We identified a novel cystic fibrosis transmembrane conductance regulator (CFTR)-associating, PDZ domain-containing protein, CAL (CFTR associated ligand) containing two predicted coiled-coiled domains and one PDZ domain. The PDZ domain of CAL binds to the C terminus of CFTR. Although CAL does not have any predicted transmembrane domains, CAL is associated with membranes mediated by a region containing the coiled-coil domains. CAL is located primarily at the Golgi apparatus, co-localizing with trans-Golgi markers and is sensitive to Brefeldin A treatment. Immunoprecipitation experiments suggest that CAL exists as a multimer. Overexpression of CAL reduces CFTR chloride currents in mammalian cells and decreases expression, rate of insertion and half-life of CFTR in the plasma membrane. The Na⁺/H⁺ exchanger regulatory factor, NHE-RF, a subplasma membrane PDZ domain protein, restores cell surface expression of CFTR and chloride currents. In addition, NHE-RF inhibits the binding of CAL to CFTR. CAL modulates the surface expression of CFTR. CAL favors retention of CFTR within the cell, whereas NHE-RF favors surface expression by competing with CAL for the binding of CFTR. Thus, the regulation of CFTR in the plasma membrane involves the dynamic interaction between at least two PDZ domain proteins.

Cystic fibrosis is a genetic disorder caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) (1–3). CFTR is a cAMP-regulated chloride channel and channel regulator (4) that mediates salt and water balance in a number of tissues, including the airways, intestines, pancreatic and sweat ducts, and kidney tubules (5). In many polarized epithelial tissues, CFTR functions in the apical cell membrane but in the sweat duct CFTR is localized to both the apical and basolateral cell membranes. Because a common mutation in CFTR, AF508, is degraded through the endoplasmic reticulum (ER)-associated processes, many studies have focused on the trafficking of CFTR through this compartment (6). Much less attention has been devoted to how CFTR is processed through the post-ER pathways.

We reported previously that the polarization of CFTR in the apical membrane of human airway and kidney epithelial cells is altered in CFTR mutants lacking the C-terminal domain (7, 8). These data suggested that the C-terminal domain plays a role either in the trafficking to and/or stability within the plasma membrane. The last three amino acids of CFTR (Thr-Arg-Leu) comprise a consensus sequence known to bind to PDZ domain proteins, a family of proteins containing a 80- to 90-amino acid motif that binds to the C terminus of a variety of ion channels and receptors (9–11).

At least two PDZ domain-containing proteins, Na⁺/H⁺ exchanger regulatory factor, NHE-RF (also called EBP50), and the CFTR-associated protein CAP70 are known to bind to the terminal amino acids of CFTR (12, 13). NHE-RF is a submembranous phosphoprotein that regulates the apical membrane Na⁺/H⁺ exchanger NHE3 (14). Because NHE-RF associates with ezrin, which itself binds to the regulatory subunit of PKA (15), it was hypothesized that NHE-RF anchors CFTR to the cytoskeleton at a subapical compartment targeting protein kinase A near CFTR (12). E3KARP, an NHE-RF-related protein, was also shown to interact with CFTR (16, 17). CAP70, a subapical protein with four PDZ domains, binds to at least two CFTR molecules simultaneously via PDZ3 and PDZ4 (18). CAP70 and NHE-RF enhance CFTR Cl⁻ channel activity in excised patches probably by cross-linking CFTR dimers (18, 19). NHE-RF, E3KARP, and CAP70 are localized to subplasma membrane regions reflecting their proposed roles in anchoring and/or regulating CFTR.

Although there is growing evidence of a functional interaction between CFTR and PDZ domain proteins at the plasma membrane, little is known about whether PDZ domain proteins play a role in intracellular trafficking of CFTR along the ER,
The Golgi, and the plasma membrane. The post-ER secretory pathway involved in the trafficking of wild type and mutant CFTR to the plasma membrane is poorly characterized. A recent study has demonstrated that CFTR has an unusually low distribution in cis-Golgi compartments compared with most membrane proteins (20), raising the possibility that CFTR may utilize novel modes of anterograde and retrograde trafficking mechanisms.

To learn more about the role of the C terminus of CFTR in processing and trafficking, we used a yeast-two hybrid assay to identify proteins that bind to CFTR. We identified a novel PDZ domain protein, CAL, that interacts with CFTR in the post-ER secretory pathway. The PDZ domain of CAL binds directly to the C terminus of CFTR. Native CFTR and CAL localize with Golgi markers in lung tissue in vivo. Overexpression of CAL suppresses CFTR expression at the cell surface and reduces CFTR currents both in Xenopus oocytes and mammalian cells. This suppression can be overcome by NHE-RF. Our data demonstrate that CAL modulates the surface expression of CFTR. Although NHE-RF alone does not enhance surface expression of CFTR, it competes with CAL for the binding of CFTR and indirectly enhances expression in the plasma membrane. Thus, the expression of CFTR in the plasma membrane involves the dynamic interaction between at least two PDZ domain proteins.

