Inhibition of calpain 1 restores plasma membrane stability to pharmacologically rescued Phe508del-CFTR variant

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Edited by Roger J. Colbran

Cystic fibrosis (CF) is a genetic disease caused by mutations in the gene encoding CF transmembrane conductance regulator (CFTR), a chloride channel normally expressed at the surface of epithelial cells. The most frequent mutation, resulting in Phe-508 deletion, causes CFTR misfolding and its premature degradation. Low temperature or pharmacological correctors can partly rescue the Phe508del-CFTR processing defect and enhance trafficking of this channel variant to the plasma membrane (PM). Nevertheless, the rescued channels have an increased endocytosis rate, being quickly removed from the PM by the peripheral protein quality-control pathway. We previously reported that rescued Phe508del-CFTR (rPhe508del) can be retained at the cell surface by stimulating signaling pathways that coax the adaptor molecule ezrin (EZR) to tether rPhe508del-Na⁺/H⁺-exchange regulatory factor-1 complexes to the actin cytoskeleton, thereby averting the rapid internalization of this channel variant. However, the molecular basis for why rPhe508del fails to recruit active EZR to the PM remains elusive. Here, using a proteomics approach, we characterized and compared the core components of wt-CFTR– or rPhe508del-containing macromolecular complexes at the surface of human bronchial epithelial cells. We identified calpain 1 (CAPN1) as an exclusive rPhe508del interactor that prevents active EZR recruitment, impairs rPhe508del anchoring to actin, and reduces its stability in the PM. We show that either CAPN1 down-regulation or its chemical inhibition dramatically improves the functional rescue of Phe508del-CFTR in airway cells. These observations suggest that CAPN1 constitutes an appealing target for pharmacological intervention, as part of CF combination therapies restoring Phe508del-CFTR function.

The cystic fibrosis (CF) transmembrane conductance regulator (CFTR) is a multspanning cAMP-regulated chloride channel primarily localized to the apical membrane of polarized epithelial cells (1). Mutations in the CFTR gene cause the complex inherited disorder CF (1, 2). The most common CFTR mutation is the deletion of phenylalanine 508 (Phe508del), with ~85% of all CF patients having at least one copy of the Phe508del mutant (Welcome to CFTR2 website, https://www.cftr2.org/) (3) (4). This mutation is mainly characterized by protein misfolding and premature degradation by the endoplasmic reticulum (ER) quality control, preventing the mutant protein from trafficking to the cell surface (5–7). Although Phe508del ER retention is not complete, the very small fraction of channels that reach the cell surface possesses only partial activity because of a gating defect (5, 8, 9) and show a severely decreased plasma membrane (PM) half-life, because of accelerated endocytosis and lysosomal degradation (10, 11).

At present, there are two approved corrector drugs that target the molecular defects in Phe508del (12–14). The first to be used in the clinic is the drug Orkambi®, which combines “corrector” VX-809 (lumacaftor) and “potentiator” VX-770 (ivacaftor). VX-809 is an extensively characterized pharmacological chaperone that promotes folding of Phe508del-CFTR during its biogenesis and processing in the ER (16–19), whereas VX-770 increases CFTR channel open probability (20). Although beneficial, results from clinical trials with this combination therapy have shown only modest efficacy in restoring Phe508del-CFTR function in patients (21–26). Not significantly different are results with the combination drug Symdeko®/Symkevi® (corrector VX-661/teza-
CAPN1 binding reduces rPhe508del-CFTR PM stability

Results

wt-CFTR and corrected rPhe508del-CFTR assemble different PM protein interactors

We first determined whether wt-CFTR and rPhe508del-CFTR have different PM protein interactors that could contribute to their distinct cell surface stabilities. For that, we developed an IP protocol that allows to biochemically capture PM CFTR-selective interactomes. Briefly, using a cell line model (mCherry–FLAG–CFTR CFBE cells) that stably expresses mCherry-fused CFTR protein with a FLAG epitope tag inserted at the fourth extracellular loop, we were able to exclusively label CFTR proteins at the PM by incubating cells at 4 °C with an anti-FLAG antibody (Ab) without permeabilization. We then used dithiobis (succinimidyl propionate) (shorter spacer) and Succinimidyl 3-(2-pyridyldithio)propionate (long spacer) cross-linkers to reversibly bind, at 4 °C, both the Ab (extracellularly) and the intracellular proteins associated with CFTR at the PM (Fig. 1, A and B). The same protocol was applied in parallel to CFBE cells expressing either wt- or Phe508del-mCherry–FLAG–CFTR, the latter rescued either by low temperature (26 °C) or by VX-809 treatment (Fig. 1C and “Experimental procedures”).

As part of the optimization process, we scaled up the number of cells in the Phe508del-CFTR experiments, so that the total amount of rescued CFTR (band C) in the IP was roughly equivalent to that of wt-CFTR (Fig. 1D). To control the experiment for background IP contaminants we used, for wt-CFTR, cell extracts incubated with G-protein agarose beads but without the anti-FLAG Ab. For Phe508del-CFTR IPs, lysates from cells incubated at 37 °C (that have none to residual levels of band C at the PM) were considered the most reliable control for proteins co-IPed with rPhe508del-CFTR (Fig. 1C). The recovered proteins were first analyzed by SDS-PAGE followed by MS-compatible silver staining (40) (Fig. 2).

Noticeably, a distinct band profile was observed for each condition, suggestive of different co-precipitate compositions (Fig. 2). These profiles were reproducible between experimental replicates, and therefore, the whole samples of three individual assays were sent to MS analysis.

