Murine colonic mucosa hyperproliferation. I. Elevated CFTR expression and enhanced cAMP-dependent Cl\(^{-}\) secretion

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Umar, Shahid, Jason Scott, Joseph H. Sellin, William P. Dubinsky, and Andrew P. Morris. Murine colonic mucosa hyperproliferation. I. Elevated CFTR expression and enhanced cAMP-dependent Cl\(^{-}\) secretion. Am J Physiol Gastrointest Liver Physiol 278: G753–G764, 2000.—Fluid transport in the large intestine is mediated by the cystic fibrosis gene product and cAMP-dependent anion channel cystic fibrosis transmembrane conductance regulator (CFTR). cAMP-mediated Cl\(^{-}\) secretion by gastrointestinal cell lines in vitro has been positively correlated with the insertion of CFTR into the apical membrane of differentiated senescent colonocytes and negatively correlated with the failure of CFTR to insert into the plasma membrane of their undifferentiated proliferating counterparts. In native tissues, this relationship remains unresolved. We demonstrate, in a transmissible murine colonic hyperplasia (TMCH) model, that (8-fold) colonocyte proliferation was accompanied by increased cellular CFTR mRNA and protein expression (8.3- and 2.4-fold, respectively) and enhanced mucosal cAMP-dependent Cl\(^{-}\) secretion (2.3-fold). By immunofluorescence microscopy, cellular CFTR expression was restricted to the apical pole of cells at the base of the epithelial crypt. In contrast, increased cellular proliferation in vivo led to increases in both the cellular level and the total number of cells expressing this anion channel, with cellular CFTR staining extending into the crypt neck region. Hyperproliferating colonocytes accumulated large amounts of CFTR in apically oriented perinuclear compartments. This novel mode of CFTR regulation may explain why high endogenous levels of cellular CFTR mRNA and protein within the TMCH epithelium were not matched with larger increases in transmucosal CFTR Cl\(^{-}\) current.

The functional expression of the cystic fibrosis (CF) gene product, cystic fibrosis transmembrane conductance regulator (CFTR), is pivotal for intestinal Cl\(^{-}\} secretion elicited by neurohormonal agonists acting both through cAMP and Ca\(^{2+}\) (3). CFTR Cl\(^{-}\} channels open after phosphorylation by protein kinase A (2). The polarized expression of CFTR within the apical membrane (13) is thereby believed to control the exit of cellular Cl\(^{-}\} into the intestinal lumen. Transcellular Cl\(^{-}\} movement, coupled to paracellular Na\(^{+}\} egress through the epithelial tight junction, constitutes the ionic basis for NaCl secretion and fluid production in the intestine and many other epithelial tissues (9). Homozygous mutations in the CFTR genome have been found to either eliminate or severely curtail this apical membrane cAMP-regulated Cl\(^{-}\} permeability pathway in CF epithelia. Extensive characterization of these genomic changes has revealed that mutations manifest their effects at multiple levels within the cell. In general they can be categorized as causing either loss of or diminished CFTR expression, inhibition in the cellular processing/targeting of CFTR protein, or attenuations in anion channel function (20). All of these cellular effects result in the same pathophysiological consequence: a lack of functional CFTR within the apical membrane of intestinal epithelial cells, which is believed to be the basic cellular defect underlying the clinical manifestations of CF (20).

The localization of CFTR mRNA and protein in gastrointestinal cell lines and the digestive tract of normal and transgenic CF mice has been correlated with the ability of individual epithelial cells/glands to secrete Cl\(^{-}\} in response to cAMP agonists. The crypt-villus and crypt-surface axes of the small and large intestines, respectively, provide some of the most startling examples of this phenomenon. High levels of CFTR mRNA and protein expression within the immature cell populations of the crypt taper off to lower or nonexistent levels in the more mature villus/luminal surface regions (7, 12). This CFTR distribution thereby identifies the intestinal crypt as the primary site of fluid secretion. However, given the proposed importance of the immature intestinal crypt cells to tissue generated cAMP-dependent Cl\(^{-}\} transport, little is known about how CFTR expression is regulated in vivo. To address this question, we employed an animal model in which changes in CFTR-dependent anion transport were investigated in native colon undergoing enhanced epithelial proliferation. Transmissible murine colonic hyperplasia (TMCH), characterized by significant epithelial cell proliferation within the epithelial mucosa of the descending colon, develops in mice infected with Citrobacter rodentium (4). In contrast to previous in vitro findings, proliferation in vivo led to an increase in both cellular CFTR anion channel expression and net...
mucosal cAMP-dependent Cl− secretion. The subcellular distribution of endogenous CFTR was also changed; CFTR accumulated in intracellular structures removed from the apical plasma membrane. This may represent a means by which the hyperproliferative epithelium can downregulate increases in the functional expression of this anion channel that are potentially deleterious for the cell.

METHODS

Antibodies

Antiserum against the CFTR protein was raised against purified bovine CFTR. A second affinity-purified murine monoclonal antibody (IgM subclass) made against whole-molecule human CFTR (TAM18) was purchased from Labvision (Fremont, CA). BODIPY-conjugated goat anti-bovine and FITC-conjugated goat anti-rabbit secondary antibodies were purchased from Molecular Probes (Eugene, OR). Cy2-conjugated IgG goat anti-mouse IgM heavy-chain isof orm-specific secondary antibody and unlabeled fab anti-IgM fragment were kindly donated by Dr. W. Stegeman (Jackson Laboratories). Goat polyclonal anti-IgA antibody was purchased from Sigma Immun ochemicals (St. Louis, MO). Finally, affinity-purified murine IgM panleukocyte CD15-specific control primary monoclonal antibody was also purchased from Labvision.

Development of a Model for Hyperplasia

TMCH was developed in male Swiss Webster mice (15–20 g; Harlan Sprague Dawley, Houston, TX) by oral inoculation with 16-h culture of Citrobacter freundii (biotype 4280, ATCC) (4). Age-matched control mice received sterile culture medium only. Biotype 4280 is a unique mouse-specific hybrid Citrobacterium strain (also known as Citrobacter rodentium) that adheres to mature surface colonocytes within the distal colon to induce histopathological changes known as attaching and effacing lesions (4). Adherent bacteria were assayed using RT-PCR for bacterial intimin in whole tissue extracts (1, 16) and were found to be absent during the period of most pronounced mucosal hyperproliferation when changes in cellular CFTR anion channel abundance and ion transport were recorded (day 12 after Citrobacter inoculation; data not shown).

To determine gross morphological changes within the colonic mucosa, animals were killed by cervical dislocation and their distal colons were removed and flushed with HEPES-buffered saline (in mM: 140 NaCl, 4.7 KCl, 1 MgCl2, 1 CaCl2, 10 glucose, and 10 HEPES, pH 7.2). Tissues were then embedded in optimum cutting temperature compound (Miles, IN), cryopreserved in liquid N2, and then sectioned and stained with hematoxylin and eosin. Goblet cell number was analyzed in the 5-µm-thick sections by counting the unstained translucent mucin-containing vacuoles. Photographic slides were digitized at high resolution (2,400 DPI), and areas were measured using Universal Imaging’s Metamorph Software (West Chester, PA). Estimates of inflammatory cell number were made by counting the total number of cells within the lamina propria. To estimate the degree of mucosal hyperproliferation, both control and infected animals were given intraperitoneal injections (160 mg/kg body wt) of 5′-bromodeoxyuridine (BrdU; Sigma) 1 h before death to label the S-phase cells. Colons were divided into proximal and distal sections, attached to a paddle, and immersed in Ca2+-free standard Krebs-buffered saline (in mM/l: 107 NaCl, 4.5 KCl, 0.2 NaH2PO4, 1.8 Na2HPO4, 10 glucose, and 10 EDTA) at 37°C for 10–20 min, gassed with 5% CO2/95% O2. Individual crypt units were then separated from the submucosal musculature by intermittent (30-s) vibration into ice-cold potassium gluconate-HEPES saline (in mM/l: 100 potassium gluconate, 20 NaCl, 1.25 CaCl2, 1 MgCl2, 10 HEPES, 10 glucose, and 5 sodium pyruvate) and 0.1% BSA. Crypt suspensions were then deposited (1,200 rpm for 1 min) onto poly-l-lysine-coated microscope slides using a Cytospin cell preparation system (Shandon, Pittsburgh, PA). For detection of incorporated BrdU in S-phase cells, isolated crypts were incubated with a 1:1,000 dilution of affinity-purified goat anti-BrdU antibody at 4°C overnight after blocking of nonspecific protein-binding sites with PBS containing 2% BSA, 0.2% nonfat dry milk, and 0.3% Triton X-100. Bound anti-BrdU antibody was subsequently visualized by immunofluorescence staining with BODIPY FL-conjugated donkey anti-goat IgG antibody. Apoptotic index was measured after incorporation of fluorescein-labeled dUTP into cellular DNA by terminal deoxynucleotidyltransferase (TdT) TUNEL assay (TdT-mediated dUTP nick end labeling). Both labels were detected and quantified by fluorescence microscopy.

Ussing Chamber Studies

The effects of the cAMP-elevating fluid secretory agonist forskolin on CFTR-mediated ion transport in normal and hyperproliferative mouse colon was studied by monitoring short-circuit current (Isc) responses by automatic voltage clamp. Unstriped 1.5-cm colonic mucosal sheets encompassing the cecal (region 1) and rectal (region 4) colonic boundaries were placed into custom-designed Ussing chambers. All experiments were carried out at 37°C; standard Krebs-bicarbonate-Ringer solutions were gassed with 95%O2 -5% CO2 by airlift circulators. Transepithelial potential difference was clamped to 0, and the Isc was continuously monitored on a pen recorder. Transepithelial resistance was calculated from the magnitude of the current deflections in response to a voltage pulse imposed on short-circuited cell sheets every 60 s with a duration of 0.5 s (14).

Northern Blot Analysis and RT-PCR

Total or poly(A)+ mRNA was isolated from whole normal and Citrobacter-infected distal colon as well as from purified crypts using TRizol reagent (GIBCO BRL, Grand Island, NY) or the micro Fast Track kit (Invitrogen, San Diego, CA) according to the manufacturers’ instructions. For Northern blot analysis, each preparation [2.5 µg poly(A)+ mRNA, 10 µg total RNA] was denatured and fractionated on a 1% agarose gel containing formaldehyde. RNA was then transferred to a GeneScreen Plus nylon membrane (DuPont NEN), and the blot was hybridized at 60°C in 10% dextran sulfate, 1 M NaCl, 1% SDS, and 100 µg/ml denatured salmon testes DNA, with the use of a [α-32P]dCTP-labeled probe encompassing the R domain of CFTR (bases 1,773–2,654, 2,022,033.3 on June 28, 2017 http://ajpgi.physiology.org/ Downloaded from by 10.220.33.3 on June 28, 2017

Downloaded from http://ajpgi.physiology.org/ by 10.220.33.3 on June 28, 2017
**Tissue Preparation for Western Blot Analysis**

Swiss Webster mice were killed by cervical dislocation after 0, 1, 3, 6, 9, 12, and 15 days after Citrobacter infection. Crude homogenates were prepared from the whole distal colon and isolated crypts from three normal and Citrobacter-infected animals were prepared for each experimental observation by homogenization in detergent containing buffer (in mM: 50 Tris·HCl, 250 sucrose, 2 EDTA, 1 EGTA, pH 7.5, 10 2-mercaptoethanol, and 0.5% Triton X-100, plus protease inhibitors) followed by a low-speed spin (15,000 g for 15 min). The clear supernatant was saved as total cell extract. Protein concentration was measured before electrophoresis. Mouse brain homogenates and purified bovine tracheal CFTR acted as positive control for the CFTR immunoblotting assay. The total cell extract (30 µg protein/lane) was subjected to 10% SDS-PAGE and electrotransferred to nitrocellulose membranes. The efficiency of electrotransfer was checked by backstaining gels with Coomassie blue and/or by reversible staining of the electrotransferred protein directly on the membranes. The crypts were then separated from the surrounding connective tissue and muscle layers by mechanical vibration for 30 s in ice-cold HEPES saline (in mmol/l: 107 NaCl, 1.25 CaCl$_2$, 1 MgCl$_2$, 10 HEPES, 10 glucose, and 5 sodium pyruvate) and 0.1% BSA, resembling the intracellular medium. Freshly isolated or carbowax-prepared fragments of the goat anti-mouse IgM µ-chain-specific antibody. Further controls involving preincubation of the crypts with unlabeled goat anti-mouse IgA and the F(ab)$_2$ fragments of the goat anti-mouse IgM µ-chain-specific antibody were also performed. Fluorescence was viewed using a Noran confocal laser scanning microscope (CLSM, Noran Instruments, Middleton, WI) equipped with an argon laser and appropriate optics and filter modules for fluorophore detection. Digital images on the CLSM were obtained at ×400, ×800, and ×1,200 using a high numerical aperture lens (Nikon ×40, 1.4 N/A). A z-axis motor attached to the inverted microscope stage was calibrated to move the plane of focus in 0.4-µm steps through the sample. Eight or sixteen-bit images collected at 512 × 480 resolution were then stored on a mass storage device (removable rewritable optical hard disk) and volumetrically reconstructed using the Image 1/Metamorph 3-D software module (Universal Imaging, West Chester, PA).

**RESULTS**

Given the marked differences in CFTR mRNA abundance along the in vivo crypt base-to-surface axis, it is possible that the expression of functional CFTR protein is regulated by or in conjunction with the proliferative status of intestinal epithelial cells (12). This would represent a clear difference with in vitro data in which CFTR expression was not affected by cellular proliferative status (reviewed in Ref. 12). We therefore investigated changes in CFTR abundance and functional expression in the TMCH model.

Transmissible Murine Colonic Hyperplasia Develops in Swiss Webster Mice

Establishment of model. Citrobacter infection induced a predictable and reproducible hyperplasia in the mouse colon (48 out of 48 animals exhibited dramatic effects). Grossly detectable thickening and rigidity of the distal part of the colon was first observed around day 6 after infection (see Refs. 1 and 17). These changes were occasionally observed in middle/proximal regions but were never as severe. To more accurately characterize this phenomenon, the entire colon, encompassing cecal and rectal boundaries, was separated into four consecutive ~1.5-cm segments, with segment 1 being the most proximal. After 12–15 days of Citrobacter infection, gross changes were most evident distally (region 4, 98%; region 3, 74%; region 2, 20%; and region 1, 0%; n = 156 mice, shown as percentages of animals exhibiting 1.5-fold increase in normal mucosal thickness). The mucosa was empty and contracted, but in no instance was the mucosa grossly thickened. Transverse fixed and hematoxylin and eosin-stained sections revealed that crypt length in the descending colon increased more than twofold (region 4); the crypt length in uninfected animals (220 ± 19 µm) was less than half that in day 12 post-Citrobacter-infected animals (460 ± 46 µm, see Fig. 1). TMCH was not associated with an increased goblet cell number (Fig. 1). In fact, the average goblet cell area/crypt decreased from 34% to

**Immunofluorescence Localization Studies**

Region 4 (late distal colon) from normal and Citrobacter-infected animals were attached to paddles and immersed in Ca$^{2+}$-free standard Krebs-buffed saline (in mmol/l: 107 NaCl, 4.5 KCl, 0.2 NaH$_2$PO$_4$, 1.8 Na$_2$HPO$_4$, 10 glucose, and 10 EDTA) at 37°C for 10–20 min, gassed with 5% CO$_2$/95%O$_2$. The crypts were then separated from the surrounding connective tissue/muscle layers by mechanical vibration for 30 s in ice-cold HEPES saline (in mmol/l: 100 potassium glutonate, 20 NaCl, 1.25 CaCl$_2$, 1 MgCl$_2$, 10 HEPES, 10 glucose, and 5 sodium pyruvate) and 0.1% BSA, resembling the intracellular medium. Freshly isolated or carbowax-prepared (3% polyoxyethylene-29% denatured ethanol-2% isopropanol; Cytospin collection fluid) crypt suspensions were then deposited (1,200 rpm for 1 min) onto poly-L-lysine-coated microscope slides using the Cytospin cell preparation system. Immunolocalization studies were carried out by permeabilizing the crypts for 1–3 h at room temperature with 3% sodium deoxycholate (wt/vol in PBS) in a humidified chamber. An extended period of detergent permeabilization and extraction was found to greatly facilitate antibody specificity and reduce background in preserved crypts. Cytosols were stained for CFTR using either commercially available mouse anti-human monoclonal antibody or rabbit anti-bovine CFTR polyclonal antibody diluted in blocking solution at 1:200 and 1:100, respectively. After incubation at room temperature for 1 h or at 4°C overnight, the slides were washed and incubated with either affinity-purified Cy2 conjugated goat anti-mouse IgM heavy-chain isofrom-specific secondary antibody or goat anti-rabbit secondary antibody conjugated with FITC diluted in blocking solution at 1:500 for 1 h at room temperature or overnight at 4°C. Between washes, slides were washed for 30 min in PBS containing 1% BSA. Control slides were incubated without the primary antibody or with affinity-purified murine IgM CD15 panleukocyte-specific monodonal antibody. Positive identification of low endogenous levels of mouse crypt IgM was accomplished using a goat anti-murine IgM antibody. Further controls involving preincubation of the crypts with unlabeled goat anti-mouse IgA and the F(ab)$_2$ fragments of the goat anti-mouse IgM µ-chain-specific antibody were also performed. Fluorescence was viewed using a Noran confocal laser scanning microscope (CLSM, Noran Instruments, Middleton, WI) equipped with an argon laser and appropriate optics and filter modules for fluorophore detection. Digital images on the CLSM were obtained at ×400, ×800, and ×1,200 using a high numerical aperture lens (Nikon ×40, 1.4 N/A). A z-axis motor attached to the inverted microscope stage was calibrated to move the plane of focus in 0.4-µm steps through the sample. Eight or sixteen-bit images collected at 512 × 480 resolution were then stored on a mass storage device (removable rewritable optical hard disk) and volumetrically reconstructed using the Image 1/Metamorph 3-D software module (Universal Imaging, West Chester, PA).
18% (n = 6 whole mount slides from 6 animals). We did not find significantly more mesenchymal cells within the submucosal cell layers. Estimates from eight sections of distal colon region 4 from both control and day 12 post-Citrobacter-infected mice revealed similar counts in both samples (14 ± 6 and 19 ± 4 cell nuclei/100 µm², respectively). The lack of any change in lamina propria cell number confirms the findings of Barthold and colleagues (4), who have demonstrated that TMCH in Swiss Webster mice was not accompanied by a significant inflammatory axis (characterized as recruitment of mononuclear leukocytes/neutrophils into the mucosal and lamina propria regions). Regions 3 and 4, encompassing the whole of the distal colon, were combined and used for all of the following biochemical and immunological assays.

Proliferative and apoptotic indices in normal and Citrobacter-infected distal colon. Isolated distal colonic crypts from day 12 post-Citrobacter-infected mouse contained more BrdU-labeled cells than controls. Proliferative index (number of BrdU-labeled S-phase cells/total number of cells in the crypt unit × 100) increased eightfold and was significantly different from control mucosa (n = 60 crypts/4 mice; P < 0.001, Student’s t-test; Fig. 2A). Apoptotic index, a measure of the fraction of cells undergoing apoptosis, detected by TUNEL assay/crypt (0.08 ± 0.04 vs. 0.12 ± 0.04, Citrobacter-infected vs. normal mice, means ± SD; n = