Keratin K18 Increases Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) Surface Expression by Binding to Its C-terminal Hydrophobic Patch

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Conductance Regulator (CFTR) Surface Expression by Keratin K18 Increases Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) Surface Expression by Binding to Its C-terminal Hydrophobic Patch*

Background: CFTR function is tightly regulated by many interacting proteins.

Results: Intermediate filament protein keratin 18 increases the cell surface expression of CFTR by interacting with the C-terminal hydrophobic patch of CFTR.

Conclusion: K18 controls the function of CFTR.

Significance: These findings offer novel insights into the regulation of CFTR and suggest that K18 and its dimerization partner, K8, may be modifier genes in cystic fibrosis.

Malfunction of the cystic fibrosis transmembrane conductance regulator (CFTR) leads to cystic fibrosis, but the regulation of CFTR is not fully understood. Here, we identified the intermediate filament protein keratin K18 (K18) as a CFTR-binding protein by various approaches. We mapped a highly conserved “hydrophobic patch” (1411FLV1416) in the CFTR C-terminus, known to determine plasmalemmal CFTR stability, as the K18-binding site. On the other hand, the C-terminal tail of K18 was found to be a critical determinant for binding CFTR. Overexpression of K18 in cells robustly increased the surface expression of wild-type CFTR, whereas depletion of K18 through RNA interference specifically diminished it. K18 binding increased the surface expression of CFTR by accelerating its apical recycling rate without altering CFTR biosynthesis, maturation, or internalization. Importantly, CFTR surface expression was markedly reduced in duodenal and gallbladder epithelia of K18−/− mice. Taken together, our results suggest that K18 increases the cell surface expression of CFTR by interacting with the CFTR C-terminal hydrophobic patch. These findings offer novel insights into the regulation of CFTR and suggest that K18 and its dimerization partner, K8, may be modifier genes in cystic fibrosis.

The lethal genetic disease cystic fibrosis (CF)7 is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR), a channel protein responsible for anion transport in various epithelial cells. Over the past decade the molecular control of CFTR channel function and cell surface localization has been investigated extensively, and these studies have shown that the C terminus of human CFTR associates with a number of proteins. These include AMP-activated protein kinase (AMPK), which binds to CFTR-(1420–1457), and protein phosphatase 2A (PP2A), which interacts with CFTR-(1451–1476) for negative modulation of channel gating (1, 2); the endocytic adaptor protein 2 (AP2), which binds to CFTR-(1424–1427) and influences its endocytosis (3, 4); and several PDZ proteins that bind to CFTR-(1477–1480) and affect various functions (5–7). The importance of these regulatory proteins in vivo, however, remains to be established. Interestingly, clinical studies suggest that the deletion of the CFTR C-terminal 26 residues by the S1455X mutation elevates the chloride concentration in sweat without producing any other CF symptoms, whereas the deletion of the C-terminal 69 residues by the Q1412X mutation results in severe CF (8–10). This implies that amino acid residues 1412–1455 are important for the positive modulation of CFTR function in vivo. In agreement, ectopic expression experiments in BHK and COS-1 cells show that the deletion of this region in CFTR, particularly the highly con-
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served hydrophobic patch of amino acid residues 1413–1416 (FLVI), drastically reduce the stability and surface expression of mature CFTR without affecting its biosynthesis, maturation, and chloride channel function (9, 11). Such impact, however, cannot be properly accounted for by the regulatory proteins known to bind to this region (residues 1412–1455), including AP2, AMPK, and PP2A, because these proteins negatively regulate CFTR surface expression or channel gating. Thus, as yet unidentified proteins may bind to this region of CFTR and positively regulate its expression.

Type I and type II keratins, which make up the intermediate filament cytoskeleton of all epithelia, are expressed in pairs with a specific tissue distribution (12–14). They form intermediate filaments from obligate heterodimers, and the down-regulation of one keratin protein often suppresses the expression of its partner. Simple or single-layer epithelia express predominantly K18 (type I) and its pairing partner K8 (type II), as well as sometimes type II K7 and type I K19, K20, and K23 (15). A longstanding view has been that keratins merely protect epithelial cells against mechanical stress. However, an emerging body of evidence suggests that K18/K8 may act as scaffold proteins, regulating the activity of numerous protein kinases and determining the subcellular localization of other proteins (16–19). Supporting this view, we recently found that keratins act upstream of AMPK and mTOR to regulate protein biosynthesis by stabilizing the surface localization of GLUT1 and GLUT3 (20, 21). Site-directed mutagenesis of the inserts was performed using two-step sequential PCR with overlapping primers (Stratagene).

Cell Culture, Transfection, and Viral Gene Transfers—Human airway epithelial Calu-3 cells were grown as described previously on plastic dishes or permeable supports (24). HEK293T and COS-7 cells were grown in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum in a 5% CO2, 95% air incubator at 37 °C. These cells were transfected using LipofectamineTM 2000 (Invitrogen).

For knockdown experiments, double-stranded shRNA oligos targeting the K18 sequence GAUCCAGAGCUUUAACC (nucleotides 1154–1173 relative to the start codon) or a control sequence, ACGCATGCATGCTTGCTTT, unrelated to K18 were cloned into the lentivirus transduction vector pLVTH (Addgene plasmid 12262). Green fluorescent protein (GFP), also cloned in pLVTH under the control of a separate promoter, was used as an indicator of the expression of the shRNAs.

