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Myosin Vb Is Required for Trafficking of the Cystic Fibrosis Transmembrane Conductance Regulator in Rab11a-specific Apical Recycling Endosomes in Polarized Human Airway Epithelial Cells*

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Cystic fibrosis transmembrane conductance regulator (CFTR)-mediated Cl⁻ secretion across fluid-transporting epithelia is regulated, in part, by modulating the number of CFTR Cl⁻ channels in the plasma membrane by adjusting CFTR endocytosis and recycling. However, the mechanisms that regulate CFTR recycling in airway epithelial cells remain unknown, at least in part, because the recycling itineraries of CFTR in these cells are incompletely understood. In a previous study, we demonstrated that CFTR undergoes trafficking in Rab11a-specific apical recycling endosomes in human airway epithelial cells. Myosin Vb is a plus-end-directed, actin-based mechanoenzyme that facilitates protein trafficking in Rab11a-specific recycling vesicles in several cell model systems. There are no published studies examining the role of myosin Vb in airway epithelial cells. Thus, the goal of this study was to determine whether myosin Vb facilitates CFTR recycling in polarized human airway epithelial cells. Endogenous CFTR formed a complex with endogenous myosin Vb and Rab11a. Silencing myosin Vb by RNA-mediated interference decreased the expression of wild-type CFTR and ΔF508-CFTR in the apical membrane and decreased CFTR-mediated Cl⁻ secretion across polarized human airway epithelial cells. A recombinant tail domain fragment of myosin Vb attenuated the plasma membrane expression of CFTR by arresting CFTR recycling. The dominant-negative effect was dependent on the ability of the myosin Vb tail fragment to interact with Rab11a. Taken together, these data indicate that myosin Vb is required for CFTR recycling in Rab11a-specific apical recycling endosomes in polarized human airway epithelial cells.

The cystic fibrosis transmembrane conductance regulator (CFTR)[2] is an ATP-binding cassette transporter and a cAMP-activated Cl⁻ channel that mediates transepithelial Cl⁻ transport in many diverse tissues, including the airways (1–3), where CFTR plays a critical role in mucociliary clearance by regulating the formation and maintenance of the airway surface liquid (reviewed in Refs. 4 and 5). Cystic fibrosis, a lethal genetic disease, is caused by mutations in the CFTR gene (1, 2), and lung disease is the major cause of morbidity and mortality in cystic fibrosis patients (6, 7). Numerous studies have revealed that CFTR-mediated Cl⁻ secretion across polarized epithelial cells is regulated by modulating channel activity and by adjusting the total number of CFTR channels in the plasma membrane (reviewed in Refs. 8 and 9). The latter is achieved by the removal (*i.e.* endocytosis) and the insertion (*i.e.* recycling) of CFTR channels into the plasma membrane. The mechanisms that regulate CFTR recycling in airway epithelial cells have not been elucidated, at least in part, because the recycling itineraries of CFTR in these cells are practically unknown.

Recycling is a dynamic trafficking event during which proteins are transported from intracellular compartments and inserted into the plasma membrane. Recycling requires formation of macromolecular complexes with numerous adaptors, including myosin motors (10, 11). The non-conventional processive myosin motors (a class of mechanoenzymes that convert the energy from ATP hydrolysis into mechanical work) take multiple steps on actin filaments (12–15) and move cargo proteins contained in specific transport organelles. The plus-end-directed movement characteristic of class V myosins supports anterograde trafficking, including recycling (reviewed in

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[2] The abbreviations used are: CFTR, cystic fibrosis transmembrane conductance regulator; WT, wild-type; VPM, Vitrogen plating medium; HEK, human embryonic kidney; RT, reverse transcription; siRNA, small interfering RNA; siMyoVb, double-stranded small interfering RNA against human myosin Vb; Non-sil.siRNA, double-stranded non-silencing control small interfering RNA; Q, quantitative; MyoVb-T, myosin Vb tail domain; GFP, green fluorescent protein; GTPγS, guanosine 5′-3-*O*-(thio)triphosphate; GST, glutathione *S*-transferase.

Refs. 16 and 17). The plasma membrane recycling in non-polarized cells and the apical plasma membrane recycling in polarized epithelial cells occur primarily in Rab11a-positive endosomes (18–20). A recent study demonstrated that the recycling of CFTR in human airway epithelial cells occurs in the Rab11a recycling compartment (21).

Myosin Vb interacts specifically with the GTP-bound (*i.e.* membrane-bound) form of Rab11a (22), suggesting that myosin Vb may be recruited to Rab11a-specific recycling endosomes. Studies using dominant-negative fragments of myosin Vb have demonstrated that, together with Rab11a, myosin Vb facilitates protein recycling in several polarized and non-polarized cell models (23–26). Furthermore, a recent study demonstrated that myosin Vb facilitates trafficking of the α-amino-3-hydroxy-5-methyl-4-isoxasole propionate-type glutamate receptor subunit GluR1 in subpopulations of neurons by a Rab11-dependent mechanism (27). However, nothing is known about the role of myosin Vb in CFTR trafficking in human airway epithelial cells.

Thus, the objective of this study was to test the hypothesis that endogenous myosin Vb facilitates CFTR recycling in Rab11a-specific apical recycling compartments in polarized human airway epithelial cells. We report that myosin Vb is required for the recycling of wild-type (WT) CFTR and ΔF508-CFTR to the apical membrane of polarized human airway epithelial cells in a Rab11a-dependent fashion.

MATERIALS AND METHODS

Cell Lines and Cell Culture—Calu-3 cells (American Type Culture Collection, Manassas, VA) were seeded at 2×10^6 on Transwell permeable supports (4.67 cm^2, 0.4-μm pore size; Corning Corp., Corning, NY) coated with Vitrogen plating medium (VPM) containing Dulbecco's modified Eagle's medium (Invitrogen), 10 μg/ml human fibronectin (BD Biosciences), 1% purified collagen (PureCol™, Inamed, Fremont, CA), and 10 μg/ml bovine serum albumin (Invitrogen) and maintained as polarized monolayers in air-liquid interface culture as described previously (28, 29). Serum was removed from the medium 24 h before experiments to augment cell polarization and cell cycle synchronization (30, 31). CFBE41o$^-$ cells (ΔF508/ΔF508) stably transduced with either WT-CFTR or ΔF508-CFTR were a generous gift from Dr. J. P. Clancy (University of Alabama at Birmingham) (32). The stably transduced CFBE41o$^-$ cells were seeded at 1×10^6 on VPM-coated Transwell (4.67 cm^2) or Snapwell (1.12 cm^2, 0.4-μm pore size; Corning Corp.) permeable supports and maintained as polarized monolayers in air-liquid interface culture as described previously (21, 32). In addition, CFBE41o$^-$ cells were seeded on VPM-coated plastic tissue culture plates (1×10^6 cells/9.4-cm^2 plate; Corning Corp.). To increase export from the endoplasmic reticulum and expression of ΔF508-CFTR at the plasma membrane, cells were cultured at 27 °C for 36 h (21, 32). Fetal bovine serum and the selection antibiotic were removed from the medium 24 h before experiments. Human embryonic kidney (HEK) 293 cells stably expressing WT-CFTR were a generous gift of Dr. Neil A. Bradbury (Rosalind Franklin University of Medicine and Science, Chicago, IL) (33). HEK293 cells were cultured in VPM-coated tissue culture flasks (3×10^6 cells/25-cm^2 flask; Corning Corp.) or plastic tissue culture plates (1×10^6 cells/9.4-cm^2 plate) and maintained as described previously (29, 34). Fetal bovine serum and the selection antibiotic were removed from the medium 24 h before experiments. COS-7 cells (American Type Culture Collection) were cultured in tissue culture dishes (0.75×10^6 cells/55-cm^2 dish) in Dulbecco's modified Eagle's medium supplemented with 100 μg/ml streptomycin, 100 units/ml penicillin, and 10% fetal bovine serum in a 5% CO_2 and 95% air incubator at 37 °C as described previously (35). Calu-3 cells were studied because they express high levels of endogenous CFTR; however, Calu-3 cells are very difficult to transfect. CFBE41o$^-$ cells were used because they are isogenic except for CFTR, and they are relatively easy to transfect. However, CFBE41o$^-$ cells express WT-CFTR and ΔF508-CFTR transgenes; thus, studies on these cells do not examine endogenous CFTR. Finally, HEK293 and COS-7 cells were also used because they are very easy to transfect and are a well accepted model for conducting biochemical studies, including co-immunoprecipitation studies. However, because these cells are fibroblasts and are not polarized, their use was limited.