Bioinformatic analysis of MS data highlighted a candidate interactor potentially involved in the PM destabilization of rPhe508del-CFTR

Qualitative nano-LC MS/MS analysis of our samples generated a set of data in which each MS spectra protein hit was characterized by a protein confidence score (PCS) given by the SCIEX proprietary ProteinPilot™ software. For PCS > 1.3, the confidence in the identification of that particular peptide is equal or higher than 95%. Given that all the experiments were performed in triplicate, we created an algorithm to generate a combined confidence score (CCS), producing five additional integrated confidence levels for the proteins detected among the different replicates: level 5 proteins are detected with PCS ≥ 1.3 in more than one replicate and not in the controls; level 4 proteins are detected in one replicate with a PCS ≥ 1.3 and not in the controls; level 3 proteins are detected in more replicates than in controls with an average PCS higher than their respec-

cator with potentiator VX-770/ivacaftor) (25–27). Given the complexity of Phe508del-CFTR protein defects (6, 18), part of the incomplete effectiveness of the existing combination therapies is likely to derive from an inability to retain sufficient CFTR levels at the apical surface of epithelial cells. Indeed, we and others have shown that the PM stability of chemical chaperone-rPhe508del-CFTR is still dramatically inferior to that of WT (wt)-CFTR, because of its targeting by the peripheral protein quality control and its deficient anchoring to the actin cytoskeleton (10, 28, 29). Notwithstanding, and despite the potential for greater clinical effectiveness of newer investigational corrector combinations (30, 31), there are no therapeutic strategies specifically designed to target the decreased PM stability of chemically rPhe508del-CFTR.

Previously, we demonstrated that the anchoring of rescued channels to the actin cytoskeleton can be enhanced by promoting the PDZ-mediated interaction of Phe508del-CFTR with the Na+/H+ exchange regulatory factor-1 (NHERF1) and the actin-binding adaptor protein ezrin (EZR) (28, 29). We have also shown that the mechanism behind this PM stabilization lies on a conformational change in NHERF1, triggered by EZR activation through RAC1 signaling, which is then able to bind and stabilize misfolded CFTR at the PM (29). This strongly indicated that the protein interactions of wt- and rPhe508del-CFTR at the cell surface can constitute major determinants of CFTR stability and retention at the PM.

Over the last years, a growing number of proteins have been reported to interact with PM CFTR, possibly participating in the assembly of large macromolecular complexes (32, 33). Most interactions occur through the N terminus, the regulatory domain, or the C-terminal tail of CFTR, either directly or mediated through various PDZ domain-containing proteins (32, 34, 35). These dynamic interactions impact on channel function, as well as on its processing and localization within cells (32, 33). Furthermore, it has been established that mutant and nonmutant CFTR show considerable differences in their whole cell interactomes, and thus these differential protein interactions may contribute to the defects observed in Phe508del-CFTR processing and function (36). Hence, although several CFTR interactor partners are known (33, 36, 37), there is no study describing specifically CFTR’s particular interactome at the PM, whether differences between wt- and pharmacologically rescued Phe508del-CFTR occur (38), and how these differences could contribute to the defective function and stability of the rescued mutant channels.

Here we propose to address these questions by identifying the core components of the CFTR PM molecular complexes in human airway cells expressing wt- or Phe508del-CFTR. For this, we developed an immunoprecipitation (IP) method that selectively captures the CFTR-containing macromolecular complexes at the PM. Our data reveal significant differences between the PM interactors of wt and rPhe508del-CFTR and allowed us to identify calpain 1 (CAPN1) protease as a key player in reducing Phe508del-CFTR actin anchoring and PM stability, thus contributing to the decreased cell surface retention of this rescued mutated channel.
tive controls; level 2 proteins are detected in one replicate with a PCS $\leq 1.3$ and not in the controls; and level 1 are proteins detected in the same number of replicates and controls, with an average replicate PCS higher than the average PCS in the corresponding controls.

Altogether, we detected 481 wt-CFTR PM putative protein interactors and 631 and 660 Phe508del-CFTR PM putative interactors, respectively, rescued by pharmacological and low temperature, most of which have low CCS scores (Fig. 2B and Tables S1–S3). Given that cross-linking agents were used, it is highly likely that many of these proteins could be indirect or nonspecific interactors present in other PM complexes that share or interact with proteins present in CFTR-containing PM complexes. Notably, most proteins from both Phe508del rescued conditions overlap, sharing 533 protein putative interactors (Fig. 2B). Interestingly, $\sim 40\%$ (337 of 855) of all identified proteins are common between all data sets (Fig. 2C and Table S4), possibly containing the proteins that form the core PM CFTR interactome. For downstream analyses, only high confidence level proteins ($>95\%$ confidence, i.e. CCS of 4 or 5) were considered. This approach restricted the list of putative CFTR interactors to 56 high confidence proteins in wt-CFTR, whereas the number of high confidence proteins in complex with rPhe508del-CFTR was considerably larger, comprising 225 in pharmacological rescued and 245 in low-temperature rPhe508del-CFTR, of which 158 were common to both data sets (Fig. 2D and Table S5). By crossing our results with the whole cells CFTR interactome obtained by Pankow et al. (36), we observed that 23 of the 56 ($41\%$) proteins detected with high confidence in wt-CFTR complexes at the PM and 49 of the 158 ($30\%$) co-detected with PM–rPhe508del-CFTR were identified by both studies (Fig. 2E and Tables S6 and S7).

Because both low temperature and VX-809 treatment produce rPhe508del-CFTR proteins with equally decreased PM half-lives (41), we postulated that it would be highly likely that any interactors influencing rPhe508del-CFTR surface stability should be among the proteins shared by PM complexes produced by either rescue approach. Our previous data (29) also indicated that these unknown destabilizing factors should be proteins interacting differentially with wt- and rPhe508del-CFTR at the PM. Therefore, we narrowed the list of proteins...
putatively influencing rPhe508del-CFTR PM stability by intersecting the groups of high-confidence proteins in the low temperature and VX-809–treatment data sets and excluding those that also co-precipitated with wt-CFTR. With this approach, we further restricted our candidate list to 150 proteins (Fig. 2 and Table S5). Next, we investigated which proteins in this narrowed list were reportedly functionally related. For that, we extracted known physical protein interactions from curated human interactome databases (see “Experimental procedures”) to build an integrated network of candidate protein interactions. We found that, from the 150 putative interactors interfering with rPhe508del-CFTR stability, 135 (90%) formed a tightly connected network around CFTR, with 539 interactions (Fig. S1 and Table S8). This means that available interactome data supports the close relationship between most of the proteins here detected as specifically interacting with rPhe508del-CFTR at the PM, both upon VX-809 and low-temperature treatment. This is not unexpected because most of the recorded interactions in public databases result from whole cell studies, and many of these proteins may, at one point, interact with CFTR or with proteins that interact directly or indirectly with CFTR.

