Targeting CAL as a Negative Regulator of ΔF508-CFTR Cell-Surface Expression

AN RNA INTERFERENCE AND STRUCTURE-BASED MUTAGENETIC APPROACH*

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PDZ domains are ubiquitous peptide-binding modules that mediate protein-protein interactions in a wide variety of intracellular trafficking and localization processes. These include the pathways that regulate the membrane trafficking and endocytic recycling of the cystic fibrosis transmembrane conductance regulator (CFTR), an epithelial chloride channel mutated in patients with cystic fibrosis. Correspondingly, a number of PDZ proteins have now been identified that directly or indirectly interact with the C terminus of CFTR. One of these is CAL, whose overexpression in heterologous cells directs the lysosomal degradation of WT-CFTR in a dose-dependent fashion and reduces the amount of CFTR found at the cell surface. Here, we show that RNA interference targeting endogenous CAL specifically increases cell-surface expression of the disease-associated ΔF508-CFTR mutant and thus enhances transepithelial chloride currents in a polarized human patient bronchial epithelial cell line. We have reconstituted the CAL-CFTR interaction in vitro from purified components, demonstrating for the first time that the binding is direct and allowing us to characterize its components biochemically and biophysically. To test the hypothesis that inhibition of the binding site could also reverse CAL-mediated suppression of CFTR, a three-dimensional homology model of the CAL-CFTR complex was constructed and used to generate a CAL mutant whose binding pocket is correctly folded but has lost its ability to bind CFTR. Although produced at the same levels as wild-type protein, the mutant does not affect CFTR expression levels. Taken together, our data establish CAL as a candidate therapeutic target for correction of post-maturational trafficking defects in cystic fibrosis.

Loss-of-function mutations in the cystic fibrosis transmembrane conductance regulator (CFTR)2 are the underlying cause of cystic fibrosis (1, 2). CFTR forms ATP-gated Cl− channels in the apical membrane of epithelial cells in a variety of tissues. In the lung, it plays an essential role in regulating the fluid and ion balance required for the correct function of mucociliary clearance mechanisms (3).

Genetic analysis has revealed over 1400 distinct disease-associated CFTR mutations, which exhibit widely varying effects at the molecular level. Some lead to the complete loss of ion channel function. Others, however, retain at least partial chloride channel conductivity, but lead to incorrect folding and/or intracellular trafficking of the protein, such that the mutant CFTR does not reach the apical membrane (4, 5). This applies in particular to the most common genetic lesion associated with CF, in which the codon for Phe508 is deleted (ΔF508) (6, 7).

Even for WT CFTR, a large fraction of newly synthesized protein is degraded before reaching the apical membrane (8), and the protein that does is subjected to continual endocytosis and endocytic recycling (9, 10). As a result, regulation of CFTR intracellular trafficking is important for its function in both physiological and pathological contexts. Genetic, biochemical, and cell biological studies have revealed a complex network of protein-protein interactions that are required for correct CFTR trafficking, including a number of PDZ (PSD-95, discs-large, zonula occludens-1) proteins, which act as adaptor molecules, coupling CFTR to other components of the trafficking and localization machinery, and to other transmembrane channels and receptors (11, 12). Class I PDZ domains typically recognize C-terminal binding motifs characterized by the sequence –(S/
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The cytoplasmic C terminus of CFTR satisfies the class I PDZ binding motif, ending in the sequence -DTRL (15–17). Earlier work in some of our laboratories had shown that the CFTR C-terminal PDZ-binding motif controls retention of the protein at the apical membrane and modulates its endocytic recycling (18, 19). PDZ proteins that have been shown to interact with CFTR include NHERF1 (Na+/H+ exchanger regulatory factor 1; also known as E3KARP), NHERF2 (aka E3KARP), NHERF3 (aka CAP70, PDZK1, or NaPi, CAP-1), NHERF4 (aka IKEPP or NaPi, CAP-2), and CAL (CFTR-associated ligand; aka PIST, GOPC, and FIG) (12, 20).

Overexpression of CAL in heterologous cells leads to a dramatic decrease in the plasma-membrane levels of CFTR (21) and of several other membrane proteins that are known to interact with it, including Clc-3 chloride channels, the β1, adrenergic receptor, and the somatostatin receptor subtype 5 (22–24). In the case of CFTR, the effect is mediated by reductions in the rate of membrane insertion and in the half-life of the channels at the cell surface (21), and can be prevented by blocking endocytosis or lysosomal degradation (25). The negative effect of CAL overexpression on CFTR expression levels can also be reversed by the simultaneous overexpression of NHERF1, which competes for the C-terminal TRl binding motif (21), or by overexpression of TC10, a Rho GTPase whose constitutively active form redistributes CAL intracellularly toward the plasma membrane (26).