Lentiviruses were prepared by transfecting three plasmids into HEK293T cells: psPAX2 (Addgene), pMD2G-VSVG (Addgene), and pLVTH-shK18 or pLVTH-shControl. The culture medium was collected on the third day after transfection, and the collected medium containing the virus was filtered (pore size 45 μm) and then concentrated by ultracentrifugation (50,000 × g). The concentrated virus soup was stored at −80 °C.

One ml of the concentrated lentivirus soup with 8 μg/ml Polybrene was added to a 35-mm dish of ∼70% confluent Calu-3 cells, which were then incubated overnight in a 5% CO2, 95% air incubator at 37 °C before replacing the virus-containing medium with fresh culture medium. At 72 h after infection, the culture was monitored for GFP fluorescence. GFP was expressed in 80–90% of the cells, which were expanded for storage and culture on Snapwells.

RT-PCR—Total mRNA was isolated from transfected HEK293T or COS-7 cells using the RNeasy® mini kit (Invitrogen). From these mRNAs, cDNAs were amplified by reverse transcription-PCR using SuperScriptTM reverse transcriptase with oligo(dT)15 and Platinum® Taq DNA polymerase (Invitrogen).

Antibody Labeling for Immunofluorescence—Cultured cells were grown on coverslips or on Transwell filters; the filters were cut out from their support using a scalpel just before starting the labeling procedure. Cells were fixed by 10-min incubation in 4% paraformaldehyde and permeabilized in 0.2 M NH4Cl/phosphate-buffered saline (PBS) plus 0.2% Triton X-100. After blocking with 3% bovine serum albumin (BSA)/PBS, cells were stained with various combinations of primary antibodies and corresponding FITC- or TRITC-conjugated secondary antibodies.

For labeling tissue samples, adult mouse intestine was dissected, embedded in Tissue-Tek OCT compound (Sakura Finetek), and dehydrated by iso-pentane and liquid nitrogen in preparation for sectioning. Sections (10 μm thick) were fixed with 4% paraformaldehyde in PBS for 10 min and immersed in 50 mM NH4Cl in PBS for 25 min. After blocking with 3% BSA and 0.1% Triton X-100 in PBS for 1 h, sections were exposed to primary antibodies diluted in the blocking solution for 48 h at 4 °C. Sections were then washed four times with PBS and then...
incubated with FITC- or TRITC-conjugated secondary antibodies for 2 h. Following four more washes with PBS, sections were serially dehydrated with 50, 75, 95, and 100% ethanol before mounting on glass slides with fluorescence mounting medium for confocal microscopy. The adult mouse gallbladder was fixed in 4% paraformaldehyde and embedded in paraffin before cutting 5-μm-thick sections. These sections, after deparaffinization and rehydration, were immunostained as described above.

**Immunofluorescence Microscopy and Image Processing**—In this study we used a Leica DM IRE2 confocal microscope equipped with krypton/argon, green helium/neon, and helium/neon lamps, which provided six separated laser lines at these wavelengths: 458, 476, 488, 514, 543, and 633 nm. We used the 488- and 543-nm channels. The objectives used were Zeiss Plan-Neofluar 40×/0.75 and Plan-Neofluar 63×/1.25 oil. The pixel size of the confocal image was dependent on the objective used and the zoom factor. To suppress noise and improve image resolution, 10 scans were averaged to record images. Scanning and image collection were controlled by the Leica confocal software, and images were processed using Adobe Photoshop 7.0 software.

**Pulldown Assays**—For generating glutathione S-transferase (GST) fusion proteins, CFTR C-terminal segments (each with a C-terminal His6 tag) were cloned into pGEX-6p-1 (GE Healthcare Life Sciences). These fusion proteins were expressed in the bacterial strain BL21(DE3) and affinity-purified using nickel-nitrilotriacetic acid-agarose (Qiagen); this step helps to eliminate contamination by GST protein, which often occurs during purification with glutathione-Sepharose beads. For pulldowns, purified GST fusion proteins or GST (control) immobilized on glutathione-Sepharose beads was incubated overnight (4 °C) with the whole-cell lysates of Calu-3 or HEK293T cells. Beads were spun down and washed three times with 3 ml of PBS plus 0.2% Triton X-100, and then the bound proteins were eluted with 1× SDS-PAGE sample loading buffer and analyzed by Western blotting.

**Immunoprecipitation Assays**—3′ 3′-Dithiobis (sulfo succinimidylpropionate) (DTSSP; Pierce) was used for protein cross-linking according to the manufacturer’s instructions. Briefly, Calu-3 cells were washed three times with PBS and then incubated in PBS containing 200 μg/ml DTSSP and 0.1% Triton X-100 for 20 min at room temperature. The cross-linking reaction was terminated by adding 50 mM glycine (5 min at room temperature). After washing, the cells were lysed in radioimmune precipitation assay buffer (25 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS plus protease inhibitor mixture (Roche Applied Science), which was supplemented with 1% Empigen® BB (Calbiochem) detergent for solubilizing K18. For immuno-precipitation, cell lysates were mixed with 1 μg of mouse monoclonal anti-CFTR antibody (C terminus-specific; R&D Systems) or control IgG plus protein A-agarose beads (Santa Cruz Biotechnology). After overnight incubation at 4 °C, the beads were spun down and washed twice with radioimmune precipitation assay buffer, and the captured proteins were then eluted with 1× SDS-PAGE sample loading buffer for analysis by 10% SDS-PAGE and Western blotting. Transfected COS-7 cells were also lysed in radioimmune precipitation assay buffer and used for immunoprecipitation assays with the anti-CFTR antibody and protein A-agarose beads as above.