**EXPERIMENTAL PROCEDURES**

**Two-hybrid Screening and Cloning of CAL—** Yeast two-hybrid screening was used to identify proteins that interact with the C terminus of CFTR (21). The bait consisted of the C-terminal 51 amino acids of rat CFTR (aa 1428–1478 of rCFTR), amplified by PCR from a rat lung MATCHMAKER cDNA library (CLONTECH, Palo Alto, CA). The PCR products were subcloned into the BamHI/PstI sites of a pAS2-1 vector, in-frame with the Gal4 DNA-binding domain. Approximately 1 × 107 recombinants of a rat lung MATCHMAKER cDNA library fused to the Gal4 activation domain (CLONTECH) were screened for interaction with the bait protein in Y190 yeast cells on triple-minus plates supplemented with 35 μM 3-amino-1,2,4-triazole (3-AT) (Leu-, Trp-, His-, 3-AT). A rat CAL (rCAL) fragment identified in yeast two-hybrid screening was used to screen a human lung cDNA library (CLONTECH) to obtain the full-length human CAL (hCAL). Cloned inserts were sequenced in both directions by automated fluorescent sequencing (Johns Hopkins University Biosynthesis & Sequencing Facility). A partial rat CAL truncated at the extreme N terminus was also obtained by hybridization screening of a rat hippocampus library (gift from Drs. A. A. Lanahan and P. F. Worley).

**Yeast Two-hybrid Assay—** rCFTR/C4 (aa 1428–1474) and rCFTR/C7 (aa 1454–1471) were subcloned into the BamHI/PstI sites of the pAS2-1 vector, in-frame with the Gal4 DNA-binding domain (CLONTECH). rCFTR/C4, rCFTR/C7, or the empty pAS2-1 vector were co-transformed with rCAL (in pGAD10 vector) into Y190 cells. Interactions were monitored by the LacZ expression on filter lifted from the transformed cells and by counting viable colonies on triple-minus plates (Leu-, Trp-, His-, 35 mM 3-AT).

**Cell Culture and Transfection—** Human embryonic kidney cell line HEK 293 and colorectal carcinoma T84 cells (obtained from American Type Tissue Culture, Manassas, VA) were maintained in DMEM-F-12 (1:1), l-glutamine, penicillin, streptomycin, and 10% FCS. African green monkey kidney cell (COS-7) and Madin-Darby Canine Kidney II cells (MDCK) (obtained from American Type Tissue Culture) were maintained in DMEM, l-glutamine, penicillin, streptomycin, and 10% FCS. African green monkey kidney cell (COS-7) and Madin-Darby Canine Kidney II cells (MDCK) (obtained from American Type Tissue Culture) were maintained in DMEM, l-glutamine, penicillin, streptomycin, and 10% FCS. Human bronchial epithelial cells (16HBE14o, gift of Dr. Dieter Gruenert) were maintained in minimal essential medium, l-glutamine, penicillin, streptomycin, and 5% FCS. LHC-8 basal medium was purchased from Biofluids. All other media and components were purchased from GIBCO-BRL (Gaithersburg, MD). HEK 293 cells were grown in 100 units/ml, and streptomycin (100 μg/ml) were used. COS-7, IB3-1, MDCK, and 16HBE14o cells were transfected using LipofectAMINE or LipofectAMINE 2000 (Invitrogen) according to the manufacturer’s instructions.

**Antibody Production—** Glutathione S-transferase (GST) fusion protein of C-terminal polypeptide (aa 216–454) from human CAL was prepared using the GST gene fusion system (Amersham Biosciences, Inc., Piscataway, NJ). The Precision protease-cleaved CAL C-terminal polypeptide was sent to a commercial producer (Covance, Princeton, NJ) to prepare rabbit polyclonal antibodies.

**Phalloidin Construction—** The HA and myc-tagged constructs were made by PCR amplification and subcloning into the SacI/XhoI sites of the pcEGFP-N1 vector (CLONTECH). The resulting constructs contain HA or myc tag at the N terminus of the fusion proteins (Fig. 1C). The GFP-tagged constructs were made by PCR amplification and subcloning into the SacI/XhoI sites of the pEGFP-N1 vector (CLONTECH). The resulting constructs contain EGFP tag at the C terminus of the fusion proteins (Fig. 1C). All constructs were sequence-verified.

Other plasmid constructs used were HA-NHE-RF (13), GFP-CFTR and GFP-CFTR/3TRIL, (7), and full-length vesicular stomatitis virus glycoprotein (VSVG) protein cloned into pcDNA3.1 (gift of Dr. C. E. Machamer).

**Western Blotting—** Multiple human tissue poly(A) RNA blots (MTB1 and MTB2, CLONTECH) containing 16 human tissue poly(A) RNAs were hybridized with full-length hCAL probes under stringent conditions according to the manufacturer’s directions.

**Immunoprecipitation and Immunoblotting—** Cells were harvested and lysed as described (22). Briefly, cross-linker and lysates were spun at 14,000 × g for 15 min to pellet insoluble material. The supernatants were immunoprecipitated with 1 μg of monoclonal Myc antibody, HA antibody (Roche Molecular Biochemicals, Indianapolis, IL), R domain CFTR antibody (Genzyme, Cambridge, MA) or polyclonal GFP antibody (CLONTECH), and CAL antibody followed by Protein G-agarose beads for R domain CFTR antibody or Protein A-agarose beads for all other antibodies. The eluted proteins were then subjected to SDS-PAGE and Western blot followed by enhanced chemiluminescence (ECL, Amersham Biosciences, Inc., Piscataway, NJ). GFP-CFTR was detected with monoclonal GFP antibody (1:1000; Roche Molecular Biochemicals). HA-CAL, HA-NHE-RF, and other HA-tagged molecules were detected with monoclonal HA antibody (1:2000; Roche Molecular Biochemicals). Endogenous CAL was detected by CAL anti- antibodies. Endogenous CFTR was detected by immunoprecipitation with R domain CFTR antibody (Genzyme) followed by protein kinase A phosphorylation as described previously (24).

