Regulated recycling of mutant CFTR is partially restored by pharmacological treatment

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Summary

Efficient trafficking of the cystic fibrosis transmembrane conductance regulator (CFTR) to and from the cell surface is essential for maintaining channel density at the plasma membrane (PM) and ensuring proper physiological activity. The most common mutation, F508del, exhibits reduced surface expression and impaired function despite treatment with currently available pharmacological small molecules, called correctors. To gain more detailed insight into whether CFTR enters compartments that allow corrector stabilization in the cell periphery, we investigated the peripheral trafficking itineraries and kinetics of wild type (WT) and F508del in living cells using high-speed fluorescence microscopy together with fluorogen activating protein detection. We directly visualized internalization and accumulation of CFTR WT from the PM to a perinuclear compartment that colocalized with the endosomal recycling compartment (ERC) markers Rab11 and EHD1, reaching steady-state distribution by 25 minutes. Stimulation by protein kinase A (PKA) depleted this intracellular pool and redistributed CFTR channels to the cell surface, elicited by reduced endocytosis and active translocation to the PM. Corrector or temperature rescue of F508del also resulted in targeting to the ERC and exhibited subsequent PKA-stimulated trafficking to the PM. Corrector treatment (24 hours) led to persistent residence of F508del in the ERC, while thermally destabilized F508del was targeted to lysosomal compartments by 3 hours. Acute addition of individual correctors, C4 or C18, acted on peripheral trafficking steps to partially block lysosomal targeting of thermally destabilized F508del. Taken together, corrector treatment redirects F508del trafficking from a degradative pathway to a regulated recycling route, and proteins that mediate this process become potential targets for improving the efficacy of current and future correctors.

Key words: CFTR, F508del, Mutant, Correctors, Trafficking, Recycling, PKA, Fluorescence microscopy, Endosomal recycling compartment

Introduction

Internalization and recycling of transmembrane proteins (e.g. channels and receptors) at the cell surface are highly dynamic cellular processes that facilitate responses to extracellular stimuli, control surface protein density and remove misfolded proteins for degradation (Moore et al., 2007; Okiyoneda et al., 2010). The density of transmembrane proteins at the cell surface is tightly controlled and critical for regulating cellular homeostasis. For instance, G-protein-coupled receptors (GPCRs) are promptly removed from the plasma membrane (PM) upon binding to their extracellular ligands in order to dampen cellular signaling in a process known as receptor desensitization. On the other hand, the epithelial anion channel, cystic fibrosis transmembrane conductance regulator (CFTR), undergoes constitutive rapid endocytosis coupled with highly efficient recycling which is necessary to preserve PM density, given its long half-life of ~16 hours (Gentzsch et al., 2004; Prince et al., 1994; Ramsey et al., 2011; Swiatecka-Urban et al., 2002).

The most common cause of cystic fibrosis (CF), deletion of the phenylalanine residue at position 508 (F508del), is present on at least one allele in 90% of patients and results in a misfolded protein that is prematurely degraded by the proteasome before reaching the cell surface. Low temperature incubation (<30°C for 24 hours) or more recently, treatment with pharmacological small molecules called correctors, can partially rescue CFTR F508del trafficking to the cell surface (Pedemonte et al., 2005; Van Goor et al., 2006). However, despite partial trafficking rescue by corrector treatment or low temperature incubation, rescued CFTR F508del still exhibits severely impaired anion transport function and significantly reduced PM density and recycling efficiency (Cholon et al., 2010; Jurkuvenaite et al., 2010; Sharma et al., 2004).

Currently available correctors have limited efficacy and have proven inadequate for restoring CFTR F508del to acceptable therapeutic levels, estimated to be 25–50% of wild-type (WT) CFTR function. Even treatment with the best available corrector compound, VX-809, reaches 10–15% of the activity of CFTR WT (Clancy et al., 2012). The folding of CFTR F508del is dependent on the conformational stability of nucleotide binding domain 1 (NBD1), the site of the common CF mutation, which then destabilizes inter-domain interactions (Mendoza et al., 2012; Rabeh et al., 2012). For this reason, it is believed that a combination of correctors may be necessary for proper folding and channel function. Consistent with this idea, combinations of correctors have proven the most effective, and they display synergistic actions in promoting the trafficking of mutant CFTR to the cell surface (Holleran et al., 2012). However, the density of F508del at the PM is greatly reduced relative to WT, and severe channel gating defects remain after corrector treatment, which indicates that significant protein folding problems persist. These findings underscore the need to improve existing correctors and continue further development of new efficacious compounds.
Identifying new targets for corrector therapy and methods of improving the efficacy of existing corrector molecules requires a better understanding of how the trafficking behavior of F508del differs from CFTR WT.

From the cell surface, CFTR WT is endocytosed into clathrin-coated pits and routed to early endosome antigen 1 (EEA1) positive early endosomes (Cholon et al., 2010; Gentzsch et al., 2004; Lukacs et al., 1997). At this point, about 50% of CFTR WT is rapidly recycled back to the PM within 5 minutes and CFTR WT which is not immediately redirected to the cell surface accumulates in a slower recycling, compartment near the nucleus, generally referred to as the endosomal recycling compartment (ERC) (Bilan et al., 2008; Gentzsch et al., 2004; Picciano et al., 2003; Sharma et al., 2004; Swiatecka-Urban et al., 2002). This biphasic, rapid and slow trafficking pattern of recycling is shared by the well-studied substrate for clathrin dependent endocytosis, the transferrin receptor (TIR) (Baravalle et al., 2005) [for a review see Mayle et al. (2012)].