It thus appears that CAL plays an important role in the intracellular trafficking and localization of CFTR. Furthermore, because high levels of CAL reduce CFTR levels, it is possible that endogenous CAL acts as a negative regulator. If so, targeted modulation of the CAL-CFTR interaction could provide a mechanism for up-regulating CFTR trafficking in a therapeutic context, in analogy to the rescue of ΔF508-CFTR seen upon overexpression of NHERF1 (27). However, previous studies have focused on the effects of CAL overexpression on WT-CFTR. As a result, no evidence has been available as to whether endogenous CAL is limiting for CFTR expression nor whether its effects apply to disease-associated mutants. Furthermore, analysis of the regulatory interactions has so far been confined to heterologous cells, even though trafficking pathways depend strongly on cellular context (28–30).

In the experiments reported here, we test the hypothesis that suppression of endogenous CAL expression levels will increase the cell-surface expression of functional ΔF508-CFTR and that it will do so in a polarized human bronchial epithelial cell line. In addition, we assess the ability of a localized mutant knock-out of the CAL PDZ binding pocket to abrogate CAL-mediated suppression of cell-surface CFTR, providing new insights into the mechanism of interaction. Taken together, our results establish the potential therapeutic relevance of pharmacological inhibition of the CAL PDZ binding domain.

EXPERIMENTAL PROCEDURES

siRNA-mediated Targeting of Endogenous CAL Expression—CFBE410- cells (31, 32) stably transduced with the ΔF508-CFTR gene under control of a cytomegalovirus promoter (“CFBE+ΔF508” cells) (33) were a generous gift of Dr. J. P. Clancy (University of Alabama, Birmingham) and were maintained in the Dartmouth CF Core Facility. Monolayers of CFBE+ΔF508 cells were grown in 6-well plates and transfected with 160 nm CAL-specific siRNA (GOPC3; Qiagen) or nonspecific siRNA (control, non-silencing siRNA; Qiagen) or an equal volume of medium, using the transfection reagent Lipofectamine 2000 (Invitrogen). After 20 h, cells were provided with fresh medium. To measure cell-surface CFTR, 72 h after transfection, cells were washed with ice-cold phosphate-buffered saline (Invitrogen), incubated with EZ-Link Sulfo-NHS-LC-Biotin (Pierce; 1 mg/ml in phosphate-buffered saline with 1 mm MgCl₂, 0.1 mm CaCl₂, pH 8.2) for 1 h at 4 °C, washed, lysed in lysis buffer (25 mm HEPES, pH 8.2, 1% (v/v) Triton X-100, 10% (v/v) glycerol, 1 Complete tablet/50 ml (Roche)), collected by using a cell scraper (Sarstedt), and centrifuged. A aliquot of clarified whole-cell lysate (WCL) was subjected to SDS-PAGE and analyzed by Western blotting with CFTR-, CAL-, and ezrin-specific antibodies. The remaining clarified WCL was incubated with streptavidin beads overnight at 4 °C, after which the beads were washed three times with lysis buffer. Proteins were eluted in Laemmli sample buffer/dithiothreitol (DTT) at 85 °C for 5 min, and resolved by SDS-PAGE. Western blotting was performed with antibodies specific for CFTR, breast cancer resistance protein (BCRP), and the Na⁺/K⁺-ATPase a1 subunit. Horseradish peroxidase-conjugated secondary antibody (Bio-Rad) and Western Lightning Chemiluminescence Reagent Plus (PerkinElmer Life Sciences) were used for visualization.

For experiments with polarized monolayers, CFBE+ΔF508 cells were seeded at low density. For biochemical experiments, 10⁵ cells were seeded on 24-mm diameter Transwell filters (Corning) and allowed to grow for 3 days prior to transfection. For electrophysiological experiments, 33 × 10⁴ cells were seeded on 12-mm diameter Snapwell filters (Corning), and allowed to grow for 4 days prior to transfection. In both cases, subconfluent monolayers were transfected overnight with 50 nm CAL-specific or nonspecific siRNA (GOPC3 or control, non-silencing siRNA, respectively; Qiagen), using HiPerFect transfection reagent (Qiagen) according to the manufacturer’s protocol. Confluent monolayers were allowed to form, and cells were serum-starved for 24 h, and switched to 27 °C for 24–36 h prior to experimentation to increase signal intensity. Monolayers were apically biotinylated using EZ-Link Sulfo-NHS-LC-Biotin (Pierce), and WCL and surface-biotinylated samples were prepared and analyzed as described above for non-polarized cells.

Electrophysiology—Seven days after seeding monolayers, Ussing chamber measurements were performed essentially as described (34), except that 50 µM amiloride was used. For these studies, 50 µM genistein was applied apically to activate temperature-rescued ΔF508-CFTR channels (34, 35). Once maximal activation was achieved, 5 µM 3-[(3-trifluoromethyl)phenyl]-5-[(4-carboxyphenyl)methylene]-2-thioxo-4-thiazolidinone (CFTRinh-172; EMD Biosciences, Refs. 36 and 37) was applied apically to inhibit CFTR-mediated chloride currents. Data are reported as the difference between the...
genistein-activated and the CFTR\textsubscript{inh}-172-inhibited short-circuit currents ($I_{sc}$).

