

Knockdown of NHERF1 Enhances Degradation of Temperature Rescued $\Delta F508$ CFTR from the Cell Surface of Human Airway Cells

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Key Words

$\Delta F508$ CFTR • Temperature correction • NHERF1 • Plasma membrane

a role in the turnover of CFTR at the cell surface, and that r $\Delta F508$ CFTR at the cell surface remains highly susceptible to degradation.

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Abstract

$\Delta F508$ CFTR can be functionally restored in the plasma membrane by exposure of the cell to lower temperature. However, restored $\Delta F508$ CFTR has a much shorter half-life than normal. We studied whether NHERF1, which binds to the PDZ motif of CFTR, might be a critical mediator in the turnover of $\Delta F508$ CFTR from the cell surface. We used RNAi to reduce the expression of NHERF1 in human airway epithelial cells. Knockdown of NHERF1 reversibly reduces surface expression of WT-CFTR without altering its total expression. As expected, temperature correction increased mature C band $\Delta F508$ CFTR (r $\Delta F508$) but unexpectedly allowed immature B band of r $\Delta F508$ to traffic to the cell surface. Both surface and total expression of r $\Delta F508$ in NHERF1 knockdown cells were reduced and degradation of surface localized r $\Delta F508$ was even faster in NHERF1 knockdown cells. Proteasomal and lysosomal inhibitor treatments led to a significant decrease in the accelerated degradation of surface r $\Delta F508$ in NHERF1 knockdown cells. These results indicate that NHERF1 plays

Introduction

Newly synthesized membrane proteins destined for the cell surface undergo multiple assessments by various quality control systems to check for correct folding and/or assembly [1]. Quite often misfolded membrane proteins are trapped at the ER, finally leading to ER-associated degradation (ERAD) by the proteasome [2]. Misfolded proteins that escape from the ERAD can further traffic to the cell surface, but often have shortened residence times at their final subcellular destinations [3]. The short half-life of misfolded proteins at the cell surface is often linked to their accelerated degradation [3, 4], thus adding another level of quality control beyond the ER. Although much progress has been made in understanding the quality control pathway of misfolded proteins at the ER, the molecular fates of these misfolded proteins at the cell surface is still largely unknown.

Cystic Fibrosis (CF) is an autosomal recessive disease primarily caused by the single deletion of residue 508 in the cystic fibrosis transmembrane conductance regulator (CFTR). The processing of Δ F508 CFTR is arrested in the ER, where it is targeted for degradation through the ERAD pathways [5, 6]. However, the processing of CFTR is a temperature-sensitive process. At a reduced temperature, CFTR bypasses the quality control mechanisms in the ER that targets it for degradation, and it successfully traffics to the cell surface [7]. Unfortunately, once the rescued Δ F508 CFTR reaches the cell surface, it is rapidly endocytosed and degraded. The consequence is a much shorter half-life [8]. It is not clear how surface localized Δ F508 CFTR is degraded subsequent to its reaching the cell surface.

Once CFTR arrives at the plasma membrane, it binds to associated proteins that help it function efficiently [9-11]. These associated proteins include NHERF1 (sodium hydrogen exchange regulatory factor 1) [12;13], NHERF2 [12], CAL (CFTR-associated ligand) [14], and CAP 70 (CFTR associated protein) [10]. All bind to the type I PDZ binding motif of CFTR. Although the precise role of the PDZ binding motif of CFTR is still debated [see [15] for a review], it has been recently shown to be required for membrane retention of WT-CFTR [16-18]. Thus when the PDZ motif is deleted, the half-life of WT-CFTR at the cell surface is reduced [17]. However, the contribution of PDZ proteins to the accelerated turnover of Δ F508 CFTR is not known. We hypothesized that NHERF1 may contribute to this process, and have investigated the role of NHERF1 in regulating the turnover of Δ F508 CFTR at the cell surface of human airway cells.

Materials and Methods

Cell culture and inhibitor treatments

CFBE cell lines (CFBE41o-WT and CFBE41o- Δ F508), stably expressing either wild-type or Δ F508 CFTR, were cultured as described [19]. To avoid clonal drift associated with CFTR expression, new batches of original cells were used during the course of the experiment. The plasmids encoding NHERF1 shRNA and control shRNA (Ambion) were transfected into CFBE cell lines using lipofectamine 2000 (Invitrogen) according to the manufacturer's instruction. Five to ten clones were pooled per transfection and maintained with 75 μ g/ml hygromycin (Roche). Protein translation inhibitor cycloheximide (88 μ M; Sigma), and proteasome inhibitor MG-132 (10 μ M; CalBiochem) or membrane-permeable endolysosomal inhibitor E-64d (75 μ M; Calbiochem) were added to CFBE cells to a final solvent concentration of 0.1% DMSO (Sigma).

Generation of construct

The plasmid expressing human NHERF1 was kindly provided by B. Cha and M. Donowitz, Johns Hopkins University, School of Medicine [20]. The target sequence against human NHERF1 cDNA (NM_004252) were designed at the Ambion website (www.ambion.com) and aligned by BLAST to avoid off-target match. The construct for shRNA was generated by annealing and ligating synthetic oligonucleotides into the pSilencer hygro vector (Ambion) at BamHI/HindIII sites. The sequences of the oligonucleotides for the NHERF1 knockdown RNAi were:

GAT CCG TGAATG TGT TCC CGT CCT TCA AGA GAG
GAC GGG AAC ACA TTC ACC TTT TTT GGAA AA and
AGC TTT TCC AAA AAA GGT GAA TGT GTT CCC
GTC CTC TCT TGA AGG ACG GGA ACA CAT TCA CCG.

