Membrane Trafficking of the Cystic Fibrosis Gene Product, Cystic Fibrosis Transmembrane Conductance Regulator, Tagged with Green Fluorescent Protein in Madin-Darby Canine Kidney Cells

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The mechanism by which cAMP stimulates cystic fibrosis transmembrane conductance regulator (CFTR)-mediated chloride (Cl⁻) secretion is cell type-specific. By using Madin-Darby canine kidney (MDCK) type I epithelial cells as a model, we tested the hypothesis that cAMP stimulates Cl⁻ secretion by stimulating CFTR Cl⁻ channel trafficking from an intracellular pool to the apical plasma membrane. To this end, we generated a green fluorescent protein (GFP)-CFTR expression vector in which GFP was linked to the N terminus of CFTR. GFP did not alter CFTR function in whole cell patch-clamp or planar lipid bilayer experiments. In stably transfected MDCK type I cells, GFP-CFTR localization was substratum-dependent. In cells grown on glass coverslips, GFP-CFTR was polarized to the basolateral membrane, whereas in cells grown on permeable supports, GFP-CFTR was polarized to the apical membrane. Quantitative confocal fluorescence microscopy and surface biotinylation experiments demonstrated that cAMP did not stimulate detectable GFP-CFTR translocation from an intracellular pool to the apical membrane or regulate GFP-CFTR endocytosis. Disruption of the microtubular cytoskeleton with colchicine did not affect cAMP-stimulated Cl⁻ secretion or GFP-CFTR expression in the apical membrane. We conclude that cAMP stimulates CFTR-mediated Cl⁻ secretion in MDCK type I cells by activating channels resident in the apical plasma membrane.

The cystic fibrosis transmembrane conductance regulator (CFTR), a CAMP-activated chloride (Cl⁻) channel, is targeted to the apical plasma membrane region in many epithelial cells, including those in the kidney (1–3), and is defective in the genetic disease cystic fibrosis (4). Stimulation of CFTR-mediated Cl⁻ secretion by cAMP has been reported to occur by the following two mechanisms that are not mutually exclusive: first, cAMP stimulates protein kinase A-mediated phosphorylation and activation of CFTR Cl⁻ channels resident in the plasma membrane (5–7); and second, cAMP stimulates trafficking of CFTR from an intracellular pool to the plasma membrane while decreasing endocytic retrieval of CFTR from the plasma membrane (8–11). The second mechanism is more controversial; in intestinal epithelial cells, some investigators have found positive effects of cAMP on CFTR trafficking to the apical membrane (12), whereas other investigators have not (2, 13). In contrast, little is known about the intracellular trafficking of CFTR or the mechanism(s) by which cAMP stimulates CFTR-mediated Cl⁻ secretion in kidney epithelia. Because CFTR Cl⁻ channels are expressed in all nephron segments of the kidney (3) and are important for transepithelial Cl⁻ transport (14, 15) and enlargement of renal cysts in polycystic kidney disease (16), it is important to elucidate the role of cAMP in the regulation of CFTR in normal and pathophysiological renal states.

The study of CFTR trafficking in many epithelial cells, including renal epithelia, is hampered by the low level of endogenous CFTR expression (1, 17). To begin to understand the trafficking of CFTR, we constructed a jellyfish green fluorescent protein (GFP)-CFTR expression vector in which GFP was ligated to the N terminus of wild-type CFTR, and we used GFP fluorescence to localize CFTR in living and fixed cells. GFP, a 27-kDa protein from the jellyfish Aequorea victoria, has emerged as an in vivo reporter protein for studying complex biological processes such as organelle dynamics and protein trafficking (18, 19). GFP generates a bright green fluorescence, is resistant to photobleaching, does not require any exogenous cofactors or substrates to fluoresce, and, when ligated to other proteins, generally does not alter fusion protein function or localization (18, 20).

The present study was conducted to test the hypothesis that cAMP stimulates CFTR-mediated Cl⁻ secretion in mammalian fibrosis transmembrane conductance regulator; GFP, green fluorescent protein; MDCK, Madin-Darby canine kidney; Isc, short circuit current; Endo H, endoglycosidase H; PNGase F, peptide N-glycosidase F; CPT-cAMP, 8-(4-chlorophenylthio)-cAMP; DIDS, 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid; DPC, diphenylamine carboxylic acid; ER, endoplasmic reticulum; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; MOPS, 4-morpholinepropanesulfonic acid.

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kidney epithelial cells by inducing a relocation of CFTR from intracellular organelles to the apical plasma membrane. By using MDCK type I cells as a model, we generated stable transfectants expressing full-length GFP-CFTR fusion protein. By using quantitative confocal fluorescence microscopy, cell-surface biotinylation, and short circuit current (Isc) analyses, we demonstrate that the predominant mechanism by which cAMP stimulates GFP-CFTR-mediated Cl− secretion is by activating channels resident in the apical membrane and not by stimulating insertion of channels into the apical membrane or inhibiting retrieval of channels from the apical membrane.