**Recombinant Protein Expression Vectors**—Full-length human CAL (GenBank\textsuperscript{TM} accession AF450008; TrEMBL accession number Q969U8) was subcloned into the pET16b expression vector (Novagen) on a Ndel/BamHI fragment generated by PCR to yield the vector pHCAL. The 5’ primer was designed to introduce a decahistidine purification tag at the N terminus of the construct. The CAL PDZ domain (amino acids 278–362) was also PCR subcloned into pET16b as an Ndel/BamHI fragment to yield the vector pHCALP5. Its 5’ primer was designed to introduce an N-terminal decahistidine tag followed by a TEV protease recognition sequence. CAL-binding site mutants were prepared using the QuikChange and Multichange protocols (Stratagene) in the eukaryotic expression vector pECFP-CAL, containing full-length CAL inserted as an EcoRI/BamHI fragment into the pECFP-C1 backbone (Clontech): “CAL-D” = S294D, T296E, K340D, K342E; “CAL-F” = K299D, K340D, K342E; and “CAL-T+L” = L291E, G292E, L295E, H341F, L348N. Full-length and PDZ domain mutant constructs were subcloned into the bacterial expression vectors described above and into the mammalian expression vector encoding HA-tagged full-length CAL (25).

pGST-CFTRC, encoding C-terminal residues 1377–1480 of CFTR as a glutathione S-transferase (GST) fusion protein in the pGEX-4T-1 vector (GE Healthcare), was obtained from the Dartmouth Cystic Fibrosis Core Facility and was originally a generous gift of Drs. P. Devarajan and A. Swiatecka-Urban. pGST-CFTRC\textsubscript{ΔTRL} was PCR subcloned as a BamHI/Sall site fragment into pGEX-4T-1. All protein expression constructs were verified by DNA sequencing.

**Protein Expression**—pHCAL1-transformed BL21(DE3) RIL cells (Novagen) were grown at 37 °C in LB medium to an $A_{600}$ of ~0.6. Protein expression was induced with 0.1 mM isopropyl 1-thiogalactopyranoside and allowed to proceed for 16 h at 20 °C. Cells were harvested, resuspended in lysis buffer T (50 mM Tris, pH 8.5, 150 mM NaCl, 10 mM Na$_2$HPO$_4$, 2 mM KH$_2$PO$_4$, pH 7.3), 1 mg/ml lysozyme, 10 μg/ml DNase I (Roche), 5 mM DTT, 5 mM MgSO$_4$, supplemented with 1 Complete tablet in 50 ml. After incubation for 30 min on ice, the cells were lysed using a French press.

**Protein Purification**—All lysates were clarified by centrifugation at 40,000 rpm in a Ti45 rotor for 1 h at 4 °C. Imidazole was added to the CAL–PDZ supernatants to a final concentration of 10 mM before application to a nickel-nitrilotriacetic acid Superflow (Qiagen) column (bed volume 10 ml), which had been pre-equilibrated with 5 column volumes (CV) of TBS-CAL (50 mM Tris, pH 8.5, 150 mM NaCl, 1 mM DTT, 0.1 mM ATP), containing 10 mM imidazole. Following sample application, the column was washed with 10 CV of TBS-CAL containing 10 mM imidazole, and protein was eluted in TBS-CAL with a linear gradient of 10–400 mM imidazole over 20 CV. Eluates were collected in tubes containing Chelex 100 Molecular Biology grade resin (Bio-Rad). CAL was purified using a similar protocol, except that TBS-CAL was supplemented for metal affinity chromatography with 10% (w/v) glycerol and 0.1% (w/v) Triton X-100. CAL- or CAL-PDZ-containing fractions were pooled, centrifuged at 3700 × g for 10 min, and filtered through a 0.45-μm polyvinylidenefluoride filter (Millipore) to remove any residual Chelex resin.

GST-CFTR fusion proteins were purified by affinity chromatography using glutathione-Sepharose 4 Fast Flow beads (Sigma) (bed volume 12 ml). The column was equilibrated with 3 CV of PBS containing 0.05% (v/v) Tween 20 (ICN; PBS/Tween). Following sample application, the column was washed with 5 CV of PBS/Tween and the fusion protein eluted with 4 CV of PBS containing 25 mM glutathione.

The pooled eluates were applied to HiLoad Superdex 200 (CAL; GST–CFTRC) or Superdex 75 (CAL–PDZ) prep grade 16/60 or 26/60 size-exclusion chromatography (SEC) columns (GE Healthcare) in TBS-CAL containing 0.02% sodium azide and 25 mM, instead of 50 mM Tris (CAL; CAL–PDZ) or in PBS/Tween (GST–CFTR fusions). The purity of all proteins was assessed by SDS-PAGE. CAL was concentrated in Amicon Ultra-15, 10,000 MWCO, CAL–PDZ in Centricon Plus 80 Biomax-5, 5,000 MWCO, and GST–CFTR fusion proteins in Amicon Ultra-15, 30,000 MWCO concentrators (Millipore). Following concentration, the oligomeric homogeneity of CAL and CAL–PDZ proteins was verified by analytical SEC.