The underlined nucleotides indicate the selected target sequence for the NHERF1 RNAi experiment.

Cell surface biotinylation analysis

CFBE cells were grown to confluence in 10 cm dishes. The cells were incubated with 0.5 mg/ml of a membrane impermeable biotin, EZ-link sulfo-NHS-SS-biotin (Pierce) at 4°C for 40 min, extensively washed, and then quenched with 20 mM Tris, pH 7.4/120 mM NaCl/1.8 mM CaCl₂ for 50 min. The sample was then lysed on ice for 30 min in lysis buffer (50 mM HEPES, pH7.5/1% NP-40/150 mM NaCl/10 mM NaMoO₄/COMPLETE protease inhibitors (Roche)). After centrifugation at 15,700 x g, biotinylated surface proteins were retrieved with Ultralink immobilized Neutravidin beads (Pierce) and eluted with 2X SDS-PAGE sample buffer. For tracking cell surface CFTR, membrane impermeable biotin-pulsed cells were incubated with growth medium at either 37°C or 26-27°C. The indicated inhibitors were added in the growth medium before cell lysis as required.

Vesicle fractionation in iodixanol gradients

Cell monolayers were homogenized in 20 mM HEPES, pH 7.4/150 mM NaCl/1 mM Na₃VO₄/50 mM NaF/COMPLETE protease inhibitors (2 vials per 50 ml). The post-nuclear supernatants were then ultracentrifuged at 140,000 x g, and the resulting pellets resuspended with the homogenization buffer and further fractionated over an 11 step optiprep gradient by centrifuging at 202,000 x g for 17 hr in SW40Ti rotor. The gradient was composed of a phase of 28% and 10 phases of 28% to 10% with 2% step gradients. Each step was 1 ml. Fractions of 720 μ l were routinely collected with fraction collector Model 2110 (Bio-Rad). One-tenth of each fraction was taken for SDS-PAGE and immunoblotting.

In gel deglycosylation

Biotin-labeled proteins bound to immobilized neutravidin beads were washed with the lysis buffer and incubated with either endoglycosidase H (EndoH) or peptide: N-Glycosidase F (PNG-F) buffer (New England BioLabs). The beads were then resuspended in 87.5 μ l of the endoglycosidase buffers, and 1,750 units of EndoH or PNG-F was added, respectively. The samples were incubated at 37°C for 2 hr, washed with the lysis