**Total Protein Extracts** and Western Blotting—Calu-3 cells and transfected HEK293T or COS-7 cells were solubilized with a lysis buffer containing 150 mM NaCl, 50 mM Tris, pH 7.4, 25 μM EDTA, 1% Triton X-100, and protease inhibitor mixture. After centrifugation to pellet-insoluble cellular material, soluble lysates were collected and subject to 10% SDS-PAGE and Western blotting. In all Western blotting assays, staining was carried out with primary antibodies and appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies for detection by enhanced chemiluminescence (ECL reagents from Pierce).

**Isolation of Keratin 8/18 from Rat Liver**—Rat liver was homogenized using a buffer containing 10 mM Tris-HCl, pH 7.6, 140 mM NaCl, 5 mM EDTA, 5 mM EGTA, 0.5% Triton X-100, and 2 mM PMSF and then centrifuged at 5000 × g for 10 min at 4 °C. The pellet was homogenized in a high salt buffer (20 ml/g pellet) containing 10 mM Tris-HCl, pH 7.6, 140 mM NaCl, 1.5 mM KCl, 5 mM EDTA, 5 mM EGTA, 1% Triton X-100, and 2 mM PMSF and incubated on ice for 30 min. After a 10-min centrifugation at 15,000 × g, the obtained pellet was resuspended in ice-cold TE buffer (10 mM Tris-Cl and 0.1 mM EDTA, pH 8.0) and respun at 15,000 × g for 10 min. The insoluble keratin was solubilized with urea buffer (8 M urea, 10 mM Tris-Cl, and 1 mM DTT, pH 8.0) at room temperature for 30 min and then dialyzed against 2 mM Tris-Cl, pH 7.4, for 3–4 h with three buffer changes.

**Filament Assembly and Co-sedimentation Assays**—Filament assembly and co-sedimentation assays were performed as described previously (25). Briefly, purified K8/K18 complexes (0.5 mg/ml) were incubated with or without GST or GST-CFTR-C for 1 h at 25 °C and then centrifuged at 20,000 × g for 30 min at 4 °C. The supernatants and the pellets were both analyzed by Western blotting.

**Cell Surface Biotinylation**—HEK293T or COS-7 cells transfected with various combinations (CFTR/K18) of plasmids were cultured for 36 h. Surface biotinylation was performed using the cell surface protein biotinylation and purification kit from Pierce with sulfo-NHS-SS-biotin, a thiol-cleavable amine-reactive biotinylation reagent, according to the manufacturer’s instructions.

Selective biotinylation assays of apical or basolateral surface in Calu-3 cells were performed as described previously (26). Polarized Calu-3 monolayers were washed three times with PBS containing 1 mM MgCl2 and 0.1 mM CaCl2 (PBS-C/M) before adding 0.5 ml of sulfo-NHS-SS-biotin (1 mg/ml in ice-cold PBS-C/M) to either the apical or basolateral compartment of the Transwells (1.13 cm2 in area). Compartment not receiving sulfo-NHS-SS-biotin were filled with an equal volume of ice-cold PBS-C/M. After 30 min of gentle shaking at 4 °C, cells were washed three times with PBS-C/M and incubated with quenching buffer (50 mM glycine in PBS-C/M) to remove excess biotin. Following two more washes with PBS-C/M, the cells were lysed and incubated overnight at 4 °C with a slurry of avidin beads. The captured biotinylated pro-
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Electrophysiology—For whole-cell voltage clamp studies, HEK293T cells were transfected with CFTR, K18, CFTR plus K18, or expression vectors alone (mock), along with GFP as an indicator of transfection; GFP-positive cells were subject to voltage clamp studies. The pipette solution contained (in mM): 40 Tris-Cl, 100 Tris gluconate, 2 MgCl₂, 5 HEPES, 1 EGTA, 0.1 CaCl₂, and 1 MgATP; pH 7.4 was adjusted with Tris base. Ca²⁺ analyzed using Clampfit 9.0 software. The cAMP mixture used was acquired at 1 kHz and filtered at 1 kHz. All data were acquired using an Axopatch 200B amplifier and Axon DigiData 1322A with Axon pClamp 9 software. Whole-cell current was acquired at 1 kHz and filtered at 1 kHz. All data were analyzed using Clampfit 9.0 software. The CAMP mixture used contained 10 μM forskolin, 200 μM cpt-cAMP, and 100 μM isobutylmethylxanthine (IBMX).

For short circuit current (Isc) measurement, the procedures were essentially the same as described previously (24). Calu-3 epithelial monolayers infected with lentiviruses were grown on Snapwells to confluence (air interface culture; resistance > 800 ohms·cm²) and then mounted in Ussing chambers for experiments. The submucosal and mucosal bathing solutions were Krebs-Ringer bicarbonate solution (in mM: 140 Na⁺, 120 Cl⁻, 5.2 K⁺, 25 HCO₃⁻, 2.4 H₂PO₄⁻, 0.4 HEPES, 1.1 Ca²⁺, 1.2 Mg²⁺, and 5.2 glucose for submucosal or mannitol for mucosal bathing solutions). Glucose was replaced by mannitol in the mucosal bathing solution to rule out electrogenic sodium-glucose co-transporter activity. The solution had a pH of 7.4 when gassed with 95% O₂, 5% CO₂ at 37 °C in the chambers.

