The Short Apical Membrane Half-life of Rescued ΔF508-Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) Results from Accelerated Endocytosis of ΔF508-CFTR in Polarized Human Airway Epithelial Cells*

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The most common mutation in the cystic fibrosis transmembrane conductance regulator (CFTR) gene in individuals with cystic fibrosis, ΔF508, causes retention of ΔF508-CFTR in the endoplasmic reticulum and leads to the absence of CFTR Cl− channels in the apical plasma membrane. Rescue of ΔF508-CFTR by reduced temperature or chemical means reveals that the ΔF508 mutation reduces the half-life of ΔF508-CFTR in the apical plasma membrane. Because ΔF508-CFTR retains some Cl− channel activity, increased expression of ΔF508-CFTR in the apical membrane could serve as a potential therapeutic approach for cystic fibrosis. However, little is known about the mechanisms responsible for the short apical membrane half-life of ΔF508-CFTR in polarized human airway epithelial cells. According to this study, it was determined that the reduced apical membrane half-life of ΔF508-CFTR in polarized human airway epithelial cells lead to the decreased apical membrane half-life of ΔF508-CFTR in polarized human airway epithelial cells. We report that in polarized human airway epithelial cells (CFBE410−) the ΔF508 mutation increased endocytosis of CFTR from the apical membrane without causing a global endocytic defect or affecting the endocytic recycling of CFTR in the Rab11a-specific apical recycling compartment.

The cystic fibrosis transmembrane conductance regulator (CFTR) is an ATP binding cassette (ABC) transporter and a CAMP-regulated Cl− channel that mediates transepithelial Cl− transport in the airways, intestine, pancreas, testis, and other tissues (1–3). Cystic fibrosis (CF), a lethal genetic disease, is caused by mutations in the CFTR gene (1, 2). The most common mutation in CFTR is ΔF508 (4, 5). ΔF508-CFTR does not fold properly, and most of the protein is retained within the endoplasmic reticulum (ER) where it is subsequently degraded (5, 6). Several studies suggest that the ER retention of ΔF508-CFTR is not complete, and some ΔF508-CFTR is constitutively expressed in the plasma membrane of primary epithelial cells from individuals homozygous for the ΔF508 mutation (7–10). Because ΔF508-CFTR retains some Cl− channel activity when expressed in the plasma membrane (5, 6, 11–14), it would be desirable to increase the expression of ΔF508-CFTR in the plasma membrane to alleviate the symptoms in CF patients. The trafficking of ΔF508-CFTR to the plasma membrane can be increased by chemical means or reduced temperature (15–21). Yet, functional and biochemical studies in heterologous cell lines demonstrate that rescued ΔF508-CFTR has a greatly reduced stability or half-life in the post-ER compartments, including the plasma membrane (13, 22–24). Very little is known about the apical membrane half-life of rescued ΔF508-CFTR in polarized human airway epithelial cells. A recent study demonstrates that the functional stability of ΔF508-CFTR in the apical membrane of differentiated respiratory epithelial cells derived from nasal polyps from individuals homozygous for the ΔF508 mutation is decreased compared with WT-CFTR (25). Furthermore, the biochemical half-life of rescued ΔF508-CFTR in the apical membrane of porcine kidney epithelial cells (LLC-PK1) is reduced (26). However, the biochemical half-life of ΔF508-CFTR in the apical membrane of polarized human airway epithelial cells has not been examined.

Regulation of the plasma membrane half-life of WT-CFTR is not completely understood but depends, at least in part, on the endocytic trafficking events such as endocytosis of CFTR from the plasma membrane and endocytic recycling of CFTR from endosomes to the plasma membrane. Endocytosis of WT-CFTR 1 is clathrin-dependent (27–29) and occurs in Rab5-specific endosomes (24), 2) is mediated by multiple endocytic motifs in the C terminus of CFTR (30, and 3) requires interactions with the endocytic adaptor complex, AP-2 (31), the large GTPase, dynamin (32), and myosin VI (33). Recycling of WT-CFTR from endosomes to the plasma membrane occurs in Rab11-specific recycling vesicles (24) and is facilitated by Rme-1 (34) and by PDZ domain interaction (35). In addition, syntaxins (36–39), the CFTR associated ligand, CAL (32), and the Rho family GTPase, TC10 (40), affect the endocytic trafficking and plasma membrane expression of WT-CFTR. How these pathways and protein interactions are affected by the ΔF508 mutation in polarized human airway epithelial cells is even less well understood.
A recent study demonstrates that in fibroblasts (BHK-21 cells) heterologously expressing CFTR, the ΔF508 mutation reduces the plasma membrane half-life of CFTR by attenuating the endocytic recycling of rescued ΔF508-CFTR without significantly affecting ΔF508-CFTR endocytosis (25). Even though non-epithelial cells possess the cellular machinery to sort proteins to specific membrane domains (41–43), it is widely accepted that trafficking of heterologously expressed plasma membrane proteins in non-epithelial cells can differ from polarized epithelial cells (43, 44). Thus, studies designed to elucidate the cellular defects that lead to the decrease in the apical membrane half-life of rescued ΔF508-CFTR need to be carried out in human airway epithelial cells.