**Full-down Assay—** Biotinylated peptides were first incubated with immobilized NeutrAvidin beads (Pierce, catalog number 53151) in Nonidet P-40 lysis buffer at 4 °C for 2 h. The beads were washed three times in Nonidet P-40 lysis buffer at 4 °C and then supplemented with 100 μM DTT at 42 °C for 30 min. The eluted proteins were subjected to SDS-PAGE and Western blot.

**Surface Biotinylation—** Biotinylated CFTR at the plasma membrane was precipitated as described in detail previously with some modifications (23). Lysates were incubated with immobilized NeutrAvidin beads (Pierce, catalog number 53151), and bound proteins were eluted with 2× Laemmli sample buffer supplemented with 100 μM DTT and 42 °C for 30 min. The eluted proteins were subjected to SDS-PAGE and Western blot. GFP-CFTR was detected with monoclonal GFP antibody (1:1000; Roche Molecular Biochemicals). For VSVG surface biotinylation, COS-7 cells were transfected with full-length VSVG. VSVG was detected with rabbit anti-VSVG polyclonal antibody (25).

**Pulse-chase and Cell Surface Biotinylation—** Two days after transfection, COS-7 cells were washed twice in methionine/cysteine free DMEM and then incubated for 60 min in methionine/cysteine free DMEM. Cells were incubated in methionine/cysteine free DMEM containing Tran35S-label (250 μCi/ml) for 30 min. Subsequently, cells were washed extensively with DMEM containing 10 mM of non-labeled methionine and 10 mM of unlabeled cysteine and chased in this solution for 0, 30, 60, 90, or 120 min. Biotinylated CFTR at the plasma membrane were then isolated and subjected to SDS-PAGE and Western blot. GFP-CFTR was analyzed using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). To examine the effect of CAL overexpression on the half-life of CFTR in the plasma membrane, cells were incubated in methionine/cysteine free DMEM containing Tran35S-label (250 μCi/ml) for 3 h. Cells were washed extensively with DMEM containing 10 mM non-labeled methionine and 10 mM unlabeled cysteine (containing pen-
icillin/streptomyacin and 10% FBS) and chased in this solution for 0, 12, 24, or 32 h. Plasma membrane proteins were biotinylated at 0, 12, 24, or 32 h, and biotinylated CFTR was immunoprecipitated using a GFP monoclonal antibody. Radiolabeled CFTR was analyzed using a PhosphorImager.

Subcellular Fractionation—For subcellular fractionation experiments, cells were trypsinized and collected by centrifugation. The pellet was resuspended and incubated in ice-cold swelling buffer (1 mm DTT, 1 mm MgCl₂, 1 mm EGTA, and Complete protease inhibitor mixture (Roche Molecular Biochemicals)) for 5 min. Cells were collected by centrifugation and resuspended in buffer containing 0.25 m sucrose, 3 mm imidazole, pH 7.4, and Complete protease inhibitor mixture. Lysate was obtained by homogenizing 10 strokes with a Dounce homogenizer. After a pre-clarification centrifugation (100 g, 4 °C, 5 min), the lysate was spun in an Ultracentrifuge at 100,000 g for 45 min at 4 °C in a centrifuge (TL-100) using a TLA-100.2 rotor (both from Beckman Coulter). α-Tubulin monoclonal antibody was obtained from Amersham Biosciences, Inc.

Confocal Microscopy—Cultured cells were plated on glass coverslips 1-day post-transfection and examined 2-days post-transfection. Cells were fixed in 4% paraformaldehyde and permeabilized in 0.2% Nonidet P-40. Nonspecific binding sites were blocked with 5% normal goat serum (NGS). Cells were stained with anti-HA monoclonal antibody (1:2000, Roche Molecular Biochemicals) or γ-adaptin (1:10, Sigma Chemical, St. Louis, MO) in 5% NGS, washed with 1% bovine serum albumin, and incubated with goat anti-mouse Cy3 secondary antibodies in 1% NGS. In some cases, cells were counter-stained with 4,6-diamidino-2-phenylindole (200 µg/ml, Sigma) to visualize nuclei. Specimens were mounted and viewed on an LSM 410 confocal microscope (Zeiss).

The immunofluorescence of rat tracheae was performed exactly as described (26). Sections of frozen trachea 6 µm thick were incubated with the following antibodies: rabbit-anti-CAL (1:500), mouse-anti-β-COP (1:50, Sigma), mouse-anti-58K (1:50, Sigma), and mouse-anti-TGN38 (1:50, Oncogene, Cambridge, MA).

