study, we examined the role of CAL on the expression of mature CFTR protein. We found that CAL regulates the expression of mature CFTR through mechanisms that are both bafilomycin A1- and dynamin 2-sensitive.

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 μg/ml streptomycin, and 10% fetal calf serum. Media and other components were purchased from (Invitrogen). The COS-7 cells were transfected using LipofectAMINE 2000 (Invitrogen) according to the manufacturer's instructions.

Immunoblotting—The cells were harvested and processed as described previously (11). Briefly, the 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 Sciences). The cell lysates were spun at 14,000 × g for 15 min at 4 °C to pellet insoluble material. The 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 FujiFilm LAS-1000 plus system with 1,300,000-pixel cooled CCD camera that has 3.7-order of magnitude linearity. Quantification was carried out within the linear range using the Image Gauge version 3.2 software (FujiFilm). GFP-CFTR and Dyn2-GFP were detected with polyclonal GFP antibody (1:1000; BD Biosciences, Boston, MA). HA-CAL was detected with monoclonal HA antibody (1:2000; Roche Applied Sciences). Tubulin was detected with monoclonal tubulin antibody (1: 1000; Sigma).

Surface Biotinylation—Surface biotinylation of CFTR at the plasma membrane was described previously (11). Briefly, the cell surface proteins were labeled with cell-impermeable EZ-Link™ Sulfo-NHS-SS-Biotin (sulfosuccinimidyl-2-(biotinamido)ethyl-1,3-dithiopropionate; Pierce) at 4 °C for 15 min. The cell surface proteins were isolated by incubating with immobilized NeutrAvidin beads at 4 °C for 2 h (Pierce; catalog number 53151). The bound proteins were eluted with 2× Laemmli sample buffer supplemented with 100 mM dithiothreitol at 42 °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 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-^{35}S-label (250 μCi/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 system (FujiFilm). 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. Nonspecific binding sites were blocked with 5% normal goat serum. The cells were stained with anti-HA monoclonal antibody (1:2000; Roche Applied Sciences) 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 GFP signal was detected by its fluorescence. The specimens were mounted and viewed on the UltraVIEW spinning disk confocal microscope (PerkinElmer Life Sciences).

Calculation of Degradation—The percentage of bafilomycin A1-sensitive CFTR degradation was calculated as follows: $[\text{CFTR}_{\text{bafilomycin A1-treated}} - \text{CFTR}_{\text{untreated}}]/\text{CFTR}_{\text{bafilomycin A1-treated}} \times 100\%$.

Statistical Analysis—The data are presented as the means ± S.E. Statistical significance was determined by Student's t test. We assigned significance at $p < 0.05$.

RESULTS

Is Expression of Mature CFTR Protein Influenced by CAL?—Co-expression of CAL reduces the total cellular CFTR (23). To confirm these observations, we co-transfected GFP-CFTR into COS-7 cells with different concentrations of either HA- or Myc-tagged CAL. GFP-CFTR was previously shown to have the single channel properties and intracellular trafficking patterns similar to that of wild type CFTR (28). Fig. 1 depicts the steady state levels of CFTR, HA-CAL, and tubulin, which is used to assess protein loading. Note that CFTR resolves into two bands that are well known to correspond to the upper mature C-band (fully glycosylated) and the lower immature B band (core glycosylated) of CFTR (29). Note that expression of either type of tagged CAL construct significantly reduced steady state levels of the mature band of CFTR assayed at 48 h post-transfection. Consistent with previous results (23), the effects of CAL on mature CFTR were dose-dependent and independent of the epitope tags. Both results argue that CAL down-regulates CFTR mature protein in a specific fashion. For example, the abundance of tubulin, a cytoskeleton protein, was not affected by either HA-CAL (Fig. 1, *A* and *C*) or Myc-CAL (Fig. 1, *B* and *C*). Likewise, co-expression of HA-CAL or Myc-CAL did not affect the expression of GFP, a cytosolic protein, or GFP-frizzled 4, a membrane protein (data not shown). Neither GFP nor frizzled 4 binds to CAL (11). All of the GFP-tagged proteins used have the same cytomegalovirus promoters, eliminating a nonspecific effect on promoter activity. In addition, NHE-RF has no effect on the abundance of mature CFTR (11, 23). These data suggest that CAL does not down-regulate GFP-CFTR by reducing its transcription (see more on pulse-chase experiments later) or by acting on the GFP moiety of the fusion proteins.

To verify these results further, the effect of CAL on CFTR was investigated in cells stably expressing a low level of CFTR. This cell line was created by transfecting COS-7 cells with GFP-CFTR, followed by selection and expansion of isolated G418-resistant colonies. One resultant clone, 4F2, was obtained that expressed one-tenth of the GFP-CFTR cells transiently transfected with 3 μg of GFP-CFTR/100-mm dish (data not shown). In these cells, HA-CAL also reduced the steady state levels of CFTR (Fig. 2*C*, *third lane* is 65.6 ± 6.8% of *first lane*; $p < 0.05$, $n = 3$).