RNA Isolation and Reverse Transcription (RT)-PCR—RT-PCRs were conducted to examine the endogenous expression of myosin Vb in human airway epithelial cells (Calu-3 and CFBE41o$^-$) and in HEK293 cells as described previously (29). Total RNA was isolated from each cell line using an RNeasy mini kit (Qiagen Inc.) and treated with DNase (DNA-free, Ambion, Inc., Austin, TX) to remove contaminating DNA. Total RNA was quantified using a NanoDrop spectrophotometer, and the quality was assessed using an Agilent 2100 bioanalyzer. Two-step RT-PCR was performed using RETROscript reverse transcriptase (Ambion, Inc.) with random decamers and *Taq* DNA Polymerase (Invitrogen). Oligonucleotide primers flanking nucleotides 3692–4396 (sense, 5′-ACCAAGCCACGCAGAATAACT-3′; and antisense, 5′-GGACCGTGACCTGCCTGTTGA-3′) were designed using Oligo 6.1 primer analysis software and synthesized by Integrated DNA Technologies (Coralville, IA). These primers were used to amplify a single 705-bp myosin Vb PCR product. The product was subcloned into pCR4-TOPO (Invitrogen), and the sequence was verified by ABI PRISM dye terminator cycle sequencing (Applied Biosystems, Foster City, CA).

RNA-mediated Interference—A sequence corresponding to a non-conserved region of human myosin Vb cDNA, 5′-AACGAGAATCTGGACCTTAAA-3′ (positions 4227–4248; GenBank™ accession number AB032945) (36), was used as a target for the small interfering RNA (siRNA) directed against human myosin Vb. The double-stranded siRNA against human myosin Vb (siMyoVb) was synthesized by Qiagen Inc. The double-stranded non-silencing control siRNA (Non-sil.siRNA) target sequence 5′-AATTCTCCGAACGTGTCACGT-3′, which showed no significant homology to any other gene known analyzed using BLAST search. Non-sil.siRNA was purchased from Qiagen Inc. (catalog no. 1022076). Transfection of siMyoVb or Non-sil.siRNA into HEK293 and CFBE41o$^-$ cells was conducted using HiPerFect (Qiagen Inc.) according to the manufacturer's instructions. HEK293 or CFBE41o$^-$ cells (1×10^6) were plated on 9.4-cm^2 tissue culture plates and incubated with the optimized transfection mixture (5 nM siMyoVb or Non-sil.

siRNA and 10 μl of HiPerFect) at 37 °C. After 24 h, the transfection mixture was replaced with fresh cell culture medium, and cells were cultured for different lengths of time. Because silencing of myosin Vb expression was maximum between 48 and 72 h, experiments were conducted at ~60 h after transfection with siMyoVb. The efficiency of silencing myosin Vb expression was assessed by quantitative (Q) RT-PCR and Western blotting. The specificity of silencing myosin Vb expression was determined by examining the expression of myosin Vc, another member of the class V myosin family expressed in epithelial cells (37), and by examining the expression of myosin VI, a myosin known to facilitate CFTR endocytosis (29), by Western blotting. A pre-designed siRNA targeting another non-conserved region of the human myosin Vb gene (GenBank™ accession number AB032945) was purchased from Qiagen Inc. (catalog no. SI00653527). Because siMyoVb (target sequence 4227–4248) silenced myosin Vb protein expression more effectively than did SI00653527, siMyoVb was used in subsequent experiments. (Myosin Vb expression was 44.3 ± 10.4 and 17.2 ± 9.2% of the control after transfection of HEK293 cells with SI00653527 and siMyoVb, respectively, as assessed by Western blotting ($n = 3$ in each group).)

Experiments were performed to silence the expression of myosin Vb in CFBE41o$^-$ cells cultured on Transwell and Snapwell permeable supports. CFBE41o$^-$ cells (1×10^6) stably expressing either WT-CFTR or ΔF508-CFTR were plated on 9.4-cm^2 tissue culture plates and incubated with the optimized transfection mixture (10 nM siMyoVb or Non-sil.siRNA and 20 μl of HiPerFect) at 37 °C. After 24 h, cells were trypsinized and plated on VPM-coated Transwell or Snapwell permeable supports and cultured for an additional 6 days to establish polarized monolayers. CFBE41o$^-$ cells stably expressing ΔF508-CFTR were cultured at 27 °C during the last 36 h to increase trafficking and expression of ΔF508-CFTR in the plasma membrane. The efficiency of silencing myosin Vb expression in polarized CFBE41o$^-$ cells was assessed by Western blotting.

Q-RT-PCR—Q-RT-PCRs were conducted to measure silencing of myosin Vb expression by siMyoVb. The sequence of human myosin Vb (GenBank™ accession number AB032945) was submitted to the Assays-by-Design services (Applied Biosystems) for the primers and probe design. The probe target was set to a predicted exon-exon splice junction. The probe was 6-carboxyfluorescein-labeled with a minor groove binding modification and non-fluorescent quencher on the 3′-end. The primers (18 μM each; 5′-GGAACCTGGTGACAGACTTGAAG-3′ (sense) and 5′-CCGGATGCACATGTAGAGGAT-3′ (antisense)), the probe (5 μM; 6-carboxyfluorescein-CTGTGCCCGACAGCAT (antisense)) combined with TaqMan Universal Master Mix (Applied Biosystems), and cDNA were placed in triplicates in a 96-well format spectrofluorometric thermal cycler (ABI PRISM 7700 sequence detection system, Applied Biosystems). Q-RT-PCR products were size-verified on a low melting point agarose gel, subcloned into pCR4-TOPO, and sequence-verified by ABI PRISM dye terminator cycle sequencing. The standard curve prepared from the myosin Vb plasmid DNA isolated from HEK293 cells (not siRNA-transfected; control) demonstrated $r^2 > 0.99$ and was linear over a 9-log range. Equivalent amplification efficiencies of standard and target molecules were observed. Gel analysis, SYBR Green melting curve dissociation analysis, and sequencing revealed a single PCR product. Raw data were analyzed; base-line and threshold values were set; and gene expression was interpolated using the standard curve. The cDNA generated during reverse transcription and used as a template was quantified using the NanoDrop spectrophotometer, and data were calculated as gene expression (fg/ng cDNA). The expression level of human myosin Vb is reported as a percent of the control.

Plasmids and Transient Transfection—cDNA encoding the entire tail domain of mouse myosin Vb (referred to as MyoVb-T) and cDNA encoding a truncated globular tail region of the mouse myosin Vb tail domain (referred to as MyoVb-GT) were amplified from the mouse cDNA library by RT-PCR as described previously (38) using the following oligonucleotides: 5′-GGATCCAGCTCCCCAGACAGCTACAGC-3′ (MyoVb-T sense primer), 5′-TCAGACTTCATTGAGAAACT-3′ (MyoVb-T/GT antisense primer), and 5′-CGGATCCGAAAAGCTGGAGAAGAATGA-3′ (MyoVb-GT sense primer). Addition of the FLAG tag to the N terminus of the myosin Vb constructs and construction of the expression vector pEF were performed as described previously (38–40). The resulting fusion fragments consisted of part of the proximal/medial tail region and the entire globular tail region of the myosin Vb tail domain (amino acids 1231–1818; FLAG-MyoVb-T) and a truncated globular tail region of the myosin Vb tail domain (amino acids 1384–1818; FLAG-MyoVb-GT). A plasmid containing the green fluorescent protein (GFP)-tagged tail domain of human myosin Vc (amino acids 902–1742; GFP-MyoVc-T) was generated using the eukaryotic expression vector pEGFP-C2 as previously described (37). Constructs were sequence-verified by ABI PRISM dye terminator cycle sequencing. Transfection of cells with plasmids was performed according to the manufacturer's instructions. HEK293 cells were transfected using Effectene® (Qiagen Inc.) or FuGENE 6 (Roche Diagnostics), and transfection of CFBE41o$^-$ cells was performed using FuGENE 6 (29). The above cells were harvested 48 h after transfection. Transfection of COS-7 cells was performed using Lipofectamine Plus (Invitrogen), and the cells were harvested 72 h after transfection (35).

Production of Affinity-purified Antibodies to Myosin Vb—Two polyclonal antibodies against myosin Vb were raised in rabbits, antibody 2506 (against sequence QDSKKVQAEPPQTDIDLDPN, amino acids 1174–1193) and antibody 2507 (against sequence AGRNAEPNINARSSWPNSE, amino acids 1278–1296), in the proximal/medial tail region of the tail domain (PickCell Laboratories BV, Amsterdam, The Netherlands). These myosin Vb sequences were absent in any other known genes as analyzed by a BLAST search. Rabbits were injected and boosted two times. The antiserum was affinity-purified, and the specificity of the anti-myosin Vb antibodies was determined by the ability of the antibodies to detect endogenous myosin Vb and the FLAG-tagged myosin Vb fragments. The selectivity of the antibodies for myosin Vb was determined by the ability of the anti-myosin Vb antibodies to detect silencing of endogenous myosin Vb.

