The deubiquitinating enzyme USP10 regulates the endocytic recycling of CFTR in airway epithelial cells

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The Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) is a cyclic AMP-regulated chloride channel that plays an important role in regulating the volume of the lung airway surface liquid, and thereby mucociliary clearance and elimination of pathogens from the lung. In epithelial cells, cell surface CFTR abundance is determined in part by regulating both CFTR endocytosis from the apical plasma membrane and recycling back to the plasma membrane. We recently reported, using an activity-based chemical screen to identify active deubiquitinating enzymes (DUBs) in human airway epithelial cells, that Ubiquitin Specific Protease-10 (USP10) is located and active in the early endosomal compartment and regulates the deubiquitination of CFTR and thereby promotes its endocytic recycling. siRNA-mediated knockdown of USP10 increased the multi-ubiquitination and lysosomal degradation of CFTR and decreased the endocytic recycling and the half-life of CFTR in the apical membrane, as well as CFTR-mediated chloride secretion.1 Overexpression of wild-type USP10 reduced CFTR multi-ubiquitination and degradation, while overexpression of a dominant-negative USP10 promoted increased multi-ubiquitination and lysosomal degradation of CFTR. In the current study, we show localization and activity of USP10 in the early endosomal compartment of primary bronchial epithelial cells, as well as an interaction between CFTR and USP10 in this compartment. These studies demonstrate a novel function for USP10 in facilitating the deubiquitination of CFTR in early endosomes, thereby enhancing the endocytic recycling and cell surface expression of CFTR.

Cystic Fibrosis is a genetic disease caused by mutations in the CFTR chloride channel. ∆F508 is the most common mutation in CFTR that leads to disease, with over 75% of CF patients carrying at least one allele of the ∆F508 mutation in CFTR. Our group and others reported that the ∆F508 mutant CFTR has reduced residence time in the apical membrane and an increased endocytic rate, leading to decreased CFTR-mediated chloride secretion.1 Through its chloride secretion function, CFTR plays an important role in innate immunity by regulating the volume of the airway surface liquid, and thereby mucociliary clearance and elimination of pathogens from the lung. The decreased function of ∆F508 mutant CFTR disrupts mucociliary clearance and is thought to result in the persistent airway infections that afflict CF patients, ultimately leading to the death of the patient.2

In epithelial cells, cell surface CFTR is determined in part by regulating both CFTR endocytosis from the apical plasma membrane and recycling of CFTR back to the plasma membrane. The endocytosis of CFTR and subsequent recycling back to the plasma membrane in human airway epithelial cells is rapid and efficient, with >75% of endocytosed wild-type CFTR recycling back to the plasma membrane.1,3-5 Ubiquitination, the process of conjugating an 8 kDa ubiquitin moiety to a target protein, regulates the endocytosis, endocytic recycling and endosomal sorting of numerous transport

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Ubiquitin is conjugated to target proteins via a series of steps that includes ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes (E2), and ubiquitin ligases (E3). Ubiquitinated proteins in the plasma membrane are internalized and are either deubiquitinated to recycle back to the plasma membrane or, via interactions with the endosomal sorting complexes required for transport (ESCRT) machinery, are targeted for lysosomal degradation. The balance between ubiquitin ligation by E3 ligases and deubiquitination by deubiquitinating enzymes (DUB) regulates the plasma membrane abundance of numerous membrane proteins, including the epithelial sodium channel (ENaC), the epidermal growth factor receptor (EGFR), the transforming growth factor-β receptor (TGFβ-R), and the cytokine receptor γc.5-10

Previous studies by Sharma et al. demonstrated that addition of ubiquitin to the C-terminus of CFTR reduced the plasma membrane abundance of CFTR in BHK cells by redirecting CFTR from recycling endosomes to lysosomes for degradation.17 However, the E3 ubiquitin ligase(s) responsible for the ubiquitination of CFTR and the DUB(s) responsible for the deubiquitination of CFTR in the endocytic pathway have not been identified in any cell type. Because the human genome encodes approximately 100 DUBs, we chose an activity-based, chemical probe screening approach developed by Dr. Hidde Ploegh to identify DUBs that regulate the intracellular trafficking of CFTR. This approach utilizes a hemagglutinin (HA)-tagged ubiquitin probe engineered with a C-terminal modification incorporating a thiol-reactive group that forms an irreversible, covalent bond with active DUBs. Using this approach, we demonstrated that Ubiquitin Specific Protease-10 (USP10) is located in early endosomes and regulates the deubiquitination of CFTR and thus its trafficking in the post-endocytic compartment.

USP10 is a member of the ubiquitin specific protease (USP) domain family of DUBs, which is comprised of over 50 members, making it the largest family of DUBs.11,12 USP DUBs have a structurally conserved catalytic domain and variable N-terminal extensions that are thought to be responsible for modulating cellular localization and protein-protein interactions, thus resulting in a high specificity of USP DUBs for their target proteins.13-15 In our recent study, we reported a novel function for USP10 in promoting the deubiquitination of CFTR in early endosomes and thereby enhancing the endocytic recycling and cell surface expression of CFTR.16 We demonstrated, using biochemistry and confocal microscopy, that USP10 is enzymatically active in the early endosomal compartment of a polarized human airway epithelial cell line. Moreover, USP10 interacted with CFTR in the early endosomes, an important requirement for a DUB to regulate a target protein. siRNA-mediated gene knockdown of USP10 increased the amount of multi-ubiquitinated CFTR in the early endosomal compartment, leading to increased lysosomal degradation of CFTR, which decreased its apical membrane half-life. Moreover, studies verified that the increased degradation of CFTR in the lysosomes was due to a dramatic reduction in the endocytic recycling of CFTR and retargeting of CFTR from recycling endosomes to late endosomes and lysosomes. Mutational analysis studies, with a catalytically dead USP10, confirmed that the enzymatic activity of USP10 was required to maintain CFTR abundance in the apical membrane. Overexpression of USP10 lacking deubiquitinating activity increased the amount of multi-ubiquitinated CFTR, and the lysosomal degradation of CFTR. Taken together, these studies demonstrated a critical role for USP10 in determining the cell surface expression of CFTR by deubiquitinating CFTR and redirecting CFTR from lysosomes to the endocytic recycling pathway.

Here, we extend our previous study, conducted using a stably transformed airway epithelial cell line, by demonstrating that USP10 associates with CFTR in primary cultures of polarized human bronchial epithelial cells. Several groups have demonstrated that protein-protein interactions that regulate intracellular trafficking can differ between cell lines and primary cultures of epithelial cells.16-17 For this reason, it was critical to confirm the localization and interaction of USP10 with CFTR in primary bronchial epithelial cultures (HEB), to augment the relevance of the detailed biochemical analysis previously done in the bronchial epithelial cell line.

The presence of USP10 in early endosomes in primary cultures of human bronchial epithelial cells was demonstrated by two independent methods: biochemical and confocal microscopy. First, early endosomes were isolated as described in experimental procedures, and USP10 was identified in the early endosomal fraction by SDS-PAGE followed by western blot analysis (Fig. 1A). The isolation of early endosomes was confirmed by the presence of Rab5a and early endosome antigen-1 (EEA-1) (Fig. 1A), and by the lack of actin and lysosomal associated protein-1 (LAMP-1) labeling, which served as negative controls (Fig. 1A). In addition, immunofluorescence microscopy confirmed the expression of USP10 in early endosomes (Fig. 1B). The co-localization between USP10 and eGFP-labeled Rab5a, an early endosomal protein, was quantified by intensity correlation analysis using Nikon Elements Software. Pearson’s correlation coefficient confirmed the expression of USP10 in early endosomes. Pearson’s correlation coefficient is a number between -1 and +1 that measures the degree of pattern similarity between two fluorochromes (+1 indicates a complete positive correlation and -1 for a negative correlation, with zero indicating no correlation). A value of 0.42 ± 0.054 indicates a strong positive correlation between USP10 (red channel) and Rab5a (green channel). Mander’s overlap coefficient also ranges from 0 to 1, with 0 indicating low co-localization and 1 indicating high co-localization. Mander’s coefficient values are independent of the pixel intensities within respective channels. A Mander’s overlap coefficient of 0.60 ± 0.14 indicates a high level of overlap between USP10 (red channel) and Rab5a (green channel). While USP10 was localized to early endosomes, its localization was also detected in other subcellular compartments, as was seen in our previous studies in a bronchial epithelial cell line (data not shown). Thus, our biochemical and immunolocalization studies demonstrate that USP10 is pres-
Figure 1. USP10 is expressed and active in early endosomes of primary human bronchial epithelial (HBE) cells. (A) Early endosomes were isolated from HBE cells using a sucrose gradient. The isolation and purification of early endosomes was confirmed by western blot analysis. The isolated fractions contained the early endosomal antigen (EEA)-1 and Rab5a, which are located in early endosomes, but did not contain LAMP-1, a lysosomal protein, or actin, a cytoplasmic protein. The early endosome fraction was positive for USP10. Lysates (LYSATE), which contains cytoplasm, lysosomes and early endosomes, were positive for USP10, EEA-1, Rab5, LAMP-1 and actin. Rab11a and Rab7 protein were not detected in the early endosomal fraction (data not shown). (B) Representative confocal images of HBE cells infected with a baculovirus Rab5a-eGFP construct, which is expressed in early endosomes (green). USP10 was immunolocalized using an anti-USP10 antibody and an Alexa-568 secondary antibody (red). Infection with the baculovirus expressing the empty vector had no effect on cellular morphology, and fluorescence in the green channel in cells infected with this baculovirus was similar to background (data not shown). Scale bar equals 10 μm. (C) Early endosomes were isolated and incubated with the HA-UbVME probe to identify active DUBs. The HA-UbVME-DUB complex was immunoprecipitated with an anti-HA antibody, and the immunoprecipitated complex was analyzed by SDS-PAGE followed by western blot analysis using an anti-USP10 antibody, an anti-USP8 antibody or an anti-HA antibody (lane labeled HA-IP). Lane labeled LYSATE represent lysates of early endosomes blotted with the anti-USP10, the anti-USP8 or the anti-HA antibody. Although USP10 and USP8 were expressed in early endosomes, only USP10 was active as determined by the chemical probe technique. The non-immune IgG did not immunoprecipitate USP10, USP8 or HA-labeled complexes, and thus served as a negative control. Experiments were performed three times. Representative blots are shown.

Accordingly, USP10 was immunoprecipitated from the early endosomal fraction isolated from HBE cells, and the immunoprecipitated proteins were separated by SDS-PAGE, and western blots were probed for CFTR. Western blot analysis demonstrated that CFTR immunoprecipitates with USP10 (Fig. 2A). Thus, USP10 and CFTR interact in the early endosomal compartment of polarized primary human bronchial epithelial cells.

Figure 2. USP10 immunoprecipitates CFTR. HBE cells were lysed, USP10 was immunoprecipitated using an anti-USP10 antibody, and western blot analysis was performed for USP10 and CFTR. LYSATE, cell lysates (2.5% of lysate run on gel). USP10 IP indicates proteins that were immunoprecipitated using the USP10 antibody. The non-immune IgG did not immunoprecipitate USP10 or CFTR, and thus served as a negative control. Experiments were performed three times. Representative blots are shown.

ent in early endosomes in primary human airway epithelial cells.

To determine if USP10 is active in early endosomes of the primary cultures of human airway epithelial cells, we used the chemical probe screening approach described above. Briefly, the HA-UbVME probe was added to early endosomes, the HA-UbVME-DUB complex was immunoprecipitated with an anti-HA antibody, and western blots of the immunoprecipitated complex were blotted with an anti-USP10 antibody. The USP10 antibody detected a 110 kDa protein in the early endosomal fraction, demonstrating that USP10 is active in early endosomes (Fig. 1C). Although USP8 (also known as UBPY) was also identified in early endosomes, USP8 was not active as determined by the chemical probe assay and western blotting (Fig. 1C). These studies are consistent with the conclusion that USP10 is expressed and active in the early endosomal compartment of HBE in primary culture.

The ability of USP10 to deubiquitinate target proteins requires interaction between USP10 and its substrate.10,28 Thus, if USP10 regulates the deubiquitination of CFTR, we would predict that USP10 should interact with CFTR in the early endosomal compartment of HBE cells.
incubator at 37°C. To establish confluent, polarized monolayers, 0.5 x 10^6 cells were seeded onto 24-mm Transwell permeable supports (0.4-µm-pore size; Corning, Corning, NY) coated with Vitrogen coating medium containing human fibronectin (10 µg/ml; Collaborative Biomedical Products, Bedford, MA), Vitrogen 100 (1%; Collagen, Palo Alto, CA), and bovine serum albumin (10 µg/ml; Sigma-Aldrich) and grown in an air-liquid interface culture at 37°C for 21 days.

Identification of active DUBs. To examine the activity of USP10 in HBE cells we used a chemical probe screening approach described in detail by Dr. Hidde Ploegh. Briefly, cells were lysed in RIPA buffer (25 mM Tris-HCl pH 7.6, 10 mM NaCl, 1% NP-40 (IGEPAL), 1% sodium deoxycholate, 0.1% SDS), and 0.1 µg of the HA-UbVME probe was added to 20 µg of early endosomal fractions (isolated as described below) isolated from HBE cells. The HA-UbVME probe forms an irreversible, covalent bond with active DUBs. Identification of DUBs covalently linked to the HA-UbVME probe was achieved by immunoprecipitation of the HA-UbVME-DUB complex(s) using an anti-HA monoclonal antibody (Santa Cruz Biotechnology) followed by SDS-PAGE and western blot analysis using a specific anti-USP10 and USP-8 antibody (Bethyl Laboratories).

Isolation of early endosomes. To determine if USP10 is expressed in early endosomes, differential centrifugation and fractionation techniques were used to isolate early endosomes from HBE cells using a previously published protocol. Western blot analysis for various Rab GTPases was used to confirm purity of the early endosomal fraction. Rab5a and EEA-1 served as markers for the early endosomal fraction, whereas LAMP-1 and actin served as negative controls (Fig. 1A).

Immunoprecipitation. To determine if CFTR interacts with USP10 in early endosomal fractions, USP10 was immunoprecipitated from early endosomal fractions isolated from the HBE cell lysate by methods described previously in detail. USP10 was immunoprecipitated by incubation with 5 µg of a polyclonal USP10 antibody (Bethyl Laboratories) and protein A-agarose complex. Immunoprecipitated

endocytosed CFTR in the early endosome to promote recycling of CFTR back to the plasma membrane. CFTR that is not deubiquitinated in early endosomes is targeted for lysosomal degradation via late endosomal trafficking. The ability of USP10 to efficiently deubiquitinate CFTR in the early endosome allows for efficient endocytic recycling of CFTR and thus the long half-life of CFTR (approximately 8–24 h in polarized human airway cells) in the plasma membrane. Studies in progress are focused on identifying the E3 ligases and possibly other DUBs that determine the amount of ubiquitinated CFTR, and to elucidate the physiological and pathological factors that regulate the activity of these E3 ligases and DUBS in order to understand how cell surface CFTR abundance is regulated. Because the ΔF508 mutation in CFTR reduces the plasma membrane half-life of chemically rescued CFTR by negatively effecting the endocytic trafficking of CFTR, a better understanding of E3 ligases and DUBS may identify new targets for rational drug design to correct defective endocytic trafficking of ΔF508-CFTR in the treatment of Cystic Fibrosis.

Methods

Cell culture. The role of USP10 in the intracellular trafficking of CFTR was studied in primary human bronchial epithelial cells (HBE). HBE cells between passages 2 and 4 were maintained in bronchial epithelial cell growth media (BGEM), available commercially from Lonza (Walkersville, MD) in a 5% CO₂–95% air
proteins were eluted from the protein A-agarose complexes in Laemmeli sample buffer (Bio-Rad) containing 80 mM diithiothreitol. Immunoprecipitated proteins were separated by SDS-page using 7.5% Tris-HCl gels (Bio-Rad) and analyzed by western blot analysis.

**Confocal microscopy.** Co-localization studies were conducted to confirm western blot studies demonstrating that endogenous USP10 is expressed in early endosomes. Briefly, HBE cells seeded at 0.5 x 10^6 on collagen-coated, glass-bottom Mat-Tek dishes, were infected 24 h after seeding with a baculovirus expressing a Rab5a-eGFP plasmid (Organelle Lights™ Endosomes-GFP, Molecular Probes, Invitrogen), according to the manufacturer’s instructions, and fixed for immunolabeling 96 h post-infection, as described previously.20 The Organelle Lights baculovirus expression system was chosen to label early endosomes due to a lack of robust staining of endogenous Rab5a in the primary HBE cells. USP10 was visualized by indirect immunofluorescence using a polyclonal antibody for USP10 (Bethyl Laboratories, Montgomery, TX) followed by an Alexa 586-labeled secondary antibody. Z-stack images (0.4 µm sections) of labeled cells were acquired with a Nikon Swepfield confocal microscope (Apo TIRF 100x oil immersion 1.49 NA objective) fitted with a QuantEM-512sc camera (Photometrics, Tuscon, AZ) and Elements 2.2 software (Nikon, Inc.) to reconstruct and render 3D images. Experiments were repeated three times, with 5 fields imaged for each experiment.

**Antibodies and reagents.** The antibodies used were: mouse anti-human CFTR antibody (clone 596; purchased through Cystic Fibrosis Foundation, UNC Cystic Fibrosis Center); mouse anti-EEA1 antibody, mouse anti-ezrin antibody, mouse anti-Rab5 antibody, mouse anti-LAMP-1 antibody, mouse anti-actin antibody (BD Biosciences, San Jose, CA); rabbit anti-USP10 antibody, rabbit anti-USP8 (Bethyl Laboratories, Montgomery, TX); horseradish peroxidase-conjugated goat anti-mouse and goat anti-rabbit secondary antibodies (Bio-Rad, Hercules, CA). All antibodies and reagents were used at the concentrations recommended by the manufacturers or as indicated in the figure legends.

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