LMTK2-mediated Phosphorylation Regulates CFTR Endocytosis in Human Airway Epithelial Cells*

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Background: The CFTR-Ser737 site has a phospho-dependent inhibitory effect on Cl− secretion.

Results: LMTK2 phosphorylation of CFTR-Ser737 facilitates endocytosis, reduces cell surface density of CFTR, and inhibits Cl− secretion.

Conclusion: Targeting LMTK2 regulates the cell surface density of CFTR Cl− channels.

Significance: Targeting LMTK2 in CF patients may stabilize ΔF508-CFTR pharmacologically rescued to the cell surface.

Cystic fibrosis transmembrane conductance regulator (CFTR) is a Cl−-selective ion channel expressed in fluid-transporting epithelia. Lemur tyrosine kinase 2 (LMTK2) is a transmembrane protein with serine and threonine but not tyrosine kinase activity. Previous work identified CFTR as an in vitro substrate of LMTK2, suggesting a functional link. Here we demonstrate that LMTK2 co-immunoprecipitates with CFTR and phosphorylates CFTR-Ser737 in human airway epithelial cells. LMTK2 knockdown or expression of inactive LMTK2 kinase domain increases cell surface density of CFTR by attenuating its endocytosis in human airway epithelial cells. Moreover, LMTK2 knockdown increases Cl− secretion mediated by the wild-type and rescued ΔF508-CFTR. Compared with the wild-type CFTR, the phosphorylation-deficient mutant CFTR-S737A shows increased cell surface density and decreased endocytosis. These results demonstrate a novel mechanism of the phospho-dependent inhibitory effect of CFTR-Ser737 mediated by LMTK2 via endocytosis and inhibition of the cell surface density of CFTR Cl− channels. These data indicate that targeting LMTK2 may increase the cell surface density of CFTR Cl− channels and improve stability of pharmacologically rescued ΔF508-CFTR in patients with cystic fibrosis.

The cysic fibrosis transmembrane conductance regulator (CFTR), 3 a member of the ATP binding cassette (ABC) transporter superfamily functions as a cAMP-activated Cl−-selective ion channel in various fluid-transporting epithelia (1–3). CFTR is present in many tissues including the airway where it plays a critical role in regulating mucociliary clearance by maintaining the homeostasis of the airway surface liquid (4, 5). CFTR-mediated Cl− secretion across polarized epithelial cells is regulated by adjusting activity and density of the CFTR Cl− channels at the cell surface (6–10). The cell surface abundance of CFTR depends on its biosynthetic processing and post-maturational trafficking, a process of endocytic uptake followed either by recycling to the plasma membrane or trafficking for lysosomal degradation (reviewed in Ref. 11). Despite inefficient biosynthetic processing, CFTR abundance at the cell surface is maintained after rapid endocytosis via clathrin-dependent pathway because CFTR is efficiently recycled (12–15). Deletion of Phe-508 (ΔF508) resulting from the most common CFTR gene mutation in patients with cystic fibrosis (CF) leads to a biosynthetic processing defect of the CFTR protein (11). Pharmacological correction of the processing defect has been highly anticipated as a disease modifying therapy because rescue of the cell surface ΔF508-CFTR abundance partially restores the CFTR Cl− channel function (16). Several small molecules called CFTR correctors have been shown to rescue the cell surface expression of ΔF508-CFTR by improving its biosynthetic processing defect in cultured cells (16–19). Correction of the processing defect revealed that rescued (r)ΔF508-CFTR is only partially functional as a Cl− channel, in part because the mutation also alters the post-maturational trafficking and decreases the plasma membrane stability of rΔF508-CFTR (15, 20). The present generation of CFTR correctors does not improve the reduced plasma membrane stability of rΔF508-CFTR, and their clinical efficacy has been limited in patients homozygous for the ΔF508 mutation (16, 21). Hence, understanding regulation of the post-maturational trafficking of CFTR is critical to design effective treatments for CF. Although numerous adaptors have

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been shown to mediate the post-maturational trafficking of CFTR little is known how this process is regulated (7, 9, 10, 22–30).

The activity of CFTR as a Cl\(^{-}\) channel is regulated by protein kinases including protein kinase PKA, PKC, and adenosine monophosphate-stimulated kinase (AMPK) (reviewed in Ref. 31). These kinase target sites in the regulatory domain of CFTR (32, 33). Two of the sites, Ser\(^{737}\) and Ser\(^{68}\) have a phospho-dependent inhibitory effect on the CFTR channel function (32). Phosphorylation of these inhibitory sites by AMPK inhibits CFTR-mediated Cl\(^{-}\) secretion by maintaining the CFTR channels in a closed state (34, 35). Previous work demonstrated that the Ser\(^{737}\) site in the recombinant CFTR regulatory domain can be phosphorylated by purified lemur tyrosine kinase 2 (LMTK2) in vitro (36). Still, it is unknown whether CFTR is an LMTK2 substrate in airway epithelial cells.

LMTK2 also known as kinase/phosphatase/inhibitor-2 (KPI), brain-enriched kinase (BREK), apoptosis-associated tyrosine kinase (AATYK2), and cyclin-dependent kinase-5 (cdk5/p35) regulated kinase, is a member of the lemur family of membrane-anchored kinases (37–41). Despite the original prediction to be a dual-specificity serine-threonine/tyrosine kinase, studies have shown that purified LMTK2 kinase domain phosphorylates only serine and threonine residues (36, 37, 39).