To this end, we studied CFTR trafficking in polarized human airway epithelial cell lines (CFBE41o−) expressing endogenous ΔF508-CFTR or stably expressing either ΔF508-CFTR or WT-CFTR. We report that the apical plasma membrane half-life of rescued ΔF508-CFTR was shorter than that of WT-CFTR. The ΔF508 mutation specifically increased endocytosis of ΔF508-CFTR from the apical membrane without causing a global endocytic defect or altering the endocytic recycling of CFTR. Furthermore, the decrease in the apical membrane half-life of ΔF508-CFTR was not mediated by a putative cryptic endocytic motif, YRSV (517–520). The endocytic recycling of WT-CFTR and ΔF508-CFTR occurred in the Rab11a-specific apical recycling compartment in polarized human airway epithelial cells. Taken together, these data indicate that in polarized human airway epithelial cells (CFBE41o−) the ΔF508 mutation reduces the apical membrane half-life of ΔF508-CFTR by specifically accelerating the endocytic retrieval of ΔF508-CFTR from the plasma membrane without affecting the endocytic recycling of ΔF508-CFTR.

**MATERIALS AND METHODS**

**Cell Lines and Cell Culture**—Stable lentiviral-based transduction of the parental CFBE41o− cells (ΔF508/ΔF508), originally immortalized and characterized by Dr. D. Gruenert and co-workers (45, 46) with either WT-CFTR or ΔF508-CFTR, was performed by Tranzyme, Inc. (Birmingham, AL). The parental and stably transduced cells were generous gifts from Dr. J. P. Clancy (47). The transduced CFBE41o− cells were maintained in minimum Eagle’s medium supplemented with 50 units/ml penicillin, 50 μg/ml streptomycin, 2 mM L-glutamine, 10% fetal bovine serum, and 1 μg/ml blasticidin (WT-CFTR) or 2 μg/ml puromycin (ΔF508-CFTR) in a 5% CO2, 95% air incubator at 37 °C. The parental CFBE41o− cells were maintained under the same culture conditions but without blasticidin or puromycin. To establish polarized monolayers, CFBE41o− cells were seeded on 24-mm-diameter Transwell permeable supports (0.4 mm pore size; Corning Corp., Corning, NY) at 2 × 104 and grown in air-liquid interface culture at 37 °C for 6–9 days and then at 27 °C for 36 h. In addition, CFBE41o− cells were seeded on 40-mm-diameter plastic tissue culture plates (Corning) at 0.55 × 105 per plate and on glass coverslips (No. 1; Corning) at 0.2 × 106/22 mm2. Calu-3 cells, obtained from the American Type Culture Collection (Manassas, VA), were seeded at 2 × 104 on Transwell permeable supports (24-mm diameter, 0.4-mm pore size; Corning) and maintained as polarized monolayers in air-liquid interface culture in minimum Eagle’s medium containing 50 units/ml penicillin, 50 mg/ml streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, and 10% fetal bovine serum in a 5% CO2, 95% air incubator at 37 °C for 14–21 days as described previously (48).

**siRNA-mediated Silencing of Rab11a Expression**—The double-stranded, small interfering RNA (siRNA) against a non-conserved region of the human Rab11a sequence (siRab11a; catalog #1022547) and the double-stranded, non-silencing siRNA control (Non-sil. siRNA; catalog #1022076) were purchased from Qiagen (Valencia, CA). Transfection of siRab11a and Non-sil. siRNA into CFBE41o− cells was conducted using LipofectamineTM 2000 (Invitrogen) according to the manufacturer’s instructions. CFBE41o− cells grown on 40-mm tissue culture plates were incubated for 24 h with the optimized transfection mixture (7.5 μg of either siRab11a or Non-sil. siRNA and 15 μl of LipofectamineTM 2000 per plate) at 37 °C. Subsequently, cells were cultured in fresh medium at 37 °C for 36 h and then at 27 °C for another 36 h to increase trafficking and expression of ΔF508-CFTR in the plasma membrane. The efficiency of siRNA-mediated silencing of Rab11a expression was assessed by measuring the expression of endogenous Rab11a by Western blotting. The specificity of siRNA-mediated silencing of Rab11a expression was assessed by measuring the expression of other Rab GTPases facilitating endocytosis and recycling (Rab5a and Rab4) by Western blotting.

**Plasmids and Transient Transfection**—A plasmid containing GFP-tagged ΔF508-CFTR (GFP-ΔF508-CFTR) was generated as previously described using the eukaryotic expression vector pcDNA3.1 (49). To construct the GFP-ΔF508-CFTR Y517A mutant, the GFP-ΔF508-CFTR cDNA sequence in pcDNA3.1 was mutated using the QuikChangeTM XL site-directed mutagenesis kit (Stratagene; La Jolla, CA). Constructs were sequence-verified by ABI PRISM dye terminator cycle sequencing (Applied Biosystems; Foster City, CA). Transient transfection of the GFP-tagged CFTR cDNAs into parental CFBE41o− cells was performed using LipofectamineTM 2000 according to the manufacturer’s instructions. CFBE41o− cells grown on 40-mm tissue culture plates were incubated for 24 h with the transfection mixture (2 μg of cDNA and 4 μl of LipofectamineTM 2000 per plate) at 37 °C. Subsequently, cells were cultured in fresh medium at 27 °C for 36 h to increase the expression of GFP-ΔF508-CFTR and GFP-ΔF508-CFTR Y517A in the plasma membrane.

A plasmid containing FLAG-tagged wild type mouse Rab11a (FLAG-Rab11a WT) was generated as described previously using the eukaryotic expression vector pEF (50–52). To construct the FLAG-Rab11a S25N and FLAG-Rab11a S20V mutants, the Rab11a cDNA sequence in pEF was mutated using the QuikChangeTM XL site-directed mutagenesis kit. Constructs were sequence-verified by ABI PRISM dye terminator cycle sequencing. Transfection of the FLAG-Rab11a cDNAs into CFBE41o− cells stably expressing ΔF508-CFTR was carried out using LipofectamineTM 2000 according to the manufacturer’s instructions. CFBE41o− cells grown on 40-mm tissue culture plates were incubated with the transfection mixture (4 μg of cDNA and 8 μl of LipofectamineTM 2000 per plate) for 24 h at 37 °C and in fresh medium for another 36 h.

**Antibodies**—The antibodies used were monoclonal anti-human CFTR C terminus-specific, clone 24–1 (R&D Systems; Minneapolis, MN), monoclonal anti-CFTR, clone M3A7 (Upstate Biotechnology; Lake Placid, NY), monoclonal anti-GFP, clone JL-8, monoclonal anti-ezrin (BD Biosciences), monoclonal anti-FLAG M2, polyclonal anti-FLAG (Sigma-Aldrich), monoclonal anti-breast cancer resistance protein (BCRP), clone BXP-21 (Ref. 53; Chemicon, Temecula, CA), polyclonal anti-Rab11a (Zymed Laboratories Inc.; South San Francisco, CA), polyclonal anti-Rab4, polyclonal anti-Rab5a (Santa Cruz Biotechnology; Santa Cruz, CA), polyclonal anti-zonula occludens-1 (ZO-1; Zymed Laboratories Inc.; South San Francisco, CA), goat anti-mouse and goat anti-rabbit horseradish peroxidase secondary antibodies (Bio-Rad), and Alexa Fluor 647 goat anti-rabbit secondary antibody (Molecular Probes; Eugene, OR). All antibodies were used at the concentrations recommended by the manufacturer.
Trafficking of ΔF508-CFTR in Human Airway Epithelial Cells

FIGURE 1. Summary of experiments performed to determine the effects of reduced temperature (27 °C for 36 h) on the expression of endogenous ΔF508-CFTR (ΔF508e) in parental CFBE41o− cells and the expression of WT-CFTR (WT) and ΔF508-CFTR (ΔF508) in stably transduced polarized CFBE41o− cells. A, summary of experiments demonstrating by domain-selective cell surface biotinylation that reduced temperature had no significant effect on the apical membrane expression of transduced WT-CFTR or endogenous ΔF508-CFTR but increased the expression of ΔF508-CFTR in stably transduced CFBE41o− cells, to levels equivalent to the expression observed in the WT-CFTR-transduced cells. Data are expressed as percent of the endogenous ΔF508-CFTR expressed in the apical membrane of parental CFBE41o− cells at 37 °C. The asterisk indicates p < 0.05. 3–4 experiments were performed per group. B, representative Western blots demonstrating that in CFBE41o− cells stably transduced with ΔF508-CFTR, reduced temperature (1) increased expression of the core glycosylated (band B) (*) and the fully glycosylated (band C) (**) form of ΔF508-CFTR in cell lysate (the left panel) and 2) increased trafficking of the ΔF508-CFTR band C to the apical plasma membrane (right panel). Reduced temperature had no effect on ezrin expression in cell lysate. It is important to note that at 37 °C a small but detectable amount of ΔF508-CFTR, predominantly band B, was observed in the lysate from stably transduced CFBE41o− cells (left panel, lower image, 3-min exposure). In addition, the presence of a small amount of ΔF508-CFTR band C was evident in the fluid phase in the ΔF508-CFTR-transduced CFBE41o− cells (right panel). Proteins were separated by SDS-PAGE using a 7.5% gel.

Biochemical Determination of the Apical Membrane CFTR and BCRP—The biochemical determination of apical membrane CFTR and BCRP at steady state was performed by domain-selective cell surface biotinylation using EZ-Link™ Biotin-LC-Hydrazide or EZ-Link™ Sulfo-NHS-LC-Biotin (Pierce), as described previously in detail (35, 54). Studies to determine the half-life of apical membrane proteins were conducted essentially as described by Heda and Marino (26). CFBE41o− cells, stably expressing either WT-CFTR or ΔF508-CFTR, were grown on Transwell permeable growth supports. Parental CFBE41o− cells transiently transfected with either GFP-ΔF508-CFTR or GFP-ΔF508-CFTR Y517A were grown on plastic tissue culture plates. Confluent cells were cultured for 36 h in a CO2 incubator at 27 °C to increase the trafficking and expression of ΔF508-CFTR in the apical plasma membrane. Incubation of cells with cycloheximide (Sigma-Aldrich; 20 μg/ml), a protein synthesis inhibitor, was performed at 37 °C, and the disassembly of CFTR or BCRP from the apical membrane was monitored over time. The half-lives were calculated by SPSS software using the one-phase exponential decay model, with plateau and span parameters constrained to 0 and 100, respectively.

Endocytic Assay and Endocytic Recycling Assay—Endocytic and endocytic recycling assays were performed in CFBE41o− cells expressing either WT-CFTR or ΔF508-CFTR grown on Transwell permeable supports, using EZ-Link™ Sulfo-SS-Biotin (Pierce), as described previously in detail (35). The temperature in the incubator was reduced (27 °C, for 36 h) to increase trafficking and expression of ΔF508-CFTR in the apical membrane of CFBE41o− cells.

Fluid-phase Endocytosis—Studies were conducted to determine the cellular uptake of Alexa 647-dextran (10,000 molecular weight; Molecular Probes), a fluorescent marker of fluid phase endocytosis (33, 55) in CFBE41o− cells stably expressing either WT-CFTR or ΔF508-CFTR. CFBE41o− cells were grown on glass coverslips at 37 °C for 48 h and then at 27 °C for 36 h to increase trafficking and expression of ΔF508-CFTR in the plasma membrane. Alexa 647-dextran (1 mg/ml) was added to the cell culture medium (37 °C) for 15 min. Subsequently, surface Alexa-647 dextran was removed by thorough washing at 4 °C. Thereafter, cells were fixed for 20 min in 4% paraformaldehyde before being mounted in antifade medium (ProLong Gold, Molecular Probes). Thirty random images of cells expressing either WT-CFTR or ΔF508-CFTR were examined from six different slides using an Olympus IX70 wide field microscope fitted with an Orca AG deep cooling CCD camera (Hamamatsu Photonics, Japan) and Openlab 4.0.3 software (Improvision, Inc.; Lexington, MA) using an 40× oil immersion objective. All images were collected using the same settings for exposure, gain, offset, and binning. Alexa 647 fluorescence was quantified using Volocity 3.5.1 software (Improvision, Inc.).

ZO-1 Immunostaining—Studies were conducted to determine the ability of CFBE41o− cells, grown on glass or plastic to polarize. Cells expressing either WT-CFTR or ΔF508-CFTR, plated on glass coverslips, were grown at 37 °C for 48 h and then at 27 °C for 36 h. Subsequently, cells were fixed in −20 °C methanol. Nonspecific binding sites were blocked with 10% normal goat serum. Cells were stained with polyclonal anti-ZO-1 antibody in 10% normal goat serum, washed with phosphate-buffered saline, postfixed in 4% paraformaldehyde, and incubated with glyoxine (100 mM) to block the aldehyde groups. After washing in phosphate-buffered saline and blocking for the second time with 10% normal goat serum, cells were incubated with Alexa Fluor 647 goat anti-rabbit secondary antibody in 10% normal goat serum. Cells were mounted with antifade medium (ProLong Gold; Molecular Probes). Z-stacks of the immunolabeled cells were acquired with an Olympus IX70 wide field microscope (40× oil immersion objective) fitted with an Orca AG deep cooling CCD camera (Hamamatsu Photonics, Japan) and Openlab 4.0.3 software (Improvision, Inc.), and the volumes...
were deconvolved (iterative restoration) using Volocity 3.5.1 software (Improvision, Inc.).

**Immunoprecipitation and Immunoblotting—** CFTR and Rab11a were immunoprecipitated from CFBE41o− and Calu-3 cell lysates by methods described previously (33). For experiments with endogenous and stably expressed proteins, cells were grown on Transwell permeable supports, and for experiments with transiently transfected FLAG-Rab11a WT, cells were grown on plastic tissue culture plates. Briefly, cells were solubilized in lysis buffer containing 150 mM NaCl, 50 mM Tris, pH 7.2, 0.1% IGEPAI (Sigma-Aldrich), 5 mM MgCl₂, 5 mM EDTA, 1 mM EGTA, 30 mM NaF, 1 mM Na₃VO₄, and Complete Protease Inhibitor mixture (Roche Applied Science). After centrifugation at 14,000 × g for 15 min to pellet insoluble material, the soluble lysates were incubated for 10 min at 30 °C with 20 μM GTPγS (Sigma-Aldrich), a non-hydrolysable analog of GTP (56–58). After cooling to 4 °C, the soluble lysates were pre-cleared by incubation with protein G conjugated to Sepharose beads (Pierce) at 4 °C. CFTR was immunoprecipitated by incubation with the M3A7 antibody-protein G-Sepharose complexes. Immunoprecipitated proteins were eluted from the protein G-Sepharose complexes by incubation at 85 °C for 5 min in sample buffer (Bio-Rad) containing 80 mM dithiothreitol. Immunoprecipitated proteins were separated by SDS-PAGE using 15% gels (Bio-Rad) and analyzed by Western blotting with an appropriate primary antibody and an anti-mouse or anti-rabbit horseradish peroxidase secondary antibody.

**Data Analysis and Statistics—** Statistical analysis of the data were performed using GraphPad Prism version 4.0 for Macintosh (GraphPad Software Inc.; San Diego, CA) and SPSS software (SPSS Inc.; Chicago, IL). The half-lives were calculated by SPSS software using the one-phase exponential decay model, with plateau and span parameters constrained to 0 and 100, respectively. The half-life means were compared by a two-tailed t test with assumed unequal variances. The means for the remaining data were compared by a two-tailed t test. A p value < 0.05 was considered significant. Data are expressed as the mean ± S.E.

**RESULTS**

The Apical Membrane Half-life of Rescued ΔF508-CFTR Compared with WT-CFTR Is Decreased in Polarized Human Airway Epithelial Cells (CFBE41o−)—First, studies were conducted to determine the relative amount of WT-CFTR and ΔF508-CFTR in CFBE41o− cells grown on permeable growth supports at 37 or at 27 °C, a temperature that, at least for some cells, increases the expression of ΔF508-CFTR in the plasma membrane (15, 16). As illustrated in Fig. 1, ΔF508-CFTR was detected in the apical plasma membrane of parental and ΔF508-CFTR-transduced cells at 37 °C. Reduced temperature (27 °C for 36 h) increased the apical membrane expression of ΔF508-CFTR in the transduced cells and had no significant effect on the expression of endogenous ΔF508-CFTR in the parental cells or WT-CFTR in the transduced cells (Fig. 1A). It is worth noting that in stably transduced CFBE41o− cells, the plasma membrane expression of WT-CFTR and ΔF508-CFTR was comparable at reduced temperature (Fig. 1A). Reduced temperature did not affect the expression of the cytoskeletal protein, ezrin, in cell lysates (Fig. 1B). Thus, reduced temperature increased the plasma membrane expression of ΔF508-CFTR to a level sufficient to perform endocytic and recycling assays and similar to the level of WT-CFTR expression.

Next, studies were conducted to determine whether the ΔF508 mutation affected the biochemical half-life of CFTR in the apical membrane of polarized human airway epithelial cells (CFBE41o−). As described above, the temperature was decreased before studies (27 °C for 36 h) to increase expression of ΔF508-CFTR in the apical membrane. The disappearance of WT-CFTR and ΔF508-CFTR from the apical membrane was monitored over time at 37 °C in the presence of 20 μg/ml cyclohexamide (CHX). The half-life of WT-CFTR (3.0 h) was significantly shorter than that of ΔF508-CFTR (1.0 h) (p < 0.05). Reduced temperature had no effect on the apical membrane expression of WT-CFTR and ΔF508-CFTR in CFBE41o− cells grown on permeable growth supports at 37 or at 27 °C, a temperature that, at least for some cells, increases the expression of ΔF508-CFTR in the plasma membrane. The asterisk indicates p < 0.05. Six experiments were performed per group in A, and three experiments were performed per group in B.
hypothesis that the ΔF508 mutation increased ΔF508-CFTR endocytosis. Endocytosis of CFTR was measured at 2.5, 5, and 7.5 min, as described under “Materials and Methods.” Endocytosis of WT-CFTR and ΔF508-CFTR increased linearly between 0 and 5 min (not shown). Thus, data are reported at the 5 min time point. The percent of ΔF508-CFTR endocytosed at 5 min was significantly greater than that of WT-CFTR (Fig. 3A). The accelerated endocytosis of ΔF508-CFTR is consistent with the decreased apical membrane half-life of rescued ΔF508-CFTR observed in polarized CFBE41o– cells.

The Endocytic Recycling of Rescued ΔF508-CFTR Is Similar to WT-CFTR in Polarized Human Airway Epithelial Cells (CFBE41o–)–As noted above, the decreased apical membrane half-life of ΔF508-CFTR could also result from inhibition of the ΔF508-CFTR recycling from endosomes to the apical membrane. Accordingly, endocytic recycling of CFTR was measured at 2.5, 5, and 7.5 min as described under “Materials and Methods.” Endocytic recycling of WT-CFTR and ΔF508-CFTR increased linearly between 0 and 5 min (not shown). Thus, data are reported at the 5 min time point. The percent of endocytosed ΔF508-CFTR that recycled back to the plasma membrane was similar to that of WT-CFTR (Fig. 3B). Our data suggest that the ΔF508 mutation reduces the half-life of CFTR in the apical membrane of polarized human airway epithelial cells by accelerating the endocytosis of CFTR from the apical membrane without affecting the endocytic recycling of CFTR.

Accelerated Endocytosis and Decreased Apical Membrane Half-life of Rescued ΔF508-CFTR Do Not Result From a Global Endocytic Trafficking Defect in Polarized CFBE41o– Cells—Studies in polarized epithelial cells demonstrate that the ΔF508 mutation causes a global endocytic trafficking defect; thus, CFTR might be a pleiotropic regulator of endocytic trafficking (59–61). However, the effects of the ΔF508 mutation on endocytic trafficking appear to be cell type-specific (62–64). Accordingly, studies were conducted to determine whether the accelerated endocytosis and decreased apical membrane half-life of rescued ΔF508-CFTR in CFBE41o– cells resulted from a generalized defect in endocytic trafficking.

CFTR (27, 28) and other ABC transporters (65) are endocytosed from the apical membrane by a clathrin-dependent pathway. Hence, a generalized defect in clathrin-mediated endocytosis should alter the apical membrane expression and half-life of another ABC transporter. The BCRP is an ABC transporter (66) highly expressed in the apical membrane of respiratory epithelial cells (67) including polarized CFBE41o– cells (Fig. 4C). As illustrated in Fig. 4A, the apical membrane expression of BCRP at steady state did not differ in polarized CFBE41o– cells expressing either WT-CFTR or ΔF508-CFTR. Furthermore, the apical membrane half-life of BCRP did not differ in CFBE41o– cells expressing WT-CFTR (4.4 h) or ΔF508-CFTR (4.6 h) as monitored by the disappearance of BCRP from the apical membrane over time at 37 °C in the presence of 20 μg/ml cyclohexamide (Figs. 4, B and C). Further-
more, the ΔF508 mutation had no effect on fluid phase endocytosis in CFBE41o− cells as determined by measuring the uptake of the Alexa 647-conjugated dextran (Fig. 5A). These data are consistent with the conclusion that increased endocytosis and decreased apical membrane half-life of rescued ΔF508-CFTR observed in polarized CFBE41o− cells did not result from a generalized defect in clathrin-mediated endocytosis or in fluid-phase endocytosis.

Rab5a and CFTR Endocytic Trafficking—The small GTPase, Rab5a, is an important regulator of clathrin-mediated endocytosis (68, 69) and fluid phase endocytosis (70–72). A recent study demonstrates that endocytosis of WT-CFTR and ΔF508-CFTR is Rab5-dependent in heterologous cells (24). Interestingly, the Rab5a gene expression is up-regulated in bronchial epithelial cells expressing ΔF508-CFTR (IB3–1) compared with the IB3–1 cells corrected with WT-CFTR (S9 cells) (73). Thus, accelerated endocytosis of ΔF508-CFTR observed in this study could result from increased expression of the Rab5a protein. However, as illustrated in Fig. 5B, the expression of endogenous Rab5a was similar in polarized CFBE41o− cells transduced with either WT-CFTR or ΔF508-CFTR. Thus, we conclude that a difference in Rab5a expression does not account for accelerated endocytosis of rescued ΔF508-CFTR in polarized CFBE41o− cells.

A Putative Cryptic Endocytic Motif YRSV (517–520) Does Not Contribute to the Decrease in the Apical Membrane Half-life of Rescued ΔF508-CFTR in Polarized CFBE41o− Cells—A recent study based on the crystal structure of the first nucleotide binding domain (NBD1) of human CFTR (74) suggests that the sequence YRSV (517–520) is a cryptic tyrosine-based endocytic motif, which becomes functional in ΔF508-CFTR due to conformational changes in the ΔF508-NBD1 (75). The sequence YRSV (517–520) conforms to the canonical YXXØ endocytic motif (Y is tyrosine, X is any amino acid, and Ø is an amino acid with a bulky hydrophobic side chain) (76) and is conserved across species. If exposed by the ΔF508 mutation, this motif may account for accelerated endocytosis of rescued ΔF508-CFTR observed in the present study. Thus, studies were conducted to test the hypothesis that the putative YRSV (517–520) endocytic motif might be responsible for increased endocytosis of ΔF508-CFTR. Mutation of Tyr-517 would be expected to reduce the endocytic retrieval and, thus, increase the expression and the half-life of rescued ΔF508-CFTR in the apical plasma membrane. To test this hypothesis, we transiently expressed the GFP-tagged ΔF508-CFTR or the GFP-ΔF508-CFTR Y517A mutant in parental CFBE41o− cells. Because the transfection efficiency is low in CFBE41o− cells grown on permeable supports, the transient expression studies were performed in CFBE41o− cells grown on plastic tissue culture plates. As demonstrated in Fig. 6, CFBE41o− cells form tight junctions and thereby polarize when grown on non-permeable growth support such as glass or plastic tissue culture plates. The apical membrane expression of GFP-ΔF508-CFTR and GFP-ΔF508-CFTR Y517A was similar at steady state (Fig. 7A). Furthermore, as illustrated in Figs. 7, B and C, the apical membrane half-life of GFP-ΔF508-CFTR Y517A (1.0 h) did not differ from that of GFP-ΔF508-CFTR (1.1 h). These data demonstrate that the putative endocytic motif, YRSV (517–520), does not contribute to the decrease in the apical membrane half-life of rescued ΔF508-CFTR.

Endogenous Rab11a Facilitates Recycling of WT-CFTR and Rescued ΔF508-CFTR to the Apical Plasma Membrane in Polarized CFBE41o− Cells—Trafficking between apical recycling endosomes and the apical plasma membrane in epithelial cells is facilitated by Rab11a, a member of the Ras-like small GTPases (77–80). Rab11a facilitates plasma membrane expression of WT-CFTR and rescued ΔF508-CFTR in heterologous cells (24). Thus, studies were conducted to determine whether Rab11a facilitates the endocytic recycling of CFTR in human airway epithelial cells. We first examined whether WT-CFTR and ΔF508-CFTR interact with endogenous Rab11a in polarized human airway epithelial cells. As illustrated in Fig. 8, endogenous Rab11a co-immunoprecipitated with endogenous ΔF508-CFTR and rescued ΔF508-CFTR in parental CFBE41o− cells and with WT-CFTR and ΔF508-CFTR in stably transduced CFBE41o− cells. In addition, endogenously expressed WT-CFTR and Rab11a co-immunoprecipitated in another polarized human airway epithelial cell line (Calu-3; Fig. 8D). These data demonstrate that Rab11a interacts with WT-CFTR and ΔF508-CFTR in recycling endosomes.

The co-immunoprecipitation studies presented above suggest a role for Rab11a in the endocytic recycling of CFTR in polarized human airway epithelial cells. To examine more directly the role Rab11a plays in endocytic recycling of WT-CFTR and ΔF508-CFTR, studies were conducted using siRNA-mediated silencing of Rab11a expression. Reduced expression of Rab11a should decrease the endocytic recycling of CFTR and, thus, reduce the apical membrane expression of WT-CFTR and rescued ΔF508-CFTR. To test these predictions, CFBE41o− cells stably expressing either WT-CFTR or ΔF508-CFTR were transfected with double-stranded small interfering RNA specific for a non-conserved region of the human Rab11a sequence (siRab11a) or with the Non-sil. siRNA as described under “Materials and Methods.” Expression of endogenous Rab11a was similar in the WT-CFTR- and ΔF508-CFTR-transduced CFBE41o− cells (Fig. 9A). siRab11a decreased the expression of Rab11a in both cell lines (Fig. 9B). Neither Rab5a nor Rab4 protein expression was altered by siRab11a (Fig. 9C). By contrast, Non-sil. siRNA had no effect on the endogenous expression of Rab11a, WT-CFTR, or ΔF508-CFTR when compared with the non-transfected cells (not shown). As predicted, siRab11a decreased the expression of WT-CFTR and ΔF508-CFTR in the apical plasma membrane (Fig. 9D). The decreased apical membrane expression of WT-CFTR and ΔF508-CFTR in CFBE41o− cells is consistent with the view that Rab11a facilitates the endocytic recycling of WT-CFTR and rescued ΔF508-CFTR in polarized human airway epithelial cells.

3 A. Swiatecka-Urban, A. Brown, S. Moreau-Marquis, J. Renuka, B. Coutermarsh, R. Barnaby, K. H. Karlson, T. R. Flotte, M. Fukuda, G. M. Langford, and B. A. Stanton, unpublished observations.
Endocytosis and endocytic recycling determine, at least in part, the expression of CFTR in the apical membrane (28, 81, 82). Thus, inhibition of the endocytic recycling of ΔF508-CFTR by siRNA-mediated silencing of Rab11a together with increased endocytosis of ΔF508-CFTR should result in lower apical membrane expression of ΔF508-CFTR compared with WT-CFTR. As expected, a similar degree of Rab11a silencing in the WT-CFTR- and ΔF508-CFTR-expressing cells had a more profound inhibitory effect on the plasma membrane expression of ΔF508-CFTR (Fig. 9D).
Rab11a Mutants Affect the Apical Membrane Expression of Rescued ΔF508-CFTR in Polarized CFBE41o− Cells—Rab GTPases alternately bind GTP and GDP and hydrolyze GTP to GDP (83). The cycling of Rab proteins between the GTP-bound (active) and GDP-bound (inactive) form, which serves as the molecular basis for their activity, has been utilized as a valuable tool to study the function of Rab proteins in the trafficking events of membrane proteins. Rab11a S25N, a mutant deficient in GTP binding (GDP-locked), has a dominant negative effect on the endocytic recycling of several plasma membrane proteins (80, 84–89). Rab11a S20V, a mutant deficient in GTP hydrolysis (GTP-locked) (86), has a dominant positive effect on the endocytic recycling of plasma membrane expression of ΔF508-CFTR (Fig. 10A). Moreover, the GTP-locked FLAG-Rab11a S20V increased the plasma membrane expression of ΔF508-CFTR (Fig. 10A). Taken together, these data indicate that Rab11a facilitates the endocytic recycling of WT-CFTR and rescued ΔF508-CFTR to the apical membrane in human airway epithelial cells.

**DISCUSSION**

The major novel observation in the present study is that the ΔF508 mutation decreases the apical membrane half-life of rescued ΔF508-CFTR in polarized human airway epithelial cells by accelerating its endocytosis from the apical membrane without inhibiting the endocytic recycling of ΔF508-CFTR. Furthermore, our data demonstrate that the ΔF508 mutation does not globally disrupt the apical clathrin-mediated endocytic pathway or fluid phase endocytosis in human airway epithelial cells (CFBE41o−). Moreover, our data demonstrate that both WT-CFTR and rescued ΔF508-CFTR undergo trafficking in the Rab11a-specific apical recycling compartment in polarized human airway epithelial cells.

We report that the reduction of the apical membrane half-life of rescued ΔF508-CFTR in polarized human airway epithelial cells is a consequence of increased endocytic retrieval of ΔF508-CFTR from the apical membrane. In all published studies on non-epithelial cells (23–25) or epithelial cells (26) heterologously expressing CFTR, the ΔF508 mutation reduced the biochemical half-life of plasma membrane CFTR. Thus, previous studies are in general agreement with our finding in polarized human airway epithelial cells in which the ΔF508 mutation reduced the biochemical half-life of ΔF508-CFTR. By contrast, previous studies on heterologous, non-epithelial cells suggest that the reduced half-life of rescued ΔF508-CFTR in the plasma membrane is due to a defect in the endocytic recycling of ΔF508-CFTR. In particular, a study in BHK-21 cells suggests that the
FIGURE 9. Summary of experiments performed to determine the effects of siRNA-mediated silencing of Rab11a on the apical membrane expression of CFTR. A, summary of Western blot experiments demonstrating that expression of endogenous Rab11a was similar in CFBE41o – cells stably transduced with either WT-CFTR or ΔF508-CFTR, CFBE41o – cells were transfected with double-stranded small interfering RNA specific for the Rab11a sequence (siRab11a) or with the non-sil. siRNA, as described under “Materials and Methods.” B, summary of Western blot experiments demonstrating that siRab11a similarly decreased the expression of endogenous Rab11a in the WT-CFTR- and ΔF508-CFTR-transduced CFBE41o – cells. C, representative Western blots demonstrating that siRab11a had no effect on the expression of endogenous Rab4, Rab5a, or ezrin. Non-sil. siRNA had no effect on the endogenous expression of Rab11a, WT-CFTR or ΔF508-CFTR when compared with non-transfected cells (not shown). D, summary of biotinylation experiments demonstrating that siRab11a decreased the plasma membrane expression of WT-CFTR and ΔF508-CFTR compared with the Non-sil. siRNA control (single asterisks). Furthermore, the amount of the plasma membrane ΔF508-CFTR was significantly lower than the amount of the plasma membrane WT-CFTR in the siRab11a-transfected cells (double asterisk), consistent with increased endocytosis of ΔF508-CFTR, in addition to the siRab11a-induced inhibition of the endocytic recycling. Asterisks indicate p < 0.05. 3 experiments/group.

