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A Stabilizing Influence: CAL PDZ Inhibition Extends the Half-Life of ΔF508-CFTR**

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From synthesis to degradation, membrane proteins navigate interwoven networks that control their localization and activity within the cell. At branchpoints within these networks, protein-protein interactions often determine the flux of individual proteins through specific pathways and thus offer targets for therapeutic modulation. The PDZ (PSD-95, Dlg, and ZO-1) proteins constitute a major family of trafficking regulators. Characterized by the presence of eponymous protein-protein interaction domains (PPIDs), PDZ proteins generally bind the C-termini of their partners and help choreograph their movements throughout the cell. The targets of PDZ regulation include the cystic fibrosis transmembrane conductance regulator (CFTR), the chloride channel mutated in patients with cystic fibrosis (CF)[1].

CF is the most common life-threatening autosomal recessive disease among people of European ancestry. In airway epithelia, loss of CFTR impairs mucociliary clearance and facilitates chronic bacterial infections[2–3]. The *ΔF508* allele, found in ~90% of patients, results in a protein that fails to fold correctly[4–5]. However, if the folding defect is overcome, the resulting ΔF508-CFTR retains limited chloride channel activity (Figure 1a) [6].

Because only 10–35% of WT activity may be required for therapeutic benefit[7], many efforts have been made to identify 'corrector' and 'potentiator' compounds that address the primary folding and gating defects of ΔF508-CFTR, respectively (Figure 1b)[8–9]. There is now a growing prospect that the maturation and specific activity of ΔF508-CFTR can be pharmacologically enhanced. However, the rescued protein remains unstable[10–12]. Optimal therapy is thus likely to require repair of all three defects: folding, open probability, and stability (Figure 1a).

To identify 'stabilizers'–a new class of reagents that extend the half-life of ΔF508-CFTR–we targeted a key regulator of its post-endocytic trafficking. The CFTR-associated ligand (CAL) negatively regulates ΔF508-CFTR cell-surface abundance through its PDZ domain[13]. However, CFTR interacts not only with CAL, but also with the Na^+/H^+ exchanger regulatory factors NHERF1 and NHERF2, which counteract CAL's effect,

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enhancing the activity and the abundance of ΔF508-CFTR at the apical membrane[14–16]. In an accompanying report[17], we describe a novel strategy that permitted elaboration of the decameric peptide inhibitor $iCAL36_{10}$ (iCAL36; ANSRWPTSII). iCAL36 targets the CAL, but not the NHERF, PDZ domains, despite their overlapping specificities. Here, we report its biochemical characterization and functional effects in CF patient-derived bronchial epithelial cells expressing ΔF508-CFTR (CFBE-ΔF cells).

To visualize the iCAL36 binding site on the CAL PDZ domain (CALP), we performed NMR heteronuclear single quantum coherence (HSQC) analyses (Figure 2a). When assigned and mapped to the surface of the protein, the chemical shift perturbations associated with iCAL36 binding highlight the same site as a CFTR C-terminal peptide, reflecting competitive inhibition (Figure 2b, c). Furthermore, compared to the $CFTR_8$ octamer (EEVQDTRL)[18], the longer $iCAL36_{10}$ decapeptide makes additional contacts at the distal end of the peptide-binding groove (Figure 2b, arrow). This stereochemical footprint is consistent with the observed contributions of N-terminal modifications to peptide affinity and selectivity[17].

We next investigated whether our PDZ domain-based approach predicts peptide interactions with full-length proteins in the context of other cellular factors. Biotinylated (*BT*-) versions of three peptides were synthesized for pull-down assays: the CFTR C-terminus (*BT*-CFTR), which binds NHERF PDZ domains strongly; the somatostatin receptor type 5 C-terminus (*BT*-SSR5), which binds both NHERF and CAL domains; and *BT*-iCAL36, which binds CALP with the highest affinity and selectivity. Fluorescence polarization (FP) analysis confirmed that the biotinylated peptides retain the relative binding profiles of the core sequences (Supporting Information, Table S1).

