Myosin VI Regulates Endocytosis of the Cystic Fibrosis Transmembrane Conductance Regulator

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The cystic fibrosis transmembrane conductance regulator (CFTR) is a cyclic AMP-regulated Cl\(^-\) channel expressed in the apical plasma membrane in fluid-transporting epithelia. Although CFTR is rapidly endocytosed from the apical membrane of polarized epithelial cells and efficiently recycled back to the plasma membrane, little is known about the molecular mechanisms regulating CFTR endocytosis and endocytic recycling. Myosin VI, an actin-dependent, minus-end directed mechanoenzyme, has been implicated in clathrin-mediated endocytosis in epithelial cells. The goal of this study was to determine whether myosin VI regulates CFTR endocytosis. Endogenous, apical membrane CFTR in polarized human airway epithelial cells (Calu-3) formed a complex with myosin VI, the myosin VI adaptor protein Disabled 2 (Dab2), and clathrin. The tail domain of myosin VI, a dominant-negative recombinant fragment, displaced endogenous myosin VI from interacting with Dab2 and CFTR and increased the expression of CFTR in the plasma membrane by reducing CFTR endocytosis. However, the myosin VI tail fragment had no effect on the recycling of endocytosed CFTR or on fluid-phase endocytosis. CFTR endocytosis was decreased by cytochalasin D, an actin-filament depolymerizing agent. Taken together, these data indicate that myosin VI and Dab2 facilitate CFTR endocytosis by a mechanism that requires actin filaments.
cytic motif (YDSI) in the cytoplasmic C terminus of CFTR is thought to facilitate CFTR endocytosis (16, 20, 24). In addition, a dileucine (LL) motif, also in the C terminus, is important for CFTR endocytosis (20, 24). In CF cells, there appears to be a general defect in endocytic trafficking, including a decrease in the endocytic recycling of the transferrin receptor (TfR) (25). Myosin motors and actin filaments play vital roles in membrane trafficking (26–33). The myosin superfamily consists of at least 18 different classes of myosin motors capable of using ATP hydrolysis to move on actin filaments (34). Myosin VI is most likely to participate in the apical membrane endocytosis in epithelial cells because, unlike most myosins, it moves toward the F-actin minus end, which is oriented against the plasma membrane (35–42). Myosin VI is enriched in endocytic sites, including plasma membrane actin networks, where its distribution overlaps with that of clathrin and AP-2 (36, 39, 41, 43–46). Recent evidence demonstrates that myosin VI regulates early steps of TfR endocytosis, including uptake of TfR into clathrin-coated pits and formation of clathrin-coated vesicles (36), as well as the later stages of TfR endocytosis, including movement of uncoated vesicles toward the early endosomes on actin filaments (41, 47).

The objective of the present study was to test the hypothesis that myosin VI plays a role in CFTR endocytosis. CFTR in the apical membrane formed a complex with myosin VI, the myosin VI adaptor protein Disabled 2 (Dab2), and clathrin. Displacement of endogenous myosin VI by the tail domain of myosin VI, a dominant-negative recombinant fragment, increased expression of CFTR in the plasma membrane by reducing CFTR endocytosis. However, the myosin VI tail had no effect on CFTR endocytic recycling or on fluid-phase endocytosis. CFTR endocytosis was decreased by cytochalasin D, an actin filament depolymerizing agent. Taken together, these data indicate that myosin VI facilitates CFTR endocytosis.