FIGURE 10. Experiments performed to determine the effects of Rab11a mutants on the plasma membrane expression of ΔF508-CFTR in stably transduced CFBE41o – cells. A, summary of biotinylation experiments performed to determine the effects of the FLAG-Rab11a WT, the GTP-locked FLAG-Rab11a S20V, and the GDP-locked FLAG-Rab11a S25N on the plasma membrane expression of ΔF508-CFTR. The asterisk indicates p < 0.05. Three to four experiments were performed per group. B, representative immunoprecipitation experiment demonstrating that ΔF508-CFTR interacts with FLAG-Rab11a WT in CFBE41o – cells. CFBE41o – cells stably expressing ΔF508-CFTR were transiently transfected with FLAG-Rab11a WT. ΔF508-CFTR was immunoprecipitated using antibody M3A7, and the immunoprecipitated complexes were blotted with an antibody against FLAG-Rab11a WT (24 kDa). The last lane demonstrates that a non-immune IgG antibody failed to co-immunoprecipitate (IP) FLAG-Rab11a WT. The nonspecific bands marked with an arrow indicate the light chain of the immunoprecipitating antibody M3A7. Proteins were separated by SDS-PAGE using 15% gels. The experiment was repeated two times from separate cultures, with similar results.
ΔF508 mutation has no significant effect on endocytosis but profoundly inhibits the endocytic recycling of CFTR (25). Furthermore, another study in BHK-21 cells demonstrates that, unlike WT-CFTR, ΔF508-CFTR does not recycle to the plasma membrane unless the wild type Rab11a is overexpressed (24). By contrast, we report that the ΔF508 mutation does not affect the endocytic recycling of CFTR in polarized human airway epithelial cells. Furthermore, both WT-CFTR and ΔF508-CFTR undergo trafficking in the Rab11a-specific apical recycling compartment. Substantial evidence indicates that membrane trafficking events are cell type-specific (42–44, 92–94). Thus, it is not surprising that the endocytic trafficking of rescued ΔF508-CFTR differs in polarized human airway epithelial cells such as CFBE41o− cells and in non-polarized fibroblasts such as BHK-21 cells. Furthermore, differences in the trafficking itineraries via the Rab11-dependent pathway in epithelial and non-epithelial cells exist (84, 86, 95). Thus, we believe that the difference in endocytic recycling of rescued ΔF508-CFTR in CFBE41o− and heterologous cells likely represents a difference in the cellular mechanisms that regulate the endocytic trafficking of plasma membrane proteins in these cells.

We conducted several studies to determine why the ΔF508 mutation increased endocytosis of ΔF508-CFTR. The ΔF508 mutation did not produce a generalized defect in clathrin-mediated endocytosis or in fluid phase endocytosis and did not affect expression of Rab5a, an important regulator of clathrin-mediated and fluid-phase endocytosis, and did not affect expression of Rab5a, an important regulator of clathrin-mediated and fluid-phase endocytosis, and did not affect expression of Rab5a, an important regulator of clathrin-mediated and fluid-phase endocytosis, and did not affect expression of Rab5a, an important regulator of clathrin-mediated and fluid-phase endocytosis, and did not affect expression of Rab5a, an important regulator of clathrin-mediated and fluid-phase endocytosis, and did not affect expression of Rab5a, an important regulator of clathrin-mediated and fluid-phase endocytosis. Furthermore, ΔF508-CFTR in polarized cells undergo trafficking in the Rab11a-specific clathrin-mediated endocytic pathway. Additional studies beyond the scope of the present work are required to identify these mechanisms.

In summary, our data provide direct evidence that in human airway epithelial cells the ΔF508 mutation reduces the apical membrane half-life of rescued ΔF508-CFTR by specifically accelerating endocytosis without decreasing the endocytic recycling of ΔF508-CFTR. Moreover, the ΔF508 mutation does not disrupt the endocytosis of BCRP, another ABC transporter, or fluid phase endocytosis. Furthermore, our data demonstrate that both WT-CFTR and ΔF508-CFTR undergo trafficking in the Rab11a-specific, apical recycling compartment. It is tempting to speculate that proteins involved in the endocytosis and/or endocytic recycling of rescued ΔF508-CFTR may become a therapeutic target to increase the apical membrane expression of ΔF508-CFTR in polarized human airway epithelial cells.

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