**Pull-down Binding Assay**—Pull-down experiments were carried out by directly mixing the two proteins under a given interaction condition described below. 500 μl of glutathione-Sepharose bead slurry (Sigma) was aliquoted into an Eppendorf tube. After a brief centrifugation (1,000 × g; 1 min), the liquid above the beads was carefully aspirated. The beads were equilibrated twice with 1 ml each of PBS/Tween. An aliquot containing 200 μg of GST or GST fusion protein was added (after removal of residual glutathione using a PD10 desalting column; GE Healthcare), and the volume adjusted to 1 ml with the same buffer. The mixture was incubated on ice for 1 h with shaking every 10 min to permit GST capture. After centrifuging the tubes for 5 min at 1000 × g, unbound material was discarded, and the beads were washed thoroughly. An aliquot containing 200 μg of CAL or CAL–PDZ proteins was added to the captured GST or GST fusion protein, and the volume adjusted to 1 ml. CAL Limits Cell-Surface Expression of ΔF508-CFTR

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CAL Limits Cell-Surface Expression of ΔF508-CFTR

The interaction was allowed to proceed for 1 h on ice with shaking every 10 min. After complex formation was completed, to remove unbound protein, the beads were repeatedly washed until the supernatant contained no protein as detected using Bradford reagent. The washed beads were resuspended with an equal volume of SDS-PAGE loading buffer, boiled for 3 min at 95 °C, and bound proteins visualized by SDS-PAGE followed by Coomassie Brilliant Blue R-250 staining.

Homology Modeling—Homology modeling was performed via the web-based SWISS-MODEL server (38). In one case, the program selected the templates automatically (Protein Data Bank entries 1I92, corresponding template (Protein Data Bank entry 1I92) corresponding to the NHERF1-PDZ1-CFTR crystal structure (39). The latter model was combined with the CFTR C-terminal peptide contributed by a symmetry-related molecule in the crystal lattice to generate the model of the CAL-CFTR complex shown in Fig. 4. Least-squares superpositions were performed using the program LSQKAB (40). Structural representations were prepared using the program MOLSCRIPT (41).

Mass Spectrometry and NMR Analysis of CAL Protein and Mutants—Following SEC purification, wild-type CAL, CAL-D, and CAL-E mutants and the corresponding PDZ domain proteins were subjected to MALDI-TOF analysis in the Dartmouth Molecular Biology & Proteomics Core Facility. The CAL-PDZ-D mutant domain was also subjected to 1H,15N-heteronuclear single quantum correlation spectroscopy (HSQC) analysis, as described (42).

Assays of CFTR Expression in the Presence of CAL-binding Site Mutants—A GFP-CFTR fusion protein was expressed in African green monkey kidney (COS-7) cells in the presence or absence of wild-type and mutant HA-CAL. Both proteins were detected by Western blotting as previously described (25).

RESULTS

Endogenous CAL Down-regulates ΔF508-CFTR Cell-Surface Expression—Our previous studies had shown that overexpression of CAL in heterologous cell lines reduces the levels of recombinant WT-CFTR found in whole cell lysates and at the cell surface. This effect could be blocked by the overexpression of NHERF1 together with CAL (21). Recently, overexpression of NHERF1 has been shown to rescue the cell-surface expression of ΔF508-CFTR in a human bronchial epithelial cell line (27). Given the apparent antagonism of CAL and NHERF1, we suspected that reduction of endogenous CAL expression could provide an alternative mechanism for increasing cell-surface levels of ΔF508-CFTR.

To test this hypothesis, we investigated a number of commercially available CAL-specific siRNA constructs for their ability to reduce CAL protein levels. Because recent studies have shown that CFTR endocytic and endocytic recycling processes depend strongly on cell type (28–30), we performed these experiments in an epithelial cell line derived from human airway. Furthermore, because rescue of cell-surface expression of CFTR is therapeutically relevant only for disease-associated mutants, we selected the CFBE+ΔF508 cell line. These cells were originally derived from a cystic fibrosis patient homozygous for the ΔF508-CFTR mutation and have been stably transduced with control siRNA (gray), CAL-specific siRNA (black), or CAL-knock down by 65% led to an increase of more than 80% in the amount of cell-surface ΔF508-CFTR detected.
by biotinylation of CFBE + ΔF508 cells following siRNA treatment (Fig. 1B). The effect was statistically significant (p < 0.01). No corresponding change was observed in the total amount of ΔF508-CFTR found in whole cell lysates (Fig. 1B), presumably reflecting the fact that only a small fraction of total ΔF508-CFTR is normally expressed at the cell surface.

To test the possibility that the siRNA treatment might have caused a nonspecific increase in membrane trafficking, cell-surface expression of two unrelated proteins was also quantified following biotinylation. Neither BCRP, an ABC transporter up-regulated in some tumors (43), nor the Na+/K+-ATPase α1 subunit (44) showed an increase in cell-surface expression following CAL-specific versus mock or nonspecific siRNA treatment (Fig. 1), indicating that the effect seen for ΔF508-CFTR was not due to a generalized up-regulation of membrane protein levels.