Having previously shown that rPhe508del-CFTR destabilization at the cell surface results from an interference with the formation of the CFTR–NHERF1–EZR retention complex at the PM (28, 29, 42), we further restricted this network to proteins annotated as direct interactors of either EZR, NHERF1, or CFTR. This approach generated a subnetwork of 22 proteins (Fig. 3 and Table S9) that we selected as the core of putative candidates involved in destabilizing rPhe508del-CFTR-containing complexes at the PM.

Gene ontology-based functional annotation analysis of these 22 hit proteins using DAVID (Database for Annotation, Visualization and Integrated Discovery, https://david.ncifcrf.gov/) (3, 15)3 revealed that, as expected, all 22 proteins are known to participate in macromolecular protein complexes. Interestingly, 52% of these proteins are annotated as being localized at the membrane, and 64% are annotated as being localized at the cytosol (Table S10). Both subcellular localizations are expected for such a large and dynamic protein complex, because both the N and C termini of CFTR are in the cytoplasm and mediate its interaction with large clusters of PDZ adaptor and functionally related proteins (32, 33).

To infer the potential relevance of each of these 22 proteins to the assembly of rPhe508del-CFTR–NHERF1–EZR complexes at the PM, we devised a scoring formula that measures the contribution of each node to the establishment of molecular bridges between these three proteins. Bridges involving more
proteins were given a lower weight in the final score (see “Experimental procedures”).

This analysis, depicted in Fig. 3 by the size of node boxes, identified proteins YWHAZ (14-3-3/H9256), CAPN1 (calcium-activated neutral proteinase 1, catalytic subunit, also calpain 1), and IPO5 (importin 5) as the most relevant in this context. IPO5 is a direct interactor of CFTR and EZR, whereas YWHAZ and CAPN1 interact directly with EZR and NHERF1. YWHAZ reaches a higher score because it interacts directly with five CFTR direct neighbors. CAPN1 can also form a complex with its small regulatory subunit—calpain small subunit 1 (CAPNS1). Remarkably, the CAPN1–CAPNS1 complex interacts directly with CFTR, EZR, and NHERF1 (Fig. 3).

The proteins in this list were also intersected with the druggable proteome at the ChEMBL database to identify the most likely druggable hits. This intersection highlighted CAPN1 as a particularly strong candidate.

CAPN1 is a strong candidate for selective interaction with rPhe508del-CFTR

CAPN1 belongs to a group of calcium-sensitive cysteine proteases that are ubiquitously expressed in human cells and associated with subcellular organelles such as the cytoskeletal actin filaments, vesicles, and the PM (43). Furthermore, CAPN1 inhibition was shown to promote the partial rescue of Phe508del-CFTR in peripheral blood mononuclear cells (PBMCs) from CF patients (44).

To assess the robustness/directness of the interaction between PM-CFTR and CAPN1, we performed the same IP protocol, but in the absence of cross-linking agents. We confirmed that CAPN1 co-precipitates with rPhe508del-CFTR but not with wt-CFTR, even in the absence of cross-linking agents, showing the strength of this interaction (Fig. 4). In contrast, tubulin, which was consistently absent from the isolated CFTR-containing PM-associated complexes, was also absent from the un–cross-linked co-precipitates, confirming the specificity of CAPN1 detection in the co-IP (Fig. 4). We therefore decided to study the effect of CAPN1 on the PM stability of rPhe508del-CFTR.

CAPN1 down-regulation increases the PM abundance and function of VX-809–rPhe508del-CFTR

Using a CAPN1-specific siRNA, we down-regulated CAPN1 expression in CFBE cells to ∼32% of its normal levels (Fig. 5A)
and then assessed the PM abundance of VX-809–rPhe508del-CFTR under these conditions. For this, we performed a cell surface protein immunolabeling assay that allows the selective labeling of CFTR proteins at the PM (Ref. 39; see also “Experimental procedures”). Briefly, Phe508del-CFTR–PM expression was rescued by VX-809 treatment (48 h at 37 °C) in mCherry–FLAG–Phe508del-CFTR CFBE cells transfected with either siLuc or siCAPN1 and co-treated, or not, with 25 μM of inh172 for 15 min prior to stimulation (or not; NS indicates no stimulation) for 30 min in PBS with Fsk and Gen, in the presence or absence of inh172, followed by continuous fluorescence recording and addition of 1 (100 μM). F, Summary of iodide influx rates calculated by fitting the iodide assay results to exponential decay curves. One-way ANOVA analysis detected significant differences between treatments (F = 44.862; p < 0.001). Post hoc Tukey’s tests were used to identify significant variations relative to siLuc (**, p < 0.001) or siCAPN1 (#, p < 0.001) alone. The figure shows representative images and quantification results of seven independent experiments. The means ± S.D. are given for all quantified data, highlighted by dotted gray rectangles in D. For A, C, and D, significance was tested using unpaired, two-tailed t tests, comparing siCAPN1 to siLuc (*, p < 0.01).
CAPN1 binding reduces rPhe508del-CFTR PM stability

low fluorescent protein (HS-YFP) (45–47). Briefly, CFBE cells co-expressing Phe508del-CFTR and the HS-YFP mutant (45) were transfected with either siCAPN1 or siLuc and incubated with VX-809 for 48 h (Fig. 5, E and F).

The cells were then stimulated for 30 min in PBS with 5 μM forskolin (Fsk) and 20 μM genistein (Gen) to induce CFTR activity. Next, a HS-YFP fluorescence baseline was recorded for 5 s, and its decay followed continuously (at 0.5-s intervals) after the rapid (<1 s) addition of iodide (Fig. 5E). The curves were fitted to an exponential decay function to derive the initial rates of iodide influx that, as previously described (29), are directly proportional to the extent of chloride effluxed from these cells via CFTR. Consistent with the observed >2-fold increase in PM abundance of VX-809–rPhe508del-CFTR, CAPN1 down-regulation increased by ~2.5-fold the iodide influx rate in these cells, which was specifically dependent on CFTR activity, because the effect was abolished upon treatment with the CFTR-specific inhibitor 172 (inh172) (Fig. 5F). Altogether, these results strongly suggest that CAPN1 plays an important role in regulating the overall PM stability of pharmacologically rPhe508del-CFTR at the PM of bronchial epithelial cells.