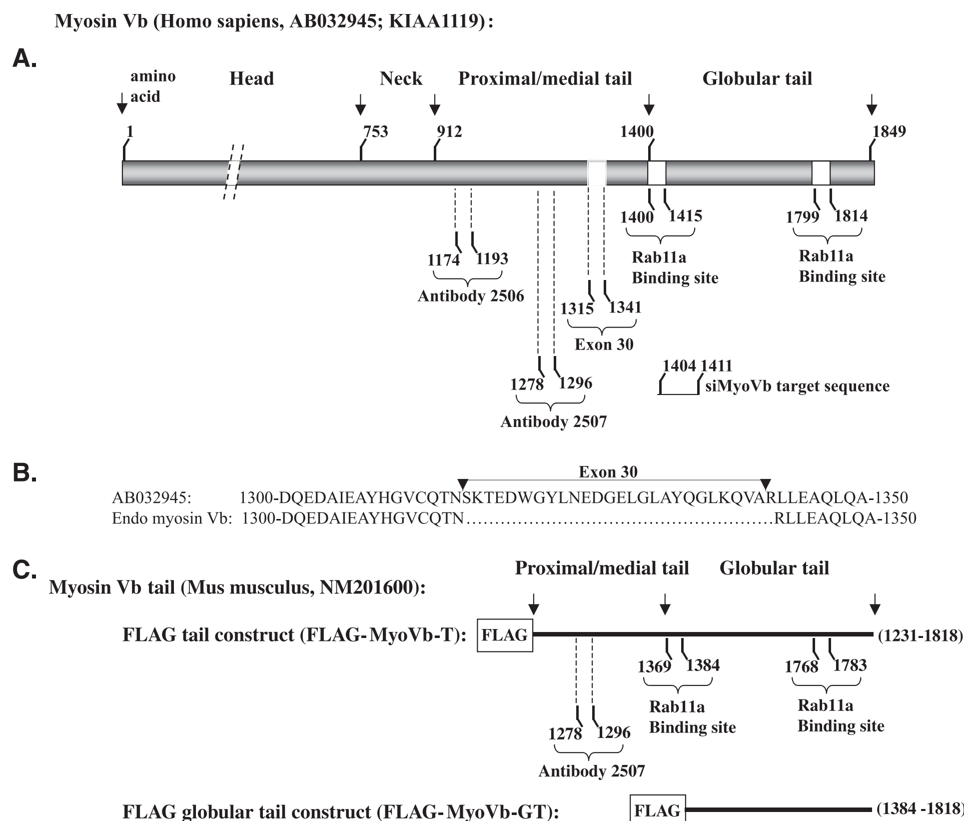

FIGURE 1. **Domain map of myosin Vb.** *A*, human myosin Vb (GenBank™ accession number AB032945 for KIAA11119 protein) (36). The tail domain consists of the proximal/medial tail region and the globular tail region. In addition, illustrated are the locations of exon 30, the Rab11a-binding sites, and the amino acid sequences used to generate anti-myosin Vb antibodies 2506 and 2507. *B*, alignment of human myosin Vb (GenBank™ accession number AB032945) and endogenous (*Endo*) myosin Vb cloned from human airway epithelial cells (CFBE41o⁻ and Calu-3) and HEK293 cells. Note that the myosin Vb cloned from the three human cell lines does not contain exon 30, as indicated by the *arrowheads*. Interestingly, this 26-amino acid region may correspond to exon D of myosin Va, a fragment of similar size spliced out of the brain form of myosin Va (37, 76, 77). *C*, FLAG-tagged fragments of the tail domain of mouse myosin Vb. FLAG-MyoVb-T contains part of the proximal/medial tail region and the entire globular tail region of the myosin Vb tail domain (amino acids 1231–1818). This fragment contains both Rab11a-binding sites (22). Furthermore, this fragment contains the amino acid sequence used to generate anti-myosin Vb antibody 2507. FLAG-MyoVb-GT spans amino acids 1384–1818 and contains a truncated globular tail region without the Rab11a-binding site located near the N-terminal end of the globular tail.

Other Antibodies—The antibodies used were as follows: rabbit anti-myosin Va antibody (41); rabbit anti-myosin Vc antibody 199 (37); rabbit anti-myosin VI tail antibody (a gift from Dr. Tama Hasson, University of California at San Diego, La Jolla, CA) (42); mouse anti-human CFTR C terminus antibody (clone 24-1; R&D Systems, Minneapolis, MN); mouse anti-CFTR antibody (clone M3A7; Upstate Biotechnology, Lake Placid, NY); mouse anti-GFP (clone JL-8) and mouse anti-ezrin antibodies (BD Biosciences); horseradish peroxidase-conjugated mouse anti-FLAG antibody M2, mouse anti-FLAG antibody M2, and rabbit anti-FLAG antibody F7425 (Sigma); rabbit anti-Rab11a antibody (Zymed Laboratories Inc., South San Francisco, CA); mouse anti-Rab8 antibody (BD Transduction Laboratories); and horseradish peroxidase-conjugated goat anti-mouse and goat anti-rabbit secondary antibodies (Bio-Rad). All antibodies were used at the concentrations recommended by the manufacturers or as indicated in the figure legends.

Immunoprecipitation and Immunoblotting—CFTR and myosin Vb were immunoprecipitated from cell lysates by the methods described previously (21, 29). Briefly, cultured cells were solubilized in immunoprecipitation buffer containing 150 mM NaCl, 50 mM Tris (pH 7.2), 0.1% Igepal (Sigma), 5 mM $MgCl_2$, 5 mM EDTA, 1 mM EGTA, 30 mM NaF, 1 mM Na_3VO_4, Complete protease inhibitor Mixture (Roche Diagnostics), and 40 μM GTPγS (EMD Biosciences, San Diego, CA), a non-hydrolyzable analog of GTP (43–46). After centrifugation at 14,000 × g for 15 min to pellet insoluble material, the soluble lysates were incubated for 10 min at 30 °C with an additional 40 μM GTPγS. After cooling to 4 °C, the soluble lysates were precleared by incubation with protein G or protein A, as appropriate, conjugated to Sepharose beads (Pierce) at 4 °C. The precleared lysates (1000 μl) were added to the antibody-protein G/A-Sepharose bead (wet volume of 120 μl) complexes. CFTR was immunoprecipitated by incubation with the mouse antibody M3A7-protein G-Sepharose complexes, and myosin Vb was immunoprecipitated by incubation with the rabbit antibody 2506-protein A-Sepharose complexes. After washing the protein G/A-Sepharose complexes with immunoprecipitation buffer, immunoprecipitated proteins were eluted by incubation at 100 °C for 3 min in sample buffer (Bio-Rad) containing 80 mM dithiothreitol. Immunoprecipitated proteins were separated by SDS-PAGE using 7.5 or 15% gels (Bio-Rad) and analyzed by Western blotting with an appropriate primary antibody and a horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibody. The immunoreactive bands were visualized with Western Lightning Chemiluminescence Reagent Plus (PerkinElmer Life Sciences). In addition, a tissue blot containing lysates from human brain (5–20% SDS-PAGE; Chemicon, Temecula, CA) was used for Western blotting as described above.

Glutathione S-Transferase (GST) Pulldown Assay—cDNA encoding mouse Rab11a was subcloned into the pGEX-4T-3 vector (Amersham Biosciences) (47). GST-Rab11a was expressed in *Escherichia coli* JM109 and purified by standard protocols. Glutathione-Sepharose beads (wet volume of 10 μl; Amersham Biosciences) coupled with 10 μg of purified GST-Rab11a were incubated for 1 h at 4 °C with COS-7 cell lysates (400 μl) containing either FLAG-MyoVb-T or FLAG-MyoVb-GT in binding buffer (50 mM HEPES-KOH (pH 7.2), 150 mM NaCl, 1 mM $MgCl_2$, 1% Triton X-100, 0.5 mM GTPγS, 0.1 mM phenylmethylsulfonyl fluoride, 10 μM leupeptin, and 10