The biological actions of LMTK2 are best described in neuronal and muscle tissues where it plays a role in intracellular trafficking (42–47). LMTK2 forms a regulatory complex with several cytosolic proteins (reviewed in Ref. 48). As shown schematically in Fig. 1A, the N-terminal transmembrane domain anchors LMTK2 at the plasma membrane and is followed by the kinase domain (37, 41). The residue Lys\(^{68}\) located upstream of the Walker A motif in the catalytic domain is critical for kinase activity (49, 50). The amino acid residues 567–773 mediate direct interaction with myosin VI (43), an actin-based minus-end directed non-conventional motor known to facilitate CFTR endocytosis (26). At the C terminus LMTK2 has a long tail domain that prompted naming the protein after the lemur, a long-tailed Madagascar primate (37). Some of the recognized LMTK2 effects may be cell-type and tissue specific. Thus far nothing is known about the role of LMTK2 in airway epithelial cells. The goals of this study were to determine the localization of LMTK2, whether it associates with and phosphorylates CFTR at residue Ser\(^{737}\) (CFTR-Ser\(^{737}\)) and whether the LMTK2-mediated phosphorylation regulates CFTR endocytic trafficking in human airway epithelial cells. We demonstrate that phosphorylation of CFTR-Ser\(^{737}\) mediated by LMTK2 facilitates endocytosis and reduces the cell surface density of CFTR Cl\(^{-}\) channels in human airway epithelial cells. Moreover, LMTK2 knockdown increases Cl\(^{-}\) secretion mediated by the wild-type (WT) and rAF508-CFTR. Thus, interfering with LMTK2 phosphorylation of ΔF508-CFTR-Ser\(^{737}\) may serve as a novel strategy to improve the cell surface stability of pharmacologically rescued ΔF508-CFTR in CF patients.

**MATERIALS AND METHODS**

**Cell Lines and Cell Culture**—Primary differentiated human bronchial epithelial (HBE) cells (WT-CFTR homozygous) were received from Dr. Joseph Pilewski (Cystic Fibrosis Research Center Epithelial Cell Core at the University of Pittsburgh School of Medicine, Pittsburgh, PA) (51–53). HBE cells were studied because they are considered a gold standard for CFTR research (54); however, these cells are not always available and cannot be passaged, so their use is severely limited. Cells were cultured on human placental collagen-coated Costar Transwell permeable supports (1.12 cm\(^{2}\) at a density \(\sim 7 \times 10^{5}\) cells/cm\(^{2}\) as previously described and used for experimentation following 6–8 weeks of culture at an air-liquid interface (53).

Human airway epithelial Calu-3 cells that express CFTR endogenously were obtained from the American Type Culture Collection (Manassas, VA). Cells were seeded on Transwell permeable supports (4.67 cm\(^{2}\) at density \(\sim 1 \times 10^{5}\) cells/cm\(^{2}\) coated with plating medium containing Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen, Carlsbad, CA) and 10% purified collagen (PureColTM; Advanced Biomatrix, San Diego, CA) and used for experimentation following 3–4 weeks of culture at an air-liquid interface as described previously (27). Calu-3 cells were studied because they express high endogenous levels of CFTR and LMTK2; however, Calu-3 cells are very difficult to transfect.

Human bronchial epithelial cells CBFE41o- stably expressing either WT-CFTR or ∆F508-CFTR cells and parental CBFE41o- (no CFTR transduction), were a generous gift from Dr. J. P. Clancy from the Department of Pediatrics, Cincinnati Children’s Hospital, Cincinnati, OH (15, 55). CBFE41o- cells were used because they are isogenic except for CFTR, and they are relatively easy to transfect. CBFE41o- cells express WT-CFTR and ∆F508-CFTR transgenes, thus, studies on these cells do not examine endogenous CFTR. However, stable expression of WT-CFTR in CBFE41o- cells is comparable to endogenous CFTR expression in Calu-3 and HBE cells (9, 26, 53), thus making it a good model of human airway epithelial cells to study regulated CFTR trafficking. CBFE41o- cells were seeded on Transwell permeable supports (4.67 cm\(^{2}\) at density \(\sim 1 \times 10^{5}\) cells/cm\(^{2}\) coated with plating medium and used for experimentation following 7–9 days of culture at an air-liquid interface as described previously (9, 10). CBFE41o- cells transfected with siRNA were seeded on collagen-coated plastic tissue culture plates (Corning Corporation) and cultured for 96 h to form monolayers. CBFE41o- cells transduced with shRNA were seeded on collagen-coated Snapwell or Transwell permeable supports (Corning Corporation) and cultured for 7–9 days to form polarized monolayers. CBFE41o- cells transfected with plasmid DNA were seeded on collagen-coated plastic tissue culture plates and cultured for 48 h to form monolayers. Fetal bovine serum (FBS) and the selection antibiotic were removed from the media 24 h before experiments to augment cell polarization and cell cycle synchronization (56, 57).

HEK293 cells from the American Type Culture Collection were transfected with plasmid DNA, seeded on plastic tissue culture plates, and used 24 h later for kinase assays. HEK293 cells were also used as a well-accepted model to express recombinant proteins and conduct biochemical studies including the kinase assay.

**Antibodies and Reagents**—The following anti-human CFTR antibodies were used: mouse monoclonal, clone 596 (Cystic Fibrosis Foundation Therapeutics, Inc.; Chapel Hill, NC) and...
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clone M3A7 (Millipore; Billerica, MA), and rabbit polyclonal Ab-737 (Assay Biotechnology Inc. San Francisco, CA). Other antibodies used were rabbit anti-LMTK2 (Sigma-Aldrich) and anti-LMTK2 kinase domain (Cocalico Biologicals Inc., Reamstown, PA), mouse anti-Na,K-ATPase (Millipore), anti-FLAG M2 (Sigma-Aldrich), and anti-ezrin (BD Biosciences, San Jose, CA), and horseradish peroxidase-conjugated goat anti-mouse, goat anti-rabbit, secondary antibodies (Bio-Rad). All antibodies were used at the concentrations recommended by the manufacturer. The following reagents were used: Complete Protease Inhibitor Mixture and PhosSTOP phosphatase inhibitor mixture tablets (Roche Applied Sciences, Indianapolis, IN), the adenylyl cyclase activator forskolin and the cAMP phosphodiesterase inhibitor IBMX (3-isobutyl-1-methylxanthine) (Sigma-Aldrich), and the inhibitor of protein serine/threonine phosphatases calyculin A (Cell Signaling Technology, Inc.; Danvers, MA).