Following immobilization on streptavidin beads, each sequence was incubated with CFBE-ΔF whole-cell lysates (WCL). Bound proteins were eluted with free peptide and probed by Western blot (Figure 3). In each case, the interactions of full-length proteins in WCL recapitulate the binding data from single PDZ domains, including the weak interaction of the CFTR C-terminus with CAL[17,19]. Our experiments confirm that iCAL36 is selective for the CAL protein, whereas the SSR5 C-terminal sequence is not.

We also tested the ability of the various peptides to bind the tetra-PDZ protein NHERF3, which has been shown to interact functionally with CFTR in intestinal epithelia[20]. NHERF3 domains were not included in the engineering phase, but the iCAL36 sequence also exhibits no interaction with NHERF3 in the pull-down assay, whereas the CFTR and SSR5 C-termini clearly do (Figure 3). Thus, iCAL36 is selective against all eight PDZ domains of the NHERF variants known to interact with CFTR.

To evaluate iCAL36 as a stabilizer of CFTR, we established an assay using patient-derived bronchial epithelial cells. Polarized monolayers of CFBE-ΔF cells were treated with peptide and BioPORTER® delivery reagent. Cells were maintained at 37 °C throughout, in order to test the effect of CAL inhibition independent of temperature-rescue effects. N-terminally fluoresceinated (*F**-) iCAL36 (*F**-ANSRWPTSII) was used, which has somewhat higher affinity for the CAL PDZ domain (1.3 μM) and comparable selectivity[17]. In order to quantitate the functionality of rescued channels, short-circuit currents (I_{SC}) were monitored in Ussing chambers. ΔF508-CFTR-mediated chloride efflux was determined as the change in I_{SC} (ΔI_{SC}) associated with acute, specific inhibition of CFTR channels by $CFTR_{inh}172$[21–22]. CFBE-ΔF monolayers treated with a scrambled control peptide (*F**-SCR; *F**-SPTINSAIWR) did not exhibit significant shifts in chloride efflux ($\Delta I_{SC} \leq 4\%$) compared to untreated cells.

Having established a physiologically relevant assay for ΔF508-CFTR rescue, we treated CFBE-ΔF monolayers with *F**-iCAL36 or *F**-SCR. Peptide uptake was confirmed by fluorescence microscopy (Supporting Information, Figure S1). Monolayers were also treated with cycloheximide (CHX), to block *de novo* protein synthesis for the final 2 h before measurement of ΔI_{SC} (Figure 4a). When normalized to baseline values obtained from monolayers washed immediately after CHX treatment, residual ΔI_{SC} values were 25% higher for monolayers treated with *F**-iCAL36 than for control monolayers (0.75 ± 0.02 vs. 0.60± 0.03, p = 0.0005; Figure 4b). Thus, iCAL36 rescues functional ΔF508-CFTR by extending the half-life of mature ΔF508-CFTR at the apical membrane. An increase was also observed in the amount of apical ΔF508-CFTR detected by surface biotinylation and Western blotting, although it was not statistically significant (p = 0.35, n = 14) due to greater variability in the biotinylation experiments. To confirm that CAL inhibition is associated with an increase in channel number, we therefore monitored cell-surface levels of ΔF508-CFTR following CAL knockdown. At the end of 2 h CHX treatment, specific CAL knockdown leads to a clear increase in the residual amount of ΔF508-CFTR channels present at the apical membrane (Figure 4c). Together, these data clearly establish CAL as a regulator of the post-maturational stability of ΔF508-CFTR.

In order to determine whether CAL inhibition also has a substantial effect on ΔF508-CFTR maturation, we treated CFBE-ΔF monolayers with *F**-iCAL36 in the absence of CHX. The resulting highly significant 26% increase in ΔI_{SC} (Figure 5) is close to the 25% increase observed in the presence of CHX (Figure 4b), indicating that iCAL36 does not strongly affect biogenesis.

The magnitude and significance of functional rescue directly correlate with the affinity and selectivity of peptide inhibitors for the CAL PDZ domain. In these studies, we also tested our NHERF-selective CFTR C-terminal mimetic 16mer ($CFTR_{16}$; AALKEETEEEVQDTRL) and the non-specific decameric CAL inhibitor *F**-SSR5 (*F**-ANGLMQTSKL). $CFTR_{16}$ did not significantly increase the magnitude of ΔI_{SC} relative to the scrambled control. Treatment with *F**-SSR5, however, increased ΔI_{SC} by 15% relative to the control, an effect intermediate between those of the NHERF-selective $CFTR_{16}$ and the CAL-selective iCAL36 inhibitors (Figure 5).