While a functional role for CFTR contained within intracellular membranes has not been clarified, several studies have demonstrated the regulated trafficking of CFTR WT in response to protein kinase A (PKA) stimulation. Phosphorylation of the regulatory (R) domain of CFTR by PKA increases whole cell conductance by increasing the channel open probability (Pₒ) as well as promoting delivery of more CFTR channels to the cell surface (Howard et al., 2000; Howard et al., 1996; Lewarchik et al., 2008; Peters et al., 1999; Rudich and Klip, 2003; Schiewert et al., 1994). As with other examples of regulated integral membrane protein trafficking, such as the H₂O channel (aquaporin-2), glucose transporter (GLUT-4), cation channel (TRPC) and the epithelial sodium channel (ENaC), this intracellular pool of CFTR may serve as reservoir to augment PM density in response to physiological stimuli (Agre et al., 1995; Butterworth et al., 2005; Rudich and Klip, 2003; Singh et al., 2004). Studies reporting regulated trafficking of CFTR in response to increased PKA activity have relied on indirect measures of stimulated trafficking such as increased membrane capacitance, exocytosis of fluorescently labeled dextran or static capacitance, exocytosis of fluorescently labeled dextran or static membrane capacitance exocytosis (Okiyoneda et al., 2004; Szent-Gyorgyi et al., 2003; Sharma et al., 2004; Swiatecka-Urban et al., 2002). This biphasic, rapid and slow trafficking pattern of recycling is shared by the well-studied substrate for clathrin dependent endocytosis, the transferrin receptor (TIR) (Baravalle et al., 2005) [for a review see Mayle et al. (2012)].

In the present study, we have applied a recently developed fluorescence detection technology called fluorogen activating proteins (FAPs) to selectively and instantaneously label CFTR at the PM in living cells in order to follow the trafficking itineraries of CFTR WT or F508del endocytosed from the cell surface (Holleran et al., 2010; Holleran et al., 2012; Szent-Gyorgyi et al., 2008). The FAP system offers several advantages over traditional biochemical or immunofluorescence techniques for studying CFTR trafficking in living cells. For instance, binding of the fluorogen is nearly instantaneous, eliminating the need for long incubation steps. Even though fluorogen–FAP complex is non-covalent, the dissociation constant (Kₒ) is extremely low (<1 nM), enabling extended time-lapse experiments (Szent-Gyorgyi et al., 2008). With this methodology, we have quantitatively measured the kinetics of CFTR internalization and PKA-stimulated CFTR translocation using high speed imaging techniques and found that the kinetics of CFTR trafficking to the PM during stimulation correlate closely with functional measurements of CFTR channel activation. We found that the regulated, trafficking-competent pool of CFTR resided in a perinuclear compartment that overlapped closely with recycling endosome markers, Rab11 and Eps15 homology domain 1 (EHD1, the mammalian homolog of the C. elegans protein Rme-1). Thermally destabilized CFTR F508del, which was rescued to the PM by low temperature then shifted to the restrictive temperature (37°C) was expected to traffic to the lysosomes via early endosome sorting based on previous studies (Okiyoneda et al., 2010; Sharma et al., 2004). Interestingly, lysosomal targeting occurred at later time points (between 1 to 3 hours), however, there was detectable accumulation of F508del CFTR in the ERC for up to 60 minutes, which also exhibited PKA-stimulated trafficking to the PM, albeit at reduced rates compared to WT CFTR. Corrector treatment stabilized CFTR F508del internalized from the PM in the ERC, which persisted for at least 3 hours and was responsive to PKA-stimulated regulated trafficking with rates comparable to that of CFTR WT levels. Finally, acute addition of the correctors C4 or C18 but not CFFT-002 were capable of partially blocking lysosomal trafficking of thermally destabilized F508del, supporting a peripheral role for these corrector molecules.

**Results**

**CFTR is endocytosed from the PM and accumulates in a perinuclear compartment**

In order to directly visualize the peripheral trafficking routes of CFTR WT in living cells, we studied internalization from the cell surface using FAP reporters fused to the N-terminus of CFTR (FAP-CFTR) together with confocal fluorescence microscopy. Stable HEK293 cell lines expressing the FAP-CFTR construct, established and validated previously (Holleran et al., 2012), were used to measure the kinetics of internalization. Importantly, these cell lines were found to have characteristic CFTR functional activity and corrector trafficking responses that correlated closely with functional data from polarized human bronchial epithelia (HBE) from CF patients (Holleran et al., 2012). To assess the rates of internalization from the surface as well as the steady-state cellular distribution, FAP-CFTR WT was labeled at the plasma membrane with malachite green containing an 11-unit polyethylene glycol linker (MG-11p), a cell-impermeant fluorogen.