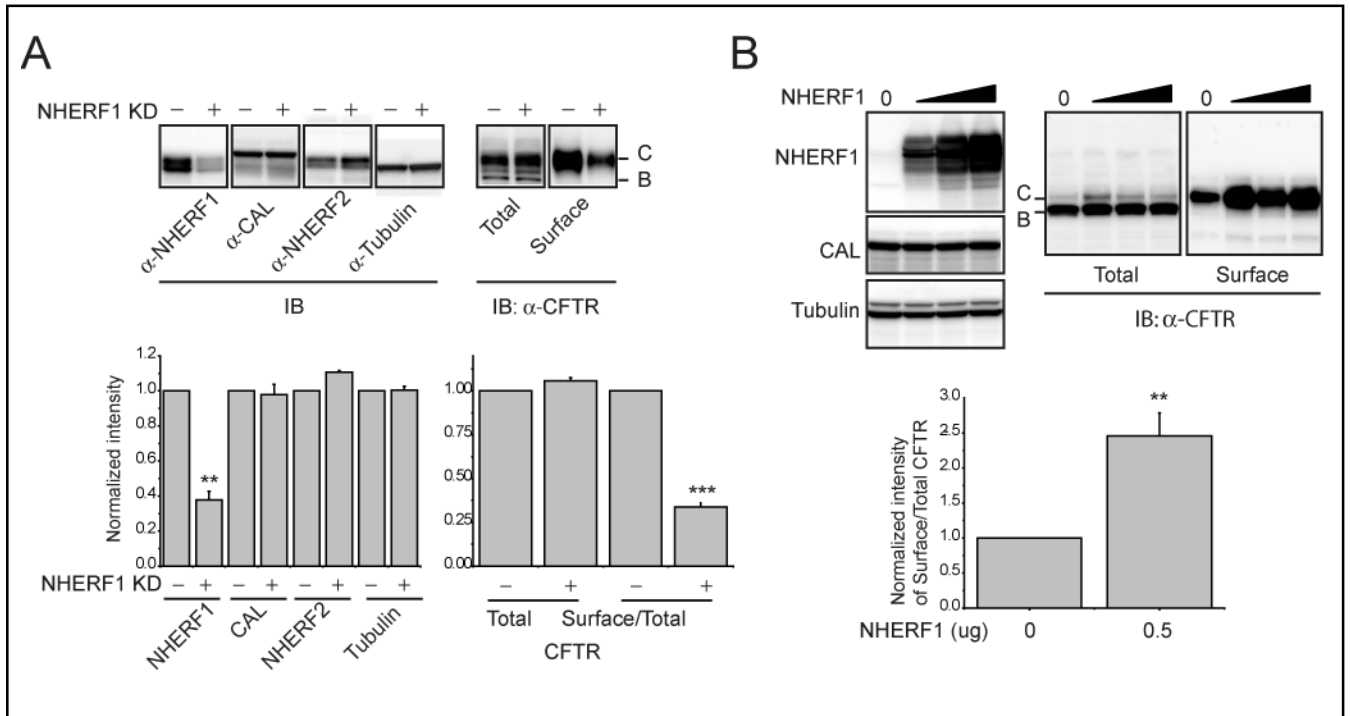


Fig. 1. NHERF1 knockdown (KD) reduces surface expression of WT-CFTR. NHERF1 added exogenously restores the surface expression of WT-CFTR in NHERF1 knockdown cells. **A.** Efficacy and specificity of NHERF1 knockdown. CFBE41o-WT CFTR cells were stably transfected with shRNA constructs directed against NHERF1 or control and were immunoblotted for the indicated endogenous proteins. Cell surface proteins of CFBE41o-WT CFTR were biotinylated, pulled down with neutravidin, and immunoblotted with CFTR antibodies (24-1). + indicates NHERF1 shRNA expression; - indicates control shRNA expression. The relative ratio of surface expression to total expression was calculated as (pixel intensity of surface)/ (pixel intensity of total) and normalized to the value calculated for the control construct. Data are presented as mean \pm std. dev. ($n=3$). **B.** Restoration of surface expression defect in NHERF1 knockdown cells by transfected human NHERF1 cDNA. CFBE41o-WT CFTR cells expressing NHERF1 shRNA were transfected with NHERF1 construct (0 μ g, 0.5 μ g, 1 μ g, and 2 μ g of NHERF1 plasmid), cell surface biotinylated as in **A**. Data are presented as means of \pm std. dev. ($n=4$). Note that the CFBE41o-WT CFTR cells contain in their genomes two endogenous copies of the Δ F508 CFTR gene in addition to the WT-CFTR transgene.

buffer, and eluted with 2X SDS-PAGE sample buffer at 37°C for 30 min.

Immunoprecipitation and Immunoblotting

CFBE cell monolayers in a 10 cm dish were lysed on ice for 30 min in 2 ml of the lysis buffer containing 2 mM iodoacetamide. The lysates were cleared by centrifugation at 14,000 \times g. CFTR in the cleared lysates was immunoprecipitated by a mixture of two anti-CFTR antibodies: 13-1 (R&D systems) and M3A7 (Upstate). Immobilized protein G beads (Amersham) were added and incubated at 4°C overnight. The beads were then extensively washed in the lysis buffer, and bound proteins were eluted with 2X SDS-PAGE sample buffer at 37°C for 30 min.

For immunoblotting, cells grown in one 10cm dish were lysed in 600 μ l of the lysis buffer and incubated on ice for 30 min. The samples were then cleared by centrifugation at 14,000 \times g for 15 min at 4°C. Protein concentration was measured by

the BCA protein assay (Pierce), and 70 μ g of the resulting cleared lysates were separated by SDS-PAGE, and transferred electrophoretically to PVDF. The blots were probed with anti-CFTR antibody 24-1 (1:2000; R&D systems), anti-NHERF1 antibody [20] (1:3000; α -5199), anti-NHERF2 antibody [20] (1:3000; α -2570), anti-CAL antibody [14] (1:2000), anti-GM130 antibody (1:1000; BD biosciences), anti-aminopeptidase N antibody [21] (1:2000), and anti-actin antibody C4 (1:3000; Chemicon). All gels for SDS-PAGE were 4-15% gradient polyacrylamide, except in Figure 1B, where they were 7.5%. Unsaturated signals on the blot were captured by LAS-1000 Plus (Fujifilm) and band intensities were quantified using Image Guage (Fujifilm).

Statistical Analysis

Statistical significance was determined by Student's *t* test. We assigned significance at * as $p < 0.05$, ** as $0.005 < p < 0.05$, and *** as $p < 0.005$.

