Supplemental Data

Spatiotemporal Coupling of cAMP Transporter to CFTR Cl^- Channel function in the Gut Epithelia

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Supplemental Experimental Procedures

Tissue Culture and Animals

Colonic epithelial cells, HT29-CL19A and T84, were cultured as described (Li et al., 2004). HEK293 cells overexpressing MRP4 were grown in DMEM (Invitrogen) supplemented with 10% FBS. For Ussing chamber experiments, cells were grown on permeable filters (6.5-mm diameter). For FRET imaging, HT29-CL19A and T84 cells were transfected with pcDNA3 (containing CFP-EPAC-YFP), and the positive colonies were selected using 400 μg/ml G418 and confirmed under fluorescence microscope. Wild-type and MRP4 knockout mice were used for the *in vivo* intestinal fluid secretion studies. MRP4 knockout mice had been established previously (Leggas et al., 2004). All animals were maintained under standard conditions, and the studies were carried out in accordance with the guidelines provided by the Institutional Animal Care and Use Committee of the University of Tennessee Health Science Center (Memphis, TN).

Antibodies, Reagents, and Constructs

CFTR monoclonal mouse antibody (R1104) and polyclonal antibody (NBD-R) have been previously described (Naren, 2002). MRP4 rat monoclonal antibody (M_4I-10) has been described before (Leggas et al., 2004). Anti-PDZK1 rabbit polyclonal antibody against the full-length PDZK1 protein and MRP4 peptide containing C-terminal 10 a.a. (MRP4-C10) were generated by

Genemed Synthesis, CA. Biotin-conjugated C-terminal 10 a.a peptide of MRP4 (biotin-MRP4-C10 and biotin-MRP4-C10-L1325A) was synthesized by the Hartwell Center at St. Jude Children's Research Hospital (Memphis, TN). GST-His-S-fusion protein for C-terminal 50 a.a. of MRP4 (GST-His-S-MRP4-C50) was generated using pET41 vector (Novagen, San Diego, CA), and His-S-fusion protein of full-length PDZK1 was generated using pET30 vector (Novagen, San Diego, CA). GST-fusion protein for full-length PDZ proteins (NHERF1, NHERF2, PDZK1 and alpha-syntrophin) were generated using pGEX vector according to manufacturer's instruction (Amersham Pharmacia). The generation of pcDNA3-CFP-EPAC-YFP was described before (Ponsioen et al., 2004). Fluorescein isothiocyanate-conjugated streptavidin, 1, N^6-etheno-cAMP, and cholera toxin (CTX) were obtained from Sigma Chemical Co. (St. Louis, MO). [^{3}H]-labeled cAMP (1 mCi/ml) was obtained from PerkinElmer LAS, Inc. (Boston, MA). MRP4-specific inhibitor MK571 was obtained from Cayman Chemical (Ann Arbor, MI). α-Toxin was obtained from Calbiochem Corp. (La Jolla, CA). The peptide delivery system (Chariot™) was procured from Active Motif (Carlsbad, CA). MRP4 siRNA (Cat# AM16704) and negative control siRNA (Cat# AM4613) were purchased from Ambion (Austin, TX). Other reagents have been described previously (Li et al., 2004).

Short-circuit Current (I_{sc}) Measurements

Polarized colonic epithelial (HT29-CL19A and T84) cell monolayers were grown to confluency on Costar Transwell permeable supports (filter area is 0.33 cm^2). Filters were mounted in a Ussing chamber setup, and short-circuit currents (I_{sc}) mediated through CFTR Cl^- channel were measured as described (Li et al., 2005). The cells were bathed in Ringer's solution (mM) (*basolateral*: 140 NaCl, 5 KCl, 0.36 K2HPO4, 0.44 KH2PO4, 1.3 $CaCl_2$, 0.5 MgCl2, 4.2 NaHCO3, 10 Hepes, 10 glucose, pH 7.2), and low Cl^- Ringer's solution (mM) (*apical*: 133.3 Na-gluconate, 5 K-gluconate, 2.5 NaCl, 0.36 K2HPO4, 0.44 KH2PO4, 5.7 $CaCl_2$, 0.5 $MgCl_2$, 4.2 $NaHCO_3$, 10 Hepes, 10 mannitol, pH 7.2) at 37 °C, gassed with 95% O_2 and 5% CO_2. MK571 (5-50 μM) was added into both the apical and basolateral sides of the cell monolayers for 30 min before adenosine (ADO) was added into the apical side to elicit a CFTR-dependent Cl^- current response. In some experiments, the polarized cell monolayers were permeabilized at the basolateral side with 100 μg/ml alpha-toxin for 30 min at 37°C. Then the cells were mounted into the Ussing chamber and preincubated with MK571 at both sides for 30 min before cAMP

was added into basolateral side to activate CFTR Cl^- channel. CFTR Cl^- channel inhibitor DPC (500 μM) or glybenclamide (500 μM) or $CFTR_{inh}$-172 (1-10 μM) were added into the apical side to inhibit the Cl^- currents toward the end of the experiment. The epithelial integrity was monitored by passing a 1-mV or 3-mV pulse across the epithelia every 1 min during the whole experiment.