**Experimental Procedures**

**GFP-CFTR and CFTR Expression Vectors**

To construct the pGFP-CFTR mammalian expression vector, human CFTR cDNA was excised from pBluescript II SK− (Stratagene, La Jolla, CA) with XbaI, treated with Klenow fragment to fill-in sticky ends, and ligated into Smal-digested and calf intestinal alkaline phosphatase-treated pS657-GFP-C1 (CLONTECH, Palo Alto, CA). To maximize GFP fluorescence, S657TGFp cDNA was exchanged for enhanced GFP cDNA using AgeI/KpnI. Enhanced GFP is codon-optimized for expression in mammalian systems and exhibits 6-fold greater levels of fluorescence than S657T-GFP (21). DNA sequence analysis of the GFP-CFTR junction confirmed the intended reading frame. Proceeding from the N′-terminal, the resultant fusion protein consists of GFP, a linker sequence of 23 amino acids, and CFTR. Based on the predicted transmembrane topology of CFTR (4), GFP resides in the cytoplasmic compartment. GFP expression vector (with no GFP tag) (22) was used to compare the function of GFP-CFTR to CFTR in whole cell patch-clamp experiments. To examine the effect of GFP on CFTR function in planar lipid bilayer experiments, GFP-CFTR was subcloned from pGFP-CFTR into pcDNA3.1 (Invitrogen, Carlsbad, CA) using NheI and EcoRV (to generate pcDNA3.1 GFP-CFTR). Similarly, CFTR was subcloned from pGFP-CFTR into pcDNA3.1 using Asp718 and XhoI (to generate pcDNA3.1 CFTR). These vectors allow synthesis of cRNA for expression in Xenopus oocytes.

**Cell Culture**

MDCK type I cells were obtained from the American Type Tissue Collection (pass number 54, CCL-34, Rockville, MD) and grown on tissue culture-treated polystyrene flasks in minimum essential medium with Earle’s salts (Life Technologies, Inc.) containing 10% fetal bovine serum (HyClone), 50 units/ml penicillin, 50 μg/ml streptomycin, and 2 mM t-glutamine (Life Technologies, Inc.). Cells were grown in 5% CO2-balanced air at 37°C. When confluent, cells were subcultured by trypsinization (0.05% trypsin, 0.53 mM EDTA in Hanks’ balanced salt solution; Life Technologies, Inc.). For short circuit current and confocal microscopic experiments, cells were seeded at 50,000–75,000 cells/3.3 cm2, and for surface biotinylation experiments, cells were seeded at 200,000/4.5 cm2 on permeable Transwell filter bottom cups (Costar, Cambridge, MA). Cells were fed daily and used 5–7 days after seeding. Because GFP fluorescence is temperature-sensitive (23, 24), we grew all transfected cell lines at 33°C for 2–3 days before patch clamp recording.

To generate MDCK cells stably transfected with pGFP-CFTR, we first optimized transient transfection efficiency with the PerFect Lipid Transfection Kit (Invitrogen) according to the manufacturer’s instructions (26). A T75 flask of MDCK cells was transfected with pGFP-CFTR plasmid. Twenty-four hours post-transfection, cells were selected with 300 μg/ml G418 (Life Technologies, Inc.) and fed every 4–5 days for 2 weeks until complete media containing G418. Surviving cell colonies were trypsinized, and single cells with bright GFP fluorescence were sorted into individual wells of 96-well plates using a FACStar PLUS flow cytometer (Becton Dickinson, San Jose, CA). GFP fluorescence was excited using the 488 nm line from an argon laser and collected with a 530/30 nm band pass filter. Clones were expanded and screened for GFP fluorescence by confocal fluorescence microscopy. Similar experimental results were found for the HC11 cell line (Hans Oberleithner) (27) stably transfected with GFP-CFTR. Following establishment of cell lines, G418 was reduced to 150 μg/ml and was removed 3–4 days prior to experimentation. MDCK stable transfectants were treated with 5 mM sodium n-butylate (Sigma) for 15–18 h prior to experimentation to increase GFP-CFTR expression levels. Sodium butyrate was removed 2 h prior to experimentation.

**Whole Cell Patch-Clamp**

Whole cell patch-clamp recording of GFP-positive cells was performed as described previously in detail (22, 28, 29). Only 50% of pGFP-CFTR and pCFTR transfected cells expressing visible levels of GFP fluorescence responded to cAMP treatment with an increase in Cl− conductance. Non-responding cells, which were probably transfected with pGFP but not pGFP-CFTR or pCFTR, exhibited currents similar to non- or mock-transfected cells, and were excluded from data analysis.

**Planar Lipid Bilayers**

Single channel properties of GFP-CFTR and CFTR Cl− channels were studied in planar lipid bilayers. Stage V–VI Xenopus oocytes were harvested and injected with 5 ng of CFTR cRNA, 5 ng of GFP-CFTR cRNA, or 50 nl of water as described previously (30). Membrane vesicles were prepared 48 h postinjection following the method of Pérez et al. (31). Thirty to forty oocytes in each group were washed and homogenized in high K+/sucrose medium containing the following protease inhibitors: aprotinin (1 μg/ml), leupeptin (1 μg/ml), pepstatin (1 μg/ml), phenylmethylsulfonyl fluoride (100 μM), and DNase I (2 μg/ml). Oocyte membranes were isolated by discontinuous sucrose gradient density centrifugation and resuspended in 300 mM sucrose, 100 mM KCl, and 5 mM MOPS (pH 6.8). Membrane vesicles were separated into 50–μl fractions and stored at −80°C until use. Planar lipid bilayers were made from a phospholipid solution containing a 1:1 mixture of diphytanoyl-phosphatidylethanolamine/diphytanoyl-phosphatidylserine (in n-octane; final phospholipid concentration of 25 mg/ml). Membrane vesicles were applied with a fire-polished glass rod to one side (trans) of a preformed bilayer bathed with symmetrical 100 mM KCl, 10 mM MOPS-Tria (pH 7.4). Acquisition and analysis of single-channel recordings were performed as described (32, 33). Channel activity was recorded in the presence of 1.85 ng/ml protein kinase A catalytic subunit (gift of Dr. Gail Johnson, University of Alabama) and 100 μM ATP.