Immunoprecipitation and Immunoblotting—Endogenous CFTR and LMTK2 were immunoprecipitated from Calu-3 or HBE cell lysates by methods described previously (10, 15, 27). Briefly, cultured cells were lysed in an immunoprecipitation (IP) buffer containing 150 mM NaCl, 50 mM Tris, pH 7.4, 1% IGEPA (Sigma-Aldrich), 5 mM MgCl$_2$, 5 mM EDTA, 1 mM EGTA, 30 mM NaF, 1 mM Na$_2$VO$_4$, and Complete Protease Inhibitor mixture (Roche Applied Sciences, Basel, Switzerland). After centrifugation at 14,000 x g for 15 min to pellet insoluble material, the soluble lysates were pre-cleared by incubation with protein G or protein A, as appropriate, conjugated to Sepharose beads (Pierce Chemical Co.) at 4°C. The pre-cleared lysates were added to the protein G- or protein A-Sepharose beads antibody complexes. CFTR was immunoprecipitated by incubation with the mouse M3A7 antibody and LMTK2 was immunoprecipitated by incubation with the rabbit anti-LMTK2 kinase domain antibody. Non-immune mouse or rabbit IgGs (DAKO North America, Inc., Carpinteria, CA) were used as controls. After washing the protein G- or protein A-Sepharose beads antibody complexes with the IP buffer, immunoprecipitated proteins were eluted by incubation at 85°C for 5 min in sample buffer (Bio-Rad) containing 100 mM DTT. Immunoprecipitated proteins were separated by SDS-PAGE using 7.5% gels (Bio-Rad) and analyzed by Western blotting. The immunoreactive bands were visualized with Western Lightning Chemiluminescence Reagent Plus (PerkinElmer LAS, Inc., Boston, MA).

RNA-mediated Interference—Transfection of CFBE41o- cells with siRNA targeting human LMTK2 gene (siLMTK2; Hs_LMTK2_6 siRNA; Qiagen, Valencia, CA) or the siRNA negative control (siCTRL; AllStars, Qiagen) was conducted using HiPerFect Transfection Reagent (Qiagen) according to the manufacturer’s instructions as we previously described (9, 10). For determination of the steady-state plasma membrane abundance of CFTR or CFTR endocytosis, CFBE41o- cells (1.0 × 10$^6$) were plated on collagen-coated tissue culture plates and incubated with the optimized transfection mixture containing 10 nM siRNA at 37°C. The transfection medium was removed after 24 h and cells were cultured on the tissue culture plates until confluent. Under these conditions cells reached confluence at 96 h, and experiments were conducted at 96 h. Silencing the target genes resulted in the corresponding protein depletion by ~70%. We aimed at such level of silencing to avoid off-target effects that may occur with more dramatic gene silencing.

For short-circuit recordings in Ussing-type chambers CFBE41o- cells (1.0 × 10$^6$) were plated on tissue culture plates and incubated with the optimized transfection mixture containing 50 nM siRNA at 37°C. After 24 h, cells were trypsinized and plated on collagen-coated Snapwell permeable supports and cultured for an additional 6 days to establish polarized monolayers (total 7 days in culture). All experiments were done under the same cell culture conditions to assure similar cellular polarization as well as protein expression and trafficking (10). LMTK2 knockdown under these conditions resulted in the corresponding protein depletion by ~70%.

Transduction of CFBE41o- cells with shRNAmir targeting the human LMTK2 gene (shLMTK2; V3LHS_345908 or V3LHS_638705) or shRNAmir negative control (RHS4348) in the lentiviral vector pGIPZ with TURBO-GFP reporter (Open Biosystems, Huntsville, AL) was carried at MOI 0.25 according to manufacturer’s instructions. Cells transduced with shRNA were selected with puromycin for 5 days, subcultured to collagen-coated Snapwell filters at 1.0 × 10$^6$ and cultured in air-liquid interface for 7–9 days to form polarized monolayers.

Plasmids and Transient Transfection—The WT-LMTK2Δ-FLAG plasmid was constructed by inserting part of the human LMTK2 sequence coding for the first 600 amino acid residues corresponding to the transmembrane and kinase domain with an engineered C-terminal FLAG into pcDNA3.1 vector (Invitrogen) as previously described (37). The human WT-CFTR was subcloned into pcDNA3.1 vector without a tag (WT-CFTR) (34). To construct the kinase-deficient KM-LMTK2Δ-FLAG fragment the WT-LMTK2Δ-FLAG cDNA was mutated to introduce the K168M substitution and to construct the phosphorylation-deficient CFTR-S737A mutant the WT-CFTR cDNA was mutated using the QuikChange™ II XL site-directed mutagenesis kit (Stratagene) and the KOD Hot Start Kit (Novagene, Darmstadt, Germany). Constructs were sequence verified by ABI PRISM dye terminator cycle sequencing (Applied Biosystems, Foster City, CA). Transfection of cells with plasmids was performed using FuGENE6 (Roche Diagnostics), according to the manufacturer’s instructions. CFBE41o- cells transfected with plasmid DNA were seeded on collagen-coated tissue culture plates until confluent. Under these conditions cells reached confluence at 48 h, and experiments were conducted at 48 h.

LMTK2 Kinase Assays—Kinase assays were performed as described previously (36). Briefly, HEK293 cells were transfected with WT-LMTK2Δ-FLAG, KM-LMTK2Δ-FLAG or empty pcDNA3.1 vector, and 24 h later, cells were lysed in RIPA buffer without sodium deoxycholate (1% (v/v) Triton X-100, 0.1% (w/v) SDS, 50 mM Tris, pH 7.4, and 150 mM NaCl). LMTK2 was immunoprecipitated with the anti-FLAG M2 antibody (Sigma) and protein G-agarose beads. The beads-antibody-protein complexes were washed three times with RIPA buffer and once with 2× kinase buffer (100 mM Hepes, pH 7.4, 40 mM MgCl$_2$, 20% glycerol, 0.02 mg/ml BSA, and 0.02% Brij 35). The kinase assay was performed by incubating the beads for 2 h at 30°C in a total reaction volume of 50 μl containing 1× kinase
buffer, 10 μM ATP, and 20 μM of a peptide corresponding to CFTR residues 733–741 (ERRSLSVPD). A control reaction was performed as above with the exclusion of cell lysates. Extent of phosphorylation was determined using the Kinase-Glo Luminescent Assay (Promega) that assesses the amount of ATP remaining after the reaction and normalized for the amount of ATP in the control reaction.

Biochemical Determination of the Plasma Membrane CFTR—

The determination of plasma membrane CFTR was performed by domain selective plasma membrane biotinylation in epithelial cell monolayers cultured on permeable growth supports or tissue culture plates using cell membrane impermeable EZ-Link™ Sulfo-NHS-LC-Biotin (Pierce Chemical Co.), followed by cell lysis in buffer containing 25 mM HEPES, pH 8.0, 1% Triton, 10% glycerol, and Complete Protease Inhibitor Mixture (Roche Applied Science), as described previously in detail (58, 59). Biotinylated CFTR was visualized by Western blotting using mouse monoclonal antibody clone 596 and an anti-mouse horseradish peroxidase antibody using the Western Lightning™ Plus-ECL detection system (Perkin Elmer Inc., Waltham, MA) followed by chemiluminescence. Biotinylated FLAG-tagged LMTK2 fragments were visualized by Western blotting using mouse monoclonal anti-FLAG M2 antibody. Quantification of biotinylated CFTR or the FLAG-tagged LMTK2 was performed by densitometry using exposures within the linear dynamic range of the film. As previously demonstrated with appropriate intracellular controls, under cell culture conditions used in our study only the cell surface proteins are accessible to biotin at 4 °C (9). Western blotting for an intracellular protein, such as ezrin was used as a quality control during each experiment to confirm absence of intracellular proteins in the biotinylated protein samples. Only experiments in which ezrin was not detected in the biotinylated protein samples were included for analysis.

Endocytic Assays—

Endocytic assays were performed in CFBE41o- cells, as described previously (10, 60). Briefly, the plasma membrane proteins were first biotinylated at 4 °C using cell membrane impermeable and cleavable EZ-Link™ Sulfo-NHS-SS-Biotin (Pierce Chemical Co.). Cells were rapidly warmed to 37 °C for different periods of time after biotinylation and, subsequently, the disulfide bonds on Sulfo-NHS-SS-biotinylated proteins remaining at the plasma membrane were reduced by 1,4-glutathione (GSH; Sigma-Aldrich) at 4 °C. At this point in the protocol, biotinylated proteins reside within the endosomal compartment. Subsequently, cells were lysed, and biotinylated proteins were isolated by streptavidin-agarose beads, eluted into SDS-sample buffer, and separated by 7.5% SDS-PAGE. The amount of biotinylated CFTR at 4 °C and without the 37 °C warming was considered 100%. The amount of biotinylated CFTR remaining at the plasma membrane after GSH treatment at 4 °C and without the 37 °C warming was considered background (<7% compared with the amount of biotinylated CFTR at 4 °C without GSH treatment) and was subtracted from the CFTR biotinylated after warming to 37 °C at each time point. CFTR endocytosis was calculated after subtraction of the background and was expressed as the percent of biotinylated CFTR at each time point after warming to 37 °C compared with the amount of biotinylated CFTR present before warming to 37 °C. We have previously shown that the culture conditions and state of epithelial cell polarization affect CFTR endocytosis in human airway epithelial cells (9, 10, 26, 27). Because endocytic assays were performed in cells cultured on permeable supports or on plastic and in cells transfected with siRNA or plasmid DNA, we determined the time course of CFTR endocytosis for each cell culture and experimental condition and compared with appropriate controls.

Short-circuit Recordings—

The short circuit currents (Isc) were measured in Ussing-type chambers (Physiological Instruments; San Diego, CA) as previously described (10, 61). In brief, monolayers of CFBE41o- cells grown on Snapwell permeable supports were mounted in an Ussing-type chamber (Physiologic Instruments) and bathed in solutions (see below) maintained at 37 °C and stirred by bubbling with 5% CO2/95% air. Short circuit current (Isc) was measured by voltage-clamping the transepithelial voltage across the monolayers to 0 mV with a voltage/current clamp (model VCC MC8, Physiologic Instruments). Transepithelial resistance was measured by periodically applying a 2.5-mV bipolar voltage pulse and was calculated using Ohm’s law. The apical bath solution contained (in mM): 115 Na-glucuronate, 5 NaCl, 25 NaHCO3, 3.3 KH2PO4, 0.8 K2HPO4, 1.2 MgCl2, 1.2 CaCl2, 10 mannitol (pH 7.4). The basolateral bath solution contained (in mM): 120 NaCl, 25 NaHCO3, 3.3 KH2PO4, 0.8 K2HPO4, 1.2 MgCl2, 1.2 CaCl2, 10.0 glucose (pH 7.4). A low Cl–, high-Na+, high-glucuronate, apical bath solution was used to prevent cell swelling due to the increased apical Cl– permeability under these conditions, as previously described (10, 61). Following an equilibration period, the baseline Isc was recorded. Amiloride (10 μM) was added to the apical bath solution to inhibit Na+ absorption through ENaC. Subsequently, Isc was stimulated with forskolin (10 μM) and IBMX (50 μM) added to the apical and basolateral bath solutions followed by thiazolidonone CFTR inhibitor CFTRinh-172 (5 μM) added to the apical bath solution to inhibit CFTR-mediated Isc. Data are expressed as forskolin/IBMX stimulated Isc, calculated by subtracting the baseline Isc from the peak stimulated Isc.

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). The means were compared by a two-tailed t test. A p value <0.05 was considered significant. Data are expressed as mean ± S.E.

RESULTS

LMTK2 Localizes at the Plasma Membrane in Primary Differentiated Human Bronchial Epithelial Cells—Little is known about the distribution of LMTK2 in human airway epithelial cells. If LMTK2 affects CFTR, it would be expected that it localized, like CFTR, at the apical plasma membrane. Thus, we first examined localization of LMTK2 in primary differentiated HBE cells by domain-selective cell surface biotinylation and Western blotting (53). HBE cells were cultured for 6–8 weeks in air-liquid interface as polarized monolayers. LMTK2 was detected at both the apical and basolateral membrane domain (Fig. 1B). Next, we examined localization of LMTK2 in CFBE41o- cells, an immortalized human airway epithelial cell model because unlike HBE these cells are constantly available. Cells were cul-
tured for 7–9 days in air-liquid interface as polarized monolayers. LMTK2 was detected at both the apical and basolateral membrane domain of similar to HBE cells (Fig. 1 C).

**Endogenous CFTR and LMTK2 Co-immunoprecipitate in Polarized Human Airway Epithelial Cells**—We predicted that LMTK2 could interact with CFTR in polarized human airway epithelial cells. We used Calu-3 cells to study immunoprecipitation because these cells express both CFTR and LMTK2 endogenously, form polarized monolayers, and are constantly available as immortalized cells. Calu-3 cells were cultured for 3–4 weeks on semi-permeable growth supports as polarized monolayers. CFTR was immunoprecipitated with a monoclonal anti-CFTR antibody (M3A7), and LMTK2 was immunoprecipitated with a polyclonal anti-LMTK2 antibody. Western blot analysis demonstrated that endogenous CFTR and LMTK2 co-immunoprecipitate in a reciprocal fashion (Fig. 1, D and E). Taken together, the co-immunoprecipitation between endogenous CFTR and LMTK2 and their plasma membrane distribu-
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LMTK2 Knockdown Increases Plasma Membrane Abundance of CFTR in Human Airway Epithelial Cells—We hypothesized that LMTK2 controls CFTR trafficking at the plasma membrane in human airway epithelial cells. To test this hypothesis, LMTK2 expression was knocked down in CFBE41o-cells stably expressing WT-CFTR by RNA interference. We used CFBE41o-cells because these immortalized cell lines localize LMTK2 at the plasma membrane similar to the primary HBE cells as shown in Fig. 1. Moreover CFBE41o-cells serve as good models for CFTR trafficking (9, 10, 15, 61, 62). Cells were transfected with siRNA specific for the human LMTK2 sequence (siLMTK2) or with a siRNA control (siCTRL) and cultured on collagen-coated tissue culture plates for 96 h as monolayers (Fig. 2). The siLMTK2 reduced LMTK2 protein abundance in whole cell lysates by ~70% without decreasing the levels of CFTR or ezrin (Fig. 2A). As determined by cell surface biotinylation and Western blotting, knockdown of LMTK2 increased the steady-state plasma membrane abundance of CFTR (Fig. 2, A and B).

LMTK2 Knockdown Increases the CFTR-mediated I sc Across Polarized Human Airway Epithelial Cells—CFTR mediated Cl− secretion is determined by the activity and number of CFTR Cl− channels at the plasma membrane. Because LMTK2 knockdown increased the plasma membrane abundance of CFTR, we analyzed the functionality of the rescued fraction, predicting that it would also increase CFTR-mediated Cl− secretion. siLMTK2 increased the forskolin/IBMX-stimulated I sc across CFBE41o-monolayers (Fig. 3). These results are consistent with the above data that siLMTK2 increased CFTR abundance at the plasma membrane and suggest that endogenous LMTK2 decreases CFTR-mediated Cl− secretion, at least in part by decreasing density of the CFTR Cl− channels at the plasma membrane in human airway epithelial cells.

LMTK2 Knockdown Decreases CFTR Endocytosis in Human Airway Epithelial Cells—Increased CFTR abundance at the plasma membrane could result from decreased endocytosis or increased recycling of CFTR. Endocytic assays were conducted to determine the mechanism of increased CFTR abundance at the plasma membrane due to LMTK2 knockdown. CFBE41o-cells stably expressing WT-CFTR were transfected with siRNA specific for human LMTK2 sequence (siLMTK2) or with a siRNA control (siCTRL) and cultured on collagen-coated tissue culture plates for 96 h as monolayers. Endocytic assays were performed by the GSH protection assay (10, 60). As illustrated in Fig. 4, siLMTK2 did not change the transepithelial resistance across the monolayers (C), * p < 0.05 versus siCTRL. 9 monolayers/group from 2 different cultures. Error bars, S.E.

FIGURE 2. Biotinylation experiments demonstrating that LMTK2 knockdown increases CFTR abundance at the plasma membrane in CFBE41o-cells. Immunoblots (A) and summary of experiments (B). CFBE41o-cells stably expressing WT-CFTR were transfected with 10 nm siRNA specific for LMTK2 (siLMTK2) or the siRNA-negative control (siCTRL) and cultured on collagen-coated tissue culture plates for 96 h to form monolayers. LMTK2 abundance in whole cell lysates (WCL) was reduced by siLMTK2 to 30.0 ± 6.7% (p < 0.05 versus siCTRL, n = 5, mean ± S.E.). By contrast siLMTK2 did not change the abundance of ezrin. siLMTK2 increased the steady-state plasma membrane (PM) abundance of CFTR without affecting WCL CFTR indicating that LMTK2 affects the post-maturational trafficking of CFTR at the plasma membrane without affecting its biosynthetic processing. Plasma membrane proteins were isolated by cell surface biotinylation, and the plasma membrane CFTR was normalized for WCL ezrin. Ezrin expression was used as a loading control. WCL represents 5% of BT sample. *, p < 0.05 versus siCTRL. Five experiments/group. Error bars, S.E.

FIGURE 3. Using chamber experiments demonstrating that LMTK2 knockdown increases CFTR mediated I sc across CFBE41o-monolayers. CFBE41o-cells stably expressing WT-CFTR were plated on tissue culture plates and incubated with the optimized transfection mixture containing 50 nm of siRNA specific for LMTK2 (siLMTK2) or the siRNA negative control (siCTRL). After 24 h, cells were trypsinized and plated on collagen-coated Snapwell permeable supports and cultured for an additional 6 days to establish polarized monolayers (total 7 days in culture). CFBE41o-cells were bathed in solutions with apical-to-basolateral Cl− gradient in the presence of amiloride (Amilo, 50 μM) in the apical bath solution to inhibit Na+ absorption through ENaC. I sc was stimulated with forskolin (FSK, 20 μM) and IBMX (50 μM) added to the apical and basolateral bath solution. Thiazolidone nonoh CFTRinh-172 (5 μM) was added to the apical bath solution to inhibit CFTR-mediated I sc. Data are expressed as net stimulated I sc, calculated by subtracting the baseline I sc from the peak stimulated I sc. siCTRL did not affect the forskolin/IBMX-stimulated I sc across CFBE41o-cells compared with the non-transfected cells (data not shown). Representative experiment (A) and summary of data (B) demonstrating that LMTK2 knockdown increased the forskolin/IBMX-stimulated I sc across CFBE41o-cells. siLMTK2 did not change the transepithelial resistance across the monolayers (C). *, p < 0.05 versus siCTRL. 9 monolayers/group from 2 different cultures. Error bars, S.E.
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**FIGURE 4.** Endocytic assays demonstrating that LMTK2 knockdown decreased CFTR endocytosis in CFBE41o-. Cells, CFBE41o- cells stably expressing WT-CFTR were transfected with 10 nM siLMTK2 or the siRNA control (siCTRL) and cultured on collagen-coated tissue culture plates for 96 h to form monolayers. We previously showed that siCTRL does not affect CFTR endocytosis under similar cell culture conditions (10). The plasma membrane proteins were first biotinylated at 4 °C using cell membrane impermeable and cleavable EZ-Link™ Sulfo-NHS-SS-Biotin (Biotin). Cells were rapidly warmed to 37 °C for different periods of time after biotinylation, and, subsequently, the disulfide bonds on Sulfo-NHS-SS-biotinylated proteins remaining at the plasma membrane were reduced by l-glutathione (GSH) at 4 °C. At this point in the protocol, biotinylated proteins reside within the endosomal compartment. The amount of biotinylated CFTR remaining after the GSH treatment at 4 °C without warming to 37 °C was considered background and was subtracted from the amount of biotinylated CFTR remaining after warming to 37 °C at each time point. CFTR endocytosis was calculated after subtraction of the background and was expressed as the percent of CFTR remaining biotinylated before and after warming to 37 °C. Immunoblots (A) and summary of experiments (B) demonstrating that siLMTK2 decreased CFTR endocytosis. Please, note the increased abundance of CFTR before endocytosis (A, the first lane) in siLMTK2 cells compared with siCTRL consistent with the increased plasma membrane abundance of CFTR at steady-state demonstrated in Fig. 2. Ezrin expression in WCL was used as a loading control (data not shown). *, p < 0.05 versus siCTRL. A–6 experiments/group. Error bars, S.E.

CFTR phosphorylation was greatly enhanced after treating cells with calyculin A (50 nM) added to the basolateral medium at 37 °C for 10 min before experiments to raise the intracellular cAMP levels and promote PKA-mediated phosphorylation (34). To further enhance phosphorylation of CFTR we treated cells with the cell-permeable phosphatase inhibitor calyculin A (50 nM), added to the basolateral medium at 37 °C for 15 min before experiments (63, 64). Low levels of CFTR were detected by the phosphosite specific antibody Ab-737 in the vehicle control (CTRL)-treated cells, representing baseline levels of CFTR phosphorylated at Ser737 (CFTR-pS737; Fig. 5A). By contrast, CFTR phosphorylation was greatly enhanced after the forskolin/IBMX and calyculin A treatment (Fig. 5A). To demonstrate specificity of the phosphosite antibody, parental CFBE41o- cells were transiently transfected with WT-CFTR or CFTR with the S737A substitution (CFTR-S737A) inactivating the phosphorylation site (31). Even with calyculin A treatment of the cells, the CFTR-S737A mutant was not detected by antibody Ab-737, unlike the WT-CFTR (Fig. 5B). Together, these results demonstrate that the phosphosite antibody Ab-737 recognizes specifically the CFTR-pS737.

LMTK2 was shown to phosphorylate CFTR Ser737 in vitro (36). We assessed the effects of altering the LMTK2 kinase on CFTR phosphorylation in CFBE41o-. The residue Lys168 located upstream of the Walker A motif in the catalytic domain of LMTK2 is predicted to be critical for catalysis (49, 50). Site-directed mutagenesis was used to produce the K168M substitution in LMTK2 kinase domain. We expressed an LMTK2 fragment containing the first 600 amino acids including the transmembrane and kinase domain with an engineered C-terminal FLAG epitope tag (WT-LMTK2Δ-FLAG) and confirmed it was expressed at the plasma membrane, similar to endogenous LMTK2 (data not shown). Next, we expressed the K168M version (KM-LMTK2Δ-FLAG). Expressing the truncated LMTK2 fragments WT-LMTK2Δ-FLAG and KM-LMTK2Δ-FLAG in human airway epithelial cells eliminated the interactions mediated by the myosin V1 binding domain and tail domain in the full-length LMTK2 and thus, allowed us to spe-
These results confirm the prediction that the residue Lys168 noblotting with anti-FLAG antibody demonstrates similar expression of the LMTK2 fragments. *, antibody CFF596. CFTR-pS737 abundance was decreased in antibody Ab-737 was normalized for total CFTR detected with antibody CFF596. The WT-LMTK2-FLAG fragment did not increase the WCL CFTR-pS737 compared with vector control. By contrast, the KM-LMTK2-FLAG mutant decreased CFTR-pS737 in WCL compared with vector control or the WT-LMTK2-FLAG. Immunoblotting with anti-FLAG antibody demonstrates similar expression of the LMTK2 fragments.

specifically examine effects mediated by the LMTK2 transmembrane and kinase domain.

To ascertain that the K168M mutation impairs LMTK2 kinase activity, we transfected HEK293 cells with the WT-LMTK2-FLAG and KM-LMTK2-FLAG or empty pcDNA3.1 vector and the resulting protein fragments were immunoprecipitated with anti-FLAG M2 antibody. The immunoprecipitated protein complexes were used to in vitro phosphorylate a peptide encompassing CFTR amino acids 733–741. The extent of phosphorylation was determined using the Kinase-Glo Luminescent Assay that assesses the amount of ATP remaining after the reaction normalized for the amount of ATP in the control reaction. The ATP level decreased in the reaction containing the WT-LMTK2-FLAG indicating that LMTK2 phosphorylated the CFTR-derived peptide. By contrast, ATP levels were similar in the reactions containing either the KM-LMTK2-FLAG or vector control indicating that the K168M mutation decreased phosphorylation of the CFTR derived peptide. Immunoblots (C) and summary of data (D) demonstrating that the kinase deficient LMTK2 fragment KM-LMTK2-FLAG (KM) decreases CFTR-pS737 abundance in whole cell lysate (WCL) of CFBE41o-cell stably expressing WT-CFTR. Forty-eight hours after transfected the WT-LMTK2-FLAG (WT), KM-LMTK2-FLAG (KM), or vector control (V) cells were treated with calyculin A and the WCL abundance of CFTR-pS737 detected with phosphosite antibody Ab-737 was normalized for the total CFTR detected with antibody CFF596. The WT-LMTK2-FLAG fragment did not increase the WCL CFTR-pS737 compared with vector control. Therefore, the KM-LMTK2-FLAG mutant decreased CFTR-pS737 in WCL compared with vector control or the WT-LMTK2-FLAG. Immunoblotting with anti-FLAG antibody demonstrates similar expression of the LMTK2 fragments.

Next, we assessed phosphorylation of CFTR-Ser737 using the phosphosite antibody Ab-737. CFBE41o-cells stably expressing WT-CFTR were transfected with WT-LMTK2-FLAG, KM-LMTK2-FLAG, or vector control (V). Forty-eight hours after transfection cells were treated with calyculin A (50 nM) added to the basolateral medium at 37 °C for 15 min before experiments. CFTR-pS737 detected by immunoblotting with antibody Ab-737 was normalized for total CFTR detected with antibody CFF596. CFTR-pS737 abundance was decreased in cells expressing KM-LMTK2-FLAG compared with WT-LMTK2-FLAG or vector control (Fig. 6, C and D). Our conclusion was that the LMTK2 fragment with inactive kinase KM-LMTK2-FLAG attenuated phosphorylation of CFTR-Ser737 because it competed with endogenous LMTK2 for phosphorylation of the CFTR-Ser737 site in CFBE41o-cells.

LMTK2-mediated Phosphorylation of CFTR Facilitates Endocytosis and Attenuates CFTR Abundance at Plasma Membrane—The above data have shown that LMTK2 facilitates CFTR endocytosis and that LMTK2 phosphorylates CFTR-Ser737 in human airway epithelial cells. Studies were conducted to determine whether CFTR endocytosis is regulated by the LMTK2 phosphorylation of CFTR-Ser737. Endocytic assays were performed to examine the effects of WT-LMTK2-FLAG and KM-LMTK2-FLAG on CFTR endocytosis. We determined the time course of CFTR endocytosis in CFBE41o-cells stably expressing WT-CFTR cultured for 48 h as monolayers on collagen-coated tissue culture plates. CFTR endocytosis reached maximum at 7.5 min (Fig. 7, A and B). Thus, in subsequent experiments we examined CFTR endocytosis at the 7.5-min time point. Differences in the magnitude and maximum of CFTR endocytosis in CFBE41o-cells cultured as monolayers on tissue culture plates in Fig. 7 are consistent with the knock effects of epithelial cell culture conditions on endocytic trafficking (65–67). CFBE41o-cells stably expressing WT-CFTR cultured for 48 h as monolayers on collagen-coated tissue culture plates. CFTR endocytosis reached maximum at 7.5 min (Fig. 7, A and B). Thus, in subsequent experiments we examined CFTR endocytosis at the 7.5-min time point. Differences in the magnitude and maximum of CFTR endocytosis in CFBE41o-cells cultured as monolayers on tissue culture plates in Fig. 7 are consistent with the knock effects of epithelial cell culture conditions on endocytic trafficking (65–67). CFBE41o-cells stably expressing WT-CFTR cultured for 48 h as monolayers on collagen-coated tissue culture plates. CFTR endocytosis reached maximum at 7.5 min (Fig. 7, A and B). Thus, in subsequent experiments we examined CFTR endocytosis at the 7.5-min time point. Differences in the magnitude and maximum of CFTR endocytosis in CFBE41o-cells cultured as monolayers on tissue culture plates in Fig. 7 are consistent with the knock effects of epithelial cell culture conditions on endocytic trafficking (65–67). CFBE41o-cells stably expressing WT-CFTR cultured for 48 h as monolayers on collagen-coated tissue culture plates. CFTR endocytosis reached maximum at 7.5 min (Fig. 7, A and B). Thus, in subsequent experiments we examined CFTR endocytosis at the 7.5-min time point. Differences in the magnitude and maximum of CFTR endocytosis in CFBE41o-cells cultured as monolayers on tissue culture plates in Fig. 7 are consistent with the knock effects of epithelial cell culture conditions on endocytic trafficking (65–67). CFBE41o-cells stably expressing WT-CFTR cultured for 48 h as monolayers on collagen-coated tissue culture plates. CFTR endocytosis reached maximum at 7.5 min (Fig. 7, A and B). Thus, in subsequent experiments we examined CFTR endocytosis at the 7.5-min time point. Differences in the magnitude and maximum of CFTR endocytosis in CFBE41o-cells cultured as monolayers on tissue culture plates in Fig. 7 are consistent with the knock effects of epithelial cell culture conditions on endocytic trafficking (65–67). CFBE41o-cells stably expressing WT-CFTR cultured for 48 h as monolayers on collagen-coated tissue culture plates. CFTR endocytosis reached maximum at 7.5 min (Fig. 7, A and B). Thus, in subsequent experiments we examined CFTR endocytosis at the 7.5-min time point. Differences in the magnitude and maximum of CFTR endocytosis in CFBE41o-cells cultured as monolayers on tissue culture plates in Fig. 7 are consistent with the knock effects of epithelial cell culture conditions on endocytic trafficking (65–67). CFBE41o-cells stably expressing WT-CFTR cultured for 48 h as monolayers on collagen-coated tissue culture plates. CFTR endocytosis reached maximum at 7.5 min (Fig. 7, A and B). Thus, in subsequent experiments we examined CFTR endocytosis at the 7.5-min time point. Differences in the magnitude and maximum of CFTR endocytosis in CFBE41o-cells cultured as monolayers on tissue culture plates in Fig. 7 are consistent with the knock effects of epithelial cell culture conditions on endocytic trafficking (65–67). CFBE41o-cells stably expressing WT-CFTR cultured for 48 h as monolayers on collagen-coated tissue culture plates. CFTR endocytosis reached maximum at 7.5 min (Fig. 7, A and B). Thus, in subsequent experiments we examined CFTR endocytosis at the 7.5-min time point. Differences in the magnitude and maximum of CFTR endocytosis in CFBE41o-cells cultured as monolayers on tissue culture plates in Fig. 7 are consistent with the knock effects of epithelial cell culture conditions on endocytic trafficking (65–67).
KM-LMTK2Δ-FLAG-transfected cells. Because expression of the WT-LMTK2Δ-FLAG did not increase phosphorylation of CFTR (Fig. 6, C and D) these conditions were not expected to alter endocytosis or the plasma membrane abundance of CFTR compared with vector control. Indeed, the WT-LMTK2Δ-FLAG had no effect on endocytosis or the plasma membrane abundance of CFTR compared with vector control (Fig. 7, C--E). Together, the above results demonstrate that the LMTK2 phosphorylation of CFTR-Ser737 facilitates CFTR endocytosis and reduces CFTR abundance at the plasma membrane.