[^{3}H]-cAMP and etheno-cAMP Transport Assay across Apical Cell Membranes

Polarized HT29-CL19A and T84 cells (grown on Transwell for 3 weeks, resistance of 1500-2000 ohm) were permeabilized at the basolateral side with 100 μg/ml alpha-toxin for 30 min at 37°C. Then the cells were incubated with MK571 or PMEA at both sides at 37°C for additional 30 min. [^{3}H]-cAMP (1 μC/ml final concentration) or etheno-cAMP (5 μM final concentration) was added into the basolateral chamber containing 4 mM ATP (Mg salt) and incubated at 4°C or 37°C. For [^{3}H]-cAMP transport assay, an aliquot of 40 μl apical solution was collected at various time points, diluted with 2.5 ml high flash-point LSC-cocktail (Ultima Gold, PerkinElmer); and the radioactivity was measured using a liquid scintillation counter (LS5000TA; Beckman Coulter, Fullerton, CA). For etheno-cAMP transport assay (a fluorescent analog of cAMP, see Secrist, 1974), an aliquot of 40 μl apical solution was collected at various time points, diluted with 260 μl HBSS, and the fluorescence was measured at wavelength ex315-nm/em420-nm using a fluorescence spectrophotometer (F-2500; Hitachi, Tokyo, Japan).

Pull-down Assay

Pull-down assay was performed as described before (Li et al., 2004; 2005). Briefly, HEK293 cells overexpressing MRP4 were lysed in lysis buffer (PBS 0.2% Triton X-100 + protease inhibitors). Cell lysates were mixed at 4^{o}C for 15 min followed by centrifugation at 15,000 *g* for 10 min at 4^{o}C. GST or GST-PDZ fusion proteins (NHERF1, NHERF2, PDZK1, and alpha-syntrophin) were added and mixed at 4^{o}C with the clear supernatant for 30-60 min. For direct binding between His-S-PDZK1 and GST-His-S-MRP4-C50, various amounts of His-S-PDZK1 (0-33.3 nM) were mixed with 5 ug of GST-His-S-MRP4-C50 at room temperature for 45 min. For MRP4-C10 peptide competitive binding, the peptide was mixed with His-S-PDZK1 at room temperature for 60 min before adding GST-His-S-MRP4-C50. After incubation, glutathione Sepharose beads (20 μl) were then added and mixed for another 2 h. Thereafter, the mixtures

were spun at 800 *g* for 2 min, and the beads were washed three times with the same lysis buffer before the proteins were eluted from beads with Laemmli sample buffer (containing 2.5% β-mercaptoethanol). Eluates were separated on 4-15% gel and immunoblotted with anti-MRP4 or anti-PDZK1 antibodies.

Co-immunoprecipitation and Immunoblotting

Cells were harvested and processed as described previously (Li et al., 2004; 2005). Colonic epithelial cells (HT29-CL19A and T84) were solubilized in RIPA buffer (+ protease inhibitors) for immunoblotting alone or PBS-0.2% Triton X-100 (+ protease inhibitors) for co-immunoprecipitation on ice for 20 min, and lysates were spun at 15,000 *g* for 15 min at 4°C to pellet insoluble material. Protein concentration of the cell lysates was determined by the Bradford assay (Pierce, Rockford, IL). For co-immunoprecipitation, CFTR monoclonal antibody (R1104 IgG, 1.0 μg) was cross-linked to 20 μl of protein A/G agarose as described before (Naren, 2002). The postnuclear supernatant of HT29-CL19A cells was incubated with the cross-linked beads overnight at 4°C under constant mixing. The beads were washed three times with PBS-0.2% Triton X-100 before the immunoprecipitated proteins were eluted with sample buffer containing 1% β-mercaptoethanol. The immunoprecipitated proteins were then separated on 4-15% gel and blotted using rabbit polyclonal anti-PDZK1 IgG, rat monoclonal anti-MRP4 IgG, and rabbit polyclonal anti-CFTR IgG (NBD-R IgG). For immunoblotting of PDZK1 from HT29-CL19A and T84 cells, cell lysates prepared as described above were separated on 4-15% SDS-PAGE, transferred to PVDF membranes, and immunoblotted using PDZK1-specific antibody. For immunoblotting of MRP4 at crude membrane and cytosol, crude membrane and cytosol fraction were prepared as described previously (Li et al., 2004). In brief, cells were homogenized in buffer A (20 mM HEPES, pH 7.4, 1 mM EDTA, 250 mM sucrose, 1 mM DTT, and protease inhibitors) and centrifuged at 800*g* for 5 min. The supernatant was collected and centrifuged at 16,000 *g* for 15 min at 4°C, and the pellet was used as crude membrane fraction. The supernatant after 16,000 *g* spinning was ultracentrifuged at 200,000 *g* for 1 h in Beckman Type 70.1 Ti rotor, and the resulting supernatant was considered the cytosolic fraction. These fractions were resolved on 4-15% SDS-PAGE, transferred to PVDF membranes, and immunoblotted using MRP4-specific antibody.

Macromolecular Complex Assembly

This *in vitro* complex formation (Li et al., 2005) was performed by mixing GST-His-S-MRP4-C terminal 50 a.a. fusion protein (20 μg) with various amounts of His-S-PDZK1 (0-40 μg) at room temperature for 1 h, followed by mixing with glutathione beads (20 μl) for another 1 h. This step, which is called pairwise binding, was done in 200 μl of lysis buffer (PBS-0.2% Triton-X 100 + protease inhibitors). The complex was washed twice with the same buffer and allowed to incubate further with 0.5 μg purified Flag-tagged wt-CFTR at 4 ^{o}C for 3 h with constant mixing. The complex was washed extensively with lysis buffer, eluted with sample buffer, and immunoblotted with anti-CFTR antibody (R1104).