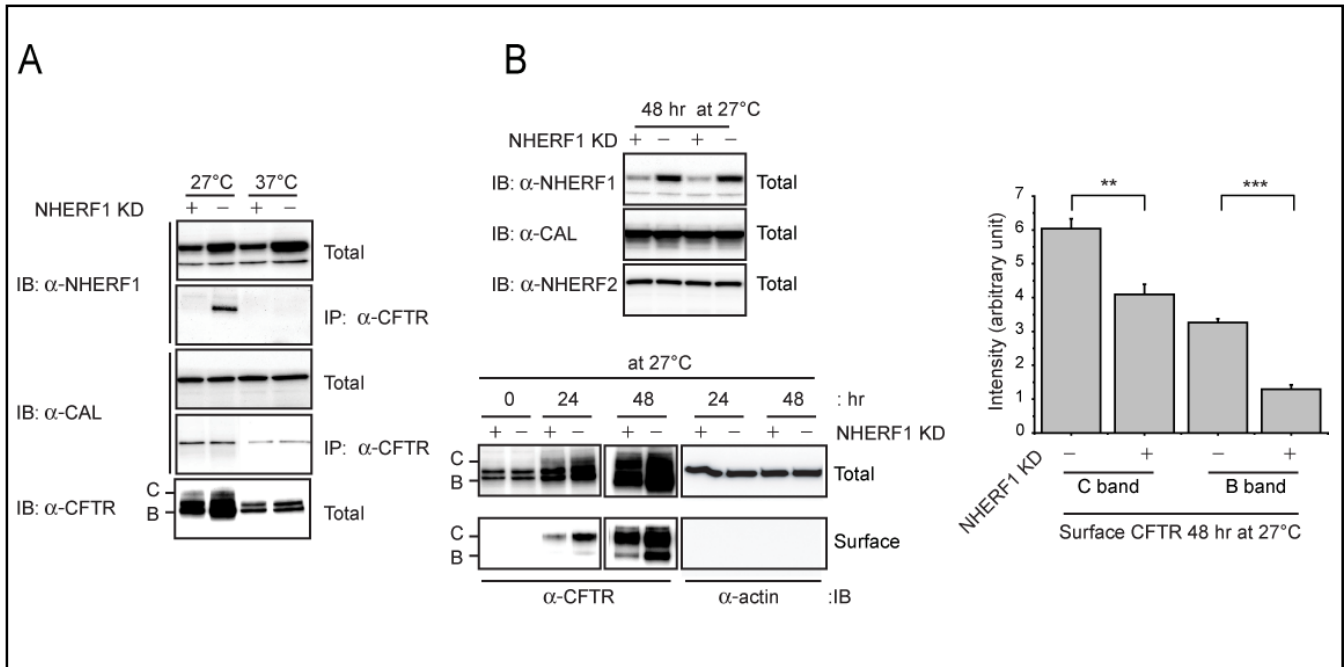


Fig. 2. NHERF1 is associated with rescued $\Delta F508$ CFTR in the post-ER compartments. NHERF1 knockdown decreases the surface expression of r $\Delta F508$ CFTR due to its reduced total expression. **A.** Interaction of endogenous NHERF1 and $\Delta F508$ CFTR in post ER compartments. Cleared lysates from CFBE41o- $\Delta F508$ CFTR cells expressing control or NHERF1 shRNA incubated at 37°C or 27°C for 24 hr, as indicated, were immunoprecipitated with CFTR antibodies (M3A7). Cleared lysates and immunoprecipitates were immunoblotted for NHERF1, CAL, and CFTR. NHERF1 coimmunoprecipitates with $\Delta F508$ CFTR from the cells grown at 27°C, but not from the cells grown at 37°C. **B.** Altered expression of $\Delta F508$ CFTR in the NHERF1 knockdown cells. Surface proteins of CFBE41o- $\Delta F508$ CFTR cells grown at the indicated temperature and incubation hours. Cells were biotinylated and the biotin pulled down with neutravidin beads. Aliquots from the cleared lysates and the pull-downed fractions were immunoblotted for the indicated endogenous proteins. Immunoblots for actin were included in experiments to assess potential permeabilization and incomplete quenching of membrane impermeable biotin. Band intensities of $\Delta F508$ CFTR rescued for 48 hours in the surface fraction were quantified. Data are presented as means of \pm std. dev. ($n = 3$). Symbols (+/-) as described in Fig. 1.

Results

NHERF1 RNAi reduces surface expression of wild type CFTR in the human airway cells

To study NHERF1, we established the human bronchial epithelial cell lines (CFBE41o-WT and CFBE41o- $\Delta F508$) stably expressing a small interfering RNA hairpin directed against 3' untranslated region (UTR) of NHERF1. As determined by western analysis, the level of endogenous NHERF1 protein was selectively reduced by 62.2% in CFBE41o-WT and 66.6% in CFBE41o- $\Delta F508$ cells, compared to cells expressing control shRNA (Fig. 1A and Fig. 2B). In contrast, the level of NHERF2 and CAL was not significantly affected by the NHERF1 RNAi. Importantly, the reduction of NHERF1 expression led to a pronounced reduction of WT-CFTR at the cell surface without affecting its overall expression (Fig. 1A).

Because our shRNA was specifically designed to target the 3' UTR, we could complement this effect by transfecting NHERF1 cDNA with no 3' UTR into the NHERF1 knockdown cells. Overexpression of NHERF1 dramatically reversed the reduction in surface expression of WT-CFTR (Fig. 1B). These results suggest that NHERF1 plays a positive role in regulating the surface expression of WT-CFTR.

Rescued $\Delta F508$ CFTR associates with NHERF1, and NHERF1 knockdown reduces total and surface expression of rescued $\Delta F508$ CFTR

To explore the role of NHERF1 in the surface expression of temperature corrected $\Delta F508$ CFTR, co-immunoprecipitation experiments were performed in the CFBE41o- $\Delta F508$ cells to determine if $\Delta F508$ CFTR binds to NHERF1 or CAL. As shown in Fig. 2A, temperature