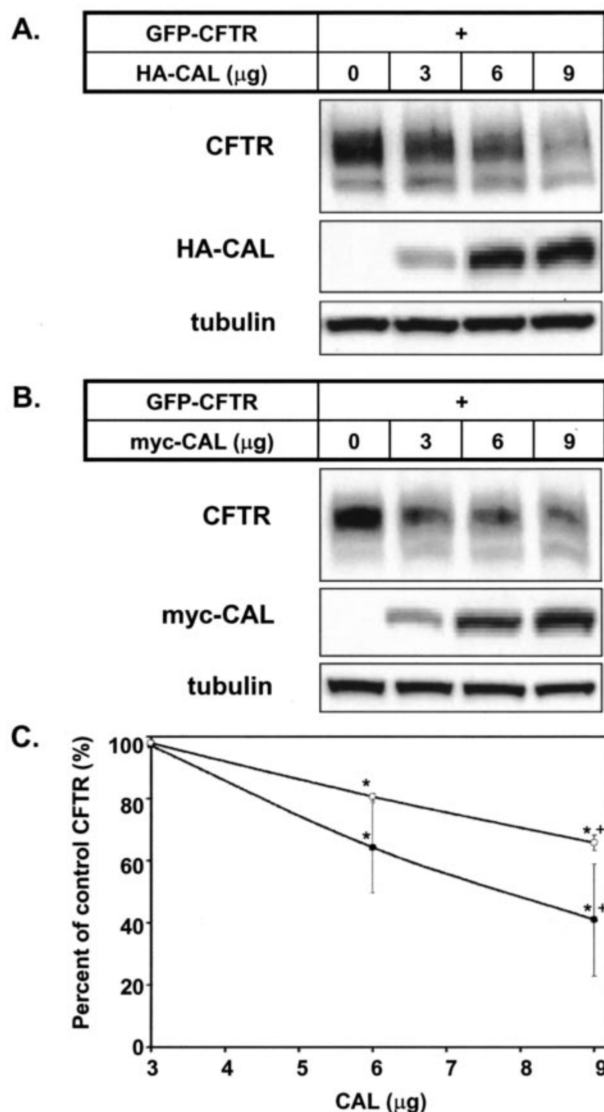

FIG. 1. **Dose-dependent degradation of CFTR by CAL.** *A*, COS-7 cells were co-transfected with GFP-CFTR (3 μg) and HA-CAL as indicated. The cells were lysed 48 h post-transfection. The proteins in cell lysates were separated on SDS-PAGE. *B*, COS-7 cells were co-transfected with GFP-CFTR (3 μg/dish) and Myc-CAL as indicated. GFP-CFTR was detected by Western blotting using an anti-GFP polyclonal antibody. HA-CAL and Myc-CAL were detected by Western blotting using anti-HA and anti-Myc monoclonal antibodies, respectively. Tubulin was detected by Western blotting using an anti-tubulin monoclonal antibody to demonstrate equal loading of total cell lysates. *C*, averages of the experiments described in *A* and *B*. The *filled circles* are values from cells co-transfected with HA-CAL. The *open circles* are values from cells co-transfected with Myc-CAL. The *error bars* are the standard deviations. *, $p < 0.05$ *versus* the control. +, $p < 0.05$ *versus* the cells transfected with 6 μg/dish CAL ($n = 3$).