To determine whether cell-surface ΔF508-CFTR rescued by CAL-specific siRNA is functional, we transfected subconfluent CFBE + ΔF508 cells grown on permeable supports and allowed them to form monolayers. Monolayers were transferred to 27 °C prior to analysis to increase release of ΔF508-CFTR from the endoplasmic reticulum. Biochemical analysis showed 89% knock-down of CAL expression compared with cells treated with a nonspecific control siRNA. This knock-down is greater than that seen in unpolarized cells, (Fig. 2A), and was associated with a correspondingly larger effect on ΔF508-CFTR. Cell-surface expression of ΔF508-CFTR was 4.4-fold greater than in control monolayers (Fig. 2A), compared with 1.8-fold in unpolarized cells (Fig. 1B). The increase in cell-surface protein was also accompanied by a smaller, 2.7-fold increase in total cellular levels of ΔF508-CFTR (Fig. 2A), again consistent with the idea that only a fraction of cellular ΔF508-CFTR is normally present at the cell surface.

Finally, electrophysiological analysis of filter-grown, temperature-rescued CFBE + ΔF508 monolayers showed that cells treated with CAL-specific siRNA had more than triple the ΔF508-CFTR-mediated chloride current compared with monolayers treated with a control siRNA (Fig. 2B). This confirms that CAL knock-down is effective in polarized cells and that the rescued ΔF508-CFTR channels are functional. The effect of CAL-specific siRNA is seen in addition to the effects of temperature rescue, suggesting that CAL inhibitors may complement therapies aimed at correcting ΔF508-CFTR biogenesis.

In Vitro Reconstitution Reveals a Direct CAL-CFTR Binding Interaction—Interference with the CAL-CFTR binding interaction could provide a potential alternative to CAL-specific RNA interference as a strategy for the stabilization of ΔF508-CFTR at the cell surface. However, such an approach requires knowledge of the biochemistry of the interaction, and in particular, whether it involves direct binding of the CAL and CFTR proteins or is mediated by additional proteins acting as adaptors. Previous investigations of the CAL-CFTR interaction have involved studies of co-localization in cells and co-immunoprecipitation from cell extracts (21, 25), and thus could not distinguish between these alternatives.

We therefore reconstituted the interaction in vitro using bacterially expressed, purified components, allowing us to characterize the interaction under rigorously defined conditions and in the absence of other proteins. Both full-length CAL and its PDZ domain were expressed with N-terminal polyhistidine tags, whereas the CFTR C terminus was expressed as a GST fusion protein (Fig. 3A). All constructs were isolated from bacterial lysates at high purity (Fig. 3B). Pull-down experiments using glutathione-Sepharose beads clearly demonstrated a specific interaction between CAL and the CFTR C terminus in the absence of any other cellular components (Fig. 3C, left-hand panel, G-CF lane). The protein concentrations used for the pull-down analysis were in the low micromolar range, consistent with the affinities typically observed for PDZ-peptide interactions (13). This provides clear evidence that the CAL and CFTR can interact directly.

To ascertain whether the reconstituted binding interaction reproduces the essential features of the functional interaction observed in cell extracts (21, 25), we also assessed the domain/motif requirements of CAL-CFTR binding in vitro. It has been shown in vivo that the CAL PDZ domain is sufficient to mediate the interaction, and that loss of the CFTR C-terminal tripeptide "TRL" dramatically reduces it (21). We therefore
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In vitro reconstitution of CAL-CFTR binding demonstrates a direct interaction and reproduces essential characteristics of the in vivo binding process. A, domain boundaries of protein constructs used in the study are shown. CAL constructs included N-terminal decahistidine (H), followed in the case of the PDZ domain by a TEV protease cleavage site (T). The CFTR cytoplasmic C-terminal domain was expressed as a GST fusion protein, with (GST-CFTR) or without (GST-CFTRΔTRL) the C-terminal residues TRL. B, purified components used to analyze the CFTR-CAL binding interaction. Coomassie-stained SDS-PAGE gels of the proteins are shown following purification. M, standards are marked to the left of each lane. For CAL and CAL-PDZ proteins, molecular mass standards are 250, 150, 100, 75, 50, 37, 25, 20, 15, and 10 kDa. For GST fusion proteins, they are 97, 66, 45, 31, 21.5, and 14.4 kDa. C, CAL binds CFTR directly in the absence of other proteins. Purified full-length CAL (left-hand panel) and CAL-PDZ domain (right-hand panel) proteins were tested for their ability to bind the CFTR C terminus by GST pull-down experiments. GST, GST-CFTRΔTRL (G-ΔT) or GST-CFTR (G-CF) were immobilized on glutathione-Sepharose beads, and then incubated with 4 μM CAL or 16 μM CAL-PDZ domain. Captured proteins were eluted and visualized by Coomassie staining of SDS-PAGE gels. M, markers are shown along the outside edge of the gels. The position of expected protein components is indicated by arrows between the gels.

expressed and purified a GST-CFTR C-terminal fusion protein, in which the C-terminal class I PDZ binding motif TRL had been deleted (Fig. 3, A and B). As expected, this ΔTRL construct was unable to pull down purified CAL (Fig. 3C, left-hand panel, G-ΔT lane). Finally, to confirm that the interaction is mediated by the CAL PDZ domain, the pull-down experiments were repeated with the purified CAL PDZ domain, which exhibited the same specificity for the intact CFTR C terminus as did full-length protein (Fig. 3C, right-hand panel).