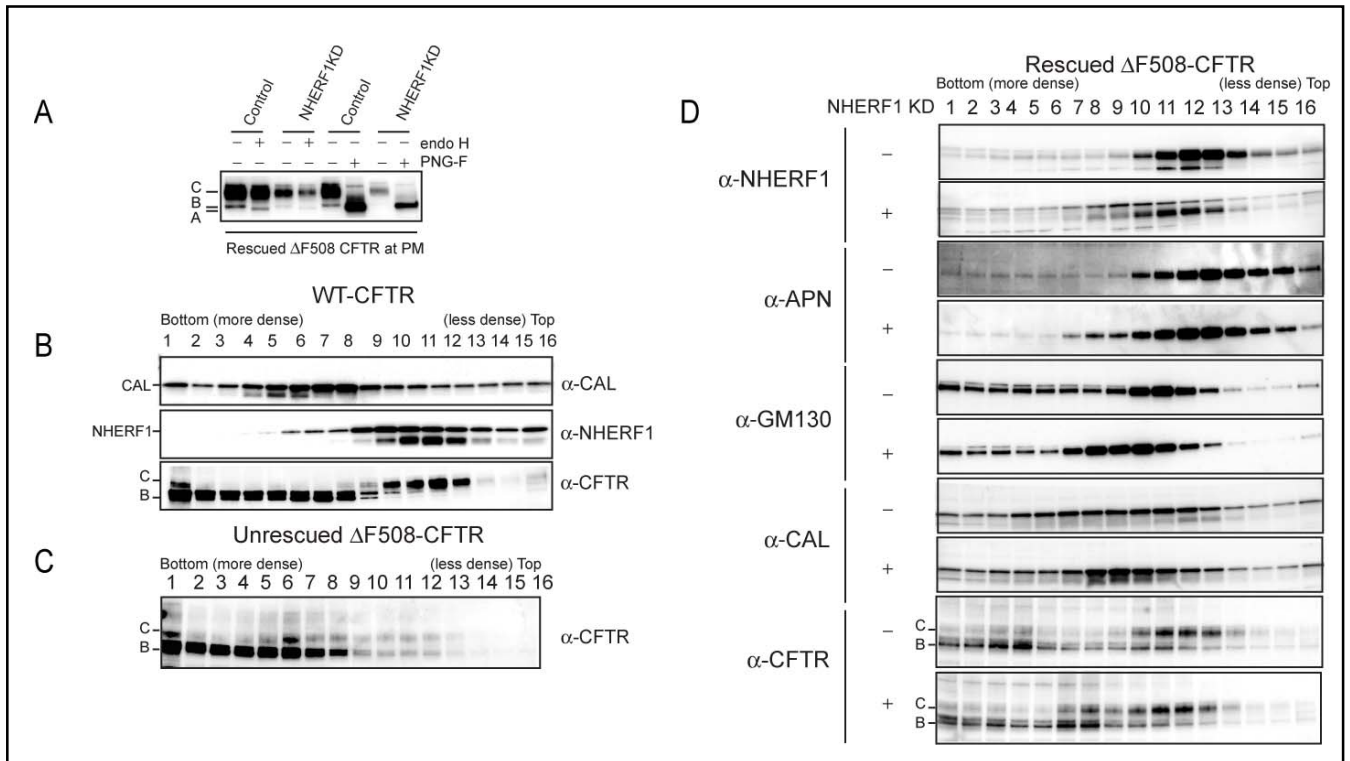


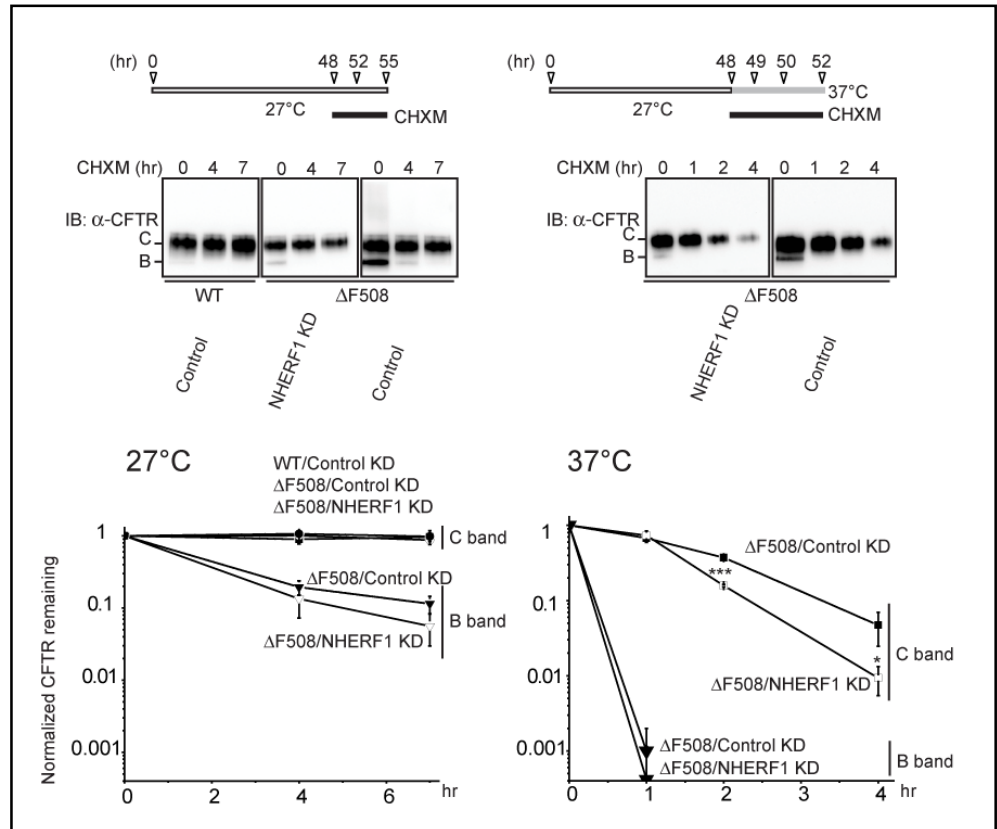
Fig. 3. Temperature rescue mis-inserts immature $\Delta F508$ CFTR at the cell surface of human bronchial epithelial cells. A. Biotinylated cell surface proteins of CFBE41o- $\Delta F508$ CFTR cells expressing control or NHERF1 shRNA (NHERF1 KD) grown at 27°C for 24 hr were collected with Neutravidin beads and were digested by endoglycosidase H (EndoH) or peptide: N-Glycosidase F (PNG-F). After endoglycosylation, the bound proteins were immunoblotted for CFTR. The data shown are representative of three experiments. Fractionation of NHERF1, CAL, and CFTR from CFBE41o-WT CFTR cells grown at 27°C for 48 hr (B), CFBE41o- $\Delta F508$ CFTR cells grown at 37°C (C; unrescued) or CFBE41o- $\Delta F508$ CFTR cells expressing control or NHERF1 shRNA grown at 27°C for 48 hr (D; rescued). Post-nuclear supernatants from the indicated cell homogenates were centrifuged at 80,000 x g. Pellets were resuspended in lysis buffer, overlaid in equal volume of 11 step Opti-Prep gradient (28%, and 28-10%, each with 2% step size), and centrifuged at 202,000 x g for 17 hr. Fractions were assessed for NHERF1, aminopeptidase N, GM130, CAL, and CFTR by immunoblotting. Note that aminopeptidase N, a plasma membrane protein and GM130, a Golgi protein were separately enriched in this gradient. NHERF1 was cofractionated with aminopeptidase N whereas CAL was mainly enriched in GM130 fractions. Due to the reduced amount of cellular expression of $\Delta F508$ CFTR in the NHERF1 knockdown cells, more membrane proteins from the knockdown cells were loaded in CFTR immunoblot to show the steady distribution of band B. Data shown are representative of at least three experiments.