The efficacy seen with the *F**-SSR5 inhibitor peptide may reflect the fundamental pharmacological susceptibility of the CAL:CFTR interaction, which broadens the spectrum of potential therapeutic CAL inhibitors. Although non-specific, the SSR5 affinities are simultaneously stronger for CAL and weaker for the NHERF proteins than are those of CFTR (Figure 3). As a result, SSR5 appears able to displace CFTR from its weak but deleterious interaction with CAL without interfering with its strong and favorable interactions with the NHERF proteins. Thus, CAL/NHERF selectivity is advantageous, but does not appear to be strictly required to achieve functional benefit.

Furthermore, the CAL-CFTR interaction is also weak compared to other CAL target interactions[19], which means that it may be possible to rescue ΔF508-CFTR using inhibitor concentrations that will not interfere with other CAL trafficking effects. Finally, CAL knockout mice are viable[23], suggesting that CAL inhibitors, particularly if delivered directly to the airway via inhalation, may be tolerated.

As CFTR stabilizers, CAL inhibitors target pathways distinct from the folding defect and thus should complement the activity of correctors. A possible caveat is that some correctors are reported to have inherent CFTR-stabilizing properties[24], although this effect has not been seen in primary human airway epithelial cells[10]. To assess the potential for additive rescue directly, we tested the ability of CAL inhibition to augment the effect of chemical

correction of the primary folding defect. CFBE-ΔF cells were treated with either the first-generation corrector corr-4a or the corresponding amount of DMSO/buffer and either *F**-iCAL36 or *F**-SCR. Relative to the double negative control (*F**-SCR and DMSO), *F**-SCR and corr-4a enhanced $CFTR_{inh}172$ ΔI_{SC} by 15%. In this experiment, cells treated with *F**-iCAL36 and vehicle exhibited an 11% increase in the magnitude of $CFTR_{inh}172$ ΔI_{SC}. The magnitude of iCAL36-mediated rescue seen in this dual-treatment experiment may be a result of higher background levels of DMSO delivery vehicle, which is known to affect CFTR expression[25]. However, in this side-by-side comparison, it is also clear that iCAL36 treatment independently provides a level of rescue comparable to that seen with the first generation corrector corr-4a (Figure 6).

When *F**-iCAL36 and corr-4a were combined, a 25% increase in $CFTR_{inh}172$ ΔI_{SC} was observed (Figure 6), demonstrating that the effects of small-molecule correctors and CAL inhibitors are essentially additive, again consistent with a model of CAL as a target for the stabilization of mature CFTR. Furthermore, our knockdown experiments demonstrate the magnitude of rescue potentially accessible to more efficacious CAL inhibitors: 71% CAL knockdown is associated with a 2.7-fold increase in cell-surface ΔF508-CFTR (Figure 4c).

To achieve post-endocytic stabilization, our goal was to identify competitive inhibitors of the CAL PDZ domain that could mimic the functional rescue provided by CAL-specific RNA interference[13]. For this work, we decided to exploit the robust inherent affinity of PDZ domains for peptide ligands[26] along with the potential of inverted peptide arrays for high-throughput screening of sequence space[27–28] to drive a thermodynamic wedge between CAL and the other binding partners, despite their target overlap. The data presented here provide proof-of-principle for selective PDZ inhibition, and establish CAL inhibitors as founding members of a class of CFTR ‘stabilizers’ specifically designed to reduce ΔF508-CFTR post-endocytic breakdown (Figure 1b).

A key therapeutic goal is the restoration of sufficient ΔF508-CFTR activity to ameliorate the chronic lung infections that currently are the major cause of CF patient mortality[29]. Our data suggest that optimal rescue will involve a combination of corrector and stabilizer activities targeting distinct protein networks. Small-molecule correctors are currently in late-stage clinical trials. Although companion pharmaceutical stabilizers are not currently available, the high-affinity peptide ligands developed in this project provide essential reagents for high-throughput screening approaches aimed at identifying small-molecule CAL inhibitors suitable for use in combination therapies.