MATERIALS AND METHODS

Cell Lines and Cell Culture—Calu-3 cells were obtained from the American Type Culture Collection (Manassas, VA) and cultured in tissue culture flasks (Costar, Corning Corporation, Corning, NY) at 37 °C in minimum Eagle's medium containing 50 units/ml of penicillin, 50 μg/ml of streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, and 10% fetal bovine serum in a 5% CO2/95% air incubator at 37 °C as described previously (48). Calu-3 cells were also grown on Transwell permeable supports (24-μm diameter, 0.4-μm pore size, Corning Corporation) in air-liquid interface culture for 14 to 21 days (48). Under these conditions, Calu-3 cells become polarized (48). Sodium n-butyrate (5 mM) was added to the Calu-3 cell culture medium 48 h before experiments to stimulate CFTR expression. Sodium n-butyrate treatment did not affect the expression of endogenous myosin VI and Dab2. HEK293 cells (American Type Culture Collection) were seeded in tissue culture flasks coated with Vitrogen plating medium and cultured at 37 °C in minimum Eagle's medium supplemented with 50 μg/ml of streptomycin, 50 units/ml of penicillin, and 2 mM L-glutamine in a 5% CO2/95% air incubator at 37 °C. HEK293 cells stably expressing wt-CFTR were a gift of Dr. Neil Bradbury (University of Pittsburgh School of Medicine, Pittsburgh, PA) (49). Cells were seeded in tissue culture flasks coated with Vitrogen plating medium and cultured at 37 °C in Dulbecco's modified Eagle's medium supplemented with 50 μg/ml of streptomycin, 50 units/ml of penicillin, and 2 mM L-glutamine, and 150 μg/ml of hygromycin in a 5% CO2/95% air incubator at 37 °C (50). Sodium n-butyrate (5 mM) was added to the HEK293 cell culture medium 24 h before experiments to stimulate CFTR expression. Sodium n-butyrate treatment did not affect the expression of endogenous myosin VI or Dab2 or the expression of the green fluorescent protein (GFP)-MyoVI-Tail and GFP-Dab2 transgenes. Plasmids—Plasmids containing GFP-Myosin VI globular tail (GFP-MyoVI-Tail), wt-CFTR (CFTR), and GFP-wt-CFTR (GFP-CFTR) constructs were generated as described (41, 51). The GFP-CFTR Y/A LLAA construct was generated from GFP-CFTR by the following alkene substitutions that disrupt the tyrosine and dileucine endocytic motifs, Y1424A, L1430A, and L1431A, using site-directed mutagenesis as described (52). All constructs were sequence verified by ABI PRISM dye terminator cycle sequencing (Applied Biosystems, Foster City, CA).

RT-PCR—The tail domain of myosin VI is alternatively spliced, leading to the generation of two predominant splice variants (36, 46, 53). RT-PCR studies were conducted to identify which myosin VI splice variants are expressed in Calu-3 and HEK293 cells. Total RNA was isolated using the Purescript RNA Isolation kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. 5′-AGAGAAGGAGAGGAGGAAAGGCG-3′; and anti-sense, 5′-GGGCGGATGGAAGGATCGGGAG-3′ were designed using Oligo6.1 Primer Analysis Software (Framingham, MN) and synthesized by the Dartmouth College Molecular Biology Core Facility. These primers were used to amplify myosin VI isoforms from polarized and nonpolarized Calu-3 and HEK293 cells. The amplified PCR products were cloned into pCR2.1 Blunt II-TOPO (Invitrogen, San Diego, CA) and the sequence was verified by ABI PRISM dye terminator cycle sequencing (Applied Biosystems).

Antibodies—Affinity-purified, polyclonal anti-myosin VI tail domain antibody was used as described previously (43). The antibodies monoclonal anti-GFP JL-8, polyclonal anti-GFP, monoclonal anti-clathrin heavy chain, and monoclonal anti-p96 Dab2 were from BD Biosciences (San Jose, CA). Other antibodies used were monoclonal anti-human CFTR C terminus, clone 24-1 (R&D Systems, Minneapolis, MN), monoclonal anti-CFTR, clone M3A7 (Upstate Biotechnology, Lake Placid, NY), polyclonal anti-Dab2, clone H-110 (Santa Cruz Biotechnology, Santa Cruz, CA), mouse IgG1 Negative Control (Dako), rabbit Immunoglobulin Fraction (normal; Dako), and goat anti-mouse and goat anti-rabbit horseradish peroxidase secondary antibodies (Bio-Rad). All purchased antibodies were used at the concentrations recommended by the manufacturer.

Immunoprecipitation and Immunoblotting—CFTR and myosin VI were immunoprecipitated from Calu-3 and HEK293 cell lysates by methods described previously (54, 55). Briefly, cells were solubilized in lysis buffer containing 150 mM NaCl, 50 mM Tris, pH 8.0, 1% Igepal (Sigma), and Complete Protease Inhibitor mixture (Roche Molecular Biochemicals, Indianapolis, IN). After centrifugation at 14,000 × g for 15 min to pellet insoluble material, the soluble lysates were preclared by incubation with protein A or protein G, as appropriate, conjugated to Sepharose beads (Pierce). Proteins were immunoprecipitated by incubation with the antibody-protein A or -protein G complex. Antibody M3A7 was used to immunoprecipitate CFTR. The antibody against the myosin VI tail domain was used to immunoprecipitate myosin VI. The polyclonal anti-GFP antibody was used to immunoprecipitate GFP fusion proteins. Immunoprecipitated proteins were eluted from the protein G or protein A-Sepharose complexes with 50 mM EDTA. The eluted fractions were analyzed by 10% SDS–PAGE and analyzed by Western blotting with an appropriate primary antibody and an anti-mouse or anti-rabbit horseradish peroxidase secondary antibody using the Western Lightning Chemiluminescence Reagent Plus detection system (Perkin Elmer, Boston, MA).