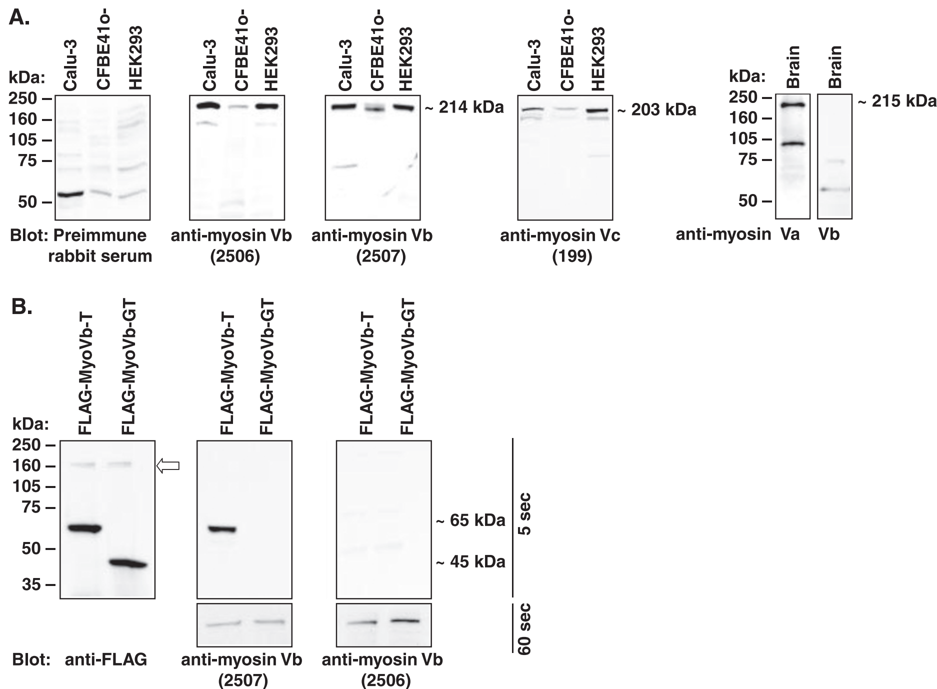

FIGURE 2. **Western blot experiments demonstrating endogenous expression of myosin Vb.** *A*, anti-myosin Vb antibodies 2506 and 2507 recognize a single endogenous product in Calu-3, CFBE41o$^-$, and HEK293 cell lysates of the appropriate molecular mass (~214 kDa). Anti-myosin Vc antibody 199 demonstrated expression of myosin Vc in these three cell lines. Anti-myosin Va antibody demonstrated expression of myosin Va in human brain lysates. In contrast, anti-myosin Vb antibodies 2506 (shown) and 2507 (not shown) failed to label myosin Va in the brain lysates, indicating that these antibodies did not cross-react with myosin Va. Equal amounts of total protein were loaded in each lane. Proteins were separated by SDS-PAGE using 7.5 or 5–20% (human brain lysate) gels. The concentrations and dilutions of the antibodies used were as follows: anti-myosin Va antibody, 1 μg/μl and 1:2000, anti-myosin Vb antibodies 2506 and 2507, 0.4 and 0.43 μg/μl, respectively, and 1:1000, anti-myosin Vc antibody, 0.1 μg/μl and 1:500; and preimmune serum, 1:1000. Experiments were repeated two to three times using separate cultures with similar results. *B*, representative Western blots demonstrating the specificity of antibodies 2506 and 2507 for myosin Vb. HEK293 cells were transiently transfected with either FLAG-MyoVb-T or FLAG-MyoVb-GT (see Fig. 1*C*). *Left panel*, shown is the expression of the FLAG-tagged fragments using anti-FLAG antibody M2. The *arrow* points to a nonspecific 160-kDa band (distinct from the molecular mass of myosin Vb) demonstrated with the anti-FLAG antibody. *Center panel*, only FLAG-MyoVb-T was recognized by antibody 2507 because only this fragment contains an epitope for this antibody. *Right panel*, neither of the myosin Vb fragments was recognized by antibody 2506 because neither of the two fragments contains an epitope for this antibody. It is important to note that the endogenous expression of myosin Vb was demonstrated by antibodies 2506 and 2507 (*lower panels*, 60-s exposure).

μM pepstatin A). After washing the glutathione-Sepharose bead-GST-Rab11a complexes with binding buffer, the bound proteins were eluted by incubation with sample buffer and analyzed by 10% SDS-PAGE followed by immunoblotting with horseradish peroxidase-conjugated anti-FLAG antibody M2. The immunoreactive bands were visualized with an enhanced chemiluminescence kit (Amersham Biosciences).

Biochemical Determination of Apical Membrane CFTR—The biochemical determination of apical membrane CFTR at steady state was performed by domain-selective cell-surface biotinylation using EZ-Link™ Sulfo-NHS-LC-Biotin (Pierce) as described previously in detail (48, 49).

Endocytic and Recycling Assays—Endocytic and recycling assays were performed in HEK293 cells essentially as described previously (49–53). For both assays, the plasma membrane proteins were first biotinylated at 4 °C using EZ-Link™ Sulfo-NHS-SS-Biotin (Pierce). For the endocytic assay, cells were warmed to 37 °C for 2.5, 5, 7.5, or 10 min after biotinylation, and the disulfide bonds on the proteins biotinylated with Sulfo-NHS-SS-Biotin and remaining in the plasma membrane were reduced by GSH (Sigma) 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 using streptavidin-agarose beads, eluted into SDS sample buffer, and separated by 7.5% SDS-PAGE. For the recycling assay, cells were warmed to 37 °C for 5 min after biotinylation to load endocytic vesicles with biotinylated proteins, including CFTR. Subsequently, cells were cooled immediately to 4 °C, and the disulfide bonds on the proteins biotinylated with Sulfo-NHS-SS-Biotin and remaining in the plasma membrane were reduced by GSH at 4 °C. Subsequently, cells were either lysed or warmed again to 37 °C for 2.5 or 5 min (to allow endocytosed and biotinylated CFTR to recycle to the plasma membrane). Cells were then cooled again to 4 °C, and the disulfide bonds on the proteins biotinylated with Sulfo-NHS-SS-Biotin and remaining in the plasma membrane were reduced with GSH. The recycling of endocytosed CFTR was calculated as the difference between the amount of biotinylated CFTR after the first and second GSH treatments.

Ussing Chamber Measurements—Ussing chamber measurements were performed as described previously (54).

Data Analysis and Statistics—Statistical analysis of the data was performed using GraphPad Prism Version 4.0 for Macintosh (GraphPad Software Inc., San Diego). The means were compared by a two-tailed t test. A p value <0.05 was considered significant. Data are expressed as the means ± S.E.

RESULTS

Determination of the Endogenous Expression of Myosin Vb in Human Airway Epithelial Cells and Development of Anti-myosin Vb Antibodies—Three members of the myosin V family encoded by three different genes are expressed in mammalian cells (37, 55–57). Myosin Va is expressed predominantly in neuronal cells. In contrast, myosins Vb and Vc are enriched in epithelial cells. RT-PCR studies revealed that human airway epithelial cells (Calu-3 and CFBE41o$^-$) and HEK293 cells express a single myosin Vb product. The sequence of the PCR product was identical to the sequence of human myosin Vb (GenBank™ accession number AB032945 for KIAA1119 protein) (36), except that the sequence in airway and HEK293 cells lacks exon 30 (Fig. 1, *A* and *B*). We developed two affinity-purified rabbit polyclonal anti-myosin Vb antibodies as

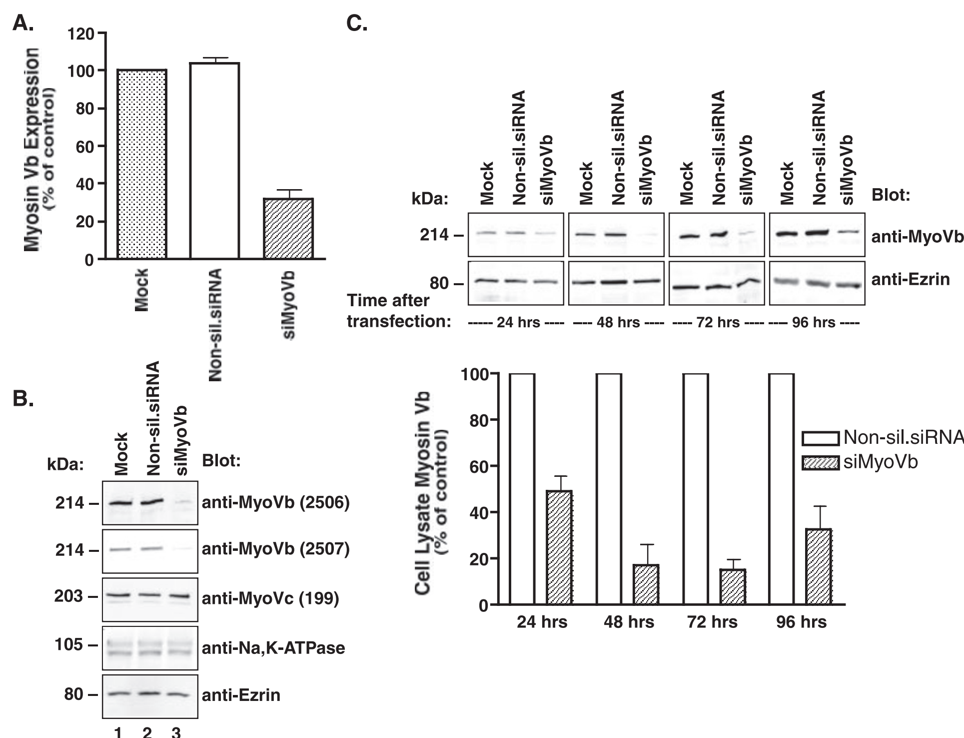

FIGURE 3. **Summary of experiments performed to determine the selectivity of antibodies 2506 and 2507 for myosin Vb using RNA-mediated interference.** HEK293 cells were transfected with siMyoVb or Non-sil.siRNA. *A*, shown is a summary of Q-RT-PCR experiments. *B*, shown are representative Western blots illustrating that antibodies 2506 and 2507 specifically recognized myosin Vb. siMyoVb did not decrease the expression of myosin Vc as determined by Western blotting with the anti-myosin Vc antibody, indicating that the anti-myosin Vb antibodies did not cross-react with myosin Vc. *C*, silencing myosin Vb reached a maximum between 48 and 72 h after transfection with siMyoVb. The data are the results of two experiments (*A*) and three experiments (*B* and *C*)/group.