CFTR present at the cell surface was labeled by addition of 50 nM MG-11p fluorogen and immediately after, cells were imaged using 3D time-lapse confocal fluorescence microscopy performed at 37°C and using media gassed with 5% CO₂/95% air. The integrated fluorescence signal across the entire Z-stack of confocal planes was collected and a ratio of intracellular to total cellular signal was determined for each time point. For clarity, a representative single confocal plane from the Z-stack is shown in Fig. 1A. At the outset (t=0), the fluorescence signal was localized exclusively at the plasma membrane. CFTR endocytic rate was reported by the initial linear uptake component (0–10 minutes) (Fig. 1A, control panel). At later times, CFTR recycled back to the PM, as will be shown below. Thus, the intracellular accumulation of CFTR from the cell surface represents a balance between endocytosis and recycling, reaching a steady state at ~25 minutes and representing ~15–20% of the surface labeled pool.

Next, the effect of pre-treatment with the adenylate cyclase agonist forskolin was tested. Cells were exposed to 10 μM M

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forskolin for 5 minutes prior to image acquisition, then confocal microscopy and subsequent image analysis was performed as described above. Both the initial rate of intracellular accumulation (endocytosis) and the steady-state distribution of surface labeled CFTR was shifted to quantitatively lower levels in the presence of forskolin compared to control conditions (Fig. 1A, Forskolin panel). By 25 minutes, both control and forskolin treated cells had reached a steady state of CFTR accumulation, but cAMP/PKA stimulation reduced the level of intracellular signal by 67%.

Intracellular pool of CFTR colocalizes with EHD-1 and Rab11

From the measurements above, it was evident that CFTR trafficked from the PM to a coalesced, central region near the nucleus. Co-staining with tubulin confirmed proximity to the microtubule organizing center (MTOC), whereas appreciable accumulation in the trans-Golgi network could be excluded (supplementary material Fig. S1). Previous reports of CFTR trafficking to recycling compartments, have investigated the behavior of CFTR in response to dominant negative (DN) endosomal protein overexpression or lacked sufficient optical resolution for conclusive compartment identification (Gentzsch et al., 2004; Picciano et al., 2003; Sharma et al., 2004). In this study, we used fluorescence colocalization analysis between FAP-CFTR and yellow fluorescent protein (YFP) tagged proteins, Rab11 and EHD1, which are markers of the endosomal recycling complex (ERC) to directly visualize the subcellular localization of CFTR in living cells (Grant and Caplan, 2008; Grant and Donaldson, 2009; Sönnechsen et al., 2000). We used several endosomal markers and to minimize the potential for mis-targeting due to fluorescent protein tagging as well as other endosomal labeling strategies such as FITC-dextran pulse chase (data not shown).

First, the ERC marker, EHD1-YFP, was transiently expressed in a HEK293 cell line stably expressing FAP-CFTR WT. Cell impermeant fluorogen was added, then quickly removed within 1 minute by three washes with pre-warmed growth media to remove unbound MG-11p. This was followed by incubation for 30, 60 or 180 minutes, which permitted tracking of CFTR from the PM to intracellular compartments. By 30 minutes, there was a detectable pool of CFTR in a juxtanuclear compartment which overlapped closely with the ERC marker, EHD1 (Fig. 2A). This pattern of highly colocalized signal increased slightly over 60-minute and 3-hour incubations and remained for at least 6 hours (data not shown), indicating the stability of CFTR WT in this recycling compartment (Pearson’s colocalization coefficients are provided in the figure legends). Like many Rab GTPases, EHD1 also regulates vesicular trafficking within the cell by facilitating vesicular scission and fusion, but it utilizes ATP preferentially and helps form tubular endosomal structures (Zhang et al., 2012). One of the primary roles of EHD1 is to control cargo movement from the ERC back to the PM, a critical process for maintaining efficient recycling. Indeed, in addition to their perinuclear colocalization, FAP-CFTR WT also colocalized with EHD1 in punctate and tubular structures near the membrane (indicated by arrows), consistent with the role of EHD1 as a recycling protein that facilitates trafficking from the ERC to the PM.

Another ERC marker, Rab11, was also tested in the same manner for internalized CFTR co-localization. Rab5 positive
endosomes deliver cargo from the cell surface to a recycling pathway via fusion with Rab11 positive compartments (Sönichsen et al., 2000). Similar to the pattern observed for EHD1, WT CFTR accumulated in a region close to the nucleus that overlapped with the Rab11 marker at 30, 60 and 180 minutes after labeling (Fig. 2B).

The lysosomes are a well-established destination for misfolded and damaged membrane proteins (Doherty and McMahon, 2009; Okiyoneda et al., 2010; Sharma et al., 2004; Sorkin and von Zastrow, 2009). We investigated many markers of lysosomal structures including fluorescently tagged proteins Lamp1 and Rab7 or fluorescent dyes such as FITC-dextran and Lysotracker (Invitrogen). After thorough testing, Lysotracker Green® was chosen as a suitable indicator of lysosomal compartments when added immediately prior to image acquisition and used at a concentration of 50 nM as others have found (Haggie and Verkman, 2009; Huynh et al., 2007). To test whether CFTR WT traffics to the lysosomes, we pulsed the surface pool with MG-11p, washed excess fluorogen away and followed CFTR trafficking to intracellular compartments in the presence of lysosomal protease inhibitors, leupeptin and pepstatin A (10 μM each), to reduce reporter protein degradation and optimize CFTR detection. In Fig. 2C, it is clear that CFTR WT which is accumulated in the ERC has very little overlap with Lysotracker at both 30 and 60 minutes. Even after 3 hours, the majority of CFTR is distributed at the PM or in the ERC and very little is present in lysosomes (Fig. 2C). This pattern of stable recycling and exclusion from lysosomes was predicted based on the slow turnover rate of CFTR WT, having a half-life of ~16 hours (Gentzsch et al., 2004; Varga et al., 2004).