Cell-attached Single-channel Recordings

Single-channel recordings were obtained from HT29-CL19A cells by using the cell-attached configuration (Li et al., 2005). Patch-clamp pipettes were obtained using quartz class (Sutter Instrument Co., Novato, CA) and a Sutter model P-2000 puller and had resistance of 6 - 8 MΩ. The extracellular (both pipette and bath) solution contained 140 mM NMDG-Cl, 2 mM $MgCl_2$, 2mM $CaCl_2$, 10 mM HEPES, and 100 μM DIDS (to pharmacologically block non-CFTR anion channel) titrated to a pH of 7.4 with NMDG. Cells were preincubated with MK571 (0-50 μM) for 30 min. CFTR channels were activated with adenosine (0-40 μM) included in the pipette as indicated. Single-channel currents were continuously recorded for 5 min at a test potential of +100 mV (referenced to the cell interior) delivered from the recording electrode and were filtered at 1 kHz and sampled at 2 kHz. All experiments were conducted at room temperature (22-24ºC) using an EPC-9 patch clamp amplifier (HEKA Electronik GmbH, Lambrecht, Germany) and using the Pulse + PulseFit V 8.65 acquisition program (HEKA Electronik GmbH, Lambrecht, Germany). Data analysis was performed using TacX4.1.5 (Bruxton Corp., Seattle, WA, USA).

FRET Imaging Microscopy and Image Analysis

Cells (expressing CFP-EPAC-YFP) for FRET imaging were seeded in the 35-mm glass bottom culture dishes (MatTek) and grown for 24 to 48 h. Cells were washed twice with Hanks' balance salt solution (HBSS) before being mounted on an Olympus microscopy system for FRET imaging. Cells were maintained in HBSS in the dark at room temperature with the addition of MK571 or DMSO, ADO, or FSK as indicated. Images were recorded with a cooled charged-

coupled device camera Hamamatsu ORCA285 (Hamamatsu, Japan) mounted on the Olympus microscope IX51 (U-Plan Fluorite 60 × 1.25 NA oil-immersion objective), and the system was controlled by SlideBook 4.1 software (Intelligent Imaging Innovations, Denver, CO) with ratio and FRET modules used to obtain and analyze the FRET images. Excitation light was provided by a 300W Xenon lamp and attenuated with a neutral density filter with 50% light transmission. Images were captured using a JP4 CFP/YFP filter set (Chroma, Brattleboro, VT) including a 430/25-nm (25-nm band-pass centered at 430-nm) excitation filter, a double dichroic beam splitter (86002v2bs), and two emission filters (470/30-nm for CFP and 535/30-nm for FRET) alternated by a high-speed filter-changer Lambda 10-3 (Sutter Instruments, Novato, CA). Time-lapse images were acquired with 4 × 4 binning mode, 200-400 ms exposure time, and 1-min intervals to reduce photobleaching of the fluorophores. Acquired fluorescent images were background-subtracted, and multiple regions of interest (ROIs) on the cell periphery were selected for quantitative data analysis (~20-30 ROIs per cell, and 4-6 cells per condition were averaged). The emission ratio images (CFP/FRET) were generated at different time points on a pixel-by-pixel basis by the SlideBook ratio module and normalized through dividing all ratios by the emission ratio right before the addition of reagents, thereby setting the basal emission ratio to 1, as formulated below,

$$R = \frac{\text{Emission intensity of background-subtracted CFP image}}{\text{Emission intensity of background-subtracted FRET image}}$$

The ratio (R) was normalized as R_t/R_0, where R_t is the ratio at time point t and R_0 is the ratio at time point = 0 (right before the addition of first test compound). The cell images are presented in pseudocolor to highlight the changes in the ratio of CFP/FRET fluorescence intensity.

Intestinal Fluid Secretion (*in vivo*) Measurement

Wild-type or *MRP4* knockout mice (body weight 20-22 g) were held off food 24 h prior to inducing anesthesia using pentobarbital (60 mg/kg) (Li et al., 2005). Mouse body temperature was maintained at 36-38 °C during surgery by using a circulating water heating pad. A small abdominal incision was made to expose the small intestine. Ileal loops (~20 mm) proximal to the cecum were exteriorized and isolated (2-4 loops per mouse). The closed loops were then injected

with 100 μl of PBS alone or PBS containing CTX (0.5 μg). Both the control and CTX-injected loops were tested in the presence and absence of MK571 (10-50 μM). In some experiments, CFTR specific inhibitor, $CFTR_{inh}$-172 (10-20 μM), was included with the test compounds. The abdominal incision and skin incision were closed with wound clips, and the mice were allowed to recover. After 6 h, the mice were sacrificed by CO_2. Intestinal loops were removed, and loop length and weight were measured to quantitate net fluid secretion.

MRP4 Peptide *in vitro* Competitive Binding

Briefly, MRP4 peptides (biotin-conjugated wild-type or L1325A mutant peptide, or non-conjugated wild-type or C10-AAA mutant peptide; 2 μM each) were mixed with various amounts of His-PDZK1 (1.3 nM and 6.6 nM) at 22-24°C for 1 hr before GST-MRP4-C50 (immobilized on glutathione beads) was added and continued to mix for another 1 hr. The beads were washed extensively with PBS-0.2% Triton X-100, and the protein complex was eluted from the beads with sample buffer containing 1% β-mercaptoethanol, separated by SDS-PAGE and immunoblotted with rabbit anti-PDZK1 polyclonal antibody as described above.