correction of $\Delta F508$ CFTR did not affect the total expression of NHERF1 and CAL but did increase the total amounts of CAL and CFTR in the immunoprecipitates. Note also that NHERF1 was still reduced in the knockdown cells rescued by low temperature. Consistent with correction of $\Delta F508$ CFTR, NHERF1 was immunoprecipitated by anti-CFTR antibodies in CFBE41o- $\Delta F508$ cells grown at reduced temperature (27°C, 24 hr), but not at normal temperature (37°C, 24 hr). Since temperature rescue allows $\Delta F508$ CFTR to exit from the ER and reach the cell surface, the coimmunoprecipitation of NHERF1 and only temperature corrected $\Delta F508$ CFTR suggests their interaction occurs

in the post-ER compartments. This is in contrast to CAL which binds to $\Delta F508$ CFTR in both corrected and uncorrected states, suggestive of pre-Golgi binding. In addition, the association between endogenous NHERF1 and r $\Delta F508$ CFTR was effectively diminished in the NHERF1 knockdown cells.

Cell surface biotinylation was performed to assess the role of NHERF1 (Fig. 2B). As expected, $\Delta F508$ CFTR is present in the plasma membrane only in temperature corrected cells. Surface $\Delta F508$ CFTR was significantly decreased in cells containing NHERF1 RNAi, compared to control cells. However, total cellular expression of $\Delta F508$ CFTR was also equally decreased in cells

Fig. 4. NHERF1 knockdown accelerates the degradation of r Δ F508 CFTR at the cell surface. After temperature rescue, cells were surface-biotinylated, left on ice to determine total biotinylated CFTR, or incubated with cycloheximide at the indicated time and temperature. Biotinylated cell surface proteins were pulled down with immobilized Neutravidin and immunoblotted for CFTR. Filled squares and triangles = control knockdown; open squares and triangles = NHERF1 knockdown; squares = bands C; triangles = bands B; filled circles = control knockdown/ wild-type CFTR. Quantification of remaining wild-type and Δ F508 CFTR after degradation are presented as means of \pm std. dev. ($n = 3$).



containing NHERF1 RNAi. The results are more prominent when cells were incubated at 27°C for a longer time, 48 hours. This observed defect is attributable to the NHERF1 knockdown because two distinct shRNAs against NHERF1 gave similar results (data not shown).

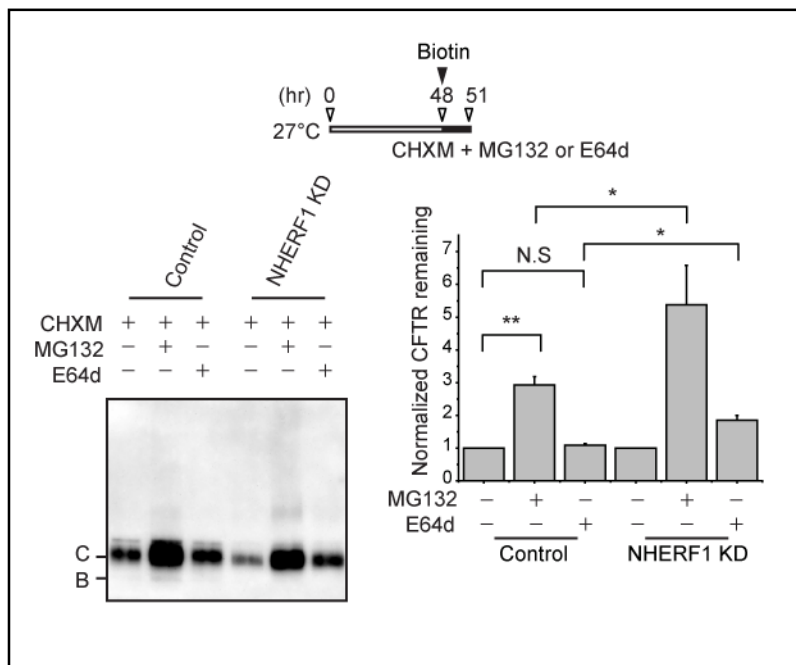
Temperature rescue leads to mislocalization of the immature Δ F508 CFTR at the cell surface of human airway cells

Interestingly, besides mature form (C band) of CFTR, another band similar in size to the immature ER form (B band) of CFTR was detected in the cell surface proteins from the rescued Δ F508 CFTR (Fig. 2B). To determine whether this band is indeed the immature B band of CFTR at the cell surface, surface biotinylated proteins were exposed to endoglycosidase H (endo H) and peptide: N-Glycosidase F (PNG-F) to study the pattern of glycosylation. As determined by western analysis (Fig. 3A), the surface band B was sensitive to both endo H and PNG-F, indicating an immature form of Δ F508 CFTR also traffics to the cell membrane subsequent to temperature correction. It is important to note that this misinsertion is not due to inefficient or retarded endoglycosylation reactions resulting from the reduced temperature because the surface expression of

the immature form of WT-CFTR at 27°C is minimal (data not shown and Fig. 4).

A 10-28% Opti-Prep gradient can be used to separate subcellular fractions enriched in ER, Golgi, and plasma membrane. To test the effect of temperature on distributions of Δ F508 CFTR into their compartments, we fractionated monolayers of CFBE41o-WT-CFTR and CFBE41o- Δ F508 CFTR cells and examined fractions for immature B band and mature C band. As shown in Fig. 3, the gradients resolve into distinct cellular compartments. B band and C band of WT-CFTR are separately enriched in fraction 1-8 and fraction 10-16, and distributions of the peripheral cell surface protein, NHERF1 enriched in fractions 10-13 and the predominately Golgi protein, CAL (Fig. 3B) enriched in fractions 4-8. Also note that in the uncorrected Δ F508 CFTR containing cells that B band of CFTR is localized sharply in fractions 1-8 a pattern of fractionation identical to that of the band B of cells containing WT-CFTR (Fig. 3C). This indicates that by using bands B and C of CFTR, NHERF, and CAL as land marks, cell fractions can be used to monitor CFTR's progression from the endoplasmic reticulum containing Δ F508 CFTR band B, to the Golgi containing CAL, and to the peripheral compartments, containing NHERF1 and band C of CFTR. The overall distribution of NHERF1

Fig. 5. The degradation of surface localized r Δ F508 CFTR is inhibited by MG-132 whereas r Δ F508 CFTR degradation in NHERF1 depleted cells is inhibited by both MG-132 and E64d. After temperature rescue (at 27°C for 48 hr), Δ F508 CFTR at the cell surface was biotinylated and then incubated with cycloheximide for 3 hr at 37°C. This procedure allowed endocytosis, recycling, and degradation to occur in the absence of protein synthesis. During the incubation, either MG-132, a proteasome inhibitor or E64d, an endolysosomal inhibitor was added. After inhibitor treatment, biotinylated Δ F508 CFTR were collected as described in Fig. 4. Quantification of remaining Δ F508 CFTR after protein synthesis and protease inhibitor treatment are presented as means of \pm std. dev. ($n = 3$). N.S represents statistically not significant.



and CAL were well conserved in CFBE41o- Δ F508 compared to CFBE41o-WT cells and in cells expressing either control or NHERF1 shRNA, and in temperature corrected CFBE41o- Δ F508 cells (Fig. 3D).

In sharp contrast, fractionation of temperature corrected CFBE41o- Δ F508 showed a more complex distribution of CFTR compared with that in CFBE41o-WT cells (Fig. 3D). Significant amounts of B band, together with C band were associated with the cell surface protein, NHERF1, providing additional evidence that B band of temperature-rescued Δ F508 is localized at the cell surface. Together with cell surface biotinylation, these results strongly indicate that an immature form of Δ F508 CFTR can traffic to the cell surface of human bronchial epithelial cells when rescued by lower temperature 27°C. This is unique to the immature form of Δ F508 CFTR because the immature band B of WT-CFTR does not traffic to the cell surface when cells are grown at reduced temperature.

Approximately 27% of Δ F508 CFTR protein including B band and C band from CFBE41o- Δ F508 expressing NHERF1 shRNA is uniquely recovered in fraction 6 though 8, compared with that in CFBE41o- Δ F508 cells expressing control shRNA, indicating that Δ F508 CFTR proteins in NHERF1 knockdown and control knockdown cells undergo different intracellular trafficking at the steady state. In addition, the rescued Δ F508 CFTR protein in CFBE41o- Δ F508 cells where NHERF1 has been knockdown is enriched in fractions 6-8 which

coincides with the major fraction of CAL. Our previous work has shown that CAL and NHERF compete for CFTR [14] so it is not surprising that when NHERF is reduced that more CFTR localizes in the CAL enriched fraction.

MG-132 and E64d inhibit the accelerated degradation of surface localized r Δ F508 CFTR caused by NHERF1 knockdown

To determine the mechanism whereby NHERF1 knockdown reduced the surface and total expression of r Δ F508 CFTR in CFBE41o- Δ F508 cells, we measured the half-life of r Δ F508 CFTR at the cell surface. Cell surface Δ F508 CFTR was biotinylated at 4°C and its degradation was monitored for the indicated time and temperatures in the presence of cycloheximide (Fig. 4). Surface-biotinylated C bands of Δ F508 CFTR from CFBE41o- Δ F508 NHERF1 knockdown and CFBE41o- Δ F508 control cells incubated at 27°C were not degraded over the time period studied (Fig. 4, left). This is significant because it is well known that C band from rescued Δ F508 CFTR at 37°C is degraded rapidly. However, the B band of Δ F508 CFTR mis-targeted to the cell surface is rapidly degraded. There was also no discernible difference within the time period in the degradation of surface Δ F508 CFTR from CFBE41o- Δ F508 cells expressing NHERF1 shRNA and control shRNA at 27°C. This is in definite contrast to the rescued C band of Δ F508 CFTR raised back to 37°C after rescue at 27°C which is rapidly degraded (Fig. 4,

right), suggesting that temperature correction is working at the level of the plasma membrane. In the same condition, the NHERF1 knockdown caused significantly faster degradation of C band of $\Delta F508$ CFTR at the cell surface.