For studying tissues in the Ussing chamber, the mouse small intestine was stripped from the muscular layer under a microscope and mounted on a homemade insert, whereas the mouse gallbladder was mounted directly. Submucosal and mucosal bathing solutions were the same as those used for Calu-3 cells, and forskolin was added only to the mucosal side in all experiments.

Pulse-Chase Experiments—COS-7 cells transfected with various combinations of plasmids and cultured for 36 h were starved in Met/Cys-free DMEM (Invitrogen) for 30 min to deplete cellular stores. After Met/Cys-free DMEM was discarded, cells were pulse-labeled for 20 min with 150 μCi of [³⁵S]methionine/cystine (Amersham Biosciences), chased with cold DMEM, and incubated in a 5% CO₂, 95% air incubator at 37 °C for different time periods. The cells were then scraped into a lysis buffer (20 mM HEPES, pH 7.4, 175 mM NaCl, 1 mM EDTA, 0.25% Nonidet P-40, 10% glycerol, 1 mM DTT, 1 mM PMSF, and protease inhibitor mixture; 350 μl/60-mm dish), and the lysates were centrifuged to separate the insoluble and soluble fractions. The soluble lysates were used for immunoprecipitation (overnight at 4 °C) with anti-CFTR antibodies (C terminus-specific; R&D Systems) and protein A-agarose beads. Immunoprecipitated proteins were resolved by SDS-PAGE, and the gels were fixed with 10% (v/v) glacial acetic acid and 30% (v/v) methanol and then incubated with EN3HANCE (PerkinElmer Life Sciences) to enhance signals. Gels were dried and exposed to Kodak Biomax MR film for 6 days for autoradiography.

Endocytic and Recycling Assays—HeLa cells were transfected with pcDNA4-V5-CFTR and pcDNA3-K18 expression plasmids as described above. V5 was tagged at the C terminus of CFTR. Cell surface proteins were biotinylated with a thiol-cleavable biotinylation reagent at 4 °C as described above, and internalization of biotinylated proteins was initiated by incubating cells at 37 °C. The biotinylated proteins remaining on the cell surface were stripped of biotin using a MESNA buffer (50 mM MESNA, 100 mM NaCl, 50 mM Tris, 1 mM MgCl₂, 0.1 mM CaCl₂, pH 8.6) for 1 h at 4 °C. Subsequently, the cells were either lysed in radioimmunoprecipitation assay buffer for assessing the amount of internalized CFTR or incubated for 5 min at 37 °C again to trigger the recycling of internalized biotin-labeled proteins to the cell surface; recycling was arrested by cooling the cells back down to 4 °C. The recycled biotinylated proteins were stripped of biotin using the MESNA buffer, and the cells were lysed to determine the biotinylated CFTR remaining in the cytosol. Biotinylated CFTR was quantitated using anti-V5 antibody capture ELISA (27) and normalized to the protein content of the cells.

Antibodies—The following commercial antibodies were used in this study: mouse monoclonal anti-CFTR C terminus antibody from R&D Systems (clone 24–1), anti-CFTR antibody from Chemicon (clone MM13–4), and anti-CFTR antibody from Abcam (clone CF3); mouse monoclonal anti-keratin 18 (L2A1) antibody (NeoMarker) and guinea pig polyclonal anti-keratin 18 (GP CK18.2) antibody (Progen); keratin 8 Ab-4 (TS1) antibody (Thermo Fisher Scientific); anti-GST antibody (Amersham Biosciences–Pharmacia); rabbit polyclonal antizO-1 antibody (Zymed Laboratories Inc.); mouse monoclonal anti-cytokeratin 4.62 (which recognizes keratin 19), anti-c-Myc, anti-AP2, and anti-Rab7 (clone Rab7–117) antibodies (Sigma); mouse monoclonal anti-V5, anti-E-cadherin, and anti-human transferrin receptor antibodies (Invitrogen); mouse anti-Rab5 (clone 1) and anti-Rab11 (clone 47) antibodies (BD Transduction Laboratories); rabbit anti-guinea pig IgG (Abcam); and donkey anti-mouse and anti-rabbit HRP secondary antibodies with fluorescein (FITC)- or rhodamine (TRITC)-conjugated AffiniPure donkey anti-mouse and anti-rabbit IgGs (The Jackson Laboratory). All antibodies were used at the concentrations recommended by their manufacturers.

Animals—K18 knock-out mice, originally generated in our laboratory (15), were kindly provided by Dr. Steve Burden (New York University). Mice were genotyped using a PCR assay according to our previous report (15).