Delivery of MRP4-specific Peptide into Polarized Epithelial Cells followed by Immunocytochemistry and Co-IP

Delivery of MRP4-specific peptide was performed using the Chariot™ system according to the manufacturer's instructions (Active Motif; Carlsbad, CA) (Li et al., 2005). Briefly, 2 μM peptides containing the PDZ motif of MRP4 [last 10 amino acids; a.a. 1316 to 1325, STLTIFETAL; wild-type peptide or mutant peptides with alanine substitution mutations at the last a.a. (MRP4-C10-L1325A), or mutations of the tri-peptide PDZ-motif (MRP4-C10-AAA); with or without biotin-conjugate at the N-terminus] was mixed with Chariot solution (total volume: 400 μl) at room temperature for 30 min, then the Chariot-peptide complex was added to both luminal and serosal sides of polarized HT29-CL19A or T84 cells grown on permeable supports and incubated for 1 h at 37 °C in a humidified atmosphere containing 5% CO_2 before mounting in a Ussing chamber for short-circuit current measurement as described above. At the end of the experiment, the epithelial cells were fixed and immunostained for the efficiency of the peptide delivery using fluorescein isothiocyanate-conjugated streptavidin and subjected to immunofluorescence confocal microscopy. In some experiments, after peptide delivery and

short-circuit current measurements, the HT29-CL19A cells in the filters were scraped into RIPA lysis buffer, and co-immunoprecipitation using rabbit PDZK1 polyclonal antibody that was cross-linked to protein A/G agarose was performed. Endogenous CFTR and MRP4 protein amounts in the co-immunoprecipitated complex were checked by immunoblotting using anti-CFTR (R1104 monoclonal) and anti-MRP4 (M_4I-10, rat monoclonal) antibodies as described above.

MRP4 siRNA to Knock Down MRP4 in Polarized Epithelial Cells

Briefly, HT29-CL19A cells were grown for 7-10 days (resistance of 2.0-2.5 kOms/cm_2) on permeable supports of transwells. MRP4 siRNA (Cat# AM16704; Ambion) and negative control siRNA (Cat# AM4613; Ambion) were dissolved with RNase-free water to make a 100 μM stock. siRNA and Lipofectamine 2000 transfection reagent were prepared and mixed with Opti-MEM medium according to the manufacturer's instruction. siRNA-Lipofectamine 2000 complexes were added to both the apical and basolateral sides of the transwell, and the cells were incubated at 37°C in a CO_2 incubator for 24 hrs before the medium was changed to serum-containing medium. After 60-72 hrs, the transwells were mounted in the Ussing chamber for the CFTR-mediated short-circuit current measurement in response to adenosine stimulation. After the Ussing chamber study, the transwells were washed with PBS, and the cells were scraped into RIPA lysis buffer. The protein levels of MRP4, CFTR, and PDZK1 were assessed by Western blotting using corresponding antibodies.

Statistical Analysis

Results are presented as mean ± SEM for the indicated number of experiments. Statistical analysis was performed using Student's *t*-test and one-way ANOVA. A value of $P < 0.05$, $P < 0.01$, or $P < 0.001$ was considered statistically significant.