Is the Degradation of CFTR Influenced by CAL?—To investigate the role of the lysosome and the proteasome in CAL-mediated down-regulation of CFTR, we examined the effect of their respective inhibitors. As shown in Fig. 2*A*, treatment with bafilomycin A1, a lysosomal proton pump inhibitor (30), increases steady state protein levels of CFTR. Our data combined with previous findings obtained with other lysosome blocking agents (10, 11) suggest that lysosomal degradation is an important process in regulating mature CFTR abundance. Important for this study is that bafilomycin A1 reverses CAL-mediated reduction in mature CFTR (Fig. 2*A*). Fig 2*A* (*first* and *third lanes*) shows the typical effect of CAL on mature CFTR. In the absence of bafilomycin A1, CAL-transfected cells have significantly less mature CFTR than in cells not transfected with CAL (Fig. 2*A*, *third lane* is 57.1 ± 4.2% of *first lane*; $p < 0.05$, $n = 3$). Importantly, after 24–48 h of bafilomycin A1 treatment, the effect of CAL is significantly reduced (Fig. 2*A*, *fourth lane*, is 75.7 ± 5.9% of *second lane*; $p > 0.05$, $n = 3$). The effect of bafilomycin is likely underestimated. Some CAL-induced reduction in mature CFTR was expected to take place prior to the addition of bafilomycin midway, at 24 h post-transfection. Thus, some CAL-induced reduction in mature CFTR was expected to take place prior to the addition of bafilomycin. Fig. 2*B* depicts the extent of CFTR bafilomycin A1 sensitivity (see "Experimental Procedures"). At 24–48 h post-transfection, ~27.93 ± 2.85% of CFTR is bafilomycin-sensitive. In contrast, in cells transfected with 6 μg of HA-CAL/100-mm dish, ~49.55 ± 7.62% of CFTR is bafilomycin-sensitive ($p < 0.01$). The reversal of CAL-mediated reduction of mature CFTR by bafilomycin A1 is also reproduced in the stable cell line 4F2 (Fig. 2*C*, *fourth lane* is 77.8 ± 9.6% of *second lane*; $p > 0.05$, $n = 3$; whereas Fig. 2*C*, *third lane* is 65.6 ± 6.8% of *first lane*, $p < 0.05$, $n = 3$). More importantly, transfection of HA-CAL leads to an increase in the extent of bafilomycin A1-sensitive degradation of CFTR (Fig. 2*D*). These results strongly suggested that CAL plays a key role in regulating the bafilomycin A1-sensitive degradation of CFTR. On the other hand, treating CAL-transfected cells with the proteasome inhibitor MG132 did not rescue mature GFP-CFTR (Fig. 2*A*) back to the control levels observed in the absence of CAL. In contrast to bafilomycin A1 treatment, in the presence of CAL, MG132 actually reduced levels of mature CFTR. It has been observed by several investigators that proteasome inhibition does not increase the maturation of CFTR (31, 32). MG132 did not increase immature CFTR in the Nonidet P-40 lysates probably because blocking of proteosomal degradation leads to the accumulation of CFTR into a detergent-insoluble polyubiquitinated form of immature CFTR (32). The failure of MG132 to inhibit CAL reduction of mature CFTR is further evidence that CAL can function downstream of the proteosome and influence the degradation of mature CFTR.

What Is the Effect of CAL on CFTR Maturation?—The steady state level of mature CFTR is determined by new protein synthesis and the trafficking and maturation of CFTR from ER to Golgi as well as post-Golgi degradation. To eliminate the possibility that, in addition to enhancing the degradation of mature CFTR in the lysosome, CAL decreases CFTR synthesis and/or maturation, we examined the synthesis and maturation of CFTR by metabolic labeling and pulse-chase experiments. In Fig. 3*A*, COS-7 cells transfected with GFP-CFTR with or without HA-CAL were metabolically pulse-labeled with [^{35}S]methionine and cysteine for 30 min at 16 h post-transfection. The cells were then chased in medium supplemented with unlabeled methionine and cysteine for 1–4 h to follow the maturation of CFTR. The *lower band* (Fig. 3*A*, *band B*) is the newly synthesized CFTR in ER that appears first. Not all newly synthesized CFTR is converted to mature CFTR (*band C*, *upper band*); because CFTR folds inefficiently, most of band B is degraded by ER-associated degradation (8). Fig. 3 shows that GFP-CFTR matures similarly whether transfected or not transfected with CAL. Therefore, CAL exerts its effects on mature CFTR and does not affect the synthesis and trafficking to Golgi. To test this hypothesis directly, the effect of CAL on mature CFTR was examined. GFP-CFTR becomes fully mature after chasing for 4 h (Fig. 3*A*). There is no significant difference in the amount of CFTR in CAL transfected and untransfected cells (40,990 ± 1840 *versus* 39,284 ± 1464 arbitrary unit/dish, $p > 0.05$, $n = 3$). The mature CFTR was then followed when cells were chased for an additional 16 h. As shown in Fig. 3*C*,