Cell Surface Biotinylation, Endocytic Assay, and Endocytic Recycling Assay—Cell surface biotinylated, endocytic assays, and endocytic recycling assays were performed on HEK293 cells as described previously (55).

Isolation of the Plasma Membrane CFTR—Apical plasma membrane vesicles in Calu-3 cells (filter grown) and the plasma membranes in HEK293 cells (culture flask grown) were biotinylated (55), cells were lysed, and biotinylated proteins were isolated by incubation with streptavidin beads. Subsequently, biotinylated proteins were eluted from the streptavidin beads by a 10-min incubation at room temperature, followed by a 5-min incubation at 85 °C in elution buffer containing 2% SDS, 50 mM Tris–HCl, pH 8.9, 10% glycerol, and 80 mM dithiothreitol. Immunoprecipitated proteins were separated by 7.5% SDS–PAGE and analyzed by Western blotting with an appropriate primary antibody and an anti-mouse or anti-rabbit horseradish peroxidase antibody using the Western Lightning Chemiluminescence Reagent Plus detection system (Perkin Elmer, Boston, MA).

Plasmids—Plasmids containing GFP-Myosin VI globular tail (GFP-MyoVI-Tail), wt-CFTR (CFTR), and GFP-wt-CFTR (GFP-CFTR) constructs were generated as described (41, 51). The GFP-CFTR Y/A LLAA construct was generated from GFP-CFTR by the following alkene substitutions that disrupt the tyrosine and dileucine endocytic motifs, Y1424A, L1430A, and L1431A, using site-directed mutagenesis as described (52). All constructs were sequence verified by ABI PRISM dye terminator cycle sequencing (Applied Biosystems).
eluted from the protein A- and G-Sepharose beads as described above.

**Fluid-phase Endocytosis**—Studies were conducted to determine whether myosin VI affects the cellular uptake of Alexa 647-dextran (10,000 molecular weight; Molecular Probes, Eugene, OR), a fluorescent marker of fluid phase endocytosis (56). HEK293 cells were transiently transfected with CFTR and either GFP-MyoVI-Tail or GFP control and grown on glass coverslips for 48 h. Alexa 647-dextran (1 mg/ml) was added to the cell culture medium (37°C) for 15 min. Subsequently, surface Alexa-647 dextran was removed by thorough washing at 4°C. Thereafter, cells were fixed for 20 min in 4% paraformaldehyde before being mounted in antifade medium (ProLong, Molecular Probes). At least 10 random fields of cells expressing CFTR with either GFP (control) or GFP-MyoVI-Tail were examined from six different slides using a Zeiss LSM 510 META laser scanning confocal microscope with a ×40 objective. All images were collected using the same setting for laser intensity, pinhole aperture, and photomultiplier gain and offset. Transfected cells expressing CFTR and GFP or GFP-MyoVI-Tail were identified by GFP fluorescence. Alexa 647 fluorescence was quantified using Velocity 2.6.1 Software (Improvision, Lexington, MA).

**F-Actin Depolymerization**—Cytochalasin D, an actin depolymerizing agent (2 μM, Sigma) or vehicle control (0.1% Me2SO) were added to the cell culture medium at 4°C for 15 min. Subsequently, cells were washed in PBS containing 1% bovine serum albumin, cells were incubated for 30 min with Alexa-647 phalloidin according to the manufacturer’s instructions (Molecular Probes). Thereafter, cells were washed in PBS and mounted in antifade medium (ProLong, Molecular Probes). Samples were observed with an Olympus BX60 light microscope fitted with a Hamamatsu ORCA 12-bit digital output CCD camera controlled by Openlab 3.5.1 Software (Improvision). In addition, DIC images of fixed cells were obtained with a Zeiss LSM 510 META laser scanning confocal microscope. The effects of cytochalasin D on fluid-phase endocytosis were monitored by measuring the uptake of Alexa-647 dextran, as described above. Ten random fields were examined from three different slides of cells treated with cytochalasin D or vehicle.