Supplemental References

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Supplemental Figures

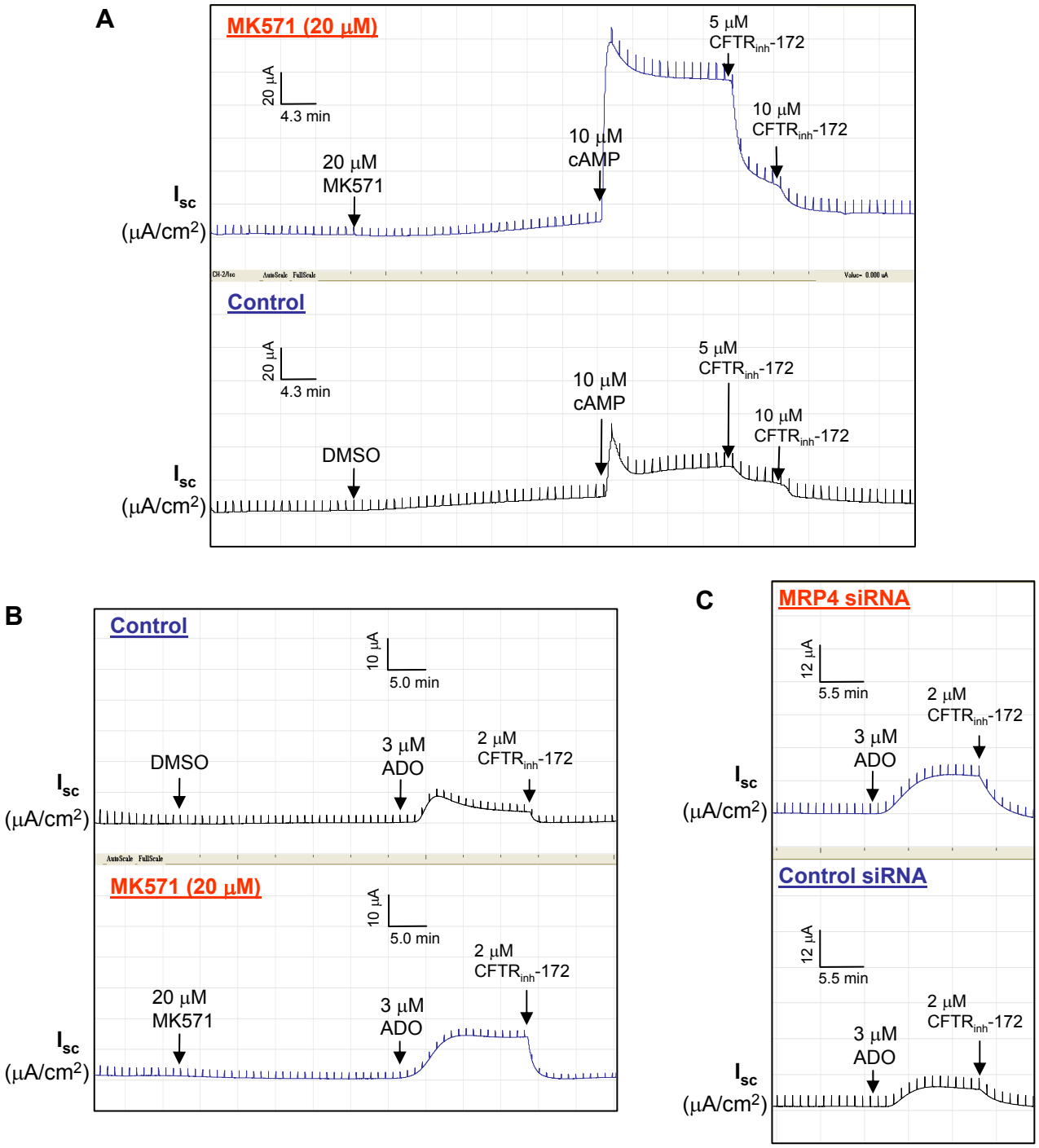

Figure S1. The Cl^- Currents Potentiated by MK571 or MRP4 siRNA were completely Inhibited by the CFTR-specific Inhibitor, CFTRinh-172.

CFTR-mediated I_{sc} in response to 10 μM cAMP (A) and 3 μM ADO (B) in HT29-CL19A cells pretreated with MK571 (20 μM) or vehicle DMSO, and in control siRNA and MRP4 siRNA transfected (C) HT29-CL19A cells stimulated by 3 μM ADO. In (A), basolateral membranes of the cells were permeabilized with 100 μg/ml alpha-toxin for 30 min, and cAMP was added to the basolateral side of the cells. ADO was added to apical sides. A CFTR specific inhibitor, CFTRinh-172, was added to the apical side.

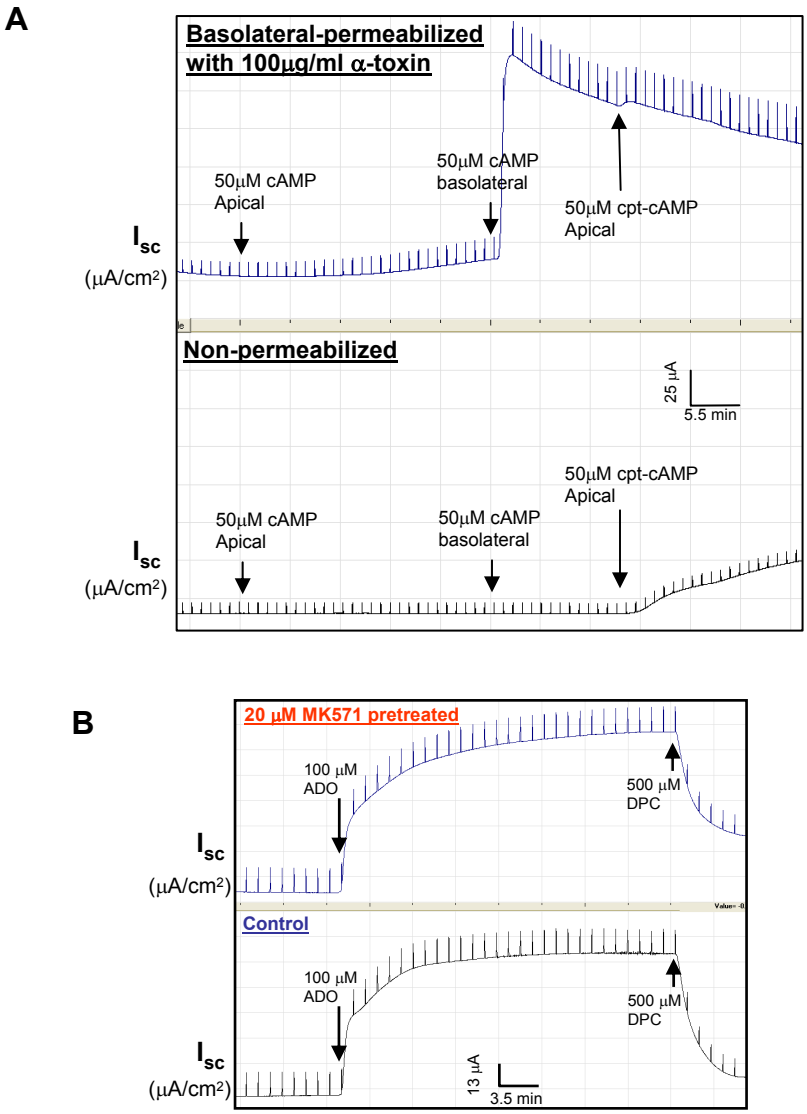

Figure S2. cAMP Activated a CFTR-mediated I_{sc} only when Added to Alpha-toxin Permeabilized Basolateral Surface of Polarized Epithelial Cells. MK571 Failed to Potentiate CFTR-mediated I_{sc} when the Channel Was Maximally Activated.

(A) CFTR-mediated I_{sc} in basolateral-permeabilized HT29-CL19A cells (top) or non-permeabilized cells (bottom). Basolateral membranes of the polarized cells were permeabilized with 100 μg/ml alpha-toxin for 30 min. cAMP or cpt-cAMP (a cell-permeant analog of cAMP) were added to apical or basolateral surfaces as indicated.

(B) CFTR-mediated *Isc* in response to 100 μM ADO in 20 μM MK571-pretreated HT29-CL19A cells (top) or untreated cells (bottom). ADO was added to the apical side of the cells. A CFTR specific inhibitor, DPC (500 μM), was added to the apical side at the end of the experiment.

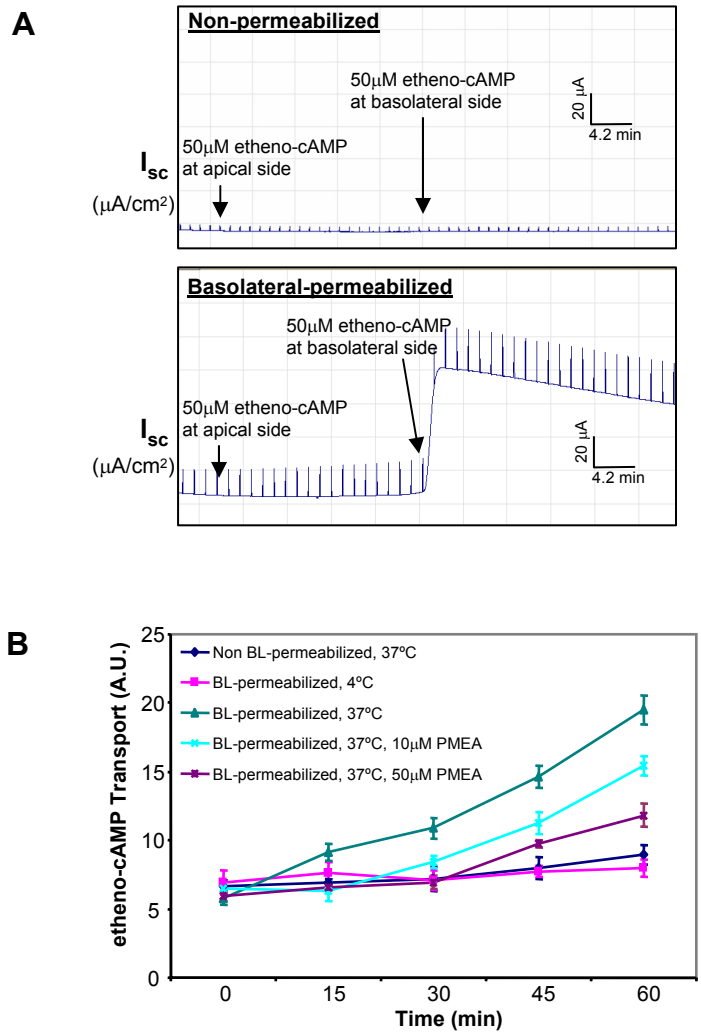

Figure S3. Etheno-cAMP Activated a CFTR-mediated I_{sc} in Alpha-toxin Permeabilized Polarized Epithelial Cells. MRP4 Substrate, PMEA, Dose-dependently Inhibited Etheno-cAMP Transport via Apical MRP4 Transporter.

(A) CFTR-mediated I_{sc} in response to etheno-cAMP (a fluorescent cell-impermeable analog of cAMP) in basolateral-permeabilized HT29-CL19A cells (bottom) or non-permeabilized cells (top).

(B) The unidirectional transport of etheno-cAMP by the apical MRP4 in basolateral-permeabilized HT29-CL19A cells pre-treated with MRP4 substrate PMEA. Data represent the mean ± SEM (n=3).

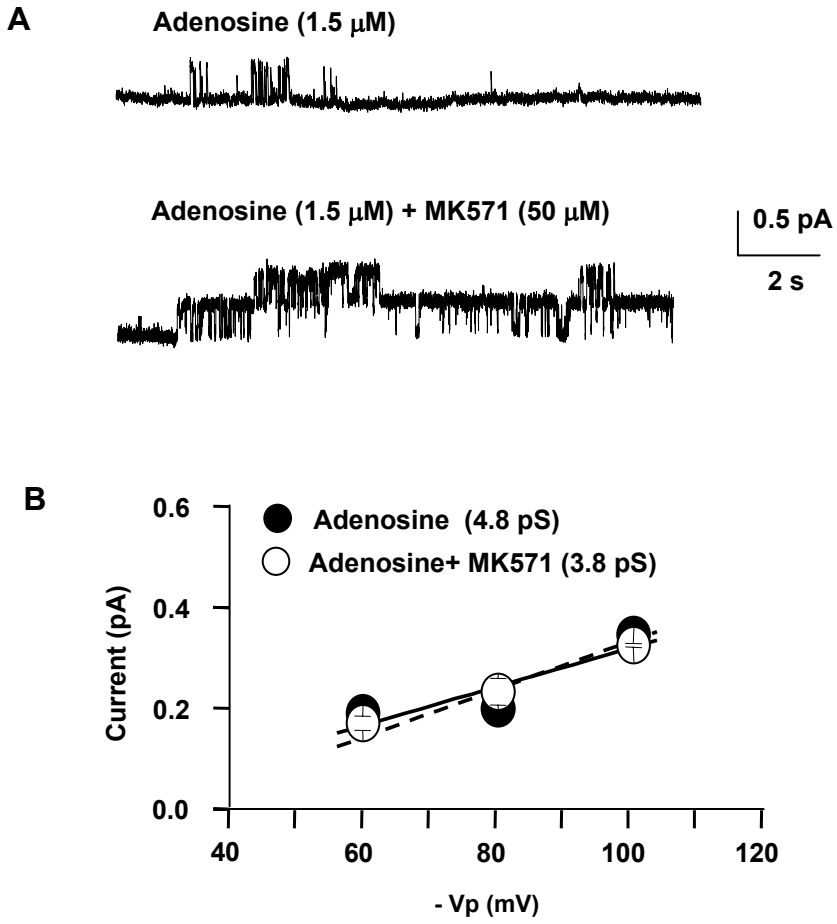

Figure S4. MK571 Potentiated CFTR Single-channel Activity

(A) Representative single-channel current recordings from cell-attached patches obtained from cultured HT29-CL19A cells. Currents were recorded at an applied potential of +100 mV.