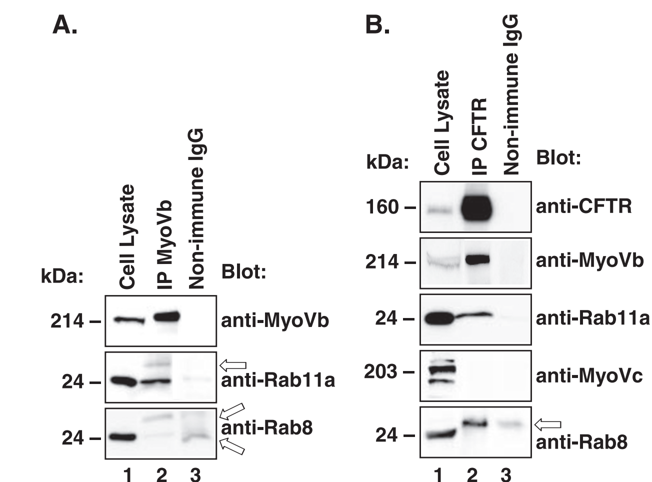

FIGURE 4. **Immunoprecipitation experiments demonstrating the interaction between a complex of proteins including myosin Vb, Rab11a, and CFTR in polarized human airway epithelial cells (Calu-3).** *A*, myosin Vb was immunoprecipitated from Calu-3 cells (*IP MyoVb*) using antibody 2506. *B*, CFTR was immunoprecipitated from Calu-3 cells using antibody M3A7 (*IP CFTR*). The nonspecific bands are marked with *arrows* and represent the light chains (~25 kDa) of the immunoprecipitating antibodies (*A* and *B*). Cell lysates represent 1% of the immunoprecipitated samples. Proteins were separated by SDS-PAGE using 7.5 or 15% gels. All experiments were repeated three times using separate cultures with similar results.

described under "Materials and Methods." Antibodies 2506 and 2507, directed against unique amino acid sequences in the proximal/medial tail region of the tail domain of myosin Vb (Fig. 1*A*), recognized a single endogenous product in Calu-3, CFBE41o^{-}, and HEK293 cell lysates of the appropriate molecular mass (~214 kDa) (Fig. 2*A*). In addition, antibody 2507 recognized the FLAG-tagged myosin Vb tail fragment containing the fragment of the proximal/medial tail region used as an epitope for this antibody (FLAG-MyoVb-T) (Figs. 1, *A* and *C*; and 2*B*). The endogenous product recognized on Western blots as myosin Vb by antibodies 2506 and 2507 did not result from cross-reacting with myosin Va (the primarily neuronal member of class V myosins) because antibodies 2506 and 2507 did not recognize myosin Va in human brain lysates (Fig. 2*A*). Because myosin Vc is enriched in epithelial cells and is expressed by Calu-3, CFBE41o^{-}, and HEK293 cells (Fig. 2*A*), experiments were conducted to examine the specificity of antibodies 2506 and 2507 for myosin Vb using RNA-mediated interference. HEK293 cells were transfected with siMyoVb (target sequence 4227–4248) or Non-sil.siRNA. siMyoVb decreased myosin Vb expression as determined by Q-RT-PCR (Fig. 3*A*). In contrast, Non-sil.siRNA had no effect on the endogenous expression of myosin Vb compared with non-transfected cells. Western blot analysis with antibodies 2506 and 2507 confirmed silencing of myosin Vb expression (Fig. 3, *B* and *C*). siMyoVb did not decrease the expression of myosin Vc as determined by Western blotting with the anti-myosin Vc antibody (Fig. 3*B*), indicating that the anti-myosin Vb antibodies specifically recognized myosin Vb and did not cross-react with myosin Vc. Taken together, the above data demonstrate that the affinity-purified rabbit anti-myosin Vb polyclonal antibodies 2506 and 2507 are specific and selective for myosin Vb.

CFTR Co-immunoprecipitates with the Endogenous Myosin Vb-Rab11a Complex in Polarized Human Airway Epithelial Cells—Members of the myosin V family are recruited to distinct transport organelles by organelle-specific Rab GTPases (58–60). Myosin Vb is thought to be specifically recruited to Rab11a-specific recycling endosomes because myosin Vb interacts specifically with the GTP-bound (*i.e.* membrane-bound) form of Rab11a (22). We examined whether endogenous myosin Vb and Rab11a interact in polarized human airway epithelial cells. Myosin Vb was immunoprecipitated from polarized Calu-3 cells using anti-myosin Vb polyclonal antibody 2506. Western blot analysis of the immunoprecipitated complexes demonstrated that endogenous myosin Vb interacted specifically with endogenous Rab11a (Fig. 4*A*).

If myosin Vb facilitates CFTR trafficking in Rab11a-specific recycling endosomes, CFTR should co-immunoprecipitate with the myosin Vb-Rab11a complex. To test this prediction, CFTR was immunoprecipitated from Calu-3 cells using anti-CFTR monoclonal antibody M3A7. Western blot analysis of the immunoprecipitated complexes demonstrated that endogenous CFTR interacted with myosin Vb and Rab11a in polarized Calu-3 cells (Fig. 4*B*). These data confirm and extend our previous observation that CFTR co-immunoprecipitates with Rab11a in polarized human airway epithelial cells (21). Myosin Vc associates with the Rab8-specific, transferrin-accessible vesicular compartment in HeLa cells but is excluded from the Rab11a-specific recycling system (37). Rab8 did not co-immunoprecipitate with myosin Vb, and neither Rab8 nor myosin Vc co-immunoprecipitated with CFTR in human airway epithelial cells (Fig. 4*B*). Taken together, these data suggest that myosin Vb is coexpressed with CFTR in Rab11a-specific recycling endosomes in polarized human airway epithelial cells.

Endogenous Myosin Vb Facilitates the Apical Plasma Membrane Expression of WT-CFTR and ΔF508-CFTR in Polarized Human Airway Epithelial Cells—The expression of CFTR in the plasma membrane is determined, in part, by the relative rates of CFTR endocytosis and recycling (61–63). If myosin Vb facilitates CFTR recycling, then reduced expression of myosin Vb should attenuate CFTR recycling and thus should decrease the expression of CFTR in the plasma membrane. To test this prediction, the expression of myosin Vb was silenced using RNA-mediated interference. CFBE41o$^-$ cells stably expressing WT-CFTR were transfected with siMyoVb or Non-sil.siRNA as described under "Materials and Methods." As predicted, silencing myosin Vb decreased the expression of WT-CFTR specifically in the plasma membrane (Fig. 5).

Compelling evidence demonstrates that protein trafficking in epithelial cells can be affected by the state of cell polarization (64, 65). Thus, additional experiments were conducted to examine the effect of myosin Vb silencing on the expression of WT-CFTR in the apical plasma membrane in polarized CFBE41o$^-$ cells. CFBE41o$^-$ cells stably expressing WT-CFTR were cultured on semipermeable growth supports for 7 days after transfection with siMyoVb as described under "Materials and Methods." Under these conditions, CFBE41o$^-$ cells form polarized monolayers (21, 32). Silencing myosin Vb in polarized CFBE41o$^-$ cells decreased the expression of WT-CFTR in the apical plasma membrane (Fig. 6, *A*–*C*).

A recent study revealed that if ΔF508-CFTR is released from the endoplasmic reticulum in human airway epithelial cells, it undergoes trafficking to the plasma membrane in Rab11a-specific recycling endosomes, similar to WT-CFTR (21). Thus, we hypothesized that myosin Vb would also facilitate the recycling of ΔF508-CFTR. CFBE41o$^-$ cells stably expressing ΔF508-CFTR were cultured on semipermeable growth supports for 7 days after transfection with siMyoVb. To increase the export of ΔF508-CFTR from the endoplasmic reticulum and thus the expression of ΔF508-CFTR in the apical membrane, cells were cultured at 27 °C for 36 h (21, 32). As with WT-CFTR, silencing myosin Vb resulted in decreased expression of ΔF508-CFTR in the apical membrane (Fig. 6, *D*–*F*). Taken together, the above data are consistent with the view that myosin Vb facilitates the endocytic recycling and apical membrane expression of WT-CFTR and ΔF508-CFTR in polarized human airway epithelial cells.

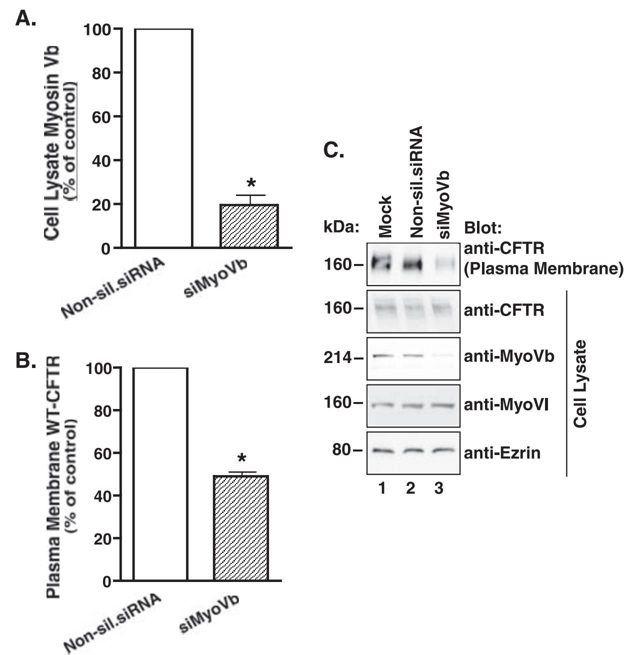

FIGURE 5. **Summary of experiments performed to determine the effects of the siRNA-mediated silencing of myosin Vb on the plasma membrane expression of WT-CFTR.** CFBE41o$^-$ cells stably expressing WT-CFTR were cultured on tissue culture plates for 60 h after transfection with siMyoVb. *A*, summary of Western blot experiments demonstrating that siMyoVb decreased the expression of endogenous myosin Vb. *B*, summary of biotinylation experiments demonstrating that siMyoVb decreased the plasma membrane expression of WT-CFTR. *C*, representative Western blots demonstrating that siMyoVb decreased the plasma membrane expression of WT-CFTR and the expression of myosin Vb in cell lysates, but did not decrease the expression of WT-CFTR, myosin VI, or ezrin in cell lysates. The cellular expression of WT-CFTR in cells transfected with siMyoVb was 94.8 ± 16.3% compared with cells transfected with Non-sil.siRNA. 45 μl of sample was loaded in each lane. *, $p < 0.05$ (five to seven experiments/group).