**Myosin VI and CFTR Endocytosis**

**RESULTS**

**CFTR Interacts with a Complex of Proteins including Myosin VI, Dab2, and Clathrin in Polarized Airway Epithelial Cells**—Studies were conducted to determine whether endogenous CFTR and myosin VI interact in polarized human airway epithelial cells (Calu-3) and to determine whether other proteins, such as Dab2 and clathrin, interact with myosin VI and CFTR. To these ends, CFTR was immunoprecipitated from Calu-3 cells using a monoclonal anti-CFTR antibody (M3A7), and the immunoprecipitated complex was analyzed by Western blotting. As illustrated in Fig. 1A, CFTR co-immunoprecipitated with myosin VI, Dab2, and clathrin. In the reciprocal experiment, myosin VI was immunoprecipitated using a polyclonal anti-myosin VI antibody, and the immunoprecipitated complex was analyzed by Western blotting. Myosin VI co-immunoprecipitated with CFTR, Dab2, and clathrin (Fig. 1B).

Dab2 is a myosin VI adaptor protein that binds directly to the tail domain of myosin VI and to clathrin associated with the plasma membrane (36, 57–59). Thus, the observation that myosin VI, Dab2, CFTR, and clathrin co-immunoprecipitate strongly suggests that Dab2 and myosin VI interact with CFTR in the plasma membrane. To provide direct evidence that CFTR forms a complex at the apical plasma membrane with myosin VI, Dab2, and clathrin, the apical membrane was selectively biotinylated, the biotinylated proteins were precipitated with streptavidin-agarose beads, biotinylated proteins were eluted from the beads, and the apical plasma membrane CFTR was immunoprecipitated with a monoclonal anti-CFTR antibody. Western blot analysis of the protein complex that co-immunoprecipitated with CFTR in the apical membrane

**Data Analysis and Statistics**—Each experiment was repeated a minimum of three to six times. Statistical analysis of the data was performed using GraphPad Prism version 4.0 for Macintosh (GraphPad Software, San Diego, CA). Means were compared by t test. p < 0.05 was considered significant. Data are expressed as mean ± S.E.
demonstrated that the apical membrane CFTR interacts with myosin VI, Dab2, and clathrin (Fig. 1C).

**Which Splice Variants of Myosin VI Are Expressed in Calu-3 Cells?**—There are two predominant splice variants of myosin VI: one that contains a 31-amino acid insert between the coiled-coil domain and the globular tail (Fig. 2A), and one that lacks this insert (Fig. 2B) (36, 46, 53). It has been reported that myosin VI containing the 31-amino acid insert is expressed predominantly in polarized epithelial cells and plays a key role in TIR endocytosis, whereas the myosin VI splice variant lacking the insert is expressed primarily in nonpolarized cells and does not play a role in TIR endocytosis (36). However, others have reported that the shorter splice variant of myosin VI is also important for TIR endocytosis (41, 47). To determine which splice variant of myosin VI is expressed in Calu-3 cells, RT-PCR experiments were conducted on polarized (i.e., filter grown) and nonpolarized (i.e., tissue culture-flask grown) cells as described in “Materials and Methods.” In both conditions, only the longer splice variant of myosin VI was detected. Taken together, our data in Calu-3 cells reveal that apical plasma membrane CFTR interacts with myosin VI containing the 31-amino acid insert.

**Validation of HEK293 Cells as a Model to Study Myosin VI Role in CFTR Endocytosis—**The experiments described above demonstrate that apical plasma membrane CFTR interacts with a complex of proteins including myosin VI (the longer splice variant), Dab2, and clathrin. However, these co-immunoprecipitation studies do not provide direct evidence that myosin VI plays a role in CFTR endocytosis. Thus, the next series of studies was conducted to determine whether myosin VI is important for CFTR endocytosis using a dominant-negative myosin VI recombinant fragment. The GFP-MyoVI-Tail disrupts the interaction between endogenous myosin VI and the adaptor-cargo complex (36, 41). Because transfection efficiency is low in Calu-3 cells, transient expression studies using the GFP-MyoVI-Tail were performed in HEK293 cells, which are relatively easy to transfet. To validate HEK293 cells as a model to study the role of myosin VI in CFTR endocytosis, experiments were conducted to identify which isoforms of myosin VI are expressed in HEK293 cells and to determine whether CFTR, myosin VI, Dab2, and clathrin also interact in HEK293 cells.