Knockdown of CAPN1 improves the PM stability of rPhe508del-CFTR through enhanced EZR binding

To determine whether the effect of CAPN1 down-regulation on the increased levels of rPhe508del-CFTR at the cell surface resulted from enhanced stability, we coupled the surface CFTR immunolabeling assay to a previously described surface thermal destabilization assay (29; see Experimental procedures). Briefly, VX-809–rPhe508del-CFTR (48 h) was stabilized at the surface by incubation of the cells at 30 °C. The cells were then shifted for 4 h to 37 °C (thermal shift (TS)), which destabilizes rPhe508del-CFTR at the cell surface causing its internalization, unless additional experimental conditions lead to the retention of the rescued channel at the PM (41, 48). CFTR at the cell surface was immunolabeled and analyzed as before (Fig. 6A). Fluorescence signal quantification revealed that upon TS to 37 °C, rPhe508del-CFTR in siLuc-transfected cells was destabilized and removed from the PM, reducing the protein cell surface abundance by ~60% (Fig. 6B). In contrast, down-regulation of CAPN1 clearly increased the surface stability of the rescued channels because ~80% of the protein remained at the PM after 4 h at 37 °C (Fig. 6B). The immunolabeling results were again confirmed by cell surface protein biotinylation (Fig. 6C), which also showed that GLUT-1 PM abundance was not influenced by CAPN1 knockdown, again supporting a CFTR-specific PM retention effect (Fig. 6C).

We have previously shown that the critical step to stabilize Phe508del-CFTR at the PM is the opening of NHERF1 PDZ2 domain by the binding of activated EZR (29). Because interaction with CAPN1 was shown to cause the inactivation of EZR (43, 49, 50), we hypothesized that the increased rPhe508del-CFTR PM stability by down-regulating CAPN1 could result from enhancement of its cytoskeletal anchoring by the promotion of rPhe508del-CFTR–NHERF1–EZR interaction. To test this hypothesis, we immunoprecipitated PM–rPhe508del-CFTR in the TS assay conditions used above and analyzed the levels of EZR and NHERF1 in the co-IP (Fig. 6D). Notably, whereas the levels of NHERF1 in the co-IP were equivalent between control (siLuc) and siCAPN1 transfected cells, the amount of EZR co-precipitating with PM–rPhe508del-CFTR upon CAPN1 down-regulation increased to levels near those found in wt-CFTR–expressing cells (Fig. 6D). These results are in accordance with our hypothesis and show that CAPN1 binding negatively influences the formation of Phe508del-CFTR–NHERF1–EZR anchoring complex, leading to a diminished stability of the pharmacological corrected channels.

Acute chemical inhibition of endogenous calpain 1 significantly increases VX-809–mediated Phe508del-CFTR functional rescue in bronchial epithelial cells

CAPN1 was identified above as a druggable candidate protein because its activity can be chemically inhibited by ALLM, a cell-permeable peptide aldehyde (51, 52). We thus tested the effect of ALLM treatment on the functional rescue of Phe508del-CFTR by VX-809 using the HS-YFP assay. CFBE Phe508del-CFTR cells co-expressing HS-YFP were incubated with 3 μM VX-809 for 48 h and co-treated with either DMSO or 250 nM of ALLM for the last 4 h. As before, the cells were then stimulated with Fsk and Gen and analyzed by live confocal imaging after 1− addition (Fig. 7A and B). Treatment with ALLM produced a striking response in fluorescence decay, which was CFTR-dependent, because it was completely inhibited by inh172 (Fig. 7A). Calculation of initial iodide influx rates (as before) revealed that treatment with ALLM increased by ~4-fold the functional response of VX-809–rPhe508del-CFTR, when compared with VX-809 + DMSO-treated cells (Fig. 7B).

Analysis of PM abundance (Fig. 7C and S2A) and surface TS (Fig. 7E and Fig. S2B) of rPhe508del-CFTR corroborate this enhanced functional response. ALLM-treated cells presented a 5-fold increase in VX-809–rPhe508del-CFTR PM levels (~8 to ~40%, compared with VX-809 + DMSO control cells; Fig. 7C).

Even considering the small enrichment in total CFTR induced by ALLM treatment, the Phe508del-CFTR retention at the PM (surface/total ratio) reached nearly 4-fold that of control, DMSO-treated cells (Fig. 7C), which translated into a full thermal stabilization of the rescued channels present at the surface of these cells (Fig. 7E). Again, these results were also observable through cell surface protein biotinylation followed by WB analysis (Fig. 7, D and F, respectively).

Discussion

So far, several studies have identified numerous CFTR interactor proteins with essential roles in the processing, localization, and function of the channel (32–36, 53, 54). Nonetheless, the present work is the first to globally characterize CFTR interactors specifically occurring at the PM. Previous studies have shown that several interactions between CFTR and its binding partners are mediated through the scaffolding protein NHERF1, which seems to be a central protein in the assembly of the CFTR-containing macromolecular complexes at the surface of airway cells (33, 55, 56). EZR is also a key component of these complexes, mediating the tethering of NHERF1-bound CFTR proteins at the PM to the actin cytoskeleton, thus stabilizing the channel at the cell surface by blocking its endocytosis (57–59). Moreover, we also showed previously that the low cell...
surface stability of chemically rescued Phe508del-CFTR was, at least in part, caused by an impaired recruitment of EZR to rPhe508del-CFTR–NHERF1 complexes at the PM, preventing their anchoring to actin and thus leading to their rapid internalization (28). Moreover, we and others showed that overexpression of constitutively active EZR coaxed the retention of rPhe508del-CFTR at the PM (29, 60), by causing a conformational change in NHERF1 that displaced CHIP E3-ubiquitin ligase, and thus prevented CFTR ubiquitination and internalization (29). This led us to hypothesize that EZR overexpression was somehow circumventing a mechanistic restraint that prevented its association with rPhe508del-CFTR–NHERF1 complexes in normal conditions. We therefore reasoned that it would be highly likely that, similarly to CHIP, additional pro-