Statistics—All data are expressed as means ± S.E. Statistical analysis was performed using Student’s t test, and values of p < 0.05 were considered statistically significant.
RESULTS

Interaction of K18 with CFTR—To search for novel binding partners of the CFTR C terminus, we performed a yeast two-hybrid screen using the human CFTR C terminus (CFTR-C, CFTR-(1407–1480)) as bait. A dozen positive clones were obtained representing ezrin-radixin-moesin-binding phosphoprotein 50 (EBP50), Na+/H+ exchanger 3 kinase A regulatory protein (E3KARP), K18, and two other proteins. EBP50 and E3KARP have been reported previously to interact with CFTR-C by multiple approaches (28, 29). Among the other three proteins, K18 appeared most relevant to CFTR function because both CFTR and K18 are expressed predominantly in simple epithelial cells (15). We thus chose K18 for further investigation.

The K18 cDNA fragment identified in the yeast two-hybrid screen represents amino acid residues 99–430. Possible non-specific interaction between K18/CFTR and vector proteins was ruled out by swapping the vectors for K18-(99–430) and CFTR-C. The major K18-binding site in CFTR-C was mapped by additional yeast two-hybrid assays (Fig. 1A). Progressive truncation from the C terminus identified CFTR-(1407–1436) to be sufficient for interacting with K18-(99–430). This region of CFTR-C contains two sequence motifs of interest: a tyrosine-based (Tyr1424) endocytic signal for binding to AP2, which in turn binds to myosin VI possibly via Dab2 protein (30); and the highly conserved hydrophobic patch1413FLVI1416 (11). Mutation of Tyr1424 to alanine did not alter CFTR-C-K18 binding, arguing against a role of AP2 in mediating the interaction between CFTR and K18. In contrast, truncations from the N terminus of CFTR-C suggested that CFTR-(1407–1416), which includes 1413FLVI1416, is necessary for CFTR-C-K18 binding. Notably, changing 1413FLVI1416 to alanines eliminated this interaction, indicating that K18-(99–430) binds to 1413FLVI1416. Also, complementing these results, progressive truncation of K18 suggested that the C-terminal tail and the coil 2 domain of K18 are important for binding to CFTR-C (Fig. 1B).

Calu-3 cells, derived from human airway epithelia, express both CFTR and K18. The endogenous K18 in Calu-3 cells could be co-immunoprecipitated with the endogenous CFTR (Fig. 1C). In addition, endogenous K18 in Calu-3 cells was captured by the GST-CFTR-(1407–1480) fusion protein but not by GST alone (Fig. 1D), and this co-precipitation was almost completely to be sufficient for interacting with K18-(99–430). This region of CFTR-C contains two sequence motifs of interest: a tyrosine-based (Tyr1424) endocytic signal for binding to AP2, which in turn binds to myosin VI possibly via Dab2 protein (30); and the highly conserved hydrophobic patch1413FLVI1416 (11). Mutation of Tyr1424 to alanine did not alter CFTR-C-K18 binding, arguing against a role of AP2 in mediating the interaction between CFTR and K18. In contrast, truncations from the N terminus of CFTR-C suggested that CFTR-(1407–1416), which includes 1413FLVI1416, is necessary for CFTR-C-K18 binding. Notably, changing 1413FLVI1416 to alanines eliminated this interaction, indicating that K18-(99–430) binds to 1413FLVI1416. Also, complementing these results, progressive truncation of K18 suggested that the C-terminal tail and the coil 2 domain of K18 are important for binding to CFTR-C (Fig. 1B).

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abolished by the mutation of FLV1 to AAAA (M1) in GST-CFTR-(1407–1480). Interestingly, the mutation of flanking sequences ENK1420 and ECQ1412 to AAAA (M2 and M3, respectively) appeared to slightly enhance the co-precipitation (Fig. 1D). These results suggest that K18 expressed by airway epithelial cells interacts with endogenous CFTR, likely through the CFTR FLV11416 sequence.

To verify the results on the CFTR-binding site in K18 from yeast two-hybrid assays (Fig. 1B), various exogenous K18 fragments were expressed in HEK293T cells and tested for their interaction with GST-CFTR-(1407–1480) (GST-CFTR-C) fusion protein. Deletion of the C-terminal tail of K18 almost eliminated the interaction of K18 and CFTR-C (Fig. 1E). Further deletion of the coil 2 domain had no additional effect (Fig. 1E). These results indicate that the C-terminal tail of K18 is critical for binding CFTR. In addition, CFTR appeared to be able to bind to K18 in a filamentous form, as CFTR-C pelleted with K18/8 filaments in co-sedimentation assays (Fig. 1F).

In polarized Calu-3 cells, K18 was localized in the apical domain (Fig. 1G, a), as in other epithelial cells (31). In contrast, actin was evenly distributed in the apical and basolateral regions (Fig. 1G, d). More importantly, K18 was colocalized with apical CFTR in Calu-3 cells (Fig. 1H), which is consistent with the idea that endogenous CFTR and K18 physically interact at or near the cell surface.

Influence of K18 on CFTR Expression in Cultured Cells and in Vivo—We next asked whether interaction with K18 affects CFTR expression or targeting. When Myc-tagged K18 was introduced into COS-7 cells, the total protein level of CFTR was markedly increased (Fig. 2A, a and b), but CFTR mRNA expression was not altered (Fig. 2A, c). This increase was completely blocked by the FLV11416, AAAA (M1) mutation in CFTR (Fig. 2A, a and b), which impairs CFTR-K18 binding (Fig. 1). Agreeing with a previous study (11), even in the absence of exogenous K18 expression, the FLV11416, AAAA mutation impaired CFTR expression so severely that the mutant CFTR could be detected only after enrichment by immunoprecipitation. This likely occurred because the mutation disrupted the CFTR interaction with endogenous K18 (Fig. 2A, a, lower panel). Taken together, these results suggest that K18 enhances
the expression of CFTR by specifically binding to its FLVI sequence.