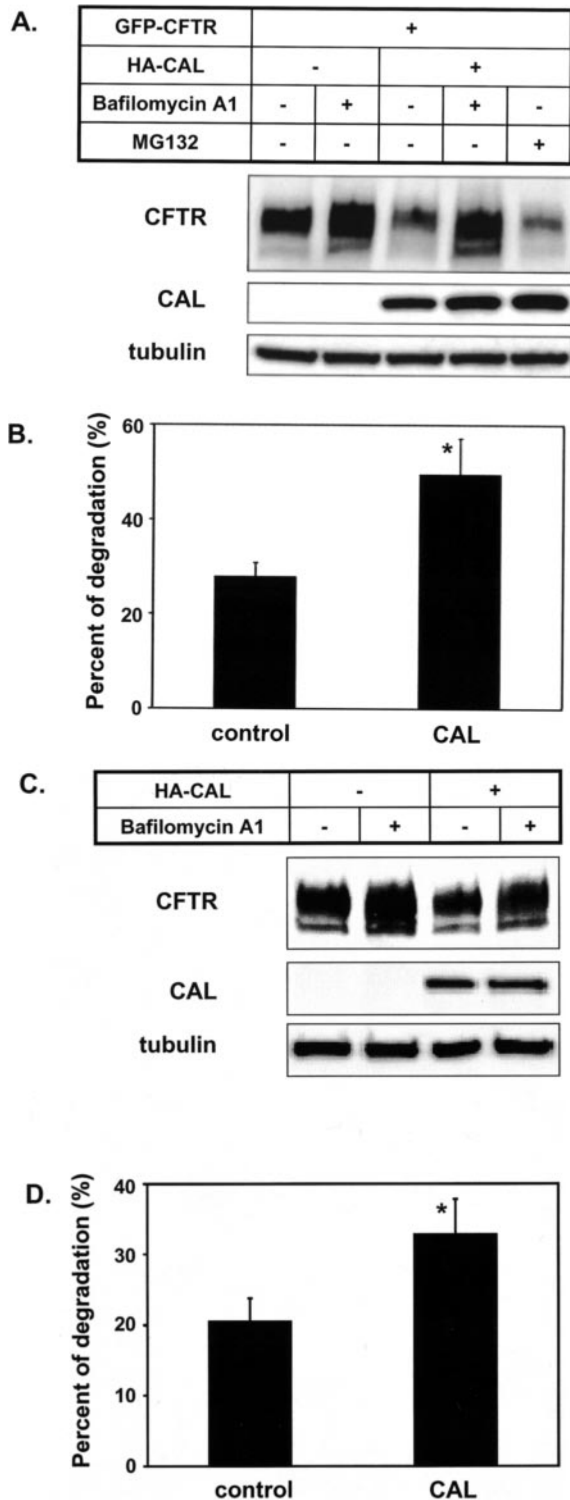

FIG. 2. **Bafilomycin A1 reverses CAL-mediated reduction of CFTR.** *A*, COS-7 Cells were co-transfected with GFP-CFTR and HA-CAL. Either the lysosome inhibitor bafilomycin A1 (1 μM) or the proteasome inhibitor MG132 (10 μM) was added 24 h post-transfection. The cells were lysed, and the proteins were detected by Western blotting as described in the legend to Fig. 1. *B*, the percentage of bafilomycin A1-sensitive CFTR degradation was calculated as the portion of CFTR protected by bafilomycin A1 (see "Experimental Procedures"). The data are the means of three experiments. The *error bars* are standard deviations. *, $p < 0.01$ *versus* the control. *C*, the COS-7 cell line, 4F2, stably expressing a low amount of GFP-CFTR was transfected with HA-CAL (6 μg/dish). Bafilomycin A1 (1 μM) was added 24 h post-transfection. The cells were lysed, and the proteins were detected as described above. *D*, the percentage of bafilomycin A1-sensitive CFTR degradation in 4F2. The data are the means of three experiments. The *error bars* are the standard deviations. *, $p < 0.01$ *versus* the control.