Homology Modeling of the CAL-CFTR Interaction—With a reconstituted binding system in hand, we wished to test the hypothesis that stereochemical interference with the CAL binding pocket could provide an alternative to CAL protein knock-down as a mechanism for increasing the amount of CFTR at the cell surface. As a result, we designed an atomic model of the CAL-CFTR interaction as a basis for site-directed mutagenesis. The crystal structure of the NHERF1 PDZ1 domain had already been determined in complex with a peptide corresponding to the CFTR C terminus (PDB entry 1I92, Ref. 39). We used this as a manually selected template for SWISS-MODEL (38), which first performs a sequence alignment (Fig. 4A), and then uses it to map the target CAL sequence onto the known NHERF1 template structure. The resulting three-dimensional model of CAL-PDZ is shown in Fig. 4, B and C. Because the CAL sequence was mapped onto a ligand-bound structural template, in silico modeling effectively folded it into position around the CFTR ligand. The homology model shown in Fig. 4 provided the first three-dimensional, structural insights into the CAL-CFTR interaction.

In general, homology modeling is challenging at the level of sequence identity between the CAL PDZ domain and the NHERF1 PDZ1 domain (26%), which is close to the threshold for the technique (38). To assess its validity, we considered two main criteria. One involved the stereochemical complementarity of the computationally apposed binding interfaces. The model preserves key features of the C-terminal CFTR binding site in the PDZ domain, including (i) the “GLGF” (in CAL: GLGI) motif that forms a binding site for the ligand carboxylate (purple ribbon in Fig. 4B); (ii) a hydrophobic pocket for the aliphatic C-terminal side chain in the class I PDZ motif (green side chains in Fig. 4C); and (iii) a conserved His side chain that interacts with the Ser/Thr−2 side chain in the motif (green side chain His341 in Fig. 4C). In addition, two polar residues Ser294 and Thr296 are positioned to interact with the Asp−3 side chain (blue side chains in Fig. 4C), and a cluster of lysines (red and purple side chains in Fig. 4C) is poised to interact with upstream acidic elements in the CFTR sequence (shown schematically as “EEE” at the lower end of the pocket in Fig. 4B, corresponding to 1472EEE1474). Overall, the modeled CAL binding site thus provides an excellent stereochemical fit to the CFTR C terminus.

A second test of the likely accuracy of the model was obtained by performing a separate homology modeling procedure, in this case allowing SWISS-MODEL to select templates automatically. The program selected five PDZ domain structures with sequence identities to CAL ranging between 37 and 49%, sufficient to support robust modeling calculations (Fig. 4A). We then compared the resulting model of the CAL domain with that generated from the NHERF1 template. Both agree very
well, with a 2.2-Å root mean square difference in Ca positions. On the basis of these assessments, we proceeded with the design and testing of binding site mutants, as described below.

**Design and Biochemical Characterization of CAL Binding-site Mutants**—The CAL-CFTR model provided a basis for investigating the role of side chains in binding, using structure-based mutagenesis. If mutations can be found that abrogate the affinity of CAL for CFTR without gross disruption of the protein fold, the physiological effects of blocking the interaction can also be probed. Working from the model shown in Fig. 4, we generated three sets of mutations designed to interfere with the binding interaction. One mutation (CAL-T/H11001L) targeted the hydrophobic pocket that accommodates the C-terminal Leu side chain in the CFTR ligand, together with the conserved His side chain (green in Fig. 4C). A second set of mutations (CAL-D) targeted CAL residues thought to interact with Asp/H11002 (blue and purple in Fig. 4C). A third set of mutations (CAL-E) targeted a cluster of lysines (red and purple in Fig. 4C) that could be responsible for binding interactions upstream of the canonical binding site, involving, e.g. the triple-glutamate motif shown in Fig. 4B.

All three CAL mutants were generated both as full-length and PDZ domain constructs. All were expressed and purified using metal-affinity chromatography and SEC. During SEC purification, the CAL-T+L construct eluted in the void volume and exhibited elevated proteolytic susceptibility, suggesting misfolding and aggregation. It was not analyzed further. In contrast, throughout purification, the CAL-D and CAL-E mutants clustered of lysines (red and purple in Fig. 4C) that could be responsible for binding interactions upstream of the canonical binding site, involving, e.g. the triple-glutamate motif shown in Fig. 4B.
CAL limits cell-surface expression of ΔF508-CFTR.

A. Pull-down experiments with the mutant CAL proteins (Fig. 5A, left-hand panel) revealed that the CAL-D mutant retained affinity for the CFTR C terminus. In contrast, the CAL-D mutant could no longer be efficiently captured by the immobilized CFTR C terminus (Fig. 5A). The same pattern was observed with the correspondingly mutated CAL-PDZ domains (Fig. 5A, right-hand panel). The contrasting affinities of the CAL-D and CAL-E mutants provide an opportunity for testing whether CAL-mediated down-regulation of CFTR levels correlates with the affinity of the PDZ binding pocket.