Endogenous Myosin Vb Facilitates CFTR-mediated Cl$^-$ Secretion across Polarized Human Airway Epithelial Cells—Because silencing endogenous myosin Vb decreased the expression of CFTR in the apical membrane, we predicted that it would also inhibit the CFTR-mediated Cl$^-$ secretion across polarized CFBE41o$^-$ cells. CFBE41o$^-$ cells stably expressing WT-CFTR were cultured on semipermeable growth supports for 7 days after transfection with siMyoVb. As predicted, siMyoVb inhibited the forskolin-stimulated short-circuit current (Fig. 7). Taken together, these data suggest that endogenous myosin Vb enhances CFTR-mediated transepithelial Cl$^-$ secretion in polarized human airway epithelial cells by a mechanism that involves increasing the total number of CFTR Cl$^-$ channels in the apical plasma membrane.

Dominant-negative FLAG-MyoVb-T Inhibits the Expression of CFTR in the Plasma Membrane—The direct interaction between the myosin Vb tail domain and Rab11a (22) that is proposed to occur upon recruitment of myosin Vb to recycling endosomes suggests that a recombinant, motorless myosin Vb fragment capable of binding to Rab11a should be able to displace endogenous myosin Vb from interacting with the Rab11a-specific recycling endosomes. The myosin Vb fragment would be expected to have a dominant-negative effect on

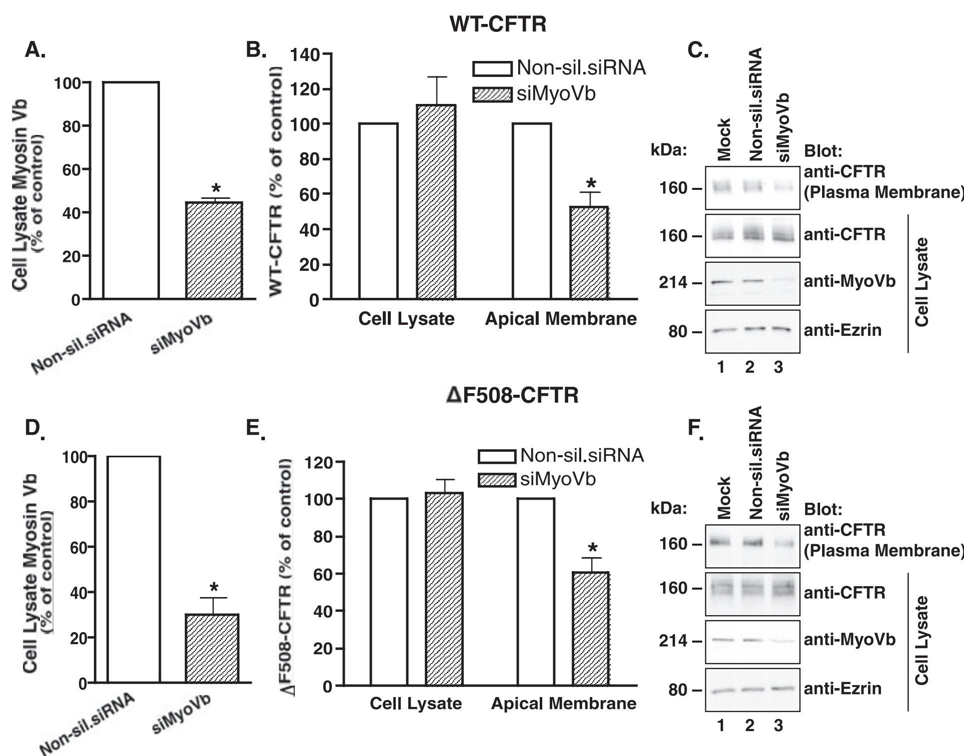

FIGURE 6. **Summary of experiments performed to determine the effects of the siRNA-mediated silencing of myosin Vb on the apical membrane expression of WT-CFTR (*A–C*) and ΔF508-CFTR (*D–F*) in polarized CFBE41o⁻ cells.** CFBE41o⁻ cells stably expressing either WT-CFTR or ΔF508-CFTR were cultured on semipermeable growth supports for 7 days after transfection with siMyoVb as described under "Materials and Methods." To increase export from the endoplasmic reticulum and expression of ΔF508-CFTR in the apical membrane, cells were cultured at 27 °C for 36 h. siMyoVb decreased myosin Vb expression in WT-CFTR-expressing (*A* and *C*) and ΔF508-CFTR-expressing (*D* and *F*) CFBE41o⁻ cells. Silencing myosin Vb attenuated the apical membrane expression of WT-CFTR (*B* and *C*) and ΔF508-CFTR (*E* and *F*) without affecting the total cellular expression of either WT-CFTR or ΔF508-CFTR in polarized CFBE41o⁻ cells. *, $p < 0.05$ (three to four experiments/group).

trafficking in Rab11a-specific recycling endosomes. Two Rab11a-binding sites located in the globular tail region of the myosin Vb tail domain are necessary for binding Rab11a (Fig. 1*A*) (22). Thus, we generated two recombinant FLAG-tagged myosin Vb fragments, the first consisting of the entire tail domain and containing both Rab11a-binding sites (FLAG-MyoVb-T) and the second consisting of a truncated globular tail region and containing only one Rab11a-binding site (FLAG-MyoVb-GT) (Fig. 1*C*). The ability of the FLAG-tagged myosin Vb fragments to interact with Rab11a was examined by pulldown experiments. Affinity-purified GST-Rab11a (47) was immobilized on glutathione-Sepharose beads and incubated with COS-7 cell lysates containing either FLAG-MyoVb-T or FLAG-MyoVb-GT. Western blot analysis of the protein complexes eluted from the beads revealed that only FLAG-MyoVb-T formed a complex with GST-Rab11a (Fig. 8*A*). These data confirm previous results that multiple sites of contact are necessary for myosin Vb binding to Rab11a (22). Furthermore, the data suggest that FLAG-MyoVb-T is sufficient for recruitment to Rab11a-specific recycling endosomes. Thus, if myosin Vb facilitates trafficking of CFTR in Rab11a-specific recycling endosomes, FLAG-MyoVb-T should have a dominant-negative effect on CFTR trafficking by displacing endogenous myosin Vb from binding to the Rab11a-specific recycling endosomes. If the above predictions are correct, FLAG-MyoVb-T (but not FLAG-MyoVb-GT) should also co-immunoprecipitate with CFTR. To test this hypothesis, HEK293 cells stably expressing WT-CFTR were transiently transfected with either FLAG-MyoVb-T or FLAG-MyoVb-GT. CFTR was immunoprecipitated with mouse anti-CFTR antibody M3A7. Western blot analysis of the immunoprecipitated complexes with rabbit anti-FLAG antibody demonstrated that, as predicted, only FLAG-MyoVb-T co-immunoprecipitated with CFTR (Fig. 8*B*). We hypothesized that dominant-negative FLAG-MyoVb-T would arrest CFTR recycling and decrease CFTR expression in the plasma membrane. To test this hypothesis, HEK293 cells stably expressing WT-CFTR were transiently transfected with either dominant-negative FLAG-MyoVb-T or FLAG-MyoVb-GT as a control. As demonstrated in Fig. 9 (*A* and *B*), FLAG-MyoVb-T decreased the plasma membrane expression of CFTR. The above data suggest that FLAG-MyoVb-T decreases the plasma membrane expression of CFTR by displacing endogenous myosin Vb from binding to Rab11a-specific recycling endosomes containing CFTR as cargo.

Myosin Vc is excluded from the Rab11-specific recycling compartment (37) and does not co-immunoprecipitate with CFTR (Fig. 4*B*). Thus, it would be expected that a dominant-negative fragment of myosin Vc would not affect the plasma membrane expression of CFTR. These experiments were conducted to examine whether the effect of FLAG-MyoVb-T on the plasma membrane expression of CFTR is specific. HEK293 cells stably expressing WT-CFTR were transfected either with the GFP-tagged myosin Vc tail fragment (GFP-MyoVc-T), shown previously to affect trafficking of the transferrin receptor (37), or with the GFP control. GFP-MyoVc-T did not decrease the plasma membrane expression of CFTR (Fig. 9, *C* and *D*).

Myosin Vb Facilitates CFTR Recycling—Dominant-negative FLAG-MyoVb-T could decrease the plasma membrane expression of CFTR either by inhibiting CFTR recycling or by stimulating CFTR endocytosis or both. Accordingly, experiments were conducted to test the hypothesis that FLAG-MyoVb-T decreases the expression of CFTR in the plasma membrane by inhibiting CFTR recycling. HEK293 cells stably expressing WT-CFTR were transfected with the GFP control, the FLAG-MyoVb-GT control, or dominant-negative FLAG-MyoVb-T. CFTR recycling was measured at 2.5 and 5 min as described under "Materials and Methods." CFTR recycling was similar in cells transfected with FLAG-MyoVb-GT and the GFP control (Fig. 10, *A* and *D*). These data confirm that this recombinant

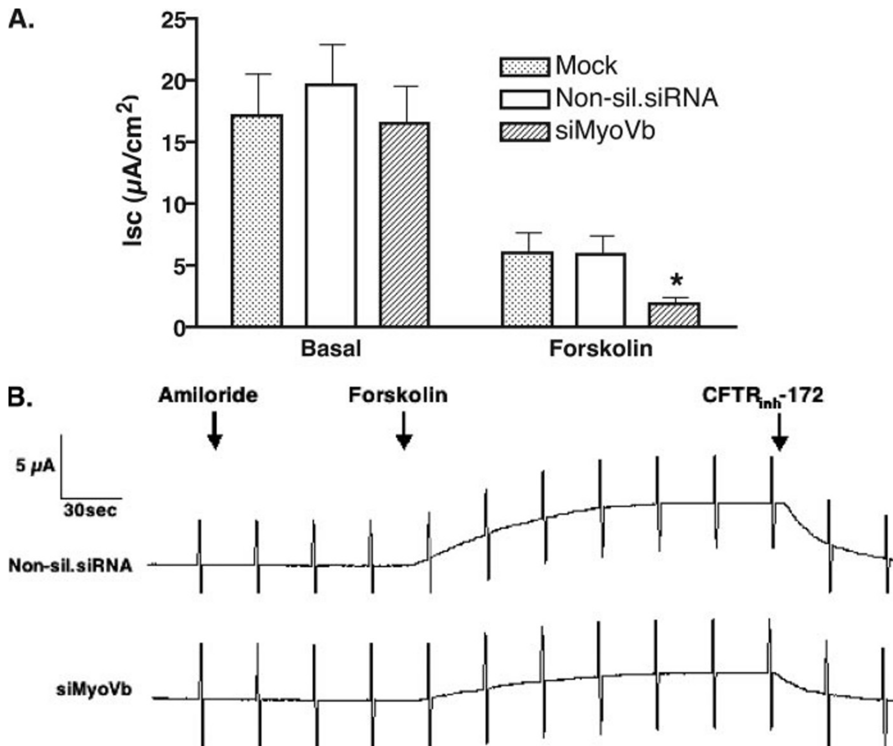

FIGURE 7. **Ussing chamber experiments performed to determine the effects of silencing myosin Vb expression on the CFTR-mediated Cl^- secretion across polarized CFBE41o$^-$ cells.** CFBE41o$^-$ cells stably expressing WT-CFTR were cultured on semipermeable growth supports for 7 days after transfection with siMyoVb. The summary of experiments (*A*) and representative recordings (*B*) demonstrate that silencing myosin Vb expression inhibited the forskolin-stimulated short-circuit current across polarized CFBE41o$^-$cells. $CFTR_{inh\text{-}172}$, thiazolidonone CFTR inhibitor (54). *, $p < 0.05$ (11 experiments/group).

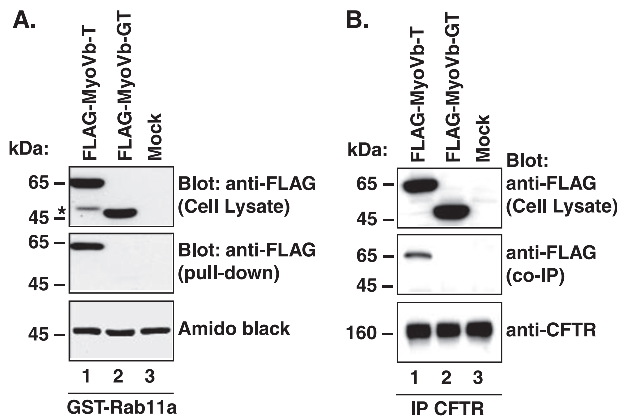

FIGURE 8. **Pulldown (*A*) and immunoprecipitation (*B*) experiments demonstrating that dominant-negative FLAG-MyoVb-T interacts with Rab11a and CFTR.** *A*, GST-Rab11a immobilized on glutathione-Sepharose beads was incubated with COS-7 cell lysates containing either FLAG-MyoVb-T (both Rab11a-binding sites present) or FLAG-MyoVb-GT (only one Rab11a-binding site present) (see Fig. 1*C*). Western blot analysis with anti-FLAG antibody M2 revealed that only FLAG-MyoVb-T formed a complex with GST-Rab11a. The *asterisk* indicates a nonspecific band demonstrated with the anti-FLAG antibody in lysates from cells transfected with FLAG-MyoVb-T. *B*, HEK293 cells stably expressing WT-CFTR were transiently transfected with either FLAG-MyoVb-T or FLAG-MyoVb-GT. CFTR was immunoprecipitated from the cell lysates using antibody M3A7 (*IP CFTR*). Western blot analysis with anti-FLAG antibody F7425 revealed that only FLAG-MyoVb-T interacted with CFTR. Cell lysates represent 5% of the pulldown or immunoprecipitated samples. The experiments were repeated twice using separate cultures with similar results. *co-IP*, co-immunoprecipitation.

myosin Vb fragment, which neither interacted with Rab11a nor co-immunoprecipitated with CFTR, served as a good negative control in our study. As illustrated in Fig. 10 (*A* and *D*), FLAG-MyoVb-T decreased CFTR recycling. The decrease in CFTR recycling is consistent with the decrease in the plasma membrane expression of CFTR observed in cells transfected with dominant-negative GFP-MyoVb-T and indicates that myosin Vb facilitates CFTR recycling.

Myosin Vb Does Not Regulate CFTR Endocytosis—As noted above, the decreased expression of CFTR in the plasma membrane caused by FLAG-MyoVb-T could also result from an increase in CFTR endocytosis. HEK293 cells stably expressing WT-CFTR were transfected with the GFP control, the FLAG-MyoVb-GT control, or dominant-negative FLAG-MyoVb-T, and CFTR endocytosis was measured as described under "Materials and Methods." CFTR endocytosis was linear between 0 and 5 min in cells transfected with the GFP control (Fig. 10*B*). Thus, data are reported at the 5-min time point. As illustrated in Fig. 10 (*C* and *D*), FLAG-MyoVb-T did not increase CFTR endocytosis. Taken together, these data indicate that dominant-negative FLAG-MyoVb-T decreases the plasma membrane expression of CFTR by specifically inhibiting the recycling of CFTR.

DISCUSSION

The major new observation in this study is that myosin Vb regulates CFTR-mediated Cl^- secretion across human airway epithelial cells by facilitating the apical membrane recycling of WT-CFTR and ΔF508-CFTR. Our data provide the first biochemical evidence that endogenous myosin Vb interacts with endogenous Rab11a in human airway epithelial cells.

Previous studies have shown that myosin Vb and Rab11a facilitate recycling (18–20, 22). Lapierre *et al.* (22) first elucidated the role of myosin Vb in the plasma membrane recycling of the transferrin and polymeric IgA receptors. These investigators established the interaction between myosin Vb and the GTP-bound (*i.e.* membrane-bound) form of Rab11a by yeast two-hybrid screening and demonstrated co-localization between GFP-tagged myosin Vb and endogenous Rab11a and between GFP-tagged Rab11a and endogenous myosin Vb in Madin-Darby canine kidney and HeLa cells. Subsequent studies confirmed the role of myosin Vb in plasma membrane recycling in Madin-Darby canine kidney and HeLa cells (66) and demonstrated that, together with Rab11a, myosin Vb facilitates the recycling of the M_4 muscarinic receptor in neuronotypic PC12 cells (23) and of the CXCR2 chemokine receptor in leukemia 2H3 cells (24) and the canalicular formation of bile in hepatic WIF-B9 cells (26). Furthermore, a recent study by Lise *et al.* (27) demonstrated that myosin Vb mediates trafficking of