Whole Cell Patch-clamp Recordings—Whole-cell recordings were performed at room temperature on COS-7 cells grown on glass coverslips 1–2 days post-transfection. Cells were treated with 0.5 mm isobutylmethylxanthine for 16 h before the experiments as described (23). Transfected cells expressing GFP-CFTR were identified under a fluorescence microscope (TE2000, Nikon). Coverslips were perfused with an extracellular solution containing (in mM): 140 NaCl, 5 KCl, 2 MgCl₂, 1 CaCl₂, 10 glucose, 20 sucrose, 0.1 DIDS, and 10 Hepes (pH 7.4 with NaOH). Sucrose and DIDS were used to prevent the activation of endogenous chloride currents. Pipette resistance, with a pipette solution (PS) containing (in mM) 150 CsCl, 1 MgCl₂, 2 Na₃ATP, 1 EGTA, 10 Hepes (pH 7.4 with Tris), ranged between 5 and 8 MΩ. The input capacitance ranged from 11 to 64 pF. The capacitance and series resistance were measured using a 2 MΩ resistor and were <2 mV. Therefore no correction for these potentials was made. Extracellular membrane-permeant cAMP analogue 8-chlorophenyl-thio-cAMP (cpt-cAMP, 250 µM) was applied using a nearby pipette of 30–35-µm diameter.

RESULTS

Identification and Tissue Distribution of CAL—To identify PDZ domain proteins that interact with CFTR, we performed a yeast two-hybrid screen using the last 51 amino acids of the C terminus of rat CFTR (21). Approximately 1 × 10⁶ recombinants of a lung cDNA library fused to the Gal4 DNA activation domain were screened. A novel protein designated as CAL (CFTR-Associated Ligand) was identified in this screening. Fifty-six clones were isolated that encoded overlapping, partial sequences of CAL. Each contained the consensus sequence for a complete PDZ domain. The interaction between CFTR and CAL was confirmed by co-transferring the recombinant cDNA into yeast cells. The interaction was bait-dependent, because it did not occur with the vector alone. The interaction was also dependent upon the terminal amino acids of CFTR, because it did not occur with CFTR C-terminal constructs missing the terminal 4 or 7 amino acids (data not shown).

To obtain the full-length CAL, we screened a human lung cDNA library (CLONTECH) with rat CAL cDNA. Seven independent and overlapping clones were identified. The largest fragment contained a 1362-bp open reading frame, preceded 132 bp upstream by an in-frame stop codon. A start codon consistent with a Kozak consensus sequence (27) was identified. Translation in this reading frame predicts a 454-amino acid polypeptide with an estimated molecular mass of 50 kDa (Fig. 1A). Hydrodynamics analysis did not detect any putative membrane-spanning regions (28). No targeting signal sequence was detected. The carboxyl half of CAL is identical to the amino half of an aberrant clone Homo sapiens RNA for ros1 oncogene (29). This aberrant clone very likely results from the rearrangement of CAL to the human ros1 oncogene. CAL mapped to chromosome 6q21A by aligning to a finished human genomic sequence (PAC 94G16) in GenBank™ (30). A rat CAL cDNA that is highly homologous to the human CAL (91% identity, 94% conserved) was obtained in a hybridization screen (Fig. 1A). Human CAL is also highly homologous (56% identity, 71% homology) to a protein identified in the Caenorhabditis elegans genome project (ZK849.2) suggesting an evolutionarily conserved function (Fig. 1A), as well as to a recently described protein (31). In addition to a PDZ domain, CAL is predicted to have two coiled-coil domains. Coiled-coil domains are also involved in protein-protein interactions (32). The coexistence of coiled-coil domains and a PDZ domain suggests that CAL can potentially interact with multiple proteins.

Northern blot analysis detected CAL in all human tissues examined, including CFTR-expressing tissues such as lung and pancreas (Fig. 1B). The major transcript is 5 kb; in addition, some tissues have less abundant transcripts of different sizes (e.g. pancreas and testis). The ubiquitous expression pattern and evolutionarily conserved protein sequence of CAL suggest an essential housekeeping role for its interaction with CFTR and other membrane proteins with similar PDZ binding motifs.

Native Properties of CAL—To study CAL in vivo, we generated a rabbit polyclonal antibody raised against the C-terminal domain of human CAL (see “Experimental Procedures”). Consistent with the wide pattern of expression of CAL mRNA, CAL protein is endogenously expressed in several cell lines, including human bronchial epithelial cells, 16HBE140 (‘Fig. 2A), the colon carcinoma T84 cell line (see related data in Fig. 3D) and in the cystic fibrosis bronchial epithelial cell line, IB3–1 (data not shown). In Western blots, 16HBE140 cells display native CAL at two molecular masses, ~50-kDa and ~100-kDa bands (Fig. 2A, Lysate), when analyzed under non-reducing conditions, but only a single ~50-kDa band (Fig. 2A, +βME and +DTT) when analyzed under reducing conditions. This raises the possibility that CAL can form a homo-multimer. To test this further, we cotransfected HEK293 cells with HA-tagged full-length CAL (HA-CAL) and with each one of the following myc-tagged CAL constructs: full-length (myc-CAL), a construct containing only the C-terminal portion, including the PDZ domain (myc-CAL-PDZ-C), or a construct containing only the N-terminal amino acids, including the coiled-coil domains but minus the PDZ domain (myc-CAL-N-CC). Only in cells containing the myc-tagged full-length CAL (first lane) and the N-terminal portion of CAL (third lane) was it possible to immunoprecipitate HA-tagged full-length CAL with anti-Myc antibodies (Fig. 2C). The myc-labeled C-terminal portion is not capable of precipitating HA-tagged full-length CAL with anti-Myc antibodies (Fig. 2C). These data demonstrate that CAL forms a homo-multimer and the self-association domain lies in the N-terminal half of CAL.