To address the physiological relevance of the above results, we examined the effect of K18 on CFTR surface expression. Surface biotinylation experiments using COS-7 and HEK293T cells showed that the expression of K18 substantially increased the cell surface levels of CFTR (Fig. 2B). K18 did not affect the expression of unrelated proteins such as A1 adenosine receptor and cytosolic protein NF/H9260B1/P105 (Fig. 2B, a and c). Furthermore, the ectopic expression of CFTR in HEK293T cells resulted in a cAMP-stimulated Cl− conductance, which was absent in mock-transfected HEK293T cells and blocked by the CFTR-specific blocker GlyH-101. This conductance was also significantly increased by K18 expression (Fig. 2C), providing functional evidence of the enhancement of CFTR surface expression by K18. In keeping with these biochemical and functional effects of K18 on CFTR surface expression, exogenous K18 colocalized with CFTR at or near the cell surface in HEK293T cells (Fig. 2D).

We next knocked down the expression of endogenous K18 in Calu-3 cells by RNA interference using a lentiviral vector. This led to nearly 50% reduction in the total protein levels of endogenous mature CFTR (Fig. 3A) and, more importantly, to about 80% decrease in the apical surface expression of CFTR (Fig. 3B). This effect appears to be specific for CFTR, because the expression of apical and basolateral transferrin receptors and basolateral E-cadherins showed no change. Further demonstrating a reduction of apical CFTR, knocking down of K18 resulted in an almost 60% decrease in forskolin-induced short circuit current (ΔIsc, peak values, a readout of cell surface CFTR function; Fig. 3C). Knockdown of K18 also decreased K8 and increased K19 expression slightly, although the changes were not statistically significant (supplemental Fig. 1).

To examine the influence of K18 on CFTR in vivo, the effect of K18 gene ablation on CFTR surface expression was investigated in the mouse. Apically distributed CFTR was colocalized with K18 in the villi of the duodenum and in the columnar epithelia of the gallbladder in wild-type mice (Fig. 4, A and B). In contrast, K18 was absent (as expected) and CFTR expression was dramatically reduced in both the duodenum and the gallbladder of K18 mice, suggesting a crucial role for K18 in CFTR surface expression in vivo. This observation was bolstered by results showing that forskolin-induced, CFTR-mediated ΔIsc currents in the duodenum and gallbladder were more than halved in K18 mice relative to those in wild-type mice (Fig. 4C).

Mechanism of K18-dependent Enhancement of CFTR Cell Surface Expression—Does K18-mediated surface expression of mature CFTR result from an increase in the biosynthesis or maturation of immature (core-glycosylated) CFTR or through the stabilization of mature (fully glycosylated) CFTR at the cell surface? To address this question, the influence of K18 on the biosynthesis, maturation and degradation of CFTR was exam-
ined by metabolic pulse-chase labeling in COS-7 cells. As shown in Fig. 5A, at time zero K18 had no effect on the density of band B, which represents the immature CFTR that is first synthesized in the endoplasmic reticulum and then converts to mature CFTR after full glycosylation. The ratio of band B in K18-expressing cells relative to that in control cells was 0.99±0.05 (p=0.79, n=3), implying that K18 does not affect CFTR biosynthesis. After 4 h of chase, CFTR proteins in both K18-expressing and control cells were present almost exclusively as band C (mature CFTR) with similar densities (Fig. 5, A and B), indicating little effect of K18 on CFTR maturation. Finally, the effect of K18 on the degradation rate of mature CFTR was assessed by comparing the band C present after 8 and 16 h of chase. More mature CFTR was detected in the presence of exogenous K18 than in its absence after both 8 and 16 h of chase (Fig. 5A), and K18 expression was calculated to increase the half-life of mature CFTR from 9 to 14 h (Fig. 5C). Taken together, these results suggest that K18 enhances the stability of mature CFTR without altering the biosynthesis or maturation of CFTR. In these experiments K18 also appeared to slightly increase immature CFTR levels after 2 h of chase (Fig. 5); it is possible that K18 confines immature CFTR in the endoplasmic reticulum.
The positive effect of K18 expression on CFTR surface stability shown above is opposite to that described for AP2 interaction with CFTR (3, 4). Because K18 could potentially increase CFTR surface stability by blocking AP2 binding and thereby obstructing the endocytosis of plasma membrane CFTR. In agreement with previous studies (4), substitution of Tyr1424 with an alanine in the CFTR C terminus (M4 mutation) strongly suppressed AP2-binding (Fig. 6A). This mutation, however, had a minimal impact on CFTR-K18 binding. On the other hand, the 1413FLVI1416-AAAA mutation (M1), which disrupts K18 binding, failed to affect CFTR interaction with AP2 (Fig. 6A). These data suggest that K18 and AP2 do not physically compete for binding to CFTR, although they may functionally counterbalance each other. One possibility is that AP2 and K18 may not be able to bind the same CFTR molecule, as their binding sites on CFTR are less than 10 residues apart. Instead, AP2 and K18 may interact with distinct CFTR molecules, with all three being or not being a part of the same multiprotein complex that contains multiple copies of CFTR.