Figure 6. Effect of CAPN1 down-regulation on the levels and thermal stability of PM-associated complexes containing EZR, NHERF1, and rPhe508del-CFTR. A: confocal images of Dox-induced CFBE mCherry–FLAG–Phe508del-CFTR cells, transfected with either siLuc or siCAPN1. Phe508del-CFTR was rescued to the PM by 48 h of incubation with VX-809 at 30 °C and subjected to a TS of 4 h at 37 °C, except for siLuc low temperature control (30 °C). Images are triple-labeled unpermeabilized cells, in which mCherry fluorescence is proportional to the total amount of CFTR, Alexa Fluor 488 fluorescence is proportional to the amount of CFTR present at the cell surface, and nuclei are stained with DAPI. Scale bars correspond to 25 μm. B: analysis of single-cell fluorescence signals in five independent experiments. The data are presented as means ± S.D. of total (T), surface (S), or S/T ratio of rPhe508del-CFTR abundance, expressed as percentages of CFTR at 30 °C (low temperature control). C: WB analysis of biotinylated cell surface proteins (biotin PD) and whole-cell lysates (WCL), treated as in A. D: WB analysis of EZR and NHERF1 levels co-precipitating with wt- or Phe508del-CFTR at the PM. Phe508del-CFTR cells were treated for 48 h with VX-809 at 30 °C and transfected with either siLuc or siCAPN1. The cells were then subjected to TS for 4 h at 37 °C to destabilize rescued CFTR at the PM. The wt-CFTR cells were maintained always at 37 °C. Surface CFTR was then labeled with anti-FLAG antibody in nonpermeabilizing conditions and immunoprecipitated. WB using specific antibodies detected co-precipitating NHERF1 and EZR proteins. The data in C and D are representative of three independent experiments, and the dotted gray rectangles highlight the respective quantification (means ± S.D.) of CFTR and GLUT-1 abundance at the PM (C), relative to siLuc at 30 °C, and of co-precipitating EZR and NHERF1 (D), relative to wt-CFTR siLuc. The data significance was tested using unpaired, two-tailed t tests, comparing TS siLuc to 30 °C siLuc (B; *, p < 0.001) and TS siCAPN1 to TS siLuc (B–D; #, p < 0.05; §, p < 0.01).
CAPN1 binding reduces rPhe508del-CFTR PM stability

Figure 7. Chemical inhibition of CAPN1 increases rPhe508del-CFTR PM abundance, function, and stability. 

A, traces of iodide-induced HS-YFP fluorescence decay of CFBE Phe508del-CFTR cells treated with 3 μM VX-809 for 48 h, nontreated (NT) or co-treated with either DMSO or 250 nM ALLM CAPN1 inhibitor in the last 4 h, co-treated or not with 25 μM of inh172 for 15 min prior to stimulation for 30 min in PBS with Fsk and Gen, in the presence or absence of inh172, followed by continuous fluorescence recording and addition of 3 μM Fsk. B, summary of iodide influx rates calculated by fitting the iodide assay results to exponential decay curves. One-way ANOVA analysis detected significant differences between treatments (F = 55.056, p < 0.001). Post hoc Tukey’s tests were used to identify significant variations relative to DMSO (***, p < 0.001) or ALLM (**, p < 0.001) alone. C, analysis of single-cell fluorescence signals in three independent experiments of Dox-induced mCherry–FLAG-wt- or Phe508del-CFTR cells, treated with 3 μM VX-809 for 48 h and co-treated with either DMSO or 250 nM ALLM CAPN1 inhibitor in the last 4 h. The data are presented as the means ± S.D. of total (T), surface (S), or S/T ratio of rPhe508del-CFTR abundance, expressed as percentages of wt-CFTR values. D, WB analysis of biotinylated cell surface proteins (biotin PD) and whole-cell lysates (WCL), treated as in C. E, analysis of single-cell fluorescence signals of Dox-induced mCherry–FLAG–Phe508del-CFTR cells, treated with 3 μM VX-809 for 48 h at 30 °C, and subjected to a TS of 4 h at 37 °C in the presence of either DMSO or 250 nM ALLM CAPN1 inhibitor, except for DMSO low temperature control (30 °C). The data are presented as total (T), surface (S), or S/T ratio of rPhe508del-CFTR abundance, expressed as percentages of CFTR at 30 °C (low temperature control); F, WB analysis of biotinylated cell surface proteins (biotin PD) and whole-cell lysates (WCL), treated as in E. The figure shows representative images and quantification results of five independent experiments. The means ± S.D. are given for all quantified data. The dotted gray rectangles in D and F highlight the respective quantification of CFTR abundance at the PM, relative to wt-CFTR with DMSO (O) and to DMSO at 30 °C (I). For C–F, significance was tested using unpaired, two-tailed t tests, comparing ALLM to DMSO (C and D; V, p < 0.05; *, p < 0.001), TS DMSO to 30 °C DMSO (F, §, p < 0.001), and TS ALLM to TS DMSO (E and F; ¶, p < 0.001).