**Short Circuit Current**

Short circuit current (Isc) was measured across MDCK monolayers as described previously (34). In all experiments, amiloride (10−5 M) was present in the apical bath solution to inhibit electrogenic Na+ absorption. Under these conditions, cAMP-stimulated Isc across monolayers of MDCK cells is referable to Cl− secretion.

**Immunocytochemistry**

Unless specifically stated otherwise, all steps were performed at room temperature in Cu2+–Mg2+-free PBS (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH2PO4, 9 mM Na2HPO4, (pH 7.1)), and antibody incubations were for 1 h.

**Na+/K-ATPase**—Cells were washed, fixed, and permeabilized with ice-cold 100% MeOH for 10 min and washed with 0.3% Triton X-100 (Sigma) and 0.1% BSA (PBS-TB). Nonspecific binding sites were blocked with 5% BSA for 30 min, and cells were incubated with 10 μg/ml anti-Na+/K-ATPase monoclonal antibody (lgG1) (Upstate Biotechnology, Lake Placid, NY) overnight at 4°C. Cells were washed with inverted fluorescence microscope intrinsic to the patch-clamp system. The luciferase plasmid was used to assess relative transfection efficiency; all transfectants displayed equivalent levels of luciferase activity. Transfected cells were transferred to 33°C for 2–5 days before patch-clamp recording.
PBS-TB and incubated with 1:100 goat anti-mouse Texas Red secondary antibody (Molecular Probes, Eugene, OR) for 3 h. Cells were washed in PBS-TB and mounted in 90% glycerol, 10% PBS containing 10 mg/ml n-propyl gallate (Sigma) to retard fading. ZO-1—Cells were washed, fixed, and permeibilized in 100% acetone for 2 min at −20 °C and incubated in 10 μg/ml anti-ZO-1 rabbit polyclonal antibody (Zymed, So. San Francisco) in 1% BSA. Cells were washed, incubated with 1:100 goat anti-rabbit Texas Red secondary antibody (Molecular Probes), and mounted as above.

**CPTR—**Cells were immunostained essentially as described previously (2) using 10–40 μg/ml anti-CPTR R domain (lgG2) or anti-CPTR C-terminal monoclonal antibodies (Genzyme, Cambridge, MA).

**Biotinylated Membranes—** Biotinylated monolayers were fixed in 3.0% paraformaldehyde for 30 min on ice and permeabilized with 0.1% Triton X-100 for 3 min, and nonspecific binding sites were blocked with 2% BSA. Biotinylated proteins were detected with 50 μg/ml Texas Red-avidin (Pierce) in 1% BSA for 30 min, washed, and mounted as above.

Control experiments in which cells were stained with nonspecific antibody of the appropriate isotype (for monoclonal antibodies), non-immune serum (for polyclonal antibodies), and/or secondary antibody only demonstrated the specificity of observed signals.

**Cryosectioning**

MDCK cells grown on permeable supports were fixed in 3.0% paraformaldehyde in Ca2+/Mg2+-free PBS for 15 min at room temperature. Monolayers were excised with a scalpel and cut into thin strips embedded in Tissue-Tek (Miles, Elkhart, IN) and frozen in liquid nitrogen-cooled liquid propane. Sections 5–7 μm in thickness were cut with a cryostat and examined by confocal microscopy.

**Confocal Microscopy**

Images were acquired using a Zeiss (Thornwood, NY) AxioSkop microscope equipped with a laser scanning confocal unit (model MRC-1024, Bio-Rad), a 15-milliwatt krypton-argon laser, and a ×63 Plan Apochromat/1.4 NA or ×40 Plan Neofluor/1.3 NA oil immersion objective. GFP fluorescence was excited using the 488-nm laser line and collected using a standard fluorescein isothiocyanate filter set (530 ± 30 nm). Fluorescence associated with Texas Red-labeled secondary antibodies and propidium iodide was simultaneously excited using the 588-nm laser line and collected using a standard Texas Red filter set (605 ± 32 nm). Three-dimensional reconstructions were rendered using LaserSharp version 2.1A (Bio-Rad) software. Acquired images were imported into National Institutes of Health Image version 1.57 software (Bethesda, MD) for quantitation and into Adobe Photoshop version 3.0 for image processing and printing. For live cell microscopy, cells were mounted in a temperature-controlled, flow-through perfusion chamber (RC21-B Chamber, Warner Instrument Corp., Hamden, CT) at 37 °C in mounted in a temperature-controlled, flow-through perfusion chamber (Bethesda, MD) for quantitation and into Adobe Photoshop version 3.0 overnight at 4 °C with end-over-end rotation. Beads were pelleted by centrifugation for 30 s at 14,000 × g and washed three times with lymph buffer. Biotinylated proteins were eluted by boiling for 5 min in 50 μl of Laemmli sample buffer (0.24 M Tris-HCl [pH 8.9], 16% glycerol, 0.005% bromphenol blue, 5.6% SDS, and 80 μM diethiothreitol).