**Rescued CFTR F508del trafficking pathways from the PM**

The most common genetic mutation that causes cystic fibrosis, F508del, leads to CFTR folding instability and consequently premature disposal by the ER associated degradation (ERAD) machinery, thereby preventing its trafficking to the cell surface. The F508del mutation is temperature sensitive, and incubation at <30°C for 24–48 hours facilitates partial folding, which permits exit from the ER, maturation in the Golgi and subsequent trafficking to the cell surface. Temperature rescue is a valuable research tool, but it is not useful therapeutically. Therefore, recent effort has focused on the discovery of small molecule corrector compounds.
An important advantage of the methods employed here is the ability to dissect individual processing steps using temporally selective labeling strategies. Accordingly, we sought to investigate how the trafficking itinerary of rescued CFTR F508del differs from WT following thermal rescue (27°C for 24 hours) or corrector treatments (C4+C18 or CFFT-002+C18). Once again, we used the fluorescently tagged ERC markers, EHD1 and Rab11, as well as the lysosomal marker Lysotracker Green® to identify intracellular trafficking routes. Based on previous reports, our expectation was that temperature rescued F508del, when shifted to 37°C, would traffic directly to the lysosomes from the PM through early and late endosomal sorting steps. Partial unfolding of the temperature rescued mutant has been documented by limited proteolysis, and this leads to its recognition by the peripheral quality control machinery mediated by the endosomal sorting complex required for transport (ESCRT) pathway (Okiyoneda et al., 2010; Sharma et al., 2004). Following temperature rescue, the same protocol used for WT CFTR, labeling of the cell surface pool followed by internalization was performed for F508del. MG-11p was added for 1 minute, unbound fluorogen was removed by washing, and the cells were incubated for 30, 60 or 180 minutes. To our surprise, we did not see colocalization of F508del with lysosomes at 30 and 60 minutes; rather, we observed accumulation in the ERC as determined by overlap of the mutant protein signal with EHD1 (Fig. 3A). However, after 3 hours at the restrictive temperature, there was extensive overlap of F508del with Lysotracker, indicating recognition of partially unfolded CFTR F508del and its targeting for degradation (Fig. 3D).

Next, we tested the effects of corrector treatment on CFTR trafficking routes and their kinetics. Correctors were added, and cells were incubated for 24 hours to deliver F508del to the PM. As with WT or temperature rescue during the first 30 and 60 minutes, corrector treatment with a combination of C4+C18 or CFFT-002+C18 resulted in the appearance of F508del in a perinuclear compartment which was highly colocalized with EHD1 (Fig. 3B,C). Similarly, colocalization of corrector treated F508del with Rab11 was also observed, as shown in supplementary material Fig. S2. Unlike the thermally destabilized condition, however, the pattern of corrector treated F508del overlap in the ERC persisted for at least 3 hours without significant accumulation in the lysosomes (Fig. 3E,F).

### Acute addition of correctors diverts F508del from lysosomal degradation

Since prolonged incubation with correctors considerably reduced the accumulation of F508del in lysosomes compared to thermal destabilization after 3 hours, we next asked whether acute addition of correctors was sufficient to rescue trafficking of thermally destabilized F508del and prevent accumulation in the lysosomes. CFTR F508del was delivered to the PM by low temperature incubation and pulsed with MG-11p fluorogen, then the correctors C18, C4 or CFFT-002 were added individually and cells were shifted to the restrictive temperature for 3 hours in the presence of leupeptin and pepstatin A. As shown in Fig. 4, acute treatment with C18 or to a lesser extent, C4, redirected F508del away from the lysosomes to a perinuclear compartment. In contrast, CFFT-002, did not prevent trafficking to the lysosomes (Fig. 4A,B). The ability of C18 or C4 to divert F508del from a degradative pathway to a perinuclear pool suggests that these correctors act, at least partially, on the peripheral quality control machinery or alter the properties of CFTR itself in a way that permits it to escape from recognition by peripheral quality control.

### Increased PKA activity depletes the intracellular pool of CFTR

We have shown that elevated levels of cAMP and PKA activity by pre-treatment with forskolin alters the steady state distribution of CFTR, resulting in its reduced accumulation in the ERC (Fig. 1). This effect may be attributed to decreased endocytosis, increased recycling and exocytosis from the intracellular compartment or a combination of these processes. The initial rate of internal compartment labeling (Fig. 1B), was reduced ～60% in response to PKA stimulation, which is consistent with earlier studies using biotinylation or fluorescent fluid phase uptake assays (Bradbury et al., 1992a; Bradbury et al., 1992b; Prince et al., 1994).

Here, we have used FAP based detection of CFTR to directly visualize the regulated trafficking of CFTR in living cells in order to monitor the recycling pathway and capture the dynamics of this process. To do this, we added MG-11p fluorogen to label the cell surface pool of FAP-CFTR and then incubated cells at 37°C for 30 minutes to allow for its steady-state intracellular distribution. Next, cells were imaged using 3D confocal microscopy at 37°C for 30 minutes. Forskolin was added to the cells after 3 minutes and was present throughout the remaining time, whereas control cells were left untreated. A striking difference between forskolin stimulated and control cells was the depletion of the intracellular compartment fluorescence. We quantified the loss of CFTR from this perinuclear compartment, previously determined to be the ERC (Fig. 2A,B) by measuring its integrated fluorescence intensity over time. Shown in Fig. 5 are example images from these time-lapse experiments (Fig. 5A) along with the rates of perinuclear fluorescence loss due to a combination of PKA-stimulated exit of CFTR from this intracellular compartment along with reduced endocytosis from the PM (Fig. 5B,C). The ERC signal of CFTR WT was depleted (55%), starting soon after the addition of PKA agonist.

The vesicular movement of CFTR to and from the ERC is a highly dynamic process. In order to visualize PKA-regulated CFTR trafficking we used high-speed time lapse imaging along with particle tracking which revealed directed movement of CFTR to the cell surface (supplementary material Fig. S3, Movie 1). In addition, we used fluorescence recovery after photobleaching (FRAP) combined with total internal fluorescence microscopy (TIRF) to observe the appearance of CFTR at the PM from intracellular compartments. Forskolin treatment promoted a 40% increase in CFTR at the cell surface and individual CFTR insertion events reflecting stimulation of exocytosis and insertion of CFTR into the PM (supplementary material Fig. S4, Movie 2).

Given that corrector rescued CFTR F508del also traffics initially to the ERC from the PM, we tested to see if this pool was also competent for PKA-stimulated translocation. In both of the corrector combinations tested, depletion of CFTR from the intracellular compartment was observed. The correctors C4+C18 or CFFT-002+C18 resulted in 43% and 48% reduction, respectively, with rates similar to those observed for CFTR WT depletion (Fig. 5B) (no statistical difference was found between corrector combinations). At 30 minutes after thermal destabilization, F508del was excluded from lysosomes and was...
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Fig. 3. See next page for legend.
detected in the ERC. Therefore, we tested to see if F508del retained regulated trafficking properties after 30 minutes of thermal destabilization. CFTR F508del which was shifted from 27°C to 37°C also exhibited depletion of the intracellular compartment upon forskolin treatment, however, its rate of depletion was slow relative to CFTR WT or corrector treated F508del (Fig. 5B). Fig. 5C quantifies this finding by comparing the maximal rates of ERC depletion under these conditions.

**Discussion**

Our goal in this study was to investigate the peripheral trafficking itineraries of CFTR WT and rescued F508del in live cells in order to gain a more detailed and direct understanding of their trafficking routes, kinetics and whether correctors exert their effects on components of this pathway. The rationale for this approach is that trafficking steps that are improved by corrector treatment, and the proteins mediating these steps in recycling, become potential targets for therapeutically stabilizing mutant CFTR at the cell surface. In addition, the developed tools can provide assays for the evaluation of compounds that improve mutant CFTR stability at the cell surface. In particular, we measured the time course of lysosomal targeting. We observed delayed targeting to the lysosomes using instantaneous detection methods. The time course of lysosomal targeting is important for the corrector set examined. Nevertheless, future studies using polarized HBE cells would be useful to determine the fidelity of internalized CFTR trafficking pathways in HBE and their responses to corrector treatment. One of the key findings in this study was our observation that corrector treated or temperature rescued CFTR F508del trafficked to recycling compartments identified by the same ERC markers colocalizing with CFTR WT. Temperature rescue followed by incubation at 37°C induces partial unfolding of F508del, thereby leading to lysosomal degradation via the ubiquitin dependent ESCRT pathway (Okiyoneda et al., 2010; Sharma et al., 2004). Those studies have shown that thermally destabilized F508del is diverted from the recycling route through sorting events that transfer the protein from early endosomes to late endosomes and ultimately to lysosomes for proteolysis. Our data supports this view, but they reveal a window during which degradation of the mutant was delayed.

Interestingly, we observed F508del trafficking to EHD1- and Rab11-positive recycling compartments for at least one hour after the mutant was destabilized by shifting the cells to the higher restrictive temperature. Only after 3 hours was a substantial pool of destabilized F508del was found in lysosomes. Other studies have reported a wide range of times for lysosomal targeting, as early as an hour and up to 6 hours after thermal destabilization (Gentzsch et al., 2004; Okiyoneda et al., 2010; Sharma et al., 2004). Several factors including cell type, detection method and protocol procedures (e.g. long incubations on ice) could influence the time course of lysosomal targeting. We observed delayed targeting to lysosomes using instantaneous detection methods. Using FAP detection, we were able to visualize destabilized F508del trafficking to the ERC for at least 60 minutes which has not been described previously.