To determine if CAL is a membrane-associated or cytosolic protein, 16HBE140 cells were fractionated by centrifugation into pellet and soluble fractions. CAL was found both in the

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pellet and soluble fractions (Fig. 2B) suggesting that CAL could associate with a membrane fraction. To isolate further the sequences responsible for membrane association, we transfected various truncation constructs of GFP-tagged CAL into HEK293 cells and assayed again after subcellular fractionation. GFP did not change the CAL membrane association (Fig. 2D). CAL containing only the N-terminal domain and lacking both the coiled-coil and PDZ domains (CAL-N) was mostly within the soluble fraction and with some in the pellet (Fig. 2D). CAL containing both the N-terminal and the coiled-coil domains but lacking a PDZ domain (CAL-N-CC) was found in the pelleted fraction (Fig. 2D). CAL containing the PDZ and the C-terminal domains (CAL-PDZ-C) was soluble (Fig. 2D). Although CAL has no predicted transmembrane sequences, the coiled-coil domains conferred membrane association, presumably by binding to resident Golgi coiled-coil proteins.

**CAL Binds to CFTR**—To test whether full-length CAL and CFTR interact in animal cells, COS-7 cells were transiently transfected with an N-terminal-tagged, full-length HA-CAL and GFP-CFTR (Fig. 3A). GFP-CFTR has single-channel and whole-cell current properties indistinguishable from wild type CFTR (23). As shown in Fig. 3A, lane 2. Removal of the last 3 amino acid residues of the full-length CFTR (GFP-CFTR/H9004TRL) substantially reduced the ability of the truncated CFTR to interact with CAL (Fig. 3A, lane 3), suggesting the importance of the extreme C-terminal PDZ binding motif. The faint band in lane 3 suggests that other weak binding sites for CAL may exist in CFTR.

To test further whether the PDZ-interacting domain of CFTR is important for the CAL/H18528CFTR interaction, we conducted studies using the C terminus of CFTR and the PDZ domain of CAL (Fig. 3B). COS-7 cells were transfected with the HA-tagged PDZ domain of CAL (HA-CAL-PDZ, aa 216–404) and the myc-tagged C-terminal half of CFTR, including the C-terminal and PDZ domains (aa 216–454). e. GFP-tagged N-terminal domain of CAL (aa 1–76). f. GFP-tagged the N-terminal and coiled-coil domains of CAL (aa 1–279). g. GFP-tagged PDZ domain and C-terminal of CAL (aa 216–454). *Blue filled circles* indicate the positions of epitope tags.
body in the absence of myc-CFTR-C (Fig. 3B, lane 2). Because truncation of C-terminal CFTR residues (TRL) abolishes the binding (Fig. 3A), and binding can occur between portions containing only the PDZ domain of CAL and the C terminus of CFTR (Fig. 3B), the PDZ domain-mediated interaction is both necessary and sufficient for the CAL-CFTR interaction.

To confirm that the C terminus of CFTR was involved in the interaction, we immobilized biotinylated peptides corresponding to the C-terminal 8 and 24 residues of CFTR on NeutrAvidin beads and incubated the peptides with whole cell lysates prepared from 16HBE14o- cells. The bound fractions were examined by Western blot using CAL antisera. Endogenous CAL bound to the immobilized CFTR peptides (Fig. 3C, lanes 1 and 2) but not to a control peptide (Fig. 3C, lane 3). The last 8 residues of CFTR bind to CAL, the interaction is likely to be direct and not mediated through other proteins.

To test whether CAL is present in a complex with CFTR in vivo, CFTR was immunoprecipitated from T84 cell lysates using an R-domain antibody from Genzyme and the precipitated fraction was analyzed by Western blot using CAL antibody. CAL was found in the CFTR immunoprecipitated fraction (Fig. 3D, lanes 1 and 3) but not in the control fraction (Fig. 3D, lane 2). In a reciprocal experiment, CAL antisera also precipitated CFTR (Fig. 3D, lane 3). Taken together, these data suggest that CFTR and CAL associate in vivo.

Golgi Localization of CAL—Next, we examined the expression of native CAL by indirect immunofluorescence staining with rabbit anti-CAL antibodies. In rat trachea, CAL immunostaining was seen in surface epithelium and submucosal glands (Fig. 4). The staining was bright in both acini (A) and the ducts (D) of submucosal glands (Fig. 4A). CAL is predominantly localized in intracellular compartments of acinar cells and is weakly expressed in cytoplasm (Fig. 4B). Under the same conditions, the pre-immune serum shows no significant staining (Fig. 4C). To examine the subcellular localization of CAL, acinar cells were double-labeled with CAL antibodies and Golgi markers. As shown in Fig. 4 (d–j), CAL colocalized with trans-Golgi network marker TGN38 (Golgi partially colocalizes with βCOP, a medial and cis-Golgi marker (Fig. 4, g–i) and 58K, a marker for ER-Golgi Intermediate Compartment (ERGIC; Fig. 4, j–l). A similar staining pattern was observed in rat kidney cells (data not shown).