**Glycosidase Digestion**

Cell lysates (30–40 μg protein) were digested with endoglycosidase H (Endo H, 1500 units) or asparaginyl-N-glycosidase F (PNGase F, 1500 units) (New England Biolabs, Beverly, MA) for 1 h at room temperature following the manufacturer’s instructions, with the exception that lysates were not denatured prior to digestion. Denaturation induced GFP-CFTR aggregation and protein failed to enter separating gels.

**SDS-PAGE and Western Blotting**

Cell lysates and biotinylated proteins were separated on 4–15% Tris-HCl gradient gels (Bio-Rad) and transferred to polyvinylidene difluoride Immobilon membranes (Millipore, Bedford, MA). Membranes were blocked overnight at 4 °C in 5% non-fat dry milk in Tris-buffered saline, 0.02% Tween 20 and incubated with either GFP (1:1000) (CLONTECH) or CFTR C-terminal (1:1000) monoclonal antibodies followed by anti-mouse horseradish peroxidase-conjugated secondary antibodies (1:5,000–1:10,000, Amersham Pharmacia Biotech). Blots were developed by enhanced chemiluminescence (Amersham Pharmacia Biotech) using Hyperfilm ECL (Amersham Pharmacia Biotech) and digitally scanned with a Silverscan III flatbed scanner (LaCie, Hillsboro, OR). Densitometric analysis of band intensities was performed with public domain NIH Image version 1.57 software.

**Statistical Analyses**

Differences between means were compared by either paired or unpaired two-tailed Student’s t test as appropriate using Instat statistical software (GraphPad, San Diego, CA). Data are expressed as mean ± S.E. Statistical significance is ascribed for p < 0.05.

**Other Materials**

8-4-Chlorophenylthio)-cAMP (CPT-cAMP, monosodium salt) was purchased from Boehringer Mannheim and Sigma. 8-Bromo-cAMP, isobutylmethylxanthine, forskolin, 4,4′-disothiocyanostilbene-2,2′-disulfonic acid (DIDS), glybenclamide, colchicine, lumiocolchicine, and propidium iodide were purchased from Sigma. Diphenylamine carboxylic acid (DPC) was purchased from Fluka (Milwaukee, WI) and Sigma.

**RESULTS**

**GFP-CFTR Functions as a cAMP-activated Cl− Channel—** Because CFTR Cl− channel function is necessary for normal CFTR trafficking (8), we performed experiments to examine whether fusion of GFP to the N terminus of CFTR affected function as a cAMP-activated Cl− channel. To this end, we performed whole cell patch-clamp experiments on transiently transfected NIH-3T3 fibroblasts, which express no detectable endogenous CFTR Cl− channels (25). As shown in Fig. 1, cAMP-activated Cl− currents were similar in cells expressing GFP-CFTR and CFTR (with no GFP tag). Currents were not sensitive to DIDS but were inhibited by glybenclamide, consistent with established CFTR pharmacology (10, 29, 36). To compare the single channel properties of GFP-CFTR to CFTR (with no GFP tag), we performed planar lipid bilayer experiments. Recordings from channels synthesized in Xenopus oocytes and incorporated into planar lipid bilayers demonstrated that GFP-CFTR single channel conductance, chloride to iodide permeability ratio, and blocker sensitivity (inhibition by DPC but not DIDS) were similar to CFTR (Table I and Fig. 2). These parameters are similar to those previously reported for CFTR channels in planar lipid bilayers (32, 33), in stably transfected cells (37, 38), and in apical membranes of cells expressing endogenous CFTR (38). Water-injected oocytes did not produce any CFTR-like Cl− channel activity. Taken together, these findings indicate that fusion of GFP to the N terminus of CFTR does not affect CFTR Cl− channel function.

**GFP-CFTR Localization Is Substratum-dependent—**To generate a cell model to study CFTR trafficking in polarized kidney epithelial cells, we stably transfected MDCK type I cells, which express low levels of endogenous CFTR Cl− channels (36), with
Mechanism of CFTR Activation in Kidney

**Fig. 1.** Whole cell patch-clamp analyses examining GFP-CFTR function as a cAMP-activated Cl\(^{-}\) channel. NIH-3T3 fibroblasts were transiently transfected with pGFP-CFTR (n = 4) (A) or pCFTR (n = 5) (B). Cells were treated with 8-bromo-cAMP (250 \(\mu\)M) and CPT-cAMP (250 \(\mu\)M) (filled circles), DIDS (100 \(\mu\)M) (open circles), and glybenclamide (100 \(\mu\)M) (×) applied to the extracellular bathing solution in a paired and sequential manner, and current (I)-voltage (V) curves were generated. I-V curves were linear, and CFTR Cl\(^{-}\) currents were not time- or voltage-dependent, in accordance with previous reports (29, 68, 69). Mock- or non-transfected cells exhibited currents similar to transfected cells following glybenclamide treatment. Error bars were often smaller than the symbols used to plot the data. C: comparison of whole cell Cl\(^{-}\) currents at \(-100\) and \(+100\) mV in cells expressing GFP-CFTR (filled bars) and CFTR (open bars). p > 0.05 for GFP-CFTR compared with CFTR at both voltages.