(B) Current-voltage relationship of CFTR currents for both control and MK571-treated cells. Data points represent means ± S.E.M obtained from Gaussian fits to all-points histograms where the number of patches is given in the parenthesis as in Figure 3B. Data was obtained in the presence of 1.5 μM adenosine. The single channel conductance was 4.8pS in the absence of MK571 and 3.8pS in the presence of MK571. Cells were pretreated with MK571 for 30 min before recording in the presence of MK571. Dotted lines connect points obtained in absence of MK571 while the solid lines connect data points obtained in the presence of MK571. The bath and pipette solution contained (in mM): 140 NMDG-Cl, 2 MgCl2, 2 CaCl2, 10 HEPES, 100μM DIDS, pH 7.4. Currents were sampled at 2 kHz and filtered for analysis at 100 Hz.

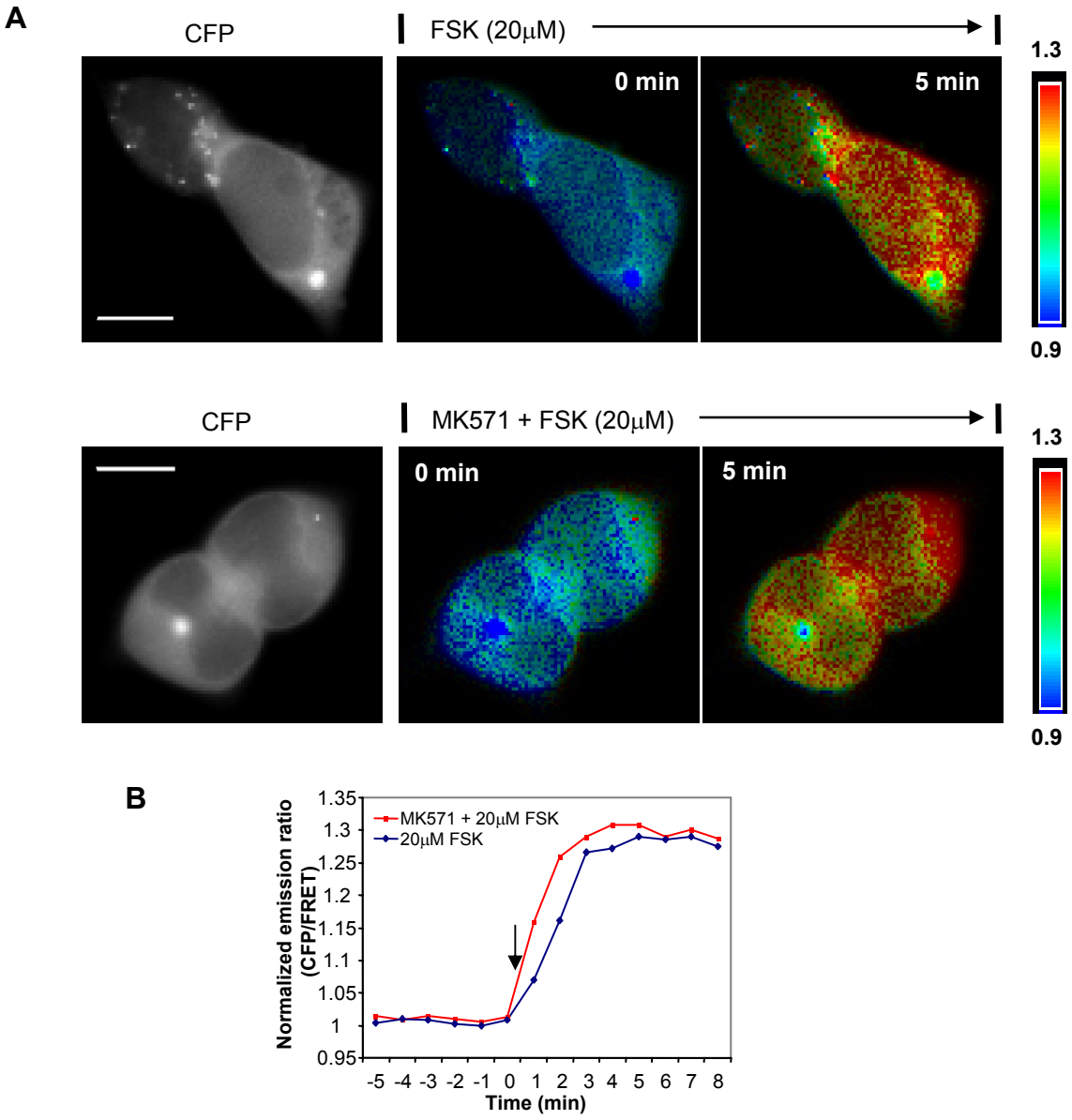

Figure S5. MK571 Failed to Elicit Additional Enhancement of cAMP Signal in the Presence of 20μM FSK

(A) A monochrome CFP image and representative pseudocolored CFP/FRET emission ratio images before (time = 0 min) and after the addition of 20 μM MK571 and/or 20 μM FSK (time = 5 min). The images in each panel were captured from the same field of view. Color bar shows magnitude of the emission ratio. Scale bar: 10 μm. The probe was excluded from the nuclear compartments, although in these nonconfocal images, a signal emanating from above and below the nucleus gives the appearance of a nuclear ratio change.

(B) Kinetics of cAMP changes (represented by the normalized CFP/FRET emission ratio) recorded in the cells shown in (A). Arrow indicates addition of the reagents. Data represent the mean ± SEM (n=4-6).

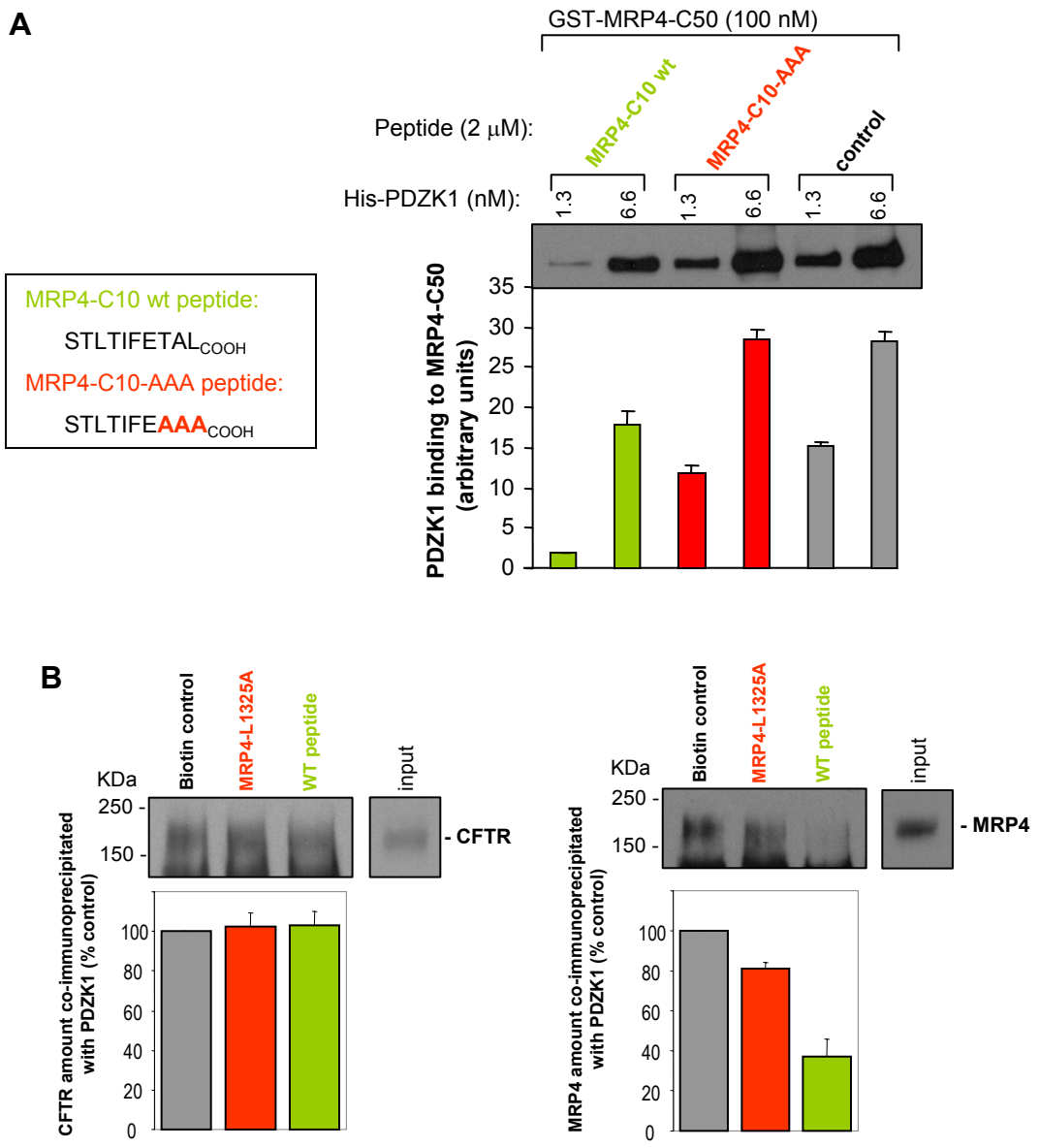

Figure S6. An MRP4 C-tail Peptide Inhibited Binding of PDZK1 to GST-MRP4-C50 Fusion Protein *in vitro,* and significantly Reduced the Endogenous MRP4 Protein Amount that was Co-immunoprecipitated with PDZK1

(A) PDZK1 binding to MRP4 in the presence of MRP4 C-tail peptides (last 10 amino acids; wild-type peptide and alanine substitution mutant of last 3 a.a.; -AAA) . The bar graphs (bottom) show the averaged band density for respective groups (n=3).

(B) After peptide delivery and short-circuit current measurements, the HT29-CL19A cells in the filters were scraped into lysis buffer, and co-immunoprecipitation using rabbit PDZK1 polyclonal antibody that was cross-linked to protein A/G agarose was performed. Endogenous CFTR (left) and MRP4 (right) protein amounts in the co-immunoprecipitated complex were checked by immunoblotting using mouse anti-CFTR (R1104) and rat anti-MRP4 (M_4I-10) antibodies. The bar graphs show the averaged band density for respective groups (n=3).