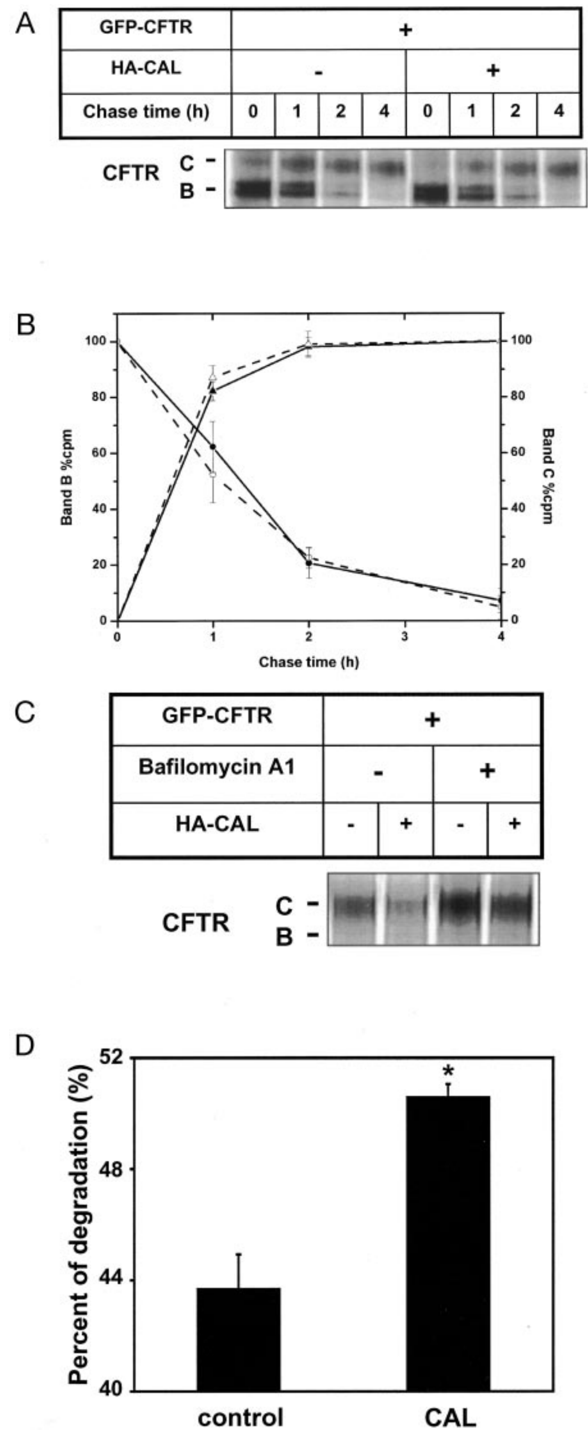

FIG. 3. **CAL enhances the degradation of mature CFTR blocked by bafilomycin A1.** *A*, COS-7 cells were transfected with GFP-CFTR and HA-CAL. The cells were metabolically labeled for 30 min with [^{35}S]methionine 16 h post-transfection. The cells were then chased in normal medium supplemented with unlabeled cysteine and methionine for 1–4 h as indicated. *B*, quantification of [^{35}S]methionine-labeled CFTR as described in *A*. The *filled symbols* are transfected with CFTR alone. The *open symbols* are co-transfected with GFP-CFTR and HA-CAL. The *circles* are immature CFTR (*band B*). The *triangles* are mature CFTR (*band C*). The data are represented as percentages of radioactivity (cpm) at 0 h for *band B* and as percentages of radioactivity (cpm) at 4 h for *band C*. The data are the mean of three experiments. The *error bars* are the standard deviations. *C*, after a 4-h chase, CFTR maturation was complete. The cells were treated with bafilomycin A1 as indicated and chased for an additional 16 h. Longer exposure was used to allow visualization. *D*, The percentage of bafilomycin A1-sensitive CFTR degradation. The data are the means of three experiments described in *C*. The *error bars* are the standard deviations. *, $p < 0.01$ *versus* the control.

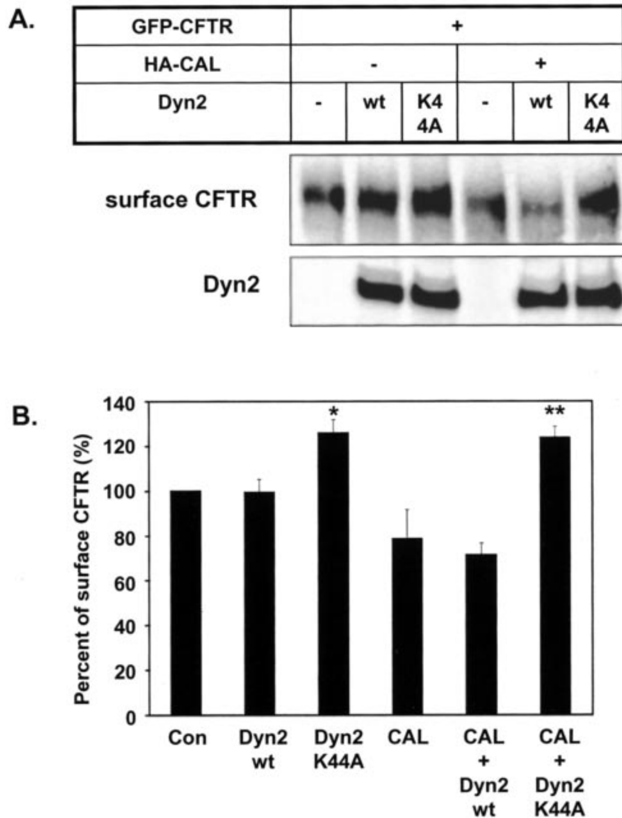

FIG. 4. **Dynamin and CAL affect CFTR surface expression.** *A*, COS-7 cells were transfected with GFP-CFTR, HA-CAL, and Dyn2-GFP or dominant-negative Dyn2K44A-GFP. Forty-eight hours post-transfection, the cell surface proteins were labeled with membrane-impermeable Sulfo-NHS-SS-Biotin at 4 °C. The cell surface proteins were isolated by NeutrAvidin beads, fractionated on SDS-PAGE, and detected by Western blotting using a GFP polyclonal antibody. *B*, averages of three experiments described in *A*. The *error bars* are the standard deviations. *, $p < 0.05$ *versus* the control. **, $p < 0.05$ *versus* the cells transfected with CAL alone. *wt*, wild type.

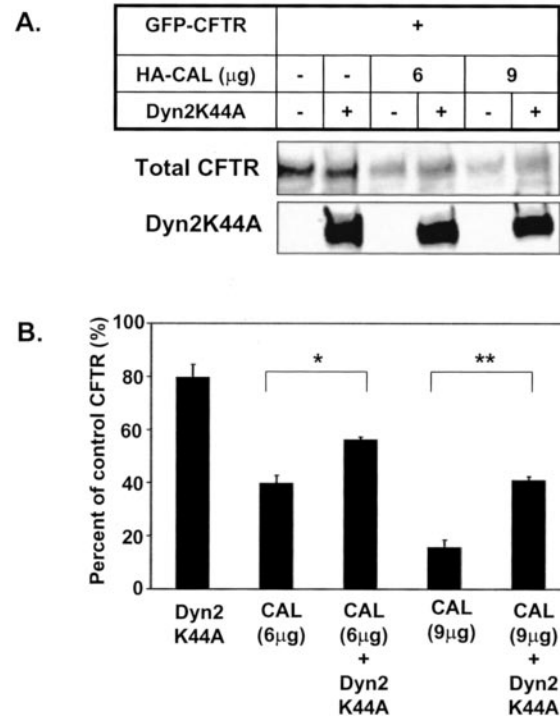

FIG. 5. **Dominant-negative dynamin 2 partially blocks CAL-mediated effects on mature CFTR.** *A*, COS-7 cells were transfected GFP-CFTR, HA-CAL, and dominant-negative Dyn2K44A-GFP. Total mature CFTR was isolated and detected by Western blotting using a GFP polyclonal antibody as described in the legend to Fig. 1*B*. *B*, averages of three experiments described for *A*. The *error bars* are the standard deviations. Dyn2K44A-transfected cells do not differ significantly from the untransfected cells. *, $p < 0.05$ *versus* the cells transfected with 6 μg of CAL. **, $p < 0.05$ *versus* the cells transfected with 9 μg of CAL.

there was less ^{35}S-labeled mature CFTR after 16 h in cells transfected with HA-CAL than with those not transfected with HA-CAL, confirming the role of CAL in the degradation of mature CFTR. On the other hand, if bafilomycin A1 was added at 4 h in the absence of CAL, followed by a 16-h chase, more labeled CFTR remains after 16 h compared with that remaining in the absence of both CAL and bafilomycin A1, suggesting that degradation was inhibited. Of note is that there is more labeled CFTR remaining in CAL-transfected cells in the presence of bafilomycin than in CAL-transfected cells in the absence of bafilomycin. Importantly, the fraction of mature CFTR sensitive to bafilomycin A1 is statistically larger in the presence of CAL *versus* the control (50.61 ± 0.45 *versus* 43.72 ± 1.2, $p < 0.01$), indicating that CAL is enhancing the degradation of mature CFTR. Taken together, these data suggest that CAL does not affect early CFTR biosynthesis in the ER or maturation to mature CFTR. CAL retains CFTR intracellularly and down-regulates mature CFTR most likely by enhancing its degradation in the post-Golgi compartments.

CAL Mediates Mature CFTR through Vesicle Formation Involving Dynamin 2—The large GTPase dynamin 2 controls the pinching of the clathrin-coated endocytic vesicles from the plasma membrane and perhaps the budding of vesicles from the Golgi (33). A dominant-negative dynamin 2 (aa), Dyn2K44A, blocks the endocytosis of a wide variety of membrane proteins (34) and is also known to affect the integrity of the Golgi in some cell types (35). Fig. 4 shows the effects of GFP-tagged Dyn2K44A and wild type Dyn2 on CFTR cell surface expression assayed by surface biotinylation. Dyn2K44A-GFP or Dyn2-GFP were co-transfected with GFP-CFTR either with or without HA-CAL. Cell surface CFTR was labeled with cell-impermeable EZ-Link^{TM} Sulfo-NHS-SS-Biotin at 4 °C, isolated with Neutravidin beads, and detected by Western blotting with polyclonal GFP antibodies. Dyn2-GFP had no effect on cell surface CFTR, whereas Dyn2K44A-GFP increased the cell surface expression of GFP-CFTR. The increased cell surface expression of mature CFTR by the dynamin mutant is consistent with previous reports that clathrin-coated pits mediate the endocytosis of CFTR (36, 37). Importantly, Dyn2K44A-GFP inhibits the ability of CAL to reduce surface expression of CFTR, thereby restoring surface expression GFP-CFTR in cells expressing HA-CAL (Fig. 4).

Next, we examined whether Dyn2K44A-GFP affects CAL-mediated effects on levels of mature CFTR. The cells were co-transfected with GFP-CFTR and increasing amounts of HA-CAL in the presence or absence of transfected Dyn2K44A-GFP (Fig. 5). The dynamin mutant does not significantly affect the levels of mature CFTR arguing against a large effect of the mutant on the maturation of CFTR through the Golgi. Again, HA-CAL reduces mature GFP-CFTR in a dose-dependent manner. Importantly, Dyn2K44A-GFP partially reverses the reduction of mature GFP-CFTR (Fig. 5) by CAL. It is not clear whether the incomplete reversal is due to incomplete co-transfection of all three plasmids or whether a portion of the CAL-mediated effect on mature CFTR occurs via a dynamin insensitive pathway. However, taken together with the lack of effect of CAL on the maturation of CFTR in Fig. 3, these data do provide further support for the notion that the degradation of

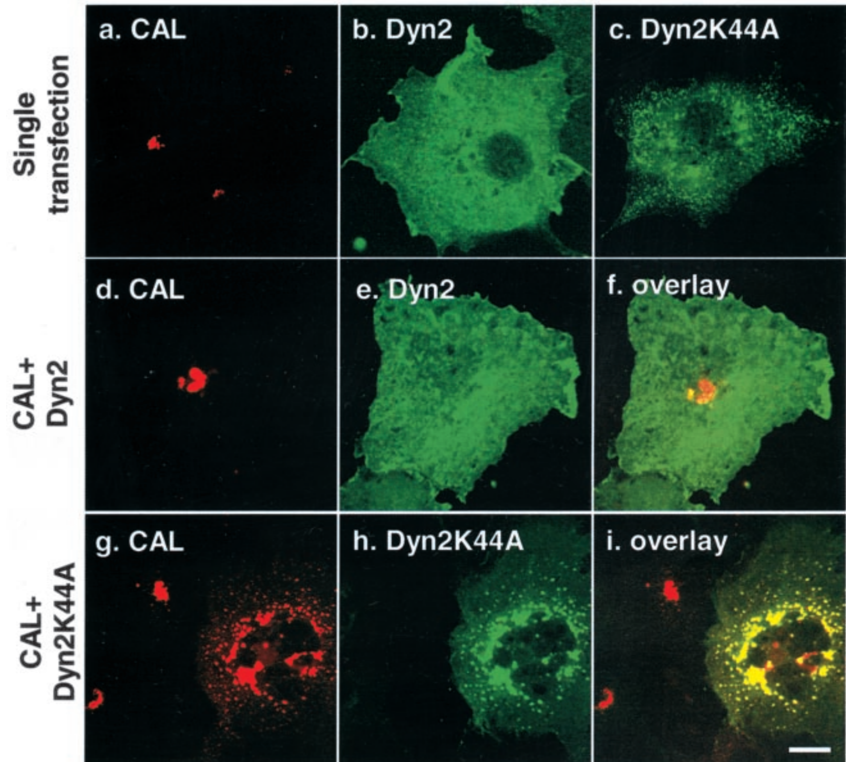

FIG. 6. **Dynamin 2 dominant-negative mutant redistributes CAL.** COS-7 cells were singly transfected with HA-CAL (*a*), wild type Dyn2-GFP (*b*), or dominant-negative Dyn2K44A-GFP (*c*) (*top row*) or doubly transfected with HA-CAL and Dyn2-GFP (*d–f*; *middle row*) or HA-CAL and Dyn2K44A-GFP (*g–i*; *bottom row*). CAL was detected by a monoclonal antibody to HA epitope (*red*) and Dyn2 were visualized by green fluorescence (*green*) on confocal microscope. CAL is typically expressed in the Golgi apparatus. Wild type Dyn2 has no effect on its intracellular localization. Dyn2K44A redistributes and co-localizes with CAL. *Scale bar*, 10 μm.

CFTR in post-Golgi compartments is enhanced by CAL causing a down-regulation of mature CFTR.

Dominant-negative Dynamin 2 Redistributes the Subcellular Localization of CAL—CAL is predominantly expressed in the Golgi apparatus, particularly at the trans-Golgi network (TGN) (11). To further study the subcellular localization of CAL, we examined the effect of dominant-negative dynamin 2 on the subcellular distribution of CAL by confocal microscopy. COS-7 cells grown on coverslips were transfected with HA-CAL, Dyn2-GFP, or Dyn2K44A-GFP. Fig. 6*a* shows the location of CAL. We have reported previously and others have also shown that CAL is located in the perinuclear region of the cell (11, 20, 21). Fig. 6*b* shows that Dyn2-GFP is expressed uniformly in COS-7 cells. Dyn2K44A-GFP, on the other hand, has a distinctly different punctuate expression pattern (Fig. 6*c*), consistent with previous report that this mutant blocks clathrin coat-mediated endocytosis and is arrested at the clathrin-coated pits at the plasma membrane (38). Whereas co-expression of wild type Dyn2 has no effect on the distribution of CAL (Fig. 6, *d–f*), expression of Dyn2K44A redistributes CAL to the sites that co-localized with Dyn2K44A (Fig. 6, *g–i*). The cells in Fig. 6 (*g–i*) transfected only with HA-CAL but not Dyn2K44A-GFP retain typical Golgi localization of CAL. These results are significant because they show that CAL traffics from Golgi and is arrested at the cell periphery, where further progression is blocked by Dyn2K44A.

DISCUSSION

In this paper, we report that co-expression of the CFTR-interacting protein CAL down-regulates expression of mature CFTR protein. We previously identified CAL as a novel CFTR-interacting protein and showed that co-expression of CAL retains CFTR in the intracellular compartment decreasing cell surface expression of CFTR (11). Here, we show that CAL causes a dose-dependent reduction in the total amount of mature CFTR (Fig. 1). The proton pump inhibitor, bafilomycin A1, reverses CAL-mediated reduction in mature CFTR (Fig. 2). Transfection of CAL leads to an increase in the extent of bafilomycin A1-sensitive degradation of CFTR (Fig. 2, *C* and *D*). The proteasomal inhibitor, MG132, on the other hand, does not reverse the effect of CAL (Fig. 2). Pulse-chase experiments indicate that CAL has no effect on CFTR maturation, suggesting that it exerts its effect on mature CFTR (Fig. 3) by enhancing the degradation of mature CFTR. As we have previously shown, CAL reduces the half-life of CFTR at the cell surface (11). Co-expression of a dominant-negative mutant of dynamin 2, Dyn2K44A-GFP, with CAL and CFTR restores cell surface CFTR and partially blocks the CAL-mediated degradation of mature CFTR (Figs. 4 and 5). These data suggest a model in which CAL retains CFTR in the cell and targets mature CFTR for degradation via mechanisms involving post-Golgi vesicle formation and trafficking.