Experimental Section

Detailed methods are provided in the Supporting Material.

NMR Studies

Isotopically labeled CALP was prepared using published protocols[18–19] and CALP backbone assignments were performed using standard NMR techniques. Labeled crosspeaks (Figure 2) exhibited a normalized chemical shift perturbation $\Delta \geq 0.15$, where

$$\Delta=[(\Delta^{1}H)^{2}+(\Delta^{15}N/6)^{2}]^{1/2} \quad (1)$$

Protein surface depictions were prepared using PYMOL[30].

Cell culture

CFBE41o- cells[31] cells stably expressing ΔF508-CFTR [32] (CFBE-ΔF cells) were grown as polarized monolayers at 37 °C in 5% CO_2.

Pull-down Assays

Biotin ("*BT*") was synthetically coupled to PDZ-binding peptides via a linker. CFBE-ΔF WCLs were incubated with peptide-loaded streptavidin beads. After washing, bound proteins were eluted by competition with the indicated BT-free peptide or the scrambled control peptide SCR (SPTINSAIWR) and analyzed by Western blotting.

Immunostaining and Microscopy

CFBE-ΔF cells were seeded on coverslips and treated with BioPORTER and the indicated peptides. The immunostaining protocol is essentially as described[12].

Ussing chamber chloride efflux experiments

CFBE-ΔF polarized monolayers were treated apically with a 500 μM peptide/BioPORTER solution and washed. 20 μg/mL CHX was applied for the final 2 h or at the completion (0 h) of peptide treatment. Short-circuit current (I_{SC}) measurements were performed as described[13], except for a temperature of 37 °C. $CFTR_{inh}172$ was applied to block CFTR-specific chloride efflux (ΔI_{SC}). For small molecule corrector experiments, 3 μM corr-4A or DMSO was added to cells 24 h before experiments.

CFTR cell-surface abundance

CFBE-ΔF cells were transfected using CAL-specific (siCAL) or control (siNEG) siRNA as described[13]. Cells were incubated at 27 °C and serum-starved. Cells were transferred to 37 °C in MEM containing 20 μg/mL CHX. At t = 0 h and t = 2 h, apical CFTR and WCL CAL and CFTR were detected as described[13,33].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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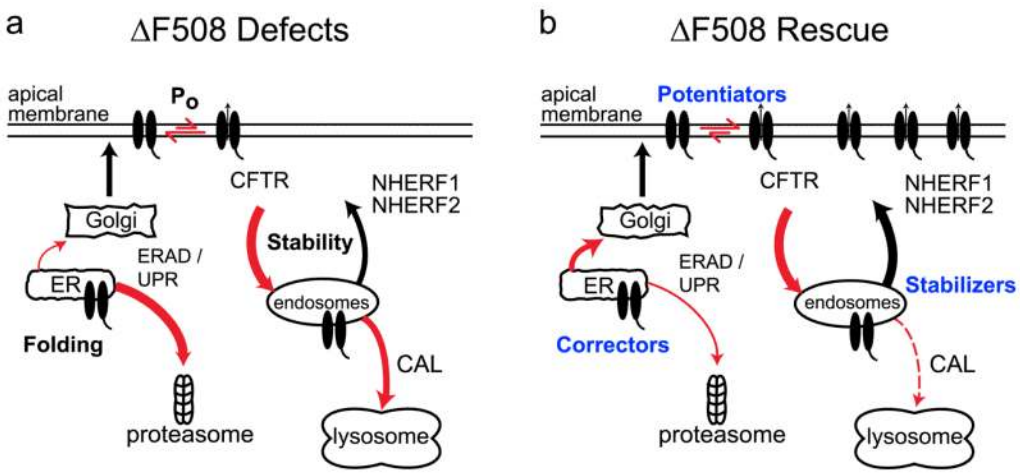

Figure 1.

Endogenous CAL limits ΔF508-CFTR half-life in polarized human airway epithelial cells and represents a potential target for CFTR 'stabilizers.' (a) ΔF508-CFTR exhibits three functional defects: (1) a failure to fold properly in the ER, leading to ER associated degradation (ERAD) ("folding"); (2) reduced open probability ("P_o") of ΔF508-CFTR channels that are found in the apical membrane; and (3) accelerated breakdown ("stability"). Aberrant flux is highlighted by red arrows. (b) Classes of therapeutic agents (blue) are being developed to address the folding defect ('correctors') and the gating defect ('potentiators'), but 'stabilizers' that specifically address the half-life deficiency have not yet been identified.

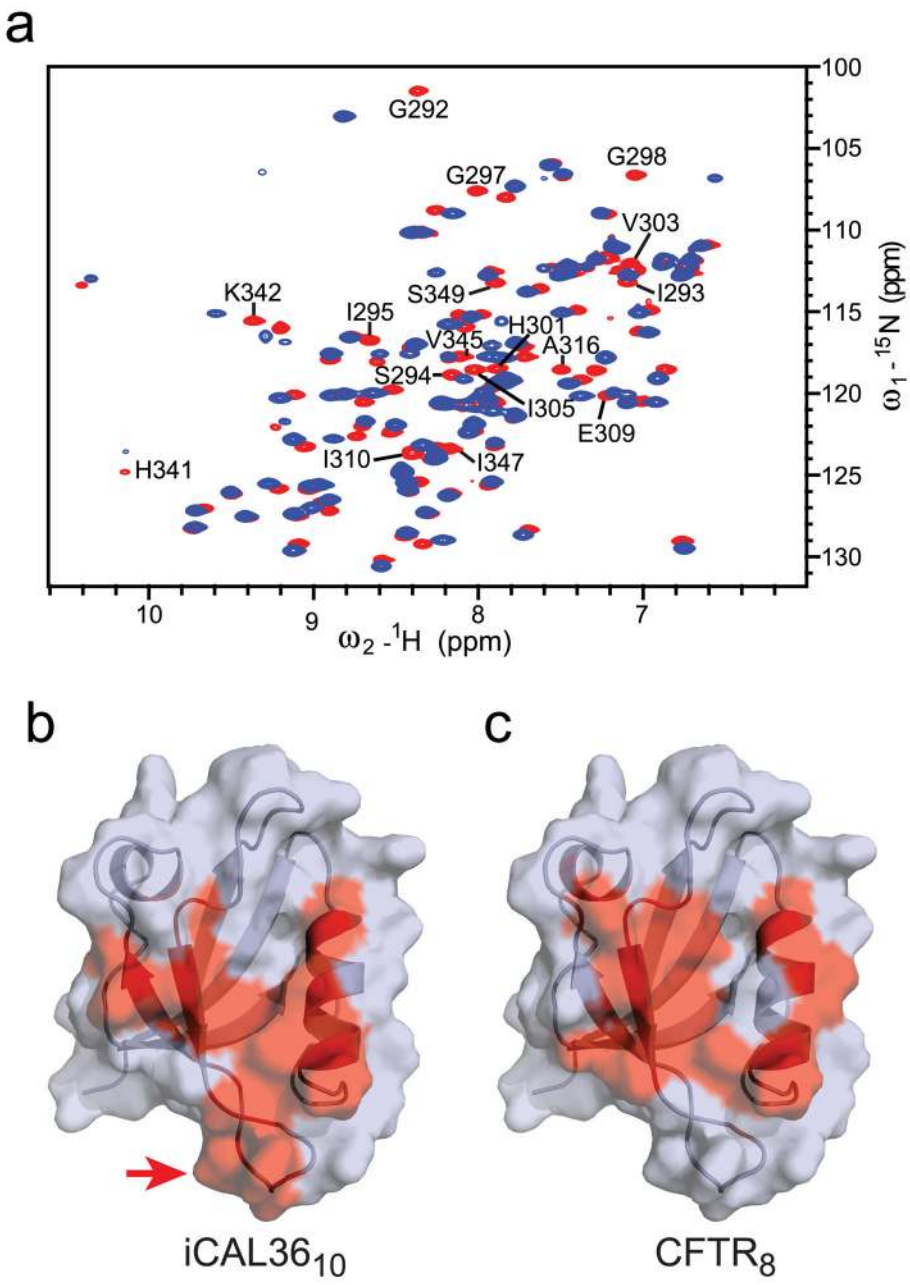

Figure 2.

iCAL36 is a competitive inhibitor. (a) HSQC spectra of ^{15}N-CALP were determined in the absence (red) and in the presence (blue) of 800 μM iCAL36. Crosspeak perturbations are labeled by residue. (b, c) Surface representations of the CAL PDZ domain (PDB entry 2DC2) show the overlapping interfaces (red) associated with binding of the iCAL36 decamer ANSRWPTSII (b) or with the CFTR C-terminal octamer EEVQDTRL (c)[18]. The binding surface of the iCAL36$_{10}$ peptide extends beyond that of CFTR$_8$ (b, arrow).

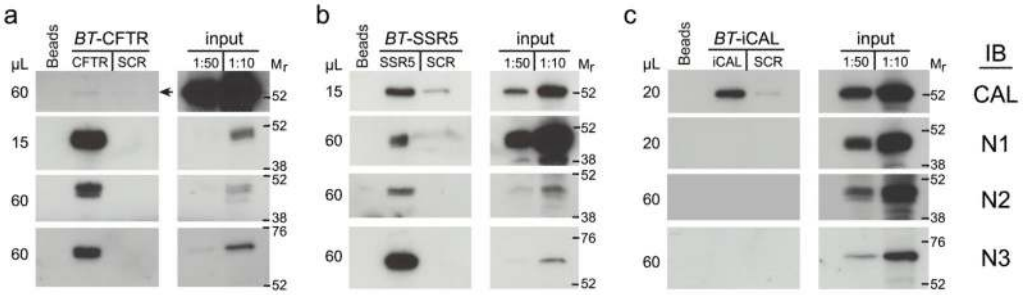

Figure 3.

Pull-down binding assays validate the specificity profiles of PDZ inhibitors for endogenous full-length targets. (a) *BT*-CFTR, (b) *BT*-SSR5, and (c) *BT*-iCAL36 (iCAL) peptides were conjugated to streptavidin beads. Control experiments were performed with unconjugated beads (Beads). Following incubation with CFBE-ΔF cell extracts, bound proteins were eluted with the corresponding unlabeled peptide or SCR, and the indicated volumes were immunoblotted (IB) with CAL-, NHERF1 (N1)-, NHERF2 (N2)-, and NHERF3 (N3)-specific antibodies. Dilutions of cell extracts were blotted on the same membrane as positive controls. Arrowhead indicates the position of the CAL band in (a). Representative blots are shown, with M_r standards at right (n = 3).

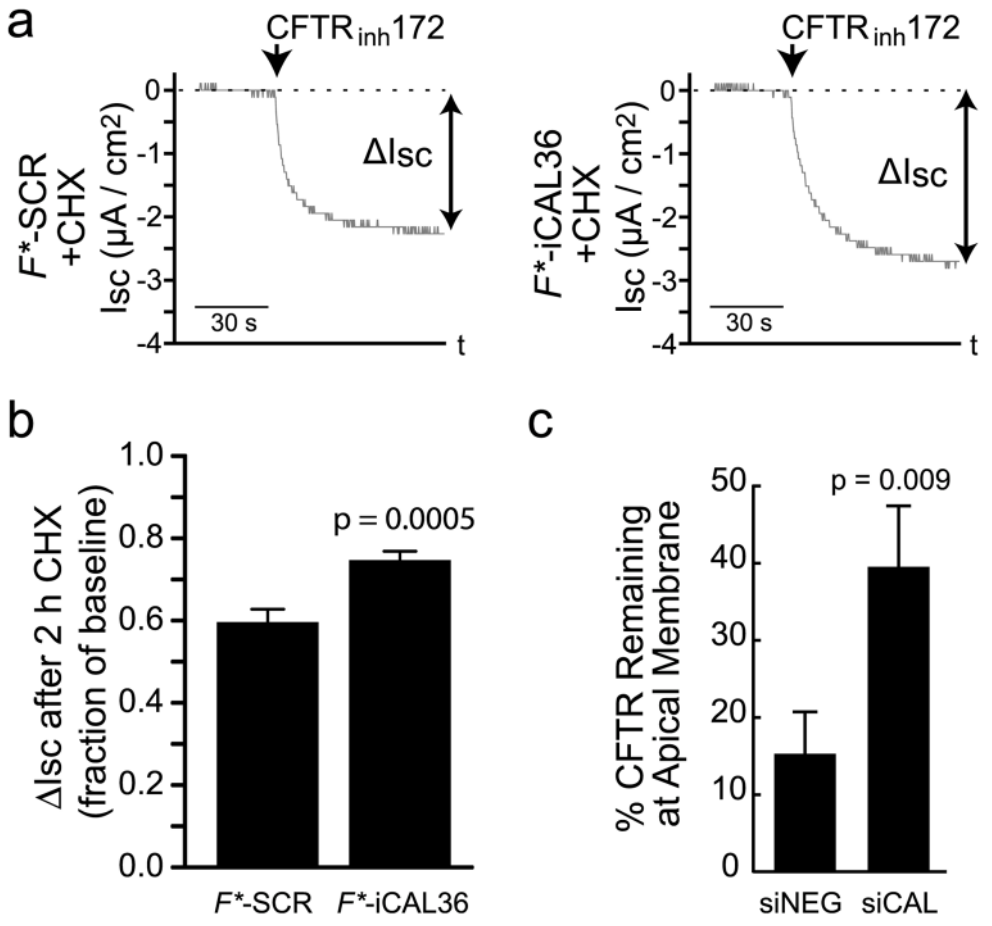

Figure 4.

CAL inhibitors enhance the functional stability of ΔF508-CFTR at the apical membrane. (a, b) Following treatment with *F**-iCAL36 or *F**-SCR, polarized CFBE-ΔF cells were treated with CHX for 0 h or 2 h, and chloride efflux (I_{SC}) was measured. (b) The residual ΔI_{SC} values after 2 h CHX exposure are plotted as a fraction of the corresponding 0 h starting value. CAL-specific inhibition increased residual ΔISC from 60% to 75% of baseline (n = 9). (c) Cell-surface retention of ΔF508-CFTR is enhanced by CAL knockdown. CFBE-ΔF cells were treated with CAL-specific (siCAL) or control (siNEG) siRNAs. Post-maturational stability of ΔF508-CFTR was determined by incubating CFBE-ΔF cells in CHX and measuring the cell-surface abundance of ΔF508-CFTR at t = 2 h as a fraction of the amount present at t = 0 h. siCAL-treated cells exhibited 71% CAL knockdown and retain 40% of ΔF508-CFTR, compared to 15% retention seen in siNEG-treated cells (n = 12 for siNEG and n = 8 for siCAL). Values shown are mean ± SEM.

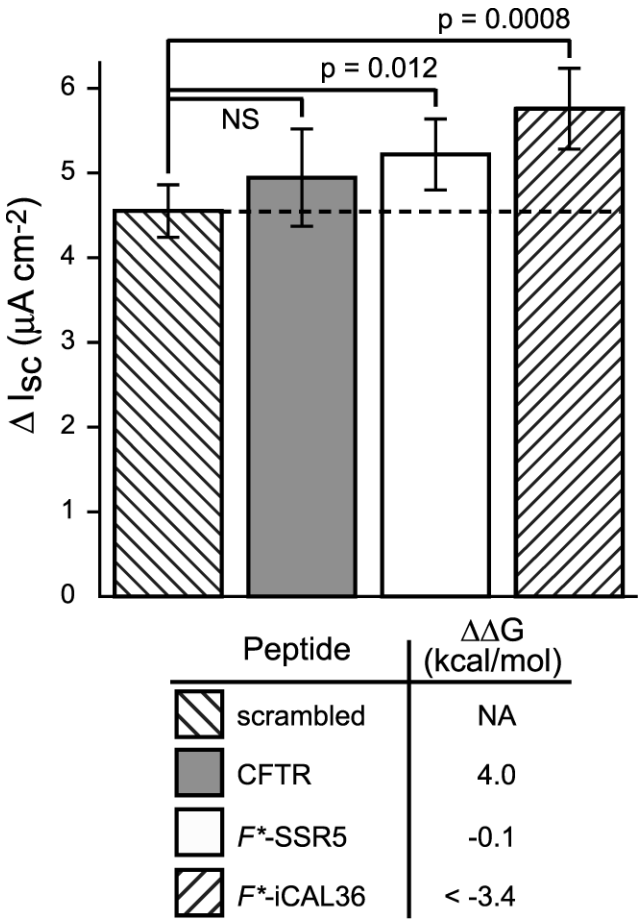

Figure 5.

CAL selectivity improves the efficacy of ΔF508-CFTR rescue. Polarized monolayers were treated with a scrambled control peptide, or else with the CFTR, SSR5, or iCAL36 peptides in the presence of BioPORTER. Following channel activation, the change in short-circuit currents (ΔI_{SC}) was monitored upon application of the CFTR-specific inhibitor $CFTR_{inh}172$[21]. The differential free energy of binding (ΔΔG) for the CAL PDZ domain vs. the NHERF1 PDZ1 domain is shown below the graph for the unlabeled decamer corresponding to each inhibitor peptide[17]. Rescue (ΔI_{SC}) increases with selectivity (ΔΔG). p-values are shown for pairwise comparisons (n ≥ 5). Values shown are mean ± SEM.

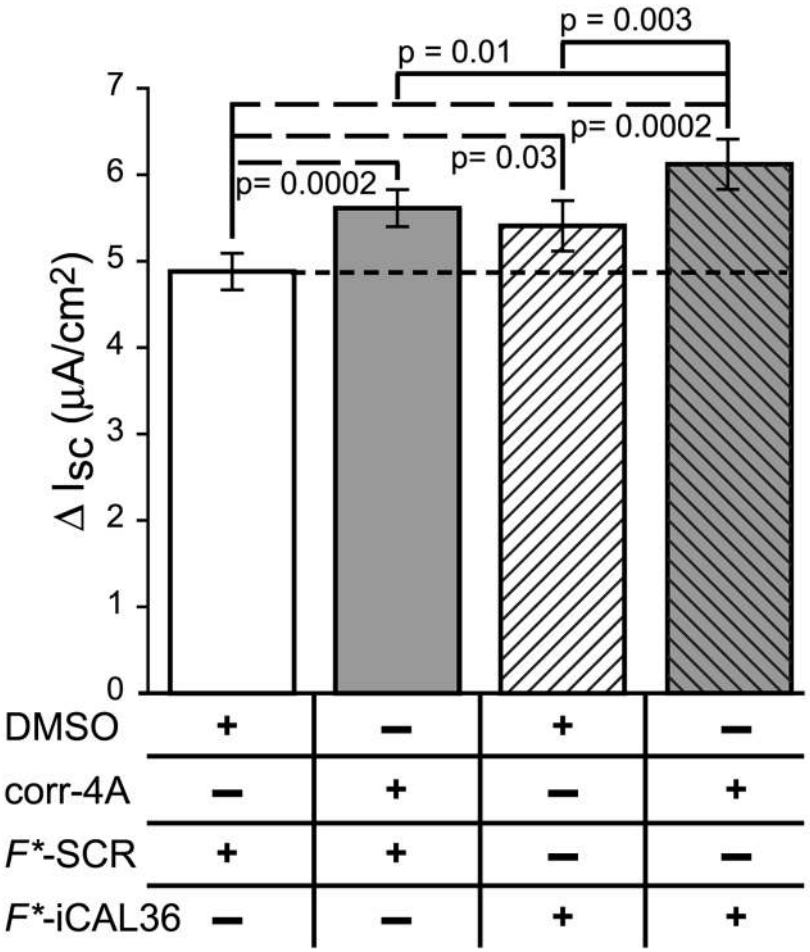

Figure 6.

iCAL36 and corr-4a represent complementary rescue strategies. CFBE-ΔF monolayers were treated with DMSO or with corr-4a[8] for 24 h, and were subsequently treated with BioPORTER and either *F**-SCR or *F**-iCAL36. Chloride efflux values (ΔI_{SC}) were determined, showing enhancements for both iCAL36 (11%) and corr-4a (15%), with nearly additive effects upon co-application (25%). p-values are shown for pairwise comparisons ($n \geq 9$). Values shown are mean ± SEM.