To further test whether K18 increases the surface stability of mature CFTR by hindering CFTR endocytosis or by increasing CFTR recycling from intracellular pools, the cell surface, internalized, and recycled levels of CFTR in HeLa cells were determined using endocytic and recycling assays. In accord with the results obtained using other approaches described above, K18 robustly increased CFTR surface expression in this assay (Fig. 6B, a). Also, whereas K18 had virtually no effect on CFTR endocytic rate (Fig. 6B, b), it nearly doubled the rate of CFTR recycling (Fig. 6B, c). Consistent with these observations, in polarized Calu-3 cells, K18 colocalized with Rab11, a recycling endosome marker, but not with Rab5, an early endosome marker (Fig. 6C). Such apically polarized early and recycling endosomes have also been observed in other epithelia (33, 34). Taken together, our data suggest that K18 increases CFTR surface expression by accelerating CFTR apical recycling, without affecting its endocytosis.

DISCUSSION

Here we have described the interaction of CFTR with the intermediate filament protein keratin 18. Previous reports have suggested that CFTR, directly or indirectly, binds to many other cytoskeletal proteins, including actin (35), filamin A (36), ezrin (37), myosin VB (38), and myosin VI (30), all of which have been implicated in regulating the trafficking or surface stability of CFTR. Keratins K8/K18 have also been suggested to modulate the expression of F508- and WT-CFTR in an earlier study (22), although several issues in that study remain to be addressed. First, Davezac et al. (22) reported the colocalization and co-immunoprecipitation of K18 with F508-CFTR and a correlated surface redistribution of K18 and F508-CFTR induced by low temperature. They also reported, however, the seemingly contrasting observation that K18 depletion by RNA interference increased the surface expression of F508-CFTR. Second, the authors did not provide any evidence of physical or functional association of K18 with WT-CFTR. Third, they originally tested only ectopically expressed CFTR (22) and more recently have presented circumstantial evidence of interaction between endogenous K18 and F508-CFTR (39). These issues demanded a critical examination of the relationship between K18 and CFTR and of the molecular mechanism underlying K18-CFTR interaction.

In this study we have demonstrated direct and functional interaction between WT-CFTR and K18 using in vitro assays, cultured airway epithelial cells, and K18 knock-out mice. Fur-
thermore, we addressed in detail whether and how K18 binding regulates the surface expression of CFTR, using multiple approaches. Although K18 significantly increased CFTR half-life, it had little effect on the rates of CFTR biosynthesis and maturation (Fig. 5). K18 increased CFTR surface expression by accelerating CFTR recycling from intracellular pools without altering the endocytic rate of CFTR (Fig. 6). Our results are reminiscent of a previous study that implicated the trafficking of late endosomes (40).

The C-terminal region of CFTR contains many protein interaction sites, including the conserved hydrophobic patch (1413FLVI1416), a tyrosine (Tyr1424), a di-leucine (Leu1430/Leu) based endocytic signal, and a PDZ binding sequence (1477DTRL1480). CFTR, through the PDZ binding motif, interacts with NHERF1/NHERF2 and forms a complex with ezrin and actin filaments, which is suggested to be important for CFTR apical localization by several studies (41–43). However, Milewski et al. (44, 45) found that the PDZ binding motif by itself is incapable of ensuring the correct polarized distribution of CFTR and that additional localization signals in the region of amino acids 1404–1425 are needed. Interestingly, Gentzsch and Riordan (11) report that 1413FLVI1416 is crucial for stabilizing mature CFTR in the apical membrane by unknown mechanisms; this in vitro study is consistent with the clinical findings that residues 1412–1455 are important for the positive modulation of CFTR function (9, 10). In this regard, our data have shed new light on these previous observations for which the underlying mechanism has remained unclear. Our results have revealed that K18 binds to the hydrophobic patch 1413FLVI1416 of CFTR and increases the surface expression of CFTR by accelerating its apical recycling. It is possible that mutation Q1412X in CFTR, but not mutation S1455X, disrupts the K18-binding site and thus leads to the loss of plasmalemmal CFTR in epithelia and causes severe CF in patients. It is noted that knocking down K18 in Calu-3 did not lead to any basolateral distribution of CFTR (Fig. 3B) and residual CFTR in K18 knock-out mice was still apically localized (Fig. 4A), suggesting that K18 binding is not required for apical targeting of CFTR although it stabilizes CFTR that is already in the apical domain.

The residues 1413FLVI1416 are at the C-terminal end of the nucleotide binding domain 2 (NBD2) of CFTR, and similar hydrophobic patches are conserved in the ATP-binding cassette (ABC) family proteins (46). In the recently resolved crystal structure of ABC transporters, the hydrophobic patch is located near the extracellular domain. It is well known that the extracellular domain of CFTR is important for its trafficking and localization (47). Therefore, it is possible that K18 binding to the hydrophobic patch 1413FLVI1416 alters the conformation of the extracellular domain of CFTR, thereby facilitating its apical targeting.

**FIGURE 6. K18 accelerates CFTR recycling without affecting its endocytosis.** A, independent effects of CFTR mutations on the binding of AP2 and K18 to CFTR. Panel a, AP2 (top blot) and K18 (middle blot) from Calu-3 cells pulled down by GST alone (GST), GST-CFTR-(1407–1480) (WT), and GST-CFTR-(1407–1480) with 1413FLVI1416-AAAA (M1) or Y1424A (M4) mutations; the bottom blot shows loading of GST proteins. Panel b, summary data of the relative protein amounts of K18 and AP2 bound to different GST fusion proteins in panel a. ***, different from WT-CFTR; p = 0.00003 for K18, p = 0.00061 for AP2, n = 5. B, the effect of K18 on the internalization and recycling of plasmalemmal CFTR in HeLa cells. Panel a, surface CFTR quantified by cell surface biotinylation and anti-V5 antibody capture ELISA (n = 3). ***, different from CFTR alone, p = 0.00005. In a–c, cell surface or endocytosed CFTR in cells expressing both CFTR and K18 (CFTR + K18) was normalized against that in cells expressing CFTR alone (CFTR); n is the number of independent experiments performed with triplicate samples. Panel b, CFTR internalized in 10 min was measured by endocytic assays and anti-V5 antibody capture ELISA (n = 4). Panel c, the endocytic CFTR remaining in the cytosol before and after recycling for 5 min, quantified by recycling assays and anti-V5 antibody capture ELISA (n = 3; **, different from CFTR alone at 5 min, p = 0.0016).

C, xz images showing immunostaining of endogenous K18 and Rab5 or Rab11 in polarized Calu-3 cells grown on permeable support. Arrowheads indicate the apical membrane. Scale bar, 5 μm.
structure of the NBD2 of human CFTR (Protein Data Bank ID, 3GD7). The 1413FLVI1416 sequence constitutes a major part of a β-strand within a β-sheet, whereas the 1417EENK1420 and 1409ECQQ1412 sequences that flank 1413FLVI1416 are parts of the random coils or turns. Because mutation of 1413FLVI1416 but not of 1417EENK1420 or 1409ECQQ1412 impaired K18 binding (Fig. 1D), it is possible that the formation of the β-sheet containing 1413FLVI1416 is essential for CFTR interaction with K18.

In the context of the physical interaction of CFTR with K18, we tested whether their binding affinity could be assessed by performing isothermal titration calorimetry (ITC). Because full-length GST-K18 was found to be insoluble in our previous performing ITC, we tested whether their binding affinity could be assessed by PreScission protease. We first found full-length GST-K18 binding to CFTR-C was shown by many other assays (Figs. 1 and 6), these negative ITC data could have resulted from: 1) K18-C fragment being unable (or insufficient though essential) to form the correct secondary structure needed for binding CFTR, especially considering that CFTR binds to filamentous K18 (Fig. 1F); or 2) the binding of CFTR and K18-C being too weak for the ITC assay to clearly detect.

CFTR knock-out mice have severe intestinal phenotypes (47). Interestingly, FVB/N mice with K8 knock-out show a loss of CFTR and some intestinal phenotypes similar to CFTR knock-out mice, including colitis, enlarged goblet cells, and diarrhea (48–50). Although the molecular link between K8 knock-out and CFTR loss was not examined in previous studies, the deletion of K8 could have led to a secondary loss or to mistargeting of its dimerization partner K18, which in turn could have caused the loss of CFTR. The present work has demonstrated the attenuation of CFTR expression in the intestinal tract of K18 knock-out mice (Fig. 4). Interestingly, these knock-out mice displayed no obvious phenotype change except for marked hepatomegaly when old, as we have shown previously (15). The most likely explanation is that K19, coexpressed with K18 and K8 in intestinal epithelium, compensates for the loss of K18. Alternatively, residual CFTR, evident in functional studies (Fig. 4C) but not in tissue immunostaining (Fig. 4, A and B), may account for the lack of apparent CF phenotype in K18/–/– mice. A previous study showed that ~90% of Cfrtm1Hgu/Cfrtm1Hgu mice survive to maturity, and these exhibit ~10% of the normal level of WT-CFTR mRNA. In contrast, Cfrtm1Vuc/Cfrtm1Vuc null mice had only a 5% survival rate (51). In addition, the Cfrtm1Hgu/Cfrtm1Hgu mice had mild (4 of 6 mice) or no histological abnormality (2 of 6 mice), although they all were defective in chloride ion transport (52). Mild or no CF phenotypes were also observed in Cfrtm2Hgu/Cfrtm2Hgu and Cfrtm1Eur/Cfrtm1Eur mice, which possess residual CFTR function (51, 53, 54). These data together suggest that just a fraction of normal CFTR function can considerably alleviate or even eliminate the phenotypes of CF mouse models. This could be one of the major reasons why there is a lack of CF phenotypes in K18 knock-out mice, in which there is 35–55% normal CFTR function (Fig. 4C).

In different mouse models of CF, in addition to the specific mutations generated and their associated residual CFTR function, CF-modifying genes and environmental factors can lead to variations in phenotypes. One must thus be cautious in making direct comparisons between various mouse models. Nevertheless, our biochemical and molecular studies and our functional assays in cultured cells and in vivo collectively raise the possibility that K8, K18, and K19 genes act as modifiers in CF disease. Also, in line with the recent finding that keratins stabilize cell surface localization of GLUT1 and GLUT3 in yolk sac endoderm (20, 21), our present study also adds strong support to a newly recognized function of keratin in protein trafficking, which may be especially relevant to the etiology of diseases such as cystic fibrosis.

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