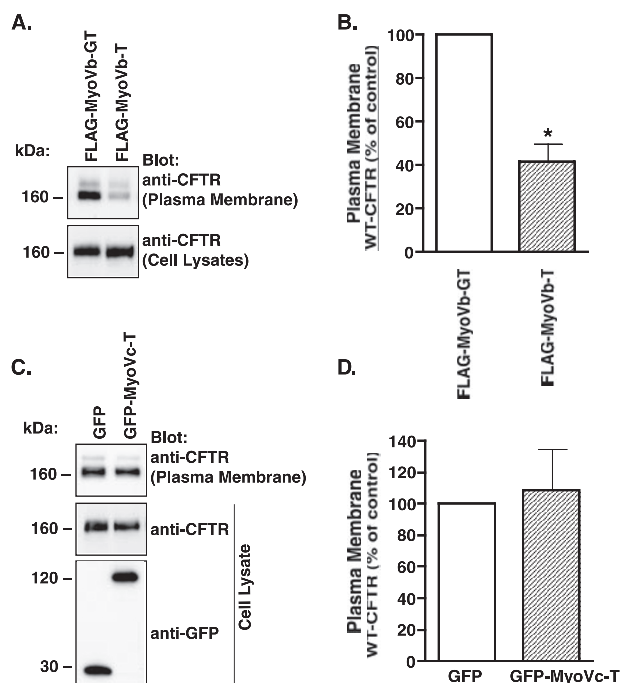

FIGURE 9. **Summary of experiments demonstrating that dominant-negative FLAG-MyoVb-T decreases the expression of CFTR in the plasma membrane.** HEK293 cells stable expressing WT-CFTR were transfected with either dominant-negative FLAG-MyoVb-T or FLAG-MyoVb-GT as a control. A representative experiment (*A*) and a summary of experiments (*B*) demonstrate that FLAG-MyoVb-T decreased the expression of CFTR in the plasma membrane. To determine the specificity of the FLAG-MyoVb-T effect on the plasma membrane expression of CFTR, additional studies were conducted using a GFP-tagged myosin Vc tail fragment (GFP-MyoVc-T). HEK293 cells were transfected with either GFP-MyoVc-T or GFP as a control. A representative experiment (*C*) and a summary of experiments (*D*) demonstrate that GFP-MyoVc-T did not decrease the plasma membrane expression of CFTR. *, $p < 0.05$ (six experiments/group).

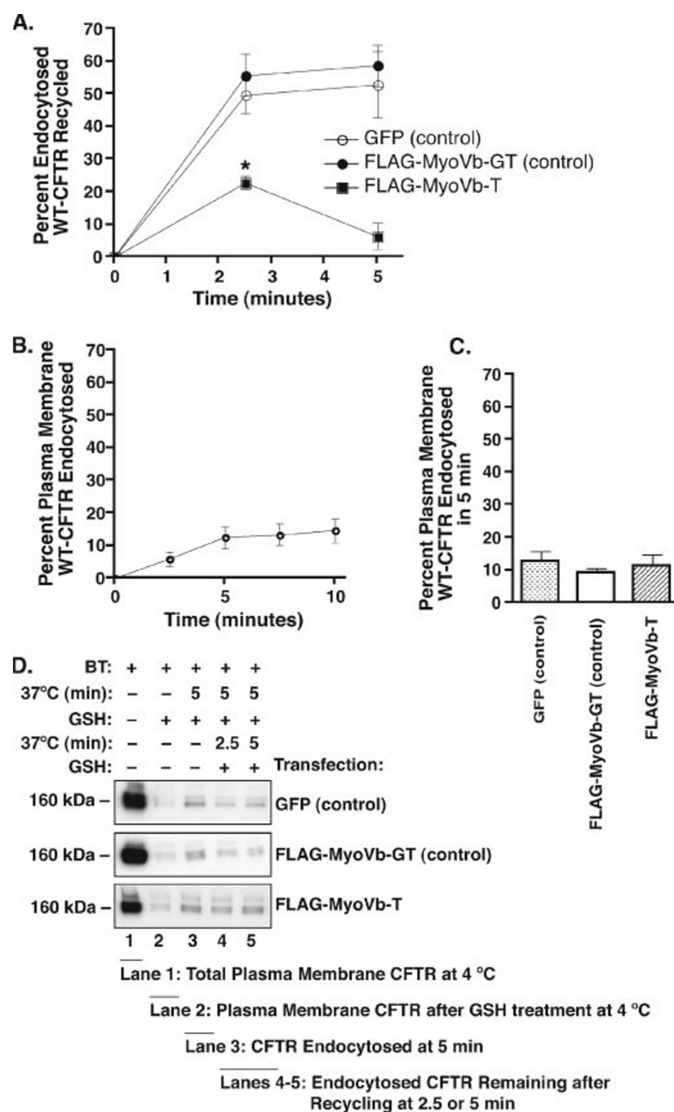

FIGURE 10. **Summary of recycling and endocytic assays performed in HEK293 cells stably expressing WT-CFTR.** *A*, the inhibition of CFTR recycling by dominant-negative FLAG-MyoVb-T is consistent with the role of myosin Vb in CFTR recycling. *B*, CFTR endocytosis was linear between 0 and 5 min in cells transfected with the GFP control. *C*, thus, CFTR endocytosis was examined at the 5-min time point. *D*, shown are the results from representative endocytic and recycling assays. The amount of biotinylated (*BT*) CFTR remaining in the plasma membrane after GSH treatment at 4 °C (*lane 2*) was subtracted from the amount of CFTR remaining biotinylated after warming to 37 °C, and this value was only 6.7 ± 0.9% (mean ± S.E.) in the experiments reported in this study. CFTR recycling was calculated as the difference between the amount of biotinylated CFTR after the first (*lane 3*) and second (*lanes 4* and *5*) GSH treatments. *, $p < 0.05$ (three experiments/group).

the glutamate receptor subunit GluR1 by a Rab11a-dependent mechanism in neuronal subpopulations. However, there is no biochemical evidence that endogenous myosin Vb and Rab11a interact (22). Furthermore, the role of myosin Vb in CFTR trafficking has not been reported in respiratory epithelial cells. We demonstrated previously that Rab11a facilitates CFTR recycling in polarized human airway epithelial cells (21). In this study, we have provided evidence that endogenous myosin Vb co-immunoprecipitates with endogenous Rab11a and facilitates CFTR recycling in human airway epithelial cells. An inability to establish biochemically the association between myosin Vb and Rab11a was attributed in the past to the weak or indirect nature of the myosin Vb-Rab11a interaction or both (22). Higher expression levels of endogenous myosin Vb and Rab11a in human airway epithelial cells (Calu-3) compared with Madin-Darby canine kidney or HEK293 cells[3] may have contributed to our success in demonstrating the co-immunoprecipitation between these proteins. Furthermore, we have shown that the interaction between myosin Vb and Rab11a is dependent on the Rab11a-binding sites in the myosin Vb tail domain (Fig. 8*A*). The complementary data from this and previous work discussed above are consistent with the conclusion that, together with Rab11a, myosin Vb facilitates recycling in several cell types. We have extended these observations and demonstrated that, together with Rab11a, myosin Vb facilitates the recycling of CFTR in human airway epithelial cells.

It remains unknown how myosin Vb interacts with Rab11a and how CFTR interacts with the myosin Vb-Rab11a complex in human airway epithelial cells. These interactions may be either direct, as demonstrated by Rab11a binding to cargo proteins (67, 68), or indirect and may be facilitated by other interacting proteins (24, 25, 66). Additional studies are needed to characterize these interactions.

[3] A. Swiatecka-Urban, L. Talebian, E. Kanno, S. Moreau-Marquis, B. Coutermarsh, K. Hansen, K. H. Karlson, R. Barnaby, R. E. Cheney, G. M. Langford, M. Fukuda, and B. A. Stanton, unpublished data.

Our results suggest that loss of myosin Vb and/or Rab11a would be expected to attenuate CFTR recycling and to decrease CFTR expression in the apical plasma membrane in airway epithelial cells. This effect could compromise the maintenance of the airway surface liquid, a situation observed in patients with cystic fibrosis (4). So far, neither mutations nor polymorphisms in the myosin Vb or *rab11a* gene have been reported in humans or animals. The effects of altered Rab11a expression, observed during treatment with chemotherapeutic agents (69), infections (70), tumorigenesis (71, 72), or hypoxia (73), on the function or plasma membrane expression of CFTR are currently unknown.

Our data do not support the role of myosin Vc in CFTR recycling. Previous data indicate that myosin Vc is recruited to the Rab8-specific vesicular compartment and is excluded from the Rab11a-specific compartment (37). Rab8 has been implicated in regulating transport of proteins from the *trans*-Golgi to the basolateral membrane (74, 75). CFTR did not co-immunoprecipitate with either myosin Vc or Rab8 in human airway epithelial cells. The tail fragment of myosin Vc (GFP-MyoVc-T), which perturbs transferrin trafficking in HeLa cells (37), had no effect on CFTR trafficking in HEK293 cells. Thus, myosin Vc may mediate trafficking of a subpopulation of endosomes from which CFTR is excluded in HEK293 cells.

In summary, our data provide direct evidence that, in polarized human airway epithelial cells, myosin Vb regulates CFTR-dependent Cl^- secretion by facilitating the recycling of WT-CFTR and ΔF508-CFTR to the apical plasma membrane. We anticipate that elucidating the mechanisms that regulate CFTR recycling will help to identify unique therapeutic targets to modulate CFTR-mediated Cl^- secretion across human airway epithelial cells.

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