Despite the limitations in our data set, supporting the selectivity of the experimental approach used here. Our hypothesis guided us to develop an assay to selectively IP and characterize CFTR-containing complexes from the PM. Notwithstanding, we showed here that CAPN1, a ubiquitous cytoskeleton- and PM-associated Ca2+-dependent cysteine protease (43), is present within the PM complex assembled around rPhe508del-CFTR, but not around wt-CFTR proteins. Moreover, we demonstrated that chemical inhibition of CAPN1 with ALLM promotes the PM stabilization of VX-809 – rPhe508del-CFTR in bronchial epithelial cells, leading to a near 4-fold increase in the functional restoration of CFTR-mediated ion transport. Although ALLM has a higher specificity to inhibit CAPN1 over other calpains, it is also an effective inhibitor of cathepsins, a group of lysosome-associated proteases (51). Because degradation of rPhe508del-CFTR internalized from the PM was described to occur mainly at the lysosome compartment (41), we cannot exclude a partial contribution of cathepsin inhibition to the increased PM levels of rPhe508del-CFTR upon ALLM treatment. Indeed, whereas siRNA-mediated CAPN1 knockdown produced a similar effect to ALLM treatment regarding PM abundance, stability, and functional rescue of Phe508del-CFTR, the overall magnitude of the effect was lower under siRNA than that achieved with the chemical inhibitor. Previous studies in PBMCs of patients...
homozygous for Phe508del-CFTR showed that elevated levels of intracellular Ca\(^{2+}\) led to an unbalance in the calpain/calpastatin system, resulting in a constitutive CAPN1 activation caused by a decrease in their specific inhibitory regulators, leading to a reduction of mature CFTR in PBMCs (44). These authors also showed that CAPN1 activity could compromise CFTR function by targeting CFTR–NHERF1 complexes at the PM and that total EZR protein levels were lower in the PBMC of CF patients (44). Although the expression of CFTR in PBMCs is still subject to controversy (61, 62), Ca\(^{2+}\) was also found elevated in epithelial airway cells of CF patients (63–66), suggesting a similar up-regulation of CAPN1 activity in these cells. Moreover, whereas the authors did not fully elucidate the mechanism of PM CFTR destabilization in PBMCs (44), here we show that knockdown of CAPN1 stabilizes Phe508del-CFTR by promoting the activation and association of EZR to CFTR–NHERF1 complexes at the PM. This result is consistent with our previous findings showing that it is possible to coax the anchoring and PM retention of chemically rescued Phe508del-CFTR by promoting the activation and association of EZR to CFTR–NHERF1 complexes at the PM, by increasing endogenous RAC1 signaling through the stimulation of airway epithelial cells with HGF (28, 42). The binding of activated EZR induces a conformational change in NHERF1 exposing a second PDZ domain for CFTR binding, which stabilizes the rescued channel at the cell surface, leading to a 3-fold increase in the functional restoration achieved by corrector drugs, such as VX-809 (28, 29, 42). An equivalent extent of functional recovery was observed with CAPN1 inhibition, either by siRNA-mediated knockdown or by chemical inhibition with ALLM. These findings indicate that the proteolytic activity of CAPN1 is required for the destabilization of rPhe508del-CFTR at the PM. Consistently, EZR is among the known CAPN1 substrates, and remarkably, it is the only member of the ezrin–radixin–moesin (ERM) family to be cleaved by this protease \textit{in vitro} (67). This supports an important function for CAPN1 in the regulation of EZR-mediated anchoring of PM proteins, namely CFTR.

All the gathered evidence led us to propose the following model (Fig. 8): elevated Ca\(^{2+}\) intracellular levels coupled to the recruitment of CAPN1 to rPhe508del-CFTR–NHERF1 PM complexes lead to the hydrolysis and displacement of EZR from the complex, preventing its activation by endogenous signaling events (such as those mediated by RAC1 (28)) and thus avoiding NHERF1 opening and the anchoring of rPhe508del-CFTR to the actin cytoskeleton. These events lead to the accelerated internalization and degradation of rescued channels, as described by us and others (19, 28, 29). Inhibition of CAPN1 allows the activation and binding of EZR to rPhe508del-CFTR–NHERF1 complexes at the PM, promoting the retention and thus stabilization of rescued channels at the cell surface.

Taken together, our results indicate that association of CAPN1 to the cell surface rPhe508del-CFTR–NHERF1 complexes plays an important role in reducing the half-life of rPhe508del-CFTR at the PM and that its pharmacological inhibition has a potentially relevant therapeutic application in combination therapies with other modulator drugs, namely correctors and potentiators. It should be noted that even though there are no CAPN1 inhibitors currently progressing into clinical trials, important advances are being made in their development, and several preclinical studies demonstrated their potential therapeutic value (68, 69). In accordance, further \textit{in vivo} studies will now be required to evaluate the therapeutic applicability of CAPN1 inhibition for the benefit of people with CF disease.

**Experimental procedures**

**Cell culture, treatment, and transfection**

CFBE41o- cells stably transduced with lentivirus encoding mCherry–FLAG–tagged, wt- or Phe508del-CFTR under the Tet-ON promoter were generated by Adv Bioscience LLC as
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described before (70). A clone of CFBE stably expressing nontagged Phe508del-CFTR cell(45) was modified to stably co-express the halide sensor YFP–F46L/H148Q/I152L (HS–YFP) by Sigma–Aldrich (Santa Cruz Biotechnology). The cells were then placed on ice, rinsed three times, and left for 15 min with Tris–HCl (50 mM Tris–HCl, pH 7.5) to quench the reaction. The cells were again washed three times with cold PBS–CM and lysed in 500 μl of lysis buffer (50 mM Tris–HCl, pH 7.5, 2 mM MgCl2, 100 mM NaCl, 10% (v/v) glycerol, 1% (v/v) Nonidet P-40, 0.01% (v/v) SDS, Protease inhibitor mixture; Sigma). Cell lysates were first cleared at 3100 × g for 5 min at 4 °C. ~450 μl of lysate were added to 45 μl of streptavidin–agarose beads (Sigma–Aldrich) to further clear the lysates and were rotated for 1 h at 4 °C, centrifuged for 1 min at 3100 × g, and recovered. ~400 μl of the cleared lysates were added to 40 μl of G-protein–agarose beads (Roche), rotated for 1 h at 4 °C, centrifuged for 1 min at 3100 × g, and washed six times in cold wash buffer (50 mM Tris–HCl, pH 7.5, 2 mM MgCl2, 300 mM NaCl, 10% (v/v) glycerol, 1% (v/v) Nonidet P-40, 0.1% (v/v) SDS). Captured proteins were recovered in 50 μl of 2× sample buffer with DTT (100 mM).