In the present study, we investigated how CFTR is redistributed to the cell surface in response to PKA activation by examining the dynamics and characteristics of the recycling competent CFTR pool. This recycling pool of CFTR was depleted upon PKA stimulation through a combination of reduced endocytosis as well as active translocation to the PM which was directly visualized for the first time using high-temporal resolution microscopy and a combination of FRAP and TIRF microscopy. Importantly,
Fig. 4. Acute corrector treatment partially diverts destabilized F508del from lysosomes. (A) Cells expressing F508del were rescued for 24 hours at 27°C. MG-11p was briefly pulsed and washed to label F508del at the surface, then cells were shifted to 37°C. Coincident with the temperature shift, individual correctors were added as well as lysosomal protease inhibitors (leupeptin and pepstatin A) or left untreated as control. After 3 hours at the restrictive temperature, cells were imaged with Lysotracker Green®. Scale bars: 10 μm. (B) Quantification of lysosomal colocalization at 3 hours after acute corrector treatment. Bars represent means±s.e. and are normalized to untreated controls (n=40 cells per condition using cells between five and 20 passages post isolation. *P<0.005, a statistically significant difference from no corrector treatment).
corrector treated F508del was also responsive to PKA-stimulated trafficking, indicating residency in a regulated endosomal compartment. It is interesting to note that the kinetics of CFTR depletion from the ERC in response to PKA activation (8–10 minutes) correlate closely with physiological anion transport rates from short circuit measurements in Ussing chambers using HBE cells and SPQ iodide efflux assays (Holleran et al., 2012; Silvis et al., 2009).

In general, there are two possibilities for the mechanism whereby correctors may permit mutant CFTR to escape cellular quality control at either the central or peripheral compartments: a direct interaction of corrector with CFTR that improves its folding, or an indirect interaction with chaperone pathways that either assist in CFTR folding or, if this does not succeed, interfere with targeting of protein to the ubiquitin–proteasome system for degradation. The pharmacological chaperone model of small molecule rescue proposes that direct binding of correctors to CFTR F508del can stabilize a foldable conformation of the protein that increases the probability that it will obtain the native state. For all correctors tested thus far, there is a lack of direct binding evidence; however, this scenario remains plausible for C18, based on cell type independent activity (Holleran et al., 2012). We found that two processes in the peripheral trafficking pathway were corrected by C18: first, F508del was diverted from lysosomal targeting, and second, F508del retained forskolin-stimulated recycling to the PM.

Importantly, rescue of F508del by correctors partially restored the normal trafficking pattern that returns CFTR to the cell surface from a recycling compartment. Furthermore, this intracellular pool maintained responsiveness to PKA stimulation with rates similar to WT. These data suggest that new therapeutic targets can be found that would optimize mutant CFTR’s stability in the peripheral recycling pathway. In contrast to thermally destabilized F508del, which traffics ultimately to lysosomes for degradation, our findings show that corrector treatment can result in the long lasting association of mutant CFTR with a recycling pathway that traverses the ERC.

Therefore, modification of existing correctors or development of new drugs that improve the efficiency of the recycling pathway from the ERC to the cell surface could greatly enhance the stability of ER corrected CFTR F508del in the PM and the overall therapeutic benefit.

Fig. 5. PKA-regulated trafficking of CFTR. CFTR WT was labeled at the cell surface and allowed to reach steady-state distribution for 30 minutes. (A) Example image montage for CFTR WT shown with forskolin treatment that promoted depletion of signal from the perinuclear compartment and was absent under control conditions. Scale bars: 10 μm. (B) Rates of perinuclear depletion of CFTR WT or rescued F508del with correctors (C4 + C18, CFFT-002 + C18) or thermal destabilization (27°C followed by 37°C) for 30 minutes. n=30 cells per condition using cells between five and 20 passages post isolation. (C) Maximum slope of PKA response for CFTR WT and rescued F508del. (*P<0.005, a statistically significant difference from WT.)
Materials and Methods

FAP-CFTR cell lines

FAP fusions to the N-terminus of CFTR WT or F508del with an additional membrane spanning segment were generated using the pBabeSacLac2 plasmid and used to generate stable cell lines in HEK 293 cells, described previously (Holleran et al., 2010; Holleran et al., 2012). Cells were grown in high glucose DMEM media containing sodium bicarbonate (Invitrogen part no. 10569-010) and supplemented with 10% FBS. The FAP-CFTR expressing cell lines were enriched for FAP expression using MG activation with fluorescence activated cell sorting on a BD FACS Diva. Cell lines were used for less than 15 passages before returning to a cryopreserved stock of FACS enriched population to maintain consistent FAP-CFTR expression levels. The passage number used for each

Fig. 6. Mutant EHD1 and Rab11 inhibit PKA-stimulated CFTR trafficking. Dominant negative (DN) YFP-tagged (A) Rab 11-N22S or (B) EHD1-K72A were transiently transfected into cells that stably express FAP-CFTR WT. FAP-CFTR WT was labeled at the cell surface with 50 nM MG-11p and allowed to reach a steady state of internal labeling for 30 minutes. CFTR accumulated strongly in both DN Rab11- and EHD1-positive compartments. PKA stimulation by forskolin (Fsk) treatment for 30 minutes did not induce depletion of CFTR in these compartments. Scale bars: 10 μm (A,B). (C) Quantification of perinuclear fluorescence over 30 minutes with forskolin stimulation (n=10 cells per condition using cells between five and 20 passages after isolation; the graph represents means±s.e.m.).
experiment was between 5 and 20 following FACS isolation, population expansion and cryo-preservation in liquid nitrogen.

Microscope image acquisition

Images were acquired with NIS-Elements software using a Nikon Ti eclipse confocal microscope with Photometrics Evolve EM CCD camera using 488 nm laser excitation with 520/50 nm emission for YFP or 640 nm laser excitation and 700/75 nm emission filter for MG. 60X (1.49NA) objectives were used to image at 37°C with DMEM + 10% FBS media with 5% CO2,95% air. Cells were plated on Mattek poly-lysine-coated dishes (part no. p35G-1.5-14-C). Scale bars representing 10 μm are indicated on each image.

Internalization assay

HEK 293 cells stably expressing the FAP-CFTR fusion were grown DMEM media with 10% FBS and plated on Mattek poly-lysine-coated dishes at ~50–60% confluency. Five minutes prior to imaging, cells were either treated with 10 μM forskolin or left as untreated controls. At T=0 minutes, the cell impermeant fluorogen, MG-11p (50 nM), was added to the cells. Time lapse confocal Z-stacks were taken at multiple stage positions every 5 minutes for 1 hour. Quantification of internalized signal was carried out using the Nikon NIS Elements ROI tracking software. CFTR which had internalized to intracellular compartments from the PM were quantified by integrating the total perinuclear fluorescence signal. The percent of internalized signal was calculated by dividing the integrated MG fluorescence intensity by the total integrated MG fluorescence per cell. A single confocal plane is shown as a montage for clarity, however, the entire Z-stack was measured for analysis.

Colocalization microscopy

Plasmids containing fluorescent protein fusions to intracellular markers were transiently transfected into the HEK293 cells which stably express the FAP-CFTR WT or F508del constructs. The YFP-EHDI and YFP-Rab11a and DN mutants of these proteins (K72A and N22S, respectively) were kindly provided by Dr Alex Sorkin (University of Pittsburgh School of Medicine). Each fluorescently tagged construct was transfected 48 hours prior to imaging using the Lipofectamine LTX reagent according to manufacturer’s instructions. Lysoosomes were labeled using Lysotracker Green® (Invitrogen) by adding 50 nM, 5 minutes prior to image acquisition. The surface pool of CFTR was labeled by addition of MG-11p followed immediately by three washes with pre-warmed growth media (DMEM + 10% FBS). Cells were then re-incubated for 30 minutes, 60 minutes or 3 hours. Colocalization was quantified using the Colocalization Threshold plugin for ImageJ to determine Pearson’s coefficient (R) and data are presented as the mean ± s.e.m.

 Trafficking of rescued CFTR F508del

Rescue of FAP-CFTR F508del was performed either by low temperature (23°C for 24 hours) or by treatment with correctors C4 (10 μM), C18 (5 μM), CFF7-002 (5 μM) used either individually or in combination for 24 hours. Vehicle treated controls received the maximum corrector volume of DMSO. HEK 293 cells expressing FAP-CFTR F508del were plated on poly-lysine-coated Mattek dishes 12–24 hours before rescue such that cells were ~50–60% confluent on the day of rescue treatment. Temperature rescued CFTR F508del cells were shifted to 37°C for 30 minutes, 60 minutes or 3 hours after MG-11p labeling and washing. Corrector treated cells were maintained at 37°C in the presence of correctors for the duration of the experiment.

Acute corrector treatment

Temperature rescued HEK293 cells expressing FAP-CFTR F508del were incubated at 27°C for 24 hours pulsed with 50 nM MG-11p and washed three times to remove unbound fluorogen. Individual correctors, C4 (10 μM), C18 (5 μM) or CFF7-002 (5 μM) were added and cells were shifted to 37°C for 3 hours. Lysotracker Green® was added 5 minutes prior to imaging to visualize lysosomal compartments. Overlap with lysosomal compartments was quantified by measuring the integrated CFTR F508del fluorescence signal coincident with Lysotracker Green® over the total CFTR signal using Metamorph software (Molecular Devices). Colocalization values were normalized to the untreated control.

Particle tracking

HEK293 cells stably expressing the FAP-CFTR WT construct were incubated in the presence of cell impermeant fluorogen for 25 minutes at 37°C. Time-lapse confocal fluorescence microscopy was performed at 37°C in a stage incubation chamber by capturing Z-stacks containing seven 1 μm slices every 5 seconds for 30 minutes. Image analysis was carried out with Imaris® particle tracking software by identifying particles by size and intensity. CFTR signals between 300 and 700 nm in diameter were classified as particles and tracked over time. The time-lapse movie and image montage was generated from a maximum intensity projection of the 3D confocal stack.

PKA-regulated trafficking

Steady-state distribution of CFTR was established by incubating HEK cells in the presence of MG-11p for 30 minutes. Imaging was performed by acquiring Z-stacks every 3 minutes for 30 minutes. After 3 minutes, cells were either treated with 10 μM forskolin or left untreated as control. Quantification of response was calculated by measuring the integrated perinuclear fluorescence over time, normalized to the T=0 minutes value then the control cells for each condition. The maximum slope of the forskolin stimulated response was calculated using SigmaPlot®.