The specific role of the CAL PDZ binding pocket in reducing CFTR expression—To exclude the possibility that the CAL-D mutant had lost affinity for CFTR due to a global disruption of the protein fold, we used NMR spectroscopy to confirm that the CAL-D PDZ domain retained its native structure. Using 1H-15N-labeled CAL-D protein, a 1H-15N HSQC spectrum was obtained, which exhibits a wide distribution of resonances across the spectral field, confirming that the protein adopts a stable fold (Fig. 5C, blue). The pattern of resonances closely resembles that of wild-type CAL (Fig. 5B, red) (42), as well as those of other PDZ domains (45). Peaks in the wild-type spectrum that have shifted in the mutant spectrum (e.g. asterisks in Fig. 5C) are associated with residues in the vicinity of the mutated side chains, as would be expected in the absence of a global conformational disruption. The NMR data confirm that the loss of CFTR binding affinity for the CAL-D mutant is due to the selective and localized disruption of the binding site, whereas the native fold of the protein is preserved. Any functional differences observed are thus attributable to the affinity of the PDZ binding pocket, making CAL-D an excellent probe of the specific role of the PDZ binding interaction in CAL function. This is particularly important given the promiscuous nature of PDZ domain scaffolding interactions, because PDZ adaptor proteins can mediate their effects either by binding to the target protein of interest directly, or by displacing interactions of other adaptor molecules with scaffolding proteins.

To test the hypothesis that the effect of CAL on CFTR protein levels requires direct CAL-CFTR binding, we took advantage of the assay initially used to characterize the interaction. HA-tagged versions of CAL and the CAL-D and CAL-E mutants were individually co-expressed in COS-7 cells transfected with a GFP-CFTR fusion protein (25). The expression of increasing amounts of wild-type CAL significantly reduced the level of mature GFP-CFTR in whole cell lysates in a dose-dependent fashion (Fig. 6A, top panels, p < 0.05), consistent with previous reports (25). In contrast, the expression of equivalent levels of the CAL-D mutant had no significant effect on GFP-CFTR expression levels (Fig. 6A, middle panel). As a control, we tested the effect of the CAL-E mutant, which shares two of the...
CAL Limits Cell-Surface Expression of ΔF508-CFTR

To understand the mechanism of the CAL-CFTR interaction better, we reconstituted it in vitro, and showed that it involves the binding of the two proteins to each other. To probe the stereochemical basis of the interaction, we pursued a structure-based mutagenesis approach. The fact that the CAL-E mutant retained its ability to bind the CFTR C terminus suggests that the cluster of three lysines (red and purple side chains in Fig. 4C) at the N-terminal end of the binding site is not essential for the CAL-CFTR interaction, although we cannot exclude a small modulatory effect on its affinity. Because the two lysines mutated in CAL-D (purple side chains in Fig. 4C) are also mutated in CAL-E, which retains CFTR binding affinity, the key difference appears to be the result of changes to Ser294 and Thr296 (blue in Fig. 4C), both of which are predicted to interact with Asp−3. Although not part of the canonical class I motif (13, 14), this side chain has been seen to play an important role in some PDZ interactions (46). Furthermore, the mutations were designed not only to abrogate potentially favorable contacts in the wild-type binding site, but also to introduce a charge incompatibility with peptides containing a negatively charged side chain at the −3 position. Regardless of the relative contributions of these two effects, the overall loss of binding affinity associated with the CAL-D mutation is clear, and in

DISCUSSION

The observation that CAL knock-down can boost the amount of functional ΔF508-CFTR at the cell surface in a patient-derived bronchial epithelial cell line provides additional evidence for the antagonistic roles of CAL and NHERF1 in regulating CFTR levels. Earlier studies had shown that overexpression of NHERF1 could reverse the effects of CAL in regulating CFTR levels. Earlier studies had shown that overexpression on WT-CFTR in a heterologous cell line (21). More recently, it was shown that NHERF1 overexpression could increase cell-surface levels of ΔF508-CFTR in airway epithelial cell lines (27). If CAL and NHERF1 compete for CFTR binding and mediate opposing effects on its cell-surface expression, we hypothesized that a similar result could be obtained by reducing CAL expression. This prediction was confirmed, and the magnitude of the CAL knock-down effect on cell-surface ΔF508-CFTR levels in polarized cells (4.4-fold) was even greater than that seen with NHERF1 overexpression (~40% increase) (27).

Our data provide the first direct evidence that endogenous CAL acts to limit cell-surface levels of ΔF508-CFTR in human airway epithelial cells. As a result, CAL may reinforce the pathophysiology of cystic fibrosis and could hinder therapeutic efforts to restore ΔF508-CFTR cellsurface expression. In terms of its negative effect on both WT and mutant CFTR cell-surface expression, CAL stands in contrast to many PDZ proteins, which, like NHERF1 (27), tend to favor the trafficking, localization, and clustering of their binding partners in the plasma membrane (13). However, because it is pharmacologically easier to block a deleterious interaction than to stabilize a beneficial one, the unfavorable influence of CAL on CFTR may actually make it a more attractive therapeutic candidate than other, more benign PDZ counterparts.