To investigate the subcellular localization of CAL further, we examined the transiently transfected HA-tagged CAL using monoclonal HA. In IB3 and MDCK cells, CAL, detected by a monoclonal antibody to the HA epitope tag, appeared predom-
Co-transfection of CFTR and CAL resulted in greatly reduced currents despite the expression of CFTR (Fig. 6, C and D). In parallel experiments, the presence of both GFP-CFTR and HA-CAL was verified to prove that green-fluorescing cells were co-transfected with both CFTR and CAL. To test this outcome further, CFTR and CAL mRNA were injected into Xenopus oocytes. In the absence of CAL, expression of CFTR mRNA resulted in cAMP-activated currents typical of CFTR. No such currents were present in non-injected oocytes. Co-expression of CAL clearly inhibited CFTR-mediated cAMP-activated Cl\(^{-}\) currents (data not shown). These data show that CAL inhibits CFTR currents in both mammalian and Xenopus oocyte expression systems.

**CAL Alters the Surface Expression of CFTR**—Because the inhibition of whole cell currents by CAL could be caused either by a direct inhibition of Cl\(^{-}\) channel activity or by a reduction in CFTR channel number at the cell surface, we investigated the effect of CAL on CFTR protein expression at the cell surface. First, we co-expressed HA-CAL and GFP-CFTR in COS-7 cells and examined how CAL affects plasma membrane expression of CFTR. HA-CAL alone was detected primarily at the Golgi apparatus with some expression in the cytosol and near the plasma membrane (Fig. 7a). Wild type GFP-CFTR alone was found at both the plasma membrane and the ER (Fig. 7b). In cells co-expressing HA-CAL and GFP-CFTR, CFTR disappeared from the cell membrane and clustered in an intracellular location (Fig. 7c). Moreover, CAL colocalized with CFTR in the same intracellular location (Fig. 7, c-e).
Next, we tested whether cell surface expression of GFP-CFTR in COS-7, as assessed by surface biotinylation, was altered by co-expression with HA-CAL. Approximately 10% of CFTR was expressed at the cell surface 40–48 h post-transfection. Overexpression of HA-CAL reduced the expression of GFP-CFTR at the cell surface (Fig. 8A, upper panel). The reduction becomes more pronounced with increasing amounts of HA-CAL (Fig. 8, A and B). In contrast, overexpression of HA-CAL had no effect on plasma membrane expression of GFP-CFTRATR (data not shown). To test whether overexpression of CAL causes a generalized suppression of membrane protein trafficking, we co-transfected COS-7 cells with HA-CAL and the vesicular stomatitis virus glycoprotein (VSVG), known to traffic to the plasma membrane through the cis-, medial, and trans-Golgi (34). Overexpression of HA-CAL (Fig. 8C) had no effect on VSVG surface expression. Likewise, CAL has no effect on P-glycoprotein surface expression, which like CFTR is an apical membrane protein (data not shown). Taken together, these data show that inhibition of surface expression of CFTR by CAL is not caused by a nonspecific effect of protein overexpression. These data demonstrate that CAL selectively inhibits the cell surface expression of CFTR.

CAL Alters the Insertion of CFTR and the Half-life of CFTR in the Plasma Membrane—To characterize the mechanism by which CAL suppresses the surface expression of CFTR, we conducted two sets of experiments. First, we measured the rate of appearance of CFTR in the plasma membrane. COS-7 cells were transfected with GFP-CFTR and HA-CAL. Two days later cells were labeled with $^{35}$S-Cys/Met (30 min) and chased for 0–120 min, and plasma membrane CFTR was detected by cell surface biotinylation. CAL inhibits the expression of CFTR at the plasma membrane at least in part by decreasing the rate of appearance of CFTR in the plasma membrane. COS-7 cells were transfected with GFP-CFTR and HA-CAL. Two days later, cells were labeled with $^{35}$S-Cys/Met (3 h) and chased for 0–32 h, and plasma membrane CFTR was then detected by cell surface biotinylation. CAL also decreased the half-life of CFTR in the plasma membrane (Fig. 9B). These data suggest that CAL may interact with components near the cell surface in determining the expression of CFTR at the plasma membrane.

The Roles of CAL and Other PDZ Domain Proteins in Determining Cell Surface CFTR Expression—NHE-RF is a sub-plasma membrane PDZ domain protein that may anchor CFTR

Fig. 7. Immunochemical characterization of CFTR and CAL interaction. COS-7 cells were transfected with HA-CAL alone (a), GFP-CFTR alone (b), or co-transfected with CAL and GFP-CFTR (c, d, e). CAL was detected by monoclonal antibody to HA epitope (red). CFTR was detected by GFP fluorescence (green). Co-localization was visualized by image overlay (yellow). Co-expression of CAL and CFTR results in the redistribution and complete colocalization of each protein into clusters. Scale bar, 10 μm.