TIRF FRAP experiments were carried out by incubation of HEK FAP-CFTR WT cells in MG-11p for 30 minutes followed by three washes with growth media (DMEM + 10% FBS) to remove unbound fluorogen. Imaging was performed using TIRF illumination to photobleach the surface of cells nearest to the cover glass with 647 nm excitation at 100% laser power from a 50 mW solid state source (Agilent Monolithic Laser Combiner) for 10–15 seconds. Next, cells were either treated with 10 μM forskolin or left untreated as control and TIRF images were acquired every 2 minutes for 12 minutes. The amount of CFTR at the cell surface was quantified by measuring the total integrated fluorescence normalized to T=0 minutes. Detection of exocytic event ‘puffs’ (Yudowsky et al., 2009) was performed the same as for the TIRF FRAP experiments; however, TIRF images were acquired every 2 seconds for 7 minutes following forskolin treatment.

Statistics

Statistical analysis was carried out using two-tailed t-tests performed using SigmaPlot® software. P values <0.05 are indicated by *. PKA responses were quantified by measuring the maximum slope of perinuclear signal depletion over time using SigmaPlot® software.

Reagents

Corrector compounds, C4, CFF7-002 and C18 were obtained from the CFF7 panel library (www.cftrfolding.org). The fluorogen MG-11p was provided by the Molecular Biosensor and Imaging Center (MBIC) reagent chemistry group (Carnegie Mellon University). Lysotracker Green® and Lipofectamine LTX were obtained from Invitrogen. The lysosomal protease inhibitors leupeptin and pepstatin A were obtained from Sigma.

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Author contributions

J.P.H. performed the research, analyzed data and contributed to writing the paper; J.Z. performed the research; R.A.F. and S.C.W. designed the research, analyzed data and contributed to writing the paper.

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References


Fig. S1. CFTR is internalized near the MTOC but not to the Golgi. HEK293 cells stably expressing FAP-CFTR WT were transfected with GFP tagged tubulin or Golgi marker, then CFTR was labeled at the cell surface with MG-11p for 30 minutes. (A) CFTR accumulates in a perinuclear compartment near the MTOC, visualized by the bright punctate signal of GFP labeled tubulin. (B) CFTR was largely excluded from the Golgi, indicated by detection of the Human Golgi-resident enzyme N-acetylgalactosaminyltransferase 2. Number of cells analyzed was n>10 using cells between 5-20 passages following isolation. Scale bars indicate 10 µm.
Fig. S2. Corrector-rescued CFTR F508del accumulates in a Rab11-positive compartment. Cells expressing CFTR F508del were treated with a combination of (A) C4 + C18 or (B) CFFT-002 + C18 for 24 hours. CFTR F508del was labeled at the cell surface, then excess fluorogen was washed. Movement of CFTR F508del was followed for 30, 60 minutes or 3 hours. Close colocalization with the ERC marker, Rab11, was observed for at least 3 hours. For each condition, n ≥ 10 cells from separate experiments using cells between 5-20 passages following isolation. Scale bars indicate 10 µm.
Fig. S3. CFTR translocates to the cell surface after PKA stimulation. CFTR WT translocation detection by live cell, high-speed time-lapse confocal microscopy. HEK 293 cells stably expressing FAP-CFTR WT were incubated with MG-11p for 25 minutes and subsequently stimulated with 10µM Forskolin. Confocal Z-stacks were acquired every 5 seconds for a period of 7 minutes. (A) Image montage from the time-lapse movie (Supplemental Movie M1). Vesicular trafficking from the ERC to the PM is indicated by the white arrows. (B) Particle tracking paths of CFTR WT upon PKA stimulation over. Scale bar indicates 10µm.
**Fig. S4. TIRF FRAP detection of PKA-induced CFTR translocation.** Cells were labeled with MG-11p for 30 minutes, then excess fluorogen was removed by three washes. The bottom surface of cells were photobleached using high intensity 640nm TIRF laser illumination for ~15s. (A) PKA stimulation by forskolin for 12 minutes resulted in increased fluorescence relative to control cells quantified in (B). Scale bar indicates 10µm. (C) High-speed TIRF image montage (from supplemental movie M2) of PKA stimulated exocytosis revealed the insertion of CFTR identified by characteristic appearance of bright structures followed by lateral diffusion into the membrane after vesicle docking and fusion, or so called “puffs”. Scale bar indicates 0.25µm.
**Movie 1.** Time-lapse movie of CFTR translocation in response to PKA stimulation from HEK 293 cells stably expressing FAP-CFTR WT were incubated with MG-11p for 25 minutes at 37°C and subsequently stimulated with 10µM Forskolin. High speed 3D confocal imaging was performed to detect FAP-CFTR translocation in response to PKA activation by maximizing temporal resolution while minimizing photobleaching. Vesicular trafficking from the ERC towards the PM described in Fig. 7 occurs over the course of ~7min. Confocal optical Z-sections were acquired every 5 seconds for a period of 7 minutes. Scale bar indicates 10 µm.

**Movie 2.** High-speed time-lapse TIRF movie of PKA stimulated CFTR exocytosis. Cells were labeled with MG-11p for 30 minutes, then excess fluorogen was removed by three washes. The bottom surface of cells were photobleached using high intensity 640nm TIRF laser illumination for ~15s. High-speed TIRF image of PKA stimulated exocytosis revealed the insertion of CFTR identified by characteristic appearance of bright structures followed by lateral diffusion into the membrane after vesicle docking and fusion, or so called “puffs”. Left panel shows a TIRF time lapse image series with expanded pseudocolored views of characteristic puff insertion events on the right to indicate signal intensity. Time is indicated in min:sec. Scale bar indicates 0.25 µm.