Next, to determine the mechanism whereby NHERF1 knockdown accelerated degradation of rescued surface $\Delta F508$ CFTR, cells with biotin-labeled cell surface $\Delta F508$ CFTR were treated with MG-132, a rather non-specific but widely used proteasome inhibitor, or with E64d, an endolysosomal inhibitor, for 3 hr at 37°C. As shown in Fig. 5, the degradation of cell surface localized $\Delta F508$ CFTR from CFBE41o- $\Delta F508$ cells expressing NHERF1 shRNA and control shRNA was remarkably retarded in the presence of MG-132 during this time period. This effect of MG-132 on surface localized $\Delta F508$ CFTR degradation was more prominent in NHERF1 depleted cells. This sensitivity to MG-132 of surface $\Delta F508$ CFTR is similar to that observed in the literature [22] for uncorrected immature $\Delta F508$ CFTR which resides in the ER. Importantly, treating NHERF1 depleted cells with E64d led to a significant decrease in the degradation rate of $\Delta F508$ CFTR. In contrast, when control cells were treated with E64d, no decrease in the degradation rate of $\Delta F508$ CFTR was observed during the same time period. This strongly suggests that NHERF1 depletion causes cell surface localized $\Delta F508$ CFTR much more susceptible to degradation, thereby reducing the steady-state surface expression of rescued $\Delta F508$ CFTR in human airway cells.

Discussion

NHERF1 is widely known to bind to the PDZ motif of CFTR [12, 13]. The functional consequences of this binding are several but overall still controversial [23, 24]. However, consensus is beginning to emerge that NHERF1 does indeed have a role in helping CFTR function efficiently at the plasma membrane [18, 25]. Although well studied with respect to WT-CFTR, the role of NHERF1 on $\Delta F508$ CFTR at the plasma membrane is seldom addressed. A recent work by Guerra et al. [18] showed that NHERF1 overexpression in CFBE41o- $\Delta F508$ and $\Delta F508$ CFT1-C2 cell monolayers induced both a significant redistribution of CFTR from the cytoplasm to the apical membrane and the appearance of PKA-dependent CFTR-dependent chloride secretion. This study was very important because it showed that NHERF1 when overexpressed could correct defective $\Delta F508$ CFTR

function in the human airway cells.

To take this one step further to explore more of the mechanism of how NHERF1 could increase surface expression of $\Delta F508$ CFTR we utilized shRNA to reduce NHERF1 protein expression. We first confirmed that NHERF1 plays a key role in the turnover of WT-CFTR at the cell surface. Our observation of the reduced half-life of WT-CFTR in NHERF1 RNAi containing cells fits well into the previous study from Swiatecka-Urban *et al.*, where they demonstrated that deletion of the PDZ motif of WT-CFTR caused the pronounced reduction of membrane retention of WT-CFTR at the cell surface [17].

Next, we studied the surface expression of $\Delta F508$ CFTR rescued by low temperature. Surprisingly, we found that temperature rescue permits the immature form of $\Delta F508$ CFTR, together with mature form, to traffic to the cell surface of human bronchial epithelial cells. As the immature B form of CFTR was not detected in human airway cells expressing WT-CFTR (Fig. 4) and human embryonic kidney cells (HEK293) expressing $\Delta F508$ CFTR (data not shown) grown at low temperature under the same experimental condition, this aberrant traffic may be mutation ($\Delta F508$)- and cell type- specific. It is currently unknown how immature B form of $\Delta F508$ CFTR reaches the cell surface of human airway cells when rescued by low temperature. We also found that the degradation of mature $\Delta F508$ CFTR at the cell surface is highly temperature-sensitive. At lower temperature, the mature form has a relatively long half-life at the plasma membrane compared to the higher temperature. The immature form of $\Delta F508$ CFTR undergoes extremely rapid degradation once delivered to the cell surface even at lower temperature. This strongly suggests that reduced temperature allows $\Delta F508$ CFTR to escape from the ER associated degradation, traffic to the plasma membrane and prolongs the half-life of the mature $\Delta F508$ CFTR if cells are kept at lower temperature. This is important because it shows that it may be feasible to correct the rapid turnover of mutant CFTR at the plasma membrane.

We also show that reduction of NHERF1 protein using RNAi enhances the degradation of the mature form of $\Delta F508$ CFTR at the plasma membrane. Thus overexpression of NHERF1 would be expected to lead to more mature $\Delta F508$ CFTR as shown by Guerra et al. [18, 26] simply by reducing its degradation.

Furthermore, we found that degradation of surface localized $\Delta F508$ CFTR is mainly blocked by MG-132. Our result obtained from biochemical assay is in good agreement with the previous report from Gentzsch *et al.* [27] where they showed by morphological assay that the

degradation of surface localized Δ F508 CFTR in baby hamster kidney cells was blocked by MG-132. However, it is becoming increasingly clear that MG-132 is not a highly selective proteasome inhibitor. It has been reported that proteasome activity is required to some internalized membrane protein to be targeted to the lysosome through multivesicular bodies, although the precise mechanisms are still unknown [28, 29]. And in addition to its inhibition of proteasomal degradation of many proteins [30], the MG-132 may also inhibit the activity of the lysosome, partially through inhibition of cathepsins [31, 32]. To exclude the latter possibility, we performed parallel experiments with E64d, which specifically inhibits endolysosomal proteases including cathepsins [33, 34]. Our work shows that degradation of Δ F508 CFTR was not dependent on E64d during the same chase period. Interestingly only when NHERF1 protein expression is decreased, the sensitivity of Δ F508 CFTR rescued to the cells surface to E64d is enhanced dramatically. Clearly our results show that NHERF1 is acting to protect Δ F508 CFTR from lysosomal degradation, but that the degree of protection at the NHERF1 expression levels present in human airway cells is not sufficient to prolong the half-

life near to that of WT-CFTR. It takes overexpression of NHERF1, as shown by Guerra et al [18], or sustained low temperature as we show here, to enhance the correction Δ F508 CFTR at the plasma membrane.

Further studies will be needed. Nevertheless, our results clearly indicate that rescued Δ F508 CFTR are recognized by cellular peripheral machineries that process this mutant for degradation, and that NHERF1 contributes to this process at the cell surface of human airway cells.

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