Fig. 6. CAL affects CFTR Cl$^-$ currents. COS-7 cells were transfected with 1 μg of GFP-CFTR (A and B); 1 μg of GFP-CFTR and 3 μg of HA-CAL (C and D); 1 μg of GFP-CFTR, 3 μg of HA-CAL, and 1 μg of HA-NHE-RF (E and F). Currents were measured by the whole cell patch clamp. Typical recordings in which application of cAMP (250 μM) evoked chloride currents (A, C, and E) and the relationships of the steady-state current to the membrane voltage are shown (B, D, and F). The cell was held at 0 mV, and 100-mV voltage steps were applied (−100 through 100 mV, increments of 20 mV). Open and solid circles indicate the basal and cAMP-stimulated current, respectively. No cAMP-induced current responses were observed in mock transfected cells (data not shown). The bar graph (G) shows the maximum deviations of the current from each basal level during stimulation with 250 μM cAMP for 1 min. Values are presented as mean ± S.E. **, p < 0.01 versus CFTR + CAL (Dunnett’s method). The numbers of experiments are given in parentheses.
to the cytoskeleton. To understand how PDZ domain proteins may work together in regulating CFTR trafficking and membrane organization, we examined the effect of overexpressing NHE-RF alone and co-expressing NHE-RF and CAL on CFTR cell surface expression. Overexpression of HA-NHE-RF did not change the plasma membrane expression of GFP-CFTR (Fig. 8, A and B, lane 5). Co-expression of HA-NHE-RF with HA-CAL, however, restored cell surface GFP-CFTR expression (Fig. 8, A and B, lane 6). Whereas CAL overexpression inhibited CFTR currents (Fig. 6, C, D, and G), these too were restored when NHE-RF was overexpressed along with CAL and CFTR (Fig. 6, E, F, and G). Addition of NHE-RF alone with CFTR did not affect CFTR currents (Fig. 6G). It has been shown that NHE-RF under certain conditions can enhance the open probability of CFTR in excised patches, but this stimulation was diminished when expression of NHE-RF was high (19). In Fig. 6, GFP-tagged CFTR and HA-tagged NHE-RF were cotransfected at a ratio of 1:1 (by weight). We also co-transfected GFP-CFTR and NHE-RF at a ratio of 1:3 (by weight) and observed the same results (data not shown). Clearly, the effect of NHE-RF on restoring CFTR currents in cells overexpressing CAL parallels the restoration of CFTR in the plasma membrane.

Because both CAL and NHE-RF bind to the same PDZ binding motif at the C terminus of CFTR, we tested whether NHE-RF competes with CAL in binding to CFTR. We co-transfected HA-tagged CAL and NHE-RF together with GFP-CFTR into COS7 cells and immunoprecipitated GFP-CFTR. Indeed, the binding of CAL to CFTR was substantially reduced by the co-expression of NHE-RF (Fig. 8D). Densitometry analysis indicated that co-expression of NHE-RF reduced the binding of CAL to CFTR by ~70%. The ratio of CAL to NHE-RF was about 1:4.3 in the lysate of cells that co-express these two proteins (lane 3, bottom panel). These data suggest that NHE-RF reverses the inhibitory effect of CAL by competing with CAL for the binding of CFTR.

**DISCUSSION**

We identified a Golgi CFTR-interacting protein, CAL, that plays a role in regulating the plasma membrane expression of CFTR. CAL is the first PDZ domain protein identified that regulates the trafficking of CFTR. Biochemical evidence suggests that CAL can exist as a multimer that is mediated by domains in the N-terminal region. CAL can form clusters of CFTR within the cell when overexpressed, suggesting that multimers of CAL may tether more than one CFTR within the cell as a mechanism to regulate export to the plasma membrane. This is consistent with data that overexpression of CAL suppresses CFTR expression at the cell surface. In contrast to its effect on CFTR, CAL does not affect VSVG surface expression (see Fig. 8C) or of P-glycoprotein expression, proteins that sort to the plasma membrane but do not have C-terminal PDZ interacting domains. CAL also does not affect surface expression of CFTRΔTRL, eliminating the possibility that the inhibition of surface expression is due simply to the nonspecific effect of protein overexpression. Thus, the role of CAL is restricted to proteins such as CFTR with C-terminal, PDZ domains capable of binding CAL. Given the wide distribution of CAL in many tissues, it is likely that CAL can regulate the surface expression of molecules other than CFTR as long as they have a C-terminal domain capable of binding to CAL. Although CAL is located primarily located within the Golgi, it is also found at the plasma membrane where it may also function (Fig. 7A).

CAL contains two putative coiled-coil domains that play a role in membrane association. Coiled-coil domains are found in SNARE proteins and a large number of Golgi resident proteins.
Recently, it has been shown that CAL can bind to the Q-SNARE protein syntaxin 6, a protein whose primary function is unknown (31). It is likely that the coiled-coil domains of CAL may mediate interactions with multiple Golgi proteins.