Nothing is known about membrane trafficking of LMTK2 in human airway epithelial cells. Thus, to gain insight, we examined the plasma membrane abundance and endocytic uptake of the LMTK2 fragments in CFBE41o- cells. Approximately 70% of the WT-LMTK2Δ-FLAG was internalized in 7.5 min (Fig. 7, C and F). By contrast, internalization of the KM-LMTK2Δ-FLAG was decreased by 50%. Compared with the WT-LMTK2Δ-FLAG, the KM-LMTK2Δ-FLAG fragment was more abundant at the plasma membrane due to decreased endocytosis. Endocytosis of the FLAG-tagged fragments was calculated as described for CFTR. The WCL expression of WT-LMTK2Δ-FLAG or KM-LMTK2Δ-FLAG was used, when appropriate as a loading control to account for small differences in the expression levels of the transiently transfected protein fragments. *, p < 0.05 versus vector control (V). **, p < 0.05 versus WT-LMTK2Δ-FLAG.

3–4 experiments/group. Error bars, S.E.
DISCUSSION

The present study discovered that in human airway epithelial cells CFTR endocytosis is regulated by the LMTK2-mediated phosphorylation of CFTR-Ser737 that decreases the cell surface density of CFTR Cl− channels and inhibits CFTR-mediated Cl− secretion. The clinical implications of our findings are that regulating LMTK2 phosphorylation of CFTR may serve as a novel approach to stabilize CFTR at the cell surface. This approach may be particularly useful to stabilize pharmacologically rescued ΔF508-CFTR in CF patients.

Several lines of evidence support these conclusions. First, endogenous LMTK2 accumulated at the apical membrane domain of polarized human airway epithelial cells, including primary differentiated HBE cells and co-immunoprecipitated with CFTR, indicating that LMTK2 and CFTR physically associate within this membrane domain (Fig. 1). Second, inactivation by mutagenesis of the kinase activity in the truncated LMTK2 fragment KM-LMTK2Δ-FLAG inhibited phosphorylation of CFTR-Ser737 in human airway epithelial cells (Fig. 6). Compared with controls, LMTK2 knockdown or substitution by mutagenesis of the LMTK2 phosphorylation site in CFTR (CFTR-Ser737A) increased the steady-state plasma membrane abundance of CFTR and decreased its endocytosis (Figs. 2, 4, 8). Conversely, inactivation by mutagenesis of the LMTK2 kinase (KM-LMTK2Δ-FLAG) increased the steady-state plasma membrane abundance of CFTR and decreased CFTR endocytosis compared with the WT-LMTK2Δ-FLAG and endogenous LMTK2 (Fig. 7). Third, endogenous LMTK2 was sufficient to phosphorylate CFTR-Ser737 and facilitate CFTR endocytosis because when compared with vector control, expression of the LMTK2 fragment with intact kinase activity WT-LMTK2Δ-FLAG did not increase CFTR-Ser737 phosphorylation, or the steady-state plasma membrane abundance of CFTR and did not attenuate CFTR endocytosis (Figs. 6 and 7).

Studies have shown that LMTK2 interacts with multiple proteins, including myosin VI (reviewed in Ref. 48). We have previously demonstrated that myosin VI facilitates CFTR endocy-
tosis (26); and others have shown that myosin VI and LMTK2 regulate endocytic trafficking in other cell model systems (42, 43). Using the truncated LMTK2 fragments without the myosin VI binding domain and the tail domain allowed us to demonstrate for the first time that the cell surface density and endocytosis of CFTR are regulated by phosphorylation mediated by the LMTK2 kinase. Future studies may determine how the protein-protein interactions mediated by other LMTK2 domains influence the cell surface density and endocytosis of CFTR.

The phosphorylation of CFTR-Ser737 by PKA and AMPK inhibits CFTR channel function (32, 33). We do not know whether LMTK2 phosphorylation of CFTR-Ser737 inhibit the CFTR channel function in addition to reducing the cell surface density of CFTR. Similarly, the effects of AMPK and PKA phosphorylation of CFTR-Ser737 on the cell surface CFTR abundance are unknown. Hence, it remains to be determined whether the phospho-dependent inhibitory effects on CFTR-Ser737 site in human airway epithelial cells because similar to mutagenesis of the Ser737 site, LMTK2 knockdown produced a 2-fold increase in the cell surface abundance of CFTR and a 3-fold decrease in CFTR endocytosis (Figs. 2B versus 8C and Figs. 4B versus 4B, respectively). When compared with the LMTK2 knockdown, the kinase deficient LMTK2 fragment produced lesser effect on the cell surface abundance and endocytosis of CFTR (Figs. 7E versus 2B and Figs. 7D versus 4B, respectively). Our interpretation is that endogenous LMTK2 diminished the effects of the kinase deficient fragment when compared with LMTK2 knockdown. Increased AMPK activity was proposed to facilitate phosphorylation of Ser737 and inhibition of CFTR in non-stimulated epithelia (34, 35). Similarly, increased activity of LMTK2 at the apical surface is expected to inhibit CFTR-mediated Cl− secretion at least in part by attenuating the cell surface density of CFTR. The LMTK2 gene polymorphisms and the protein kinase cdk5/p35 modulate LMTK2 function (68, 69); although, their effects on CFTR are unknown. By contrast, cigarette smoke exposure and chronic obstructive pulmonary diseases (COPD) reduce CFTR function by poorly
defined mechanisms (reviewed in Ref. 70). Interestingly, cigarette smoke inhalation modifies the LMTK2 kinase domain. Because rescued ΔF508-CFTR has decreased plasma membrane stability we propose that targeting LMTK2 phosphorylation of CFTR may offer a novel approach to investigate the stability defect and to design pharmacological approaches to stabilize rescued ΔF508-CFTR. Our findings may also point to a new direction in elucidating the mechanisms of decreased CFTR function in lung diseases resulting from cigarette smoke inhalation.

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