RT-PCR studies revealed that HEK293 cells express only the shorter splice variant of myosin VI. It has been suggested that this splice variant of myosin VI interacts poorly with Dab2 and does not target well to the plasma membrane (36), implying that HEK293 cells may not be an appropriate model to study the role of myosin VI in CFTR endocytosis. Our co-immunoprecipitation experiments revealed, however, that CFTR expressed in the plasma membrane in HEK293 cells interacts with endogenous myosin VI, Dab2, and clathrin (Fig. 3). Thus, similar to Calu-3 cells, HEK293 cells serve as a good model to study the role of myosin VI in CFTR endocytosis.

**Myosin VI Facilitates CFTR Endocytosis—**If myosin VI plays a role in CFTR endocytosis, it can be predicted that expression of the dominant-negative GFP-MyoVI-Tail fragment will: (1) displace endogenous myosin VI from binding to the Dab2-CFTR-clathrin complex; (2) increase expression of CFTR in the plasma membrane; and (3) decrease CFTR endocytosis. Experiments were conducted to test these predictions. To determine whether the GFP-MyoVI-Tail fragment is sufficient to displace endogenous myosin VI from the Dab2-CFTR-clathrin complex, HEK293 cells were transfected with CFTR without the GFP tag and with the GFP-MyoVI-Tail lacking the large splice insert. The GFP-MyoVI-Tail was immunoprecipitated with a polyclonal anti-GFP antibody. Western blot analysis of the immunoprecipitated complex demonstrated that the GFP-MyoVI-Tail interacts with endogenous myosin VI from binding to the Dab2-CFTR-clathrin complex.

To determine whether the GFP-MyoVI-Tail increased expression of CFTR in the plasma membrane, HEK293 cells were transfected with GFP-CFTR and either the GFP-MyoVI-Tail or GFP, and the plasma membrane expression of CFTR was measured. The GFP-MyoVI-Tail increased the plasma membrane expression of CFTR by >50% compared with the GFP control (Fig. 5).

The GFP-MyoVI-Tail could increase plasma membrane expression of CFTR by inhibiting CFTR endocytosis and/or by stimulating CFTR endocytic recycling. Accordingly, studies were conducted to test the hypothesis that GFP-MyoVI-Tail increased expression of CFTR in the plasma membrane by inhibiting CFTR endocytosis. Endocytosis of CFTR was measured at 2.5, 5, 7.5, and 10 min as described in “Materials and Methods.” Preliminary studies revealed that there was a linear increase in CFTR endocytosis between 0 and 5 min; thus, data are reported at the 5-min time point. As illustrated in Fig. 6A and B, the GFP-MyoVI-Tail decreased CFTR endocytosis by >50% compared with the GFP control. The decrease in CFTR endocytosis is consistent with the increase in the plasma membrane expression of CFTR observed in cells transfected with the GFP-MyoVI-Tail compared with the GFP control.

If the GFP-MyoVI-Tail increased expression of CFTR in the plasma membrane by inhibiting CFTR endocytosis, we predicted that the GFP-MyoVI-Tail should have no effect on the plasma membrane expression of a CFTR mutant that is unable to undergo endocytosis. As described previously, point mutations in the tyrosine and dileucine endocytic motifs in the cytoplasmic C terminus of CFTR completely inhibit endocytosis of a TIR/CFTR C terminus chimera (24). As demonstrated in Fig. 6B, the same point mutations in a full-length CFTR (CFTR Y/A LL/AA) dramatically decreased CFTR endocytosis. Moreover, the GFP-MyoVI-Tail had no effect on the endocytosis of CFTR Y/A LL/AA (Fig. 6B). In addition, the GFP-MyoVI-Tail did not affect the plasma membrane expression of CFTR Y/A LL/AA compared with the GFP control (Fig. 5). Taken together, these data indicate that the GFP-MyoVI-Tail increased the plasma membrane expression of CFTR by specifically inhibiting CFTR endocytosis from the plasma membrane. Moreover, these studies suggest that the YD51 and LL motifs may play an important role in the interaction between CFTR and myosin VI.

Additional studies were conducted to determine whether the GFP-MyoVI-Tail had an effect on fluid-phase endocytosis (56). HEK293 cells were transfected with CFTR (no GFP tag) and Fig. 2. Model of myosin VI splice variants (A and B) and the dominant-negative myosin VI construct used in this study (C). A, myosin VI has three highly conserved functional domains: the N-terminal motor domain (Motor domain), a single IQ domain (IQ), and a tail domain (Globular tail). The tail domain of myosin VI contains a coiled-coil region (Coiled-coil) followed by a C-terminal globular tail region. The tail of myosin VI is alternatively spliced, leading to two forms of myosin VI, a longer (A) and a shorter (B) form, which differ by 31 amino acids (LG, large insert) between the coiled-coil and the globular region (36). C, the dominant-negative GFP fusion construct of porcine myosin VI globular tail (GFP-MyoVI-Tail; Globular tail) used in this study.
Myosin VI and CFTR Endocytosis

**FIG. 3.** Representative immunoprecipitation experiments performed to determine whether CFTR forms macromolecular complexes with myosin VI, Dab2, and clathrin in HEK293 cells. A, HEK293 cells were transiently transfected with GFP-CFTR, the fusion protein was immunoprecipitated with a polyclonal anti-GFP antibody, and the immunoprecipitated complex (IP) was blotted for CFTR, myosin VI (MyoVI), Dab2, and clathrin. B, endogenous myosin VI was immunoprecipitated from HEK293 cells transiently transfected with GFP-CFTR and blotted for myosin VI (MyoVI), Dab2, and clathrin. C, GFP-CFTR was immunoprecipitated from the plasma membrane in HEK293 cells transiently transfected with GFP-CFTR. The immunoprecipitated protein complex (IP) was blotted with antibodies against myosin VI (MyoVI) and Dab2. CFTR in the plasma membrane interacts with myosin VI and Dab2 in HEK293 cells. Molecular weights are shown in kDa. These experiments were repeated three times from separate cultures.

**FIG. 4.** Representative immunoprecipitation experiments performed to determine whether the GFP-MyoVI-Tail, a dominant-negative fragment without the large splice insert, is capable of forming complexes with CFTR, Dab2, and clathrin. HEK293 cells were transfected with GFP-CFTR and the GFP-MyoVI-Tail. The fusion protein was immunoprecipitated with a polyclonal anti-GFP antibody, and the immunoprecipitated complex (IP) was blotted for CFTR, myosin VI (MyoVI), Dab2, and clathrin. *, non-immune rabbit IgG antibody failed to immunoprecipitate the GFP-MyoVI-Tail. The GFP-MyoVI-Tail forms a complex with CFTR, Dab2, and clathrin in HEK293 cells. Molecular weights are shown in kDa. These experiments were repeated three times from separate cultures.

**FIG. 5.** Summary of biotinylation experiments performed to determine the effects of the GFP-MyoVI-Tail on the plasma membrane expression of wt-CFTR and CFTR Y/A LL/AA. HEK293 cells were transfected with GFP-CFTR or GFP-CFTR Y/A LL/AA and either GFP-MyoVI-Tail or GFP. The asterisk indicates $p < 0.05$. The number of experiments was six in each group.

either the GFP-MyoVI-Tail or GFP. Consistent with the previous report (41), the GFP-MyoVI-Tail had no effect on fluid-phase endocytosis as determined by measuring the uptake of the Alexa 647-conjugated dextran (Fig. 6C). These data are in agreement with observations in the Snell’s waltzer mice, which do not express myosin VI and have no defect in fluid-phase endocytosis (44, 60, 61).

**Myosin VI Does Not Play a Role in the Endocytic Recycling of CFTR**—As noted above, the increase in plasma membrane expression of CFTR induced by the GFP-MyoVI-Tail could also result from an increase in the endocytic recycling of CFTR. Accordingly, endocytic recycling of CFTR was measured at 2.5 and 5 min as described in “Materials and Methods.” In preliminary studies, endocytic recycling was linear between 0 and 2.5 min; thus, data are reported at the 2.5-min time point. There was no difference in the endocytic recycling of CFTR in cells expressing the GFP-MyoVI-Tail compared with the GFP control (34.9 ± 14.2% versus 35.8 ± 10.9%, respectively; n = 3, p > 0.05). Thus, myosin VI selectively facilitates CFTR endocytosis.