FIGURE 6. Effect of CAL on CFTR protein expression requires a stereochemically compatible PDZ binding site. A, COS-7 cells were co-transfected with GFP-CFTR (3 μg of plasmid DNA) and wild-type HA-CAL, HA-CAL-D, or HA-CAL-E as indicated (0, 3, 6, or 9 μg of plasmid DNA). Cells were lysed 48 h post-transfection. Cell lysates were subjected to Western blot analysis. GFP-CFTR was detected with an anti-GFP polyclonal antibody. HA-CAL and mutants were detected with an anti-HA polyclonal antibody. B, dose-dependent expression of CAL. The CAL WT and mutant expression levels detected by Western blotting were quantitated, normalized to the expression in the absence of recombinant CAL, and averaged (n = 3; *, p < 0.05 versus control CFTR). Mean values ± S.E. are shown for cells co-transfected with wild-type HA-CAL (white), HA-CAL-D (gray), or HA-CAL-E (black). C, dose-dependent suppression of CFTR expression by CAL WT and mutant, but not CAL-D. CFTR expression levels were quantitated, normalized to the expression in the absence of recombinant CAL, and averaged (n = 3; *, p < 0.05 versus control CFTR). Mean values ± S.E. are shown for cells co-transfected with wild-type HA-CAL (white), HA-CAL-D (gray), or HA-CAL-E (black).
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stark contrast to the retention of binding function by the CAL-E mutant.

Because the CAL-D mutant domain retains its native three-dimensional conformation, its loss of affinity for the CFTR C terminus allowed us to establish the functional importance of the PDZ binding pocket, independent of any gross changes in the tertiary structure of the protein, such as those associated with truncation mutants (21). The highly localized and specific disruption of the CAL-CFTR binding interaction preserves CFTR expression levels in the presence of CAL protein (Fig. 6), presumably by suppressing the degradation of mature CFTR protein (25). This mutagenetic uncoupling mimics that which would be achieved by the design of small-molecule competitive inhibitors specifically tailored to block the CAL PDZ binding site.

Our success in designing a PDZ binding mutant of CAL also serves to validate the homology model. Even though structural templates with higher sequence identity were available in the data base, the model was developed using a particular template with only borderline (26%) identity (38), because that template had been crystallized in the presence of our ligand of interest (39). This allowed us to model the interaction, rather than just the structure of the isolated binding pocket. As the focus of structural biology shifts increasingly from individual proteins to protein-ligand and protein–protein complexes, this dilemma is likely to recur, in which the template structure with the most relevant binding partners may not be the same as that with the highest homology and thus the greatest likelihood of accuracy. Our approach was to generate models using either the most biologically relevant or the most structurally plausible templates and to compare them. In the case of CAL, both models were very similar, increasing confidence in the predicted interaction. However, in cases where the models diverge, an alternative strategy could involve least-squares superposition of the high-homology model onto the biologically relevant template. In either situation, perhaps the most important information about plausibility is provided by the stereochemical compatibility of the modeled binding interaction, which was clearly satisfied by the CAL model.

 Whereas mutagenesis and functional characterization experiments were underway, we also pursued the three-dimensional structure determination of the CAL PDZ domain by NMR spectroscopy (42). NMR data retrospectively confirmed our homology model, as shown in Fig. 4D, yielding a strong DALI similarity score to the model (z = 10.5, Ref. 47) and an overall 1.9-Å root mean square difference in Cα positions, excluding two peripheral loops (marked by asterisks, Fig. 4D) whose conformations are flexible. Chemical shift data obtained in the presence of a peptide corresponding to the C terminus of CFTR also confirmed our identification of CAL side chains that interact with the ligand (Fig. 4C and Ref. 42).

These studies provide a detailed molecular basis for future screening and design approaches to identify CAL-specific small molecule inhibitors. CAL inhibitors should prove useful in dissecting the multiple potential trafficking pathways involved in CFTR regulation (21, 25, 26). Given that CAL acts as a negative regulator of ΔF508-CFTR cell-surface levels, such compounds could also help to stabilize mutant CFTR expression levels at the apical membranes of lung epithelia. This could provide an important alternative or complementary approach to current efforts aimed at correcting folding defects (48, 49), especially because ΔF508-CFTR trafficking defects include not only inefficient maturation (8), but also a reduced biochemical half-life (30, 50–53). The potential for such complementarity is underscored by the ability of CAL knock-down to enhance functional, cell-surface expression of ΔF508-CFTR beyond the levels induced by low temperature rescue alone.

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Targeting CAL as a Negative Regulator of ΔF508-CFTR Cell-Surface Expression: AN RNA INTERFERENCE AND STRUCTURE-BASED MUTAGENETIC APPROACH

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