MS-compatible silver staining and nano-LC MS/MS

Captured membrane CFTR-associated protein complexes were separated on 9% (w/v) SDS–PAGE gels and run for 1 cm. The gels were then silver-stained through a MS-compatible protocol, as described in Ref. 40. Briefly, the gels were transferred to a glass tray and fixed for 1 h at room temperature in fixer solution (40% ethanol, 10% glacial acetic acid, 50% ddH2O), and washed overnight in ddH2O. The gels were sensitized in 0.02% sodium thiosulfate for 1 min and washed three times with ddH2O. The gels were then incubated in cold 0.1% silver nitrate solution with 0.02% formaldehyde (added just before use) for 20 min and washed twice with ddH2O. The gels were transferred to a new tray and washed for 1 min before developing in 3% sodium carbonate solution, with 0.05% formaldehyde (added just before use) until distinct bands were visible. After ddH2O washing, staining was fixed in 5% acetic acid for 5 min, and the gels were stored at 4 °C in 1% acetic acid until MS analysis. For MS analysis, the entire 1-cm lanes corresponding to each sample run were excised from the gels and sent in ddH2O for nano-LC MS/MS analysis on a SCIEX TripleTOF 6600 system, outsourced to UniMS (Instituto de Tecnología Química e Biológica António Xavier Institute, Oeiras, Portugal).

Nano-LC–MS/MS analyses

Nano-LC–MS/MS analysis was performed on an ekspert™ Nano-LC 425 chPLC® system coupled with a TripleTOF® 6600 with a NanoSpray® III source (Sciex). Peptides were separated through reversed-phase LC in a trap-and-elute mode. Trapping was performed at 2 μl/min on a Nano chPLC Trap column (Sciex 200 μm × 0.5 mm, ChromXP C18-CL, 3 μm, 120 Å) with 100% A (0.1% formic acid) for 10 min. The separation was performed at 300 nl/min, on a Nano chPLC column (Sciex 75 μm × 15 cm, ChromXP C18-CL, 3 μm, 120 Å). The gradient was as follows: 0–1 min, 5% B (0.1% formic acid in acetonitrile, Fisher Chemicals); 1–91 min, 5–30% B; 91–93 min,
30–80% B; 93–108 min, 80% B; 108–110 min, 80–5% B; and 110–127 min, 5% B. The peptides were sprayed into the MS through an uncoated fused-silica PicoTipTM emitter (360-μm outer diameter, 20-μm inner diameter, 10 ± 1.0-μm tip inner diameter; New Objective). The source parameters were set as follows: 15 ion source gas 1, 0 ion source gas 2, 30 curtain gas, 2.5 keV ion spray voltage floating, and 100 °C interface heater temperature. An information-dependent acquisition method was set with a TOF–MS survey scan of 400–2000 m/z. The 50 most intense precursors were selected for subsequent fragmentation, and the MS/MS were acquired in high sensitivity mode for 40 ms. The obtained spectra were processed and analyzed using ProteinPilotTM software, with the Paragon search engine (version 5.0, Sciex). A UniProt TrEMBL database (20422 entries, accessed in 29 April 2015) containing the sequences of the proteins from the organism Homo sapiens (Taxon ID: 9606) was used. The following search parameters were set: iodoacetamide, as Cys alkylation; trypsin, as digestion; TripleTOF 6600, as the instrument; ID focus as biological modifications and amino acid substitutions; search effort as thorough; and an false discovery rate analysis. Based on the data set size, the false discovery rate was determined to be below 5% for proteins with a calculated unused protein score (ProteinPilotTM software) above 1.3 (95% confidence).

Bioinformatics analyses of MS data
Confidence in protein detection
Precipitation of CFTR membrane association complex was performed in three replicates. In each replicate, identification of each protein in MS spectra was characterized by a PCS, detected with ProteinPilotTM Software (Sciex). For PCS of >1.3, the confidence in the identification of that particular protein in the sample is equal or higher than 95%. To conjugate this individual score with the detection of the same protein across replicates, we generated a CCS, defined by five levels of confidence for protein detection: level 5 proteins are detected with PCS ≥ 1.3 in more than one replicate and not in the controls; level 4 proteins are detected in one replicate with PCS ≥ 1.3 and not in the controls; level 3 proteins are detected in more replicates than in controls and with an average PCS higher than their respective the controls; level 2 proteins are detected in one replicate with PCS ≤ 1.3 and not in the controls; and level 1 proteins are detected in the same number of replicates and controls, with a replicate average PCS higher than the corresponding controls. All other cases are included in level 0, where proteins are not considered to be consistently present in the set of replicates.

Human interactome network
Interactions between detected proteins were retrieved from a human interactome network, built from two data sources: Agile Protein Interactomes DataServer and the Human Reference Protein Interactome Mapping Project (http://interactome.baderlab.org). APID collects physical protein interactions reported in the literature. We used all the interactions available in APID between human proteins. The Human Reference Protein Interactome Mapping Project gives access to interactions detected through unbiased high-throughput pairwise protein interaction experimental detection. Network analyses were conducted in R using functions from the iGraph package. Network visualizations were produced with Cytoscape.

CFTR–EZR–NHERF1 interaction interference score
To measure the capability of individual proteins to interfere with the formation of the interactions between CFTR–EZR–NHERF1, we developed a quantitative score. First, for a given subnetwork containing these three proteins, the number of paths linking two of these proteins through two, three, or four interactions (path length) is computed. For each protein in the network it is determined the fraction of paths of each length that pass through its node. The final score is a weighted sum of these fractions, where fractions associated with higher lengths have lower weights (Equation 1).

\[
\text{Score} = (\% \text{ path length } 2) + 0.5 \times (\% \text{ path length } 3) + 0.25 \times (\% \text{ path length } 4) \quad (\text{Eq. 1})
\]

Validation of hit proteins by co-IP of PM CFTR-associated proteins
CFBE mCherry-FLAG-wt and Phe508del-CFTR cells were incubated at 37 °C for 48 h after Dox-induced CFTR expression (1 μg/ml, Sigma–Aldrich), with mCherry–FLAG–Phe508del-CFTR cells being additionally treated with VX-809 (3 μM, Gentaur). For both conditions, the cells were placed on ice, washed three times with ice-cold PBS-CM, and left for 5 min in cold PBS-CM. The cells were then incubated with soft agitation for 2 h with anti-FLAG M2 Ab (F3165, Sigma–Aldrich) and rinsed three times with ice-cold PBS-CM. No cross-linking agents were used. The cells were then lysed in 500 μl of lysis buffer, and lysates were processed as described under “Co-immunoprecipitation of membrane CFTR-associated complexes.” Captured proteins were analyzed by SDS-PAGE and immunoblotting (see SDS-PAGE and immunoblotting) with the indicated antibodies.