Both intracellular retention and degradation of mature CFTR by CAL contributes to the CAL-mediated down-regulation of CFTR. To date, no enzymatic activity has been identified in CAL. Examining the protein domains and motifs suggests that CAL is a scaffolding protein with multiple protein-binding domains. Like other scaffolding proteins, CAL operates as an adaptor to couple multiple proteins. Other than the PDZ domain that binds to CFTR, the second coiled-coil domain binds to the small GTPase TC10 and the SNARE protein syntaxin 6 (20, 22). Both TC10 and syntaxin 6 are known to be involved in intracellular trafficking. TC10 is a member of the Rho-GTPase family. In adipose tissues, TC10 is involved in regulating the trafficking of the insulin-stimulated glucose transporter, GLUT4, to the plasma membrane. As an adaptor protein, CAL is likely to link CFTR to the intracellular trafficking processes carried out by TC10 or syntaxin 6 in CFTR-expressing epithelial tissues.

At steady state, CAL is localized to the Golgi complex, particularly in the TGN and cytosol. TC10 is localized at the plasma membrane and intracellular vesicles; syntaxin 6 is primarily in Golgi and endosomes. CFTR spans all of these compartments. For these proteins to work in concert on CFTR, they must physically interact. One possibility is that they in-

teract at the interface of their respective compartments. Alternatively, one or all of these proteins could cycle among different compartments. For example, CAL may traffic from TGN/Golgi to the plasma membrane and then to endosomes and to the lysosome, or it may traffic directly from the TGN to the lysosome. Fig. 6 depicts evidence supporting this notion. The data show that expression of a dominant-negative dynamin 2 mutant redistributes CAL to the cell periphery into punctuate structures that co-localize with the mutant dynamin 2. In this circumstance CAL is arrested at the sites where dynamin regulates vesicle budding (Fig. 6). The dynamin 2 mutant Dyn2K44A is well known to inhibit endocytosis and to affect the Golgi (33, 35). The observation that the dynamin 2 mutant Dyn2K44A enhances surface expression of mature CFTR and does not have a large effect on the total amount of mature CFTR supports the notion that the primary effect of Dyn2K44A is post-Golgi. Thus, it is likely that CAL can traffic with CFTR past the Golgi and target CFTR for degradation either through endocytosis at the plasma membrane or via direct trafficking from a post-Golgi compartment to the lysosome. However, it is important to note that our data do not exclude other ways of regulating mature protein and cell surface expression CFTR.

The evidence that CAL is involved in trafficking in tissues other than epithelia was suggested by two recent papers. Neuronal CAL contributes to the pathology of Lurcher mice (39). Lurcher mutant mice are ataxic because of degeneration of the cortex of the cerebella. Constitutive activation of the GluRd2Lc receptor causes an inward current and the death of Purkinje cells. Neuronal CAL has an additional 8 amino acids at the beginning of the second coiled-coil domain. CAL binds to GluRd2 and Beclin1 and links receptor activation to autophagy and cell death (39). Autophagy involves trafficking membrane compartments to the lysosome for degradation. Therefore, CAL maybe involved in lysosomal trafficking in autophagy.

More information on the function of CAL came from knockout mouse experiments (40). In CAL knockout mice, the males are infertile. The mature sperm of male mice missing CAL lack acrosome, a specialized lysosomal structure derived from the Golgi that is required for oocyte activation. In normal mice, CAL is located in the TGN and TGN-derived vesicles of the round spermatids. These data suggest that CAL is involved in acrosome biogenesis, perhaps by facilitating protein cargo transport to this organelle.

New therapies are under development to promote ΔF508 CFTR maturation. Interfering with the degradation of ΔF508 CFTR in the post-Golgi compartments may be an attractive method to boost levels of mature CFTR. Our current study suggests a method to accomplish this by inhibiting the degradation of mature CFTR. More importantly, a more specific way of blocking the interaction between CAL and CFTR may potentially allow more CFTR to be trafficked to the plasma membrane.

Acknowledgments—We thank Dr. Mark A. McNiven for dynamin 2 (aa) constructs: Dyn2-GFP and Dyn2K44A-GFP. We thank Drs. Carolyn Machamer and Laura Roman for helpful discussions. We thank Dr. Peying Fong for critical reading of this manuscript.

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