**Table 1**

Comparison of electrophysiological properties of GFP-CFTR and CFTR Cl\(^{-}\) channels

| Parameter                      | GFP-CFTR | CFTR  |
|-------------------------------|----------|-------|
| Single channel conductance    | 11.0 ± 1.9 | 10.6 ± 2.0 |
| (n = 5)                       | (n = 5)   |
| Cl\(^{-}\):I\(^{-}\) permeability ratio | 1.8 ± 0.4 | 1.8 ± 0.3 |
| (n = 4)                       | (n = 3)   |

pGFP-CFTR expression plasmid. By using laser scanning confocal fluorescence microscopy, GFP-CFTR was localized to the basolateral plasma membrane region in cells grown on glass coverslips (Fig. 3). Acute treatment with cAMP did not stimulate detectable trafficking of GFP-CFTR to apical or basolateral membranes (Fig. 3). By contrast, GFP-CFTR fluorescence was predominantly localized to the apical plasma membrane region and sub-apical vesicles in stably transfected, fully polarized cells cultured on permeable supports (Fig. 4, A and B). Similar results were obtained in MDCK cells transiently transfected with pCFTR (with no GFP tag) and stained with R domain or C-terminal CFTR antibodies, indicating that fusion of GFP to CFTR does not alter CFTR subcellular localization or trafficking to the apical membrane (Fig. 4C). We confirmed that GFP-CFTR was polarized to the apical membrane region by performing double-labeling experiments in fixed cells. By using a monoclonal antibody against the Na/K-ATPase to label basolateral membranes, GFP-CFTR did not colocalize with the Na/K-ATPase (Fig. 5A). By using a polyclonal antibody against ZO-1, a protein localized to the cytoplasmic face of tight junctions, GFP-CFTR was expressed in a horizontal plane between tight junctions (Fig. 5B). Because CFTR is polarized to the apical plasma membrane of many epithelial cells in vivo (1, 2), we performed all subsequent trafficking experiments using cells grown on permeable supports to simulate the physiologically relevant situation.

**GFP-CFTR Mediates Transepithelial Cl\(^{-}\) Secretion**—We performed short circuit current experiments to examine whether GFP-CFTR functioned as an apical membrane, cAMP-activated Cl\(^{-}\) channel in polarized cells by measuring transepithelial Cl\(^{-}\) secretion across monolayers of parental untransfected and stably transfected MDCK cells. In all experiments, amiloride (10 \(^{-5}\) M) was present in the apical bath solution to inhibit electrogenic Na\(^{+}\) secretion. Under these conditions, cAMP-stimulated \(I_{sc}\) is referable to Cl\(^{-}\) secretion. A cAMP-stimulating mixture (100 \(\mu\)M CPT-cAMP, 100 \(\mu\)M isobutylmethylxanthine, and 20 \(\mu\)M forskolin) elicited a rapid and small increase in \(I_{sc}\) in parental, untransfected MDCK cells (Table II), consistent with activation of endogenous CFTR Cl\(^{-}\) channels. In GFP-CFTR stable transfectants, CAMP-stimulating mixture elicited a rapid and large increase in \(I_{sc}\) which reached a peak value at 2 min, remained elevated for the duration of CAMP treatment (up to 20 min), and decreased following treatment with DPC (10 \(\mu\)M), an inhibitor of CFTR Cl\(^{-}\) channels (29) (Table II). These data demonstrate that GFP-CFTR functions as a CAMP-stimulated apical membrane Cl\(^{-}\) channel and mediates transepithelial Cl\(^{-}\) secretion in polarized MDCK type I cells.

**Effect of CAMP on GFP-CFTR Trafficking**—We next tested the hypothesis that CAMP increases \(I_{sc}\) by stimulating CFTR trafficking from an intracellular pool to the apical plasma membrane. Monolayers were treated with CAMP-stimulating mixture for 10 min, at which time CAMP-stimulated \(I_{sc}\) has peaked and reached an elevated state, and the distribution of GFP-CFTR fluorescence along the apical to basal axis was quantitated in optical sections using confocal fluorescence microscopy. Apical and basal cell-surface boundaries were identified by labeling surface glycoproteins with wheat germ agglutinin-Texas Red at 4 °C. As shown in Fig. 6, CAMP did not affect GFP-CFTR distribution. Approximately, 70% of GFP-CFTR fluorescence was localized to the apical membrane and sub-apical membrane regions, comprising the first three optical sections in Fig. 6, in vehicle and CAMP-treated monolayers. Similar results were obtained in cells treated with CAMP for 60 min. Qualitatively similar results were obtained using confocal microscopy to localize GFP-CFTR in longitudinal cryosections sectioned along the apical-basal axis (Fig. 7).

Because of the limited resolution of the confocal microscope in the vertical dimension (which corresponds to 0.5 \(\mu\)M under optimal scanning conditions), it is conceivable that CAMP stimulates the trafficking of GFP-CFTR, localized in sub-apical
vesicles less than 0.5 \( \mu m \) from the apical surface, to the apical plasma membrane. To address this possibility, we biotinylated apical cell-surface GFP-CFTR in monolayers treated with vehicle or cAMP-stimulating mixture using biotin-LC-hydrazide, a membrane-impermeant reagent which labels glycoproteins on sugar residues previously oxidized with sodium periodate. Biotinylations were performed at 4 °C to inhibit membrane trafficking and ensure that only cell-surface GFP-CFTR was labeled. Fig. 8A shows that the amount of surface-biotinylated GFP-CFTR on apical plasma membranes did not increase following 10 or 60 min of cAMP treatment. Densitometric analyses, which referenced biotinylated GFP-CFTR to total cell lysate GFP-CFTR (Fig. 8B), to account for filter to filter variability in GFP-CFTR expression levels, confirmed these observations. Similar results were obtained in a stably transfected cell line expressing 20-fold less GFP-CFTR and in cells not induced with sodium butyrate, which express 30-fold less GFP-CFTR, indicating that results were independent of GFP-CFTR expression levels.

It is conceivable that cAMP equally affects the rate of endocytosis of GFP-CFTR from the apical membrane and the rate of recycling of endosomes containing GFP-CFTR to the apical membrane such that the net cell-surface GFP-CFTR pool remains constant. To examine this possibility, GFP-CFTR internalization and recycling were followed in the presence and absence of cAMP using a two-step biotinylation procedure. Cells were placed on ice, and apical membrane glycoproteins were first oxidized with sodium periodate. Cells were then incubated at 37 °C for various times in the presence and absence of cAMP to allow GFP-CFTR internalization (at early time points) or internalization and recycling (at later time points). Cells were returned to ice, and periodate-oxidized GFP-CFTR molecules at the apical surface were next labeled with biotin-LC-hydrazide. As shown in Fig. 8C, 15–20% of GFP-CFTR was endocytosed from the apical membrane following a 3-min incubation at 37 °C in the presence or absence of cAMP. This time point reflects removal of GFP-CFTR from the apical membrane and accumulation in an endosomal pool. Longer incubations at 37 °C, which allow further internalization of apical membrane GFP-CFTR as well as recycling of GFP-CFTR internalized at earlier time points, did not change the amount of labeled GFP-CFTR at the apical membrane, and cAMP did not significantly affect the number of labeled apical membrane GFP-CFTR Cl\(^{-}\) channels. These findings suggest that after 3 min, the rate of apical membrane GFP-CFTR internalization is
equivalent to the rate of endosomal GFP-CFTR recycling such that net apical membrane GFP-CFTR remains constant.

Surface-biotinylated GFP-CFTR migrated as two bands at 240 and 300 kDa (Fig. 8A, labeled C and *, respectively), whereas GFP-CFTR from cell lysates migrated as three bands at 210, 240, and 300 kDa (Fig. 8B, labeled B, C, and *, respectively). These bands correspond to core-glycosylated (210 kDa; band B), mature-glycosylated (240 kDa; band C), and detergent-induced high molecular mass (300 kDa and as discussed below) GFP-CFTR. Endo H, which cleaves sugar residues from N-linked glycoproteins in pre-Golgi organelles and the cis-Golgi apparatus, had no apparent effect on the mobility of the 240-kDa band but increased the mobility of the 210-kDa band, which migrated at 200 kDa (band A) following Endo H digestion (Fig. 8D). PNGase F, which cleaves sugar residues from N-linked glycoproteins regardless of their localization in the secretory pathway, increased the mobility of both the 240- and the 210-kDa bands, which migrated as a single band at 200 kDa following PNGase F digestion (Fig. 8D). Detection of core-glycosylated GFP-CFTR by Western blotting but lack of substantial GFP-CFTR fluorescence in the endoplasmic reticulum (ER) by confocal microscopy in z-axis vertical scans using low laser power settings is likely due to dilution of GFP fluorescence throughout the extensive tubulovesicular elements of the ER. GFP-CFTR was detected in the ER by confocal microscopy using higher laser power settings. The predicted molecular
Control experiments, which included monolayers that were not biotinylated, monolayers that were not oxidized with sodium periodate, and monolayers from parental, untransfected MDCK cells, were all negative for apical membrane CFTR immunoreactivity in Western blot analyses (Fig. 8A). These results demonstrate that GFP-CFTR was not binding nonspecifically to streptavidin-agarose beads and that all CFTR immunoreactivity was derived from exogenously expressed GFP-CFTR protein. Our inability to detect endogenous CFTR in MDCK type I cells is likely due to low expression levels (36).

Role of Microtubules in GFP-CFTR Function and Localization—Microtubules are frequently involved in the trafficking of transport proteins to the plasma membrane upon agonist stimulation (42). To examine the role of microtubules in cAMP-stimulated GFP-CFTR Cl⁻ channel function and GFP-CFTR localization to the apical membrane region, we treated monolayers for 5–7 h with colchicine to depolymerize the microtubular cytoskeleton or lumicolchicine, an inactive colchicine analog that does not depolymerize microtubules. GFP-CFTR function was measured by short circuit current analysis, and GFP-CFTR localization was examined by confocal fluorescence microscopy. Results from short circuit current experiments (Table III) demonstrated that colchicine had no effect on basal or cAMP-stimulated peak and elevated Isc. Similar results were obtained in parental, untransfected MDCK cells, suggesting that microtubule disruption does not affect endogenous CFTR function (Table III). Following short circuit current experiments, monolayers were fixed and stained with a monoclonal antibody against α-tubulin. Microtubules were depolymerized in colchicine-treated but not in lumicolchicine-treated monolayers; however, GFP-CFTR polarization to the apical membrane region was unaltered by colchicine treatment (data not shown). This observation was confirmed by quantitative confocal fluorescence microscopy and apical surface biotinylation (densitometric analysis of apical membrane GFP-CFTR in colchicine-treated cells, 0.90 ± 0.15 (n = 6) compared with 0.95 ± 0.15 (n = 6) of parental, untransfected MDCK cells) (43, 44). Microtubule depolymerization was independently verified by analyzing the ratio of soluble (depolymerized) and insoluble (polymerized) tubulin fractions by Western blotting. The insoluble:soluble tubulin ratio was 97:3 following lumicolchicine treatment compared with 2:98 following colchicine treatment (n = 3). Taken together, these findings suggest that both steady state GFP-CFTR localization at the apical membrane region and acute activation of transepithelial Cl⁻ secretion mediated by GFP-CFTR Cl⁻ channels are independent of an intact microtubular cytoskeleton. Furthermore, these findings suggest that the half-life of GFP-CFTR at the apical membrane and in apical endosomes is long (greater than 7 h), in accordance with previous reports of CFTR half-life in heterologous cells overexpressing CFTR and in epithelial cells expressing endogenous CFTR (45, 46).