**Actin Filaments Are Required for CFTR Endocytosis**—Because myosin VI is an actin-based molecular motor, the conclusion that myosin VI facilitates CFTR endocytosis predicts that fragmentation of F-actin will reduce CFTR endocytosis. Studies were conducted to examine the effects of cytochalasin D, an actin depolymerizing agent, on CFTR endocytosis in HEK293 cells stably expressing CFTR. Cytochalasin D (2 μM) decreased the amount of F-actin (Fig. 7A) without affecting cell attachment or cell morphology (Fig. 7, A and B) and did not affect fluid-phase endocytosis (Fig. 7C). CFTR endocytosis was decreased in cells treated with cytochalasin D compared with the vehicle control (Fig. 7D).

**DISCUSSION**

In the present study, we have shown that CFTR in the apical plasma membrane in polarized human airway epithelia cells interacts with a complex of proteins including myosin VI, Dab2, and clathrin, and that myosin VI facilitates CFTR endocytosis. Our data provide the first direct evidence demonstrating a role for myosin VI in facilitating the endocytosis of a cargo protein other than the TIR (36, 41, 47). Our data indicate that, similar to TIR, CFTR endocytosis is assisted by myosin VI.

Our studies suggest that loss of myosin VI would be expected to slow down CFTR endocytosis and to increase CFTR expression in the apical plasma membrane in airway epithelial cells.
This effect should increase airway fluid secretion, which should not lead to any untoward change in airway physiology. Consistent with this prediction, humans with defective myosin VI as well as myosin VI knock-out mice have no reported airway disease (44, 60).

**How Does Myosin VI Facilitate CFTR Endocytosis?**—It has been proposed that myosin VI may regulate clathrin-mediated endocytosis by: (1) selecting and clustering membrane proteins into clathrin-coated pits; (2) enhancing the budding of clathrin-coated vesicles; and (3) facilitating the trafficking of uncoated vesicles on actin filaments. Our data that apical plasma membrane CFTR interacts with myosin VI, Dab2, and clathrin suggest that myosin VI may regulate selection and clustering of CFTR into clathrin-coated pits and/or budding of the clathrin-coated vesicles containing CFTR. Additional studies, beyond the scope of the present study, are required to determine which of the two stages of CFTR endocytosis is regulated by myosin VI.

**Is the Large Splice Insert in the Myosin VI Tail Required for Interaction with the Dab2-CFTR-Clathrin Protein Complex?**—The role of the large splice insert in myosin VI function is controversial (36, 41). It has been suggested that the large splice insert is required for myosin VI to interact with Dab2 and to target myosin VI to the plasma membrane (36). However, we demonstrate that the large splice insert is not necessary for myosin VI to interact with a complex of proteins including CFTR, Dab2, and clathrin (Figs. 3 and 4). Moreover, we find that myosin VI without the large splice insert interacts with Dab2 and clathrin, while myosin III does not (42).
with the Dab2-CTF complex in the plasma membrane (Fig. 3).

We speculate that this insert may play a role in regulating the affinity of myosin VI binding to adaptor proteins including Dab2.

Working Model of the Myosin VI Role in CTF Endocytosis—The data in this report suggest that myosin VI is important for efficient endocytosis of CTF. In addition, the Dab2-myosin VI complex may be important for targeting CFTR cargo into a distinct vesicles and that adaptor molecules regulate the selection of different cargo proteins at the plasma membrane (65, 66).

We postulate that Dab2 interacts with CTF indirectly via AP-2 because both CTF and Dab2 bind to the AP-2 complex. Dab2 binds to the α subunit of AP-2 (59), and CTF, via the endocytic YDSI motif, binds directly to the Dab2.

The data in this report suggest that myosin VI is important for plasma membrane expression of CFTR. However, at the present time, we cannot exclude a direct interaction between CTF and Dab2. Additional studies, beyond the scope of this manuscript, are required to determine the interaction between CTF and Dab2.

In summary, our data provide direct evidence that myosin VI and its adaptor protein Dab2 facilitate selection and clustering of CTF into clathrin-coated pits and/or budding of the clathrin-coated vesicles containing CTF. Current work is focusing on establishing which step of the early endocytic pathway of CTF requires myosin VI and elucidating the Dab2-CTF interaction.

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