CAL overexpression also reduces the rate of appearance of CFTR in the plasma membrane suggesting that CAL may reduce the anterograde trafficking of CFTR to the plasma membrane. CAL also reduces the half-life of CFTR in the plasma membrane. There are at least two possible scenarios to explain how CAL may interact with CFTR. First, CAL may be part of an anterograde trafficking complex that controls CFTR movement from ER to Golgi and to the plasma membrane. CAL with two coiled-coil domains may tether CFTR to the Golgi as a requisite step in moving CFTR to the cell surface. The overexpression of CAL may disrupt the stoichiometry of such an anterograde trafficking complex and favor retention of the CAL-CFTR complex in the Golgi. Second, CAL may also regulate the retrograde trafficking of CFTR from the Golgi to ER and perhaps promote the recycling of CFTR to the ER for degradation. Thus CAL, like the PDZ protein GRASP 55/65, may play a role in vesicle budding and cargo selection in ER to Golgi and Golgi to membrane transport (35, 36). As shown in Fig. 8a, overexpression of CAL enhances the ER-localized, immature, and core-glycosylated form of CFTR (band B), which is localized to the ER.

If the role of CAL is to tether CFTR to the Golgi, then what promotes CFTR trafficking to the plasma membrane? Clearly, the majority of CAL is located within the Golgi, yet some is also found near the plasma membrane (Fig. 7a). Also, membrane-biotinylated CFTR can be co-immunoprecipitated with CAL (data not shown) indicating that some CAL-CFTR complexes do travel to the plasma membrane. Unlike CAL, other PDZ domain proteins such as NHE-RF and E3KARP are associated with the plasma membrane (12), suggesting that they play a key role in the function of CFTR at the plasma membrane. Our data show that, when CAL is overexpressed, CFTR trafficking to the plasma membrane is reduced but that suppression can be reversed by NHE-RF. We hypothesize that, because CAL is more abundant in the Golgi, binding of CFTR to CAL may be favored. As a CAL-CFTR complex traffics to the plasma membrane where NHE-RF is abundant, CFTR may then bind to NHE-RF, which tethers CFTR to the actin-based cytoskeleton. This tethering of CFTR to the plasma membrane increases the half-life of CFTR in the plasma membrane resulting in the apical polarization of CFTR. Overexpression of CAL increases its expression both in the Golgi and at the plasma membrane (data not shown), shifting the stoichiometry of the interaction of the CFTR with NHE-RF toward CAL and decreasing the half-life of CFTR in the plasma membrane. Our data answer a critical question as to why more than one PDZ domain protein interacts with CFTR. At least for CAL and NHE-RF, we show that both are concentrated in different locations within the cell and only transiently interact, most likely within the plasma membrane, to regulate the surface expression of CFTR. Thus, this regulation by two or more PDZ domain proteins may allow for the precise regulation of CFTR surface expression.

Other membrane proteins are found to bind to multiple PDZ domain proteins. For example, the C termini of the GluR2/3 subunits of AMPA receptors bind to both GRIP/ABP, which contains 7 PDZ domains (37), and to PICK1, which contains a single PDZ domain as well as a domain that interacts with protein kinase C (38). Phosphorylation of the C terminus of GluR2 by protein kinase C prevents its binding to GRIP but not to PICK1, which may contribute to the protein kinase C-induced rapid internalization of surface GluR2 subunits (39). Likewise, the C terminus of the β1-adrenergic receptor binds to a pair of PDZ proteins, PSD-95 and MAGI-2 (40, 41). Although the agonist-induced internalization of the β1-adrenergic receptor is attenuated by PSD-95 co-expression, it is markedly increased by co-expression with MAGI-2 (41). These reports, as well as our present findings, support the notion that multiple PDZ domain proteins interact with the same short C terminus of a membrane protein to regulate its function.

The precise mechanism of how CAL may hand off CFTR to NHE-RF remains unknown. It is not clear whether it is driven strictly by mass balance, by affinity, or by a combination of these two. In our co-expression system, NHE-RF expression is ~4.3-fold that of CAL (Fig. 8D). Under these conditions, NHE-RF may sufficiently compete with CAL for CFTR binding strictly by mass action. Immunofluorescence localization studies suggested that CFTR is present in both the apical membrane and in the subapical vesicles (42). These findings were confirmed by cryoimmunogold electron microscopy (43). The translocation of CFTR from the intracellular pool to the plasma membrane may be regulated by protein kinase A as observed by some investigators (44, 45) but not by others (23, 46). It is possible that CAL may pass CFTR off to NHE-RF in the subapical vesicles. If so, it is important to determine the endogenous levels of CAL, NHE-RF and other CFTR binding proteins in these locations, as well as their relative affinity to CFTR in native tissues that express CFTR. It is likely that these data, together with their regulation by protein kinase A and/or other physiological and pathophysiological stimuli, will provide a more comprehensive understanding of CFTR trafficking and its regulation in different native tissues.

In summary, we have identified a novel CFTR-interacting protein, CAL, that plays a role in the surface expression of CFTR. Recent evidence suggests that some ΔF508 CFTR escapes from the ER and into the early secretory pathway. Understanding the role of interacting proteins such as CAL in CFTR trafficking may prove useful in devising strategies to increase the plasma membrane expression of ΔF508 CFTR. The wide tissue distribution of CAL suggests that it may be important for trafficking of other ion channels and receptors with C-terminal sequences similar to that of CFTR.

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A Golgi-associated PDZ Domain Protein Modulates Cystic Fibrosis Transmembrane Regulator Plasma Membrane Expression

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