DISCUSSION

We have generated a cell model of MDCK epithelial cells stably expressing a GFP-CFTR fusion protein which functions as a cAMP-activated Cl⁻ channel and is targeted to the apical plasma membrane in cells cultured on permeable supports. Quantitative confocal fluorescence microscopy, apical surface biotinylation, and short circuit current experiments showed that acute treatment with a cAMP-stimulating mixture increased GFP-CFTR-mediated Cl⁻ secretion by activating channels resident in the apical plasma membrane and that cAMP-
A. Apical Surface Biotinylated GFP-CFTR

B. Total Cell Lysate GFP-CFTR

C. GFP-CFTR Endocytosis and Recycling

D. Glycosidase Digestion of GFP-CFTR

E. Avidin Labeling of Biotinylated Apical Membrane

**Fig. 8.** A and B, Western blot analyses examining amount of apical membrane-biotinylated GFP-CFTR in MDCK cells grown on permeable supports and treated with vehicle or cAMP-stimulating mixture. A, apical surface-biotinylated GFP-CFTR. B, one-fourth of total cell lysate GFP-CFTR. Lane 1, 10-min vehicle; lane 2, 10-min cAMP; lane 3, 60-min cAMP; lane 4, no biotinylation; lane 5, no periodate oxidation; lane 6, parental, untransfected MDCK cells. GFP-CFTR was detected with C-terminal antibody. Identical banding patterns were obtained with GFP monoclonal antibody. * high molecular weight GFP-CFTR; C, 240-kDa mature-glycosylated GFP-CFTR band C; and B, 210-kDa core-glycosylated GFP-CFTR band B. Densitometric analysis of apical membrane GFP-CFTR; 2-min cAMP = 1.04 ± 0.12 (n = 4), 10-min cAMP = 0.97 ± 0.14 (n = 9), and 60-min cAMP = 0.95 ± 0.20 (n = 4) compared with vehicle-treated cells. p > 0.05 for vehicle compared with all cAMP-treated monolayers. C, endocytosis and recycling of GFP-CFTR. Cells were treated with vehicle (closed circles) or cAMP mixture (open circles), and the fraction of GFP-CFTR remaining at the apical surface was determined by a two-step biotinylation procedure as described under “Experimental Procedures.” Because biotinylation efficiency was variable in different experiments, the fraction of apical membrane GFP-CFTR at each time point was normalized to apical membrane GFP-CFTR at time 0, which is defined as 1. p > 0.05 for vehicle compared with cAMP-treated monolayers at all time points.

**Table III**

| Cell line | Pre-cAMP | cAMP peak | cAMP elevated state |
|-----------|----------|-----------|---------------------|
| Lumicolchicine (n = 4) | 3.2 ± 0.3 | 10.2 ± 0.7 | 8.6 ± 0.4 |
| Colchicine (n = 4) | 2.6 ± 0.3 | 11.9 ± 0.4 | 8.7 ± 1.2 |
| Parental | | | |
| Lumicolchicine (n = 3) | 0.6 ± 0.1 | 5.5 ± 0.5 | 3.2 ± 0.2 |
| Colchicine (n = 3) | 0.6 ± 0.1 | 6.8 ± 0.5 | 3.5 ± 0.2 |

*p < 0.05 compared to pre-cAMP in same row.*

Our findings are in agreement with numerous studies in epithelial and non-epithelial cells in which cAMP did not acutely stimulate CFTR trafficking to the plasma membrane (2, 7, 13, 48). Thus, it should not be considered dogma that CFTR traffics to the cell surface following cAMP treatment or that CFTR regulates its own trafficking. Instead, CFTR trafficking should be considered cell type-specific (49), because cAMP stimulates CFTR translocation to the plasma membrane in some cells (10–12, 50, 51) but not others (2, 7, 13, 48). In contrast to our findings, very recent reports have found positive effects of cAMP on CFTR trafficking in kidney epithelial cells. For instance, Morris et al. (10) recently reported that arginine vasotocin, a hormone which increases cellular cAMP levels, mobilized CFTR from an intracellular compartment to the apical membrane in amphibian kidney A6 cells. Similarly, in preliminary results, Howard et al. (50, 51) demonstrated that CFTR containing a FLAG-epitope tag trafficked to the apical plasma membrane following acute (10 min) forskolin treatment in MDCK type II cells. In reports quantitating surface expression of CFTR following cAMP treatment, plasma membrane CFTR increased 100% in T84 cells (12), 50–200% in HeLa cells (50), and 100–600% in MDCK type II cells (51). The detection systems used in the present study are sensitive enough to detect changes of these magnitudes.

We speculate that these conflicting results may be due to cell type-specific CFTR trafficking patterns. Agonist-stimulated trafficking of polytopic membrane transport proteins is often cell type-specific. For example, aquaporin-2 traffics from intracellular vesicles to the apical plasma membrane following treatment with cAMP-stimulating agents in collecting duct.
principal cells (52) but not in Xenopus oocytes (53). Similarly, insulin stimulates GLUT-4 trafficking from an intracellular pool to the plasma membrane in adipocytes and skeletal muscle but not in heterologous expression systems (54). The absence of agonist-stimulated protein trafficking in these systems has been attributed to cell-specific expression of signaling proteins and/or trafficking factors. Thus, we consider it likely that cAMP stimulates FLAG-CFTR trafficking to the apical membrane in MDCK type II cells but not GFP-CFTR trafficking to the apical membrane in MDCK type I cells, because of differential expression of trafficking proteins (i.e. SNAREs, annexins, Rab GTPases, etc.) and glycosphingolipids (55).

Because MDCK type I cells exhibit electrophysiological and morphological properties similar to cells in the collecting duct (56, 57), whereas MDCK type II cells partially resemble cells in proximal tubule (56) and thick ascending limb (58), it is conceivable that CAPB relocates CFTR to the apical membrane in cells derived from proximal tubule and/or thick ascending limb but not collecting duct. It is unlikely that MDCK type I cells lack factors required to traffic GFP-CFTR appropriately, as these cells express low levels of endogenous, functional CFTR Cl− channels in the apical membrane (36). We have confirmed that the MDCK type I cells used in this study express endogenous CFTR by reverse transcriptase-polymerase chain reaction (data not shown). In contrast, MDCK type II cells do not express detectable levels of CFTR by reverse transcriptase-polymerase chain reaction, Western blotting, or functional analyses (36). We consider it unlikely that GFP inhibits the ability of cAMP to stimulate CFTR trafficking to the apical membrane, because GFP-CFTR and exogenously expressed CFTR (without any GFP tag) were polarized to the apical plasma membrane region under steady state conditions, and GFP did not interfere with CFTR Cl− channel function in whole cell patch-clamp and planar lipid bilayer experiments. In addition, fusion of GFP to other ion channels does not inhibit protein trafficking or function (59–61).

The initial internalization rate of GFP-CFTR from the apical membrane was 5% per min, similar to the internalization rate of CFTR in stably transfected Chinese hamster ovary cells (9) which do not traffic CFTR to the cell surface following cAMP treatment (7). In contrast, in T84 intestinal epithelial cells the initial rate of endogenous CFTR internalization was 50% per min (8), and CFTR endocytosis and trafficking to the apical membrane were regulated by cAMP (8, 12). Thus, similar to CFTR trafficking to the plasma membrane, CFTR endocytosis and recycling are also cell type-specific.

GFP-CFTR polarity was substrate-dependent. When cells were grown to confluency on glass coverslips, a condition in which MDCK cells do not adopt a fully polarized morphology (57), GFP-CFTR was sorted to the basolateral membrane domain. In contrast, when cells were grown as fully polarized monolayers on permeable supports, GFP-CFTR was sorted to the apical membrane domain. Coating glass coverslips with various extracellular matrix proteins, including collagen, fibronectin, and laminin, to promote cellular differentiation and polarization, did not alter basolateral GFP-CFTR localization.² We speculate that substrate-dependent GFP-CFTR polarity is attributable to differences in cell differentiation in glass-grown versus filter-grown cells. Significant amounts (up to 50%) of membrane proteins which are distributed in a polarized fashion in cells grown on permeable supports are found on the “opposite” membrane domain in MDCK cells grown on glass coverslips (62, 63). Our findings emphasize the need to study trafficking of CFTR and other polarized membrane proteins in physiologically relevant settings. When using cultured epithelial cells as a model system, experiments should be performed using fully polarized monolayers grown on permeable supports. Examination of trafficking in non-physiological experimental systems (i.e. cells grown on glass coverslips or plastic dishes) may lead to conclusions that are not relevant to the in vivo situation.

In conclusion, our data support a model in which cAMP activates CFTR Cl− channels resident in the apical plasma membrane in MDCK type I cells, a model of renal distal tubule, and collecting duct. Because CFTR functions not only as a Cl− channel, but also as a regulator of other cAMP-responsive apical membrane ion channels including the epithelial sodium channel (64, 65), an outwardly rectifying chloride channel (28), and a renal potassium channel (66), we speculate that apical membrane resident CFTR Cl− channels serve at least two functions in distal nephron: first, mediation of transepithelial Cl− transport (14, 15), and second, regulation of sodium chloride reabsorption/secretion as well as potassium secretion (64). Given that cAMP did not acutely stimulate CFTR trafficking in this study, it is unlikely that CFTR regulates these other ion channels by regulating their trafficking to the apical membrane. It is more likely that CFTR regulates these channels by membrane-delimited pathway(s) involving direct interactions (67) or indirect autocrine signaling cascades (28). In this manner, apical membrane resident CFTR may control overall electrolyte homeostasis in renal distal tubule.

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