Immunofluorescence and confocal microscopy
CFBE mCherry-FLAG-wt and Phe508del-CFTR cells, grown on coverslips, were induced with Dox (1 μg/ml, Sigma–Aldrich) and treated as indicated. Afterward, the cells were rinsed on ice with cold PBS-CM (PBS, pH 8.0, containing 0.1 mM CaCl2 and 1 mM MgCl2) and incubated with anti-FLAG M2 Ab (F3165, Sigma–Aldrich) and rinsed three times with ice-cold PBS-CM. No cross-linking agents were used. The cells were then fixed with 4% formaldehyde for 15 min, stained with 4',6-diamidino-2-phenylindole (DAPI), and mounted on microscope slides with Vectashield (Vector Laboratories). Images were acquired on a Leica TCS-SPE confocal microscope.

Biotinylation of cell surface proteins
CFBE mCherry-FLAG-wt and Phe508del-CFTR cells were induced with Dox (1 μg/ml, Sigma–Aldrich) and treated as...
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indicated. Afterward, the 0cells were placed on ice, washed three times with ice-cold PBS-CM, and left for 5 min in cold PBS-CM. The cells were then incubated for 45 min with 0.5 mg of EZ-Link Sulfo-NHS-SS-Biotin (Santa Cruz Biotechnology) to label all cell surface proteins. The cells were rinsed twice and then left for 15 min on ice with ice-cold Tris-Q (100 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1 mM CaCl₂, 1 mM MgCl₂, 10 mM glycine, 1% (w/v) BSA) to quench the reaction. The cells were again washed three times with cold PBS-CM and lysed in 250 μl of pulldown (PD) buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1% (v/v) glycerol, 1% (v/v) Nonidet P-40, Protease inhibitor mixture; Sigma–Aldrich). The cell lysates were cleared at 7000 × g for 5 min at 4 °C, and an aliquot of 40 μl, representing input CFTR levels, was removed, whereas 200 μl of lysate were added to 20 μl of G-protein–agarose beads (Roche) to further clear the lysates. These were rotated for 1 h at 4 °C, centrifuged for 1 min at 3100 × g, and recovered. The cleared lysates were added to 20 μl of streptavidin–agarose beads (Sigma–Aldrich), previously incubated for 1 h in 1 ml of cold pulldown buffer containing 2% (w/v) milk, and washed three times in PD buffer. Lysate and beads were rotated for 1 h at 4 °C, centrifuged for 1 min at 3100 × g, and washed five times in cold wash buffer (100 mM Tris-HCl, pH 7.5, 300 mM NaCl, 1% (v/v) Triton X-100). Captured proteins were recovered in 20 μl of sample buffer containing 2% (w/v) DTT and analyzed by SDS-PAGE and immunoblotting, as described above.

Protein thermal destabilization assay (TS assay)

CFBE mCherry–FLAG–Phe508del-CFTR cells were induced with Dox (1 μg/ml, Sigma–Aldrich) and incubated for 48 h at 30 °C with VX-809 (3 μM, Gentaur). These conditions were previously optimized to achieve rPhe508del-CFTR PM levels near to those of wt-CFTR (29). The cells were then transferred to 37 °C for 4 h to desaturate the rPhe508del-CFTR at the PM, as described in Ref. 41. The cells were then placed on ice, washed three times with ice-cold PBS-CM, and left 5 min in cold PBS-CM. The cells were then analyzed by confocal immune fluorescence or biotinylation of surface proteins, as described above.

CFTR functional assay by HS-YFP

CFBE Phe508del-CFTR cells constitutively expressing HS-YFP-F46L/H148Q/I152L (45) were seeded in 8-well chamber slides and treated as described. The cells were then carefully washed twice with isomolar PBS (WPBS; 137 mM NaCl, 2.7 mM KCl, 0.7 mM CaCl₂, 1.1 mM MgCl₂, 1.5 mM KH₂PO₄, 8.1 mM Na₂HPO₄, pH 7.4) and incubated for 30 min with WPBS containing compounds for CFTR stimulation/inhibition (5 μM Fsk, 20 μM Gen, and 25 μM inh172, all from Sigma–Aldrich), as indicated. The slides were then transferred to a Leica TCS-SPE confocal microscope for time-lapse analysis. Each well was assayed individually for iodide influx by recording fluorescence continuously (500 ms/point) for 5 s (baseline) and then for 35 s after the rapid (≤1 s) addition of isomolar PBS in which 137 mM CI⁻ was replaced by I⁻ (final NaI concentration of 100 mM/plate well). The cells were kept at 37 °C up until assay at room temperature. After background subtraction, HS-YFP fluorescence recordings (F) were normalized to the initial average value measured before addition of I⁻ (F₀). Quantification of fluorescence decay was performed on at least 24 individual cells/well, using ImageJ (National Institutes of Health) as previously described (42). The average fluorescence decay was fitted to an exponential decay function to derive the maximal slope that corresponds to initial influx of I⁻ into the cells. Maximal slopes were converted into I⁻ variation rates (in nm/s) using the equation d[I⁻]/dt = K_d [d(F/F₀)/dt], where K_d is the affinity constant of YFP for I⁻ (29).

Statistical analyses

Statistical analysis was performed using GraphPad Prism 5 software. The quantitative results are shown as means ± S.E. of at least three independent experiments. To compare sets of data, we used either one-way ANOVA followed by Tukey’s test (for multiple treatments) or two tailed Student’s t tests (comparison between two treatments) and considered significant differences when p values < 0.05.

Acknowledgment—We acknowledge Dr. P. Haggie (University of California, San Francisco, School of Medicine) for the kind gift of halide-sensitive YFP-F46L/H148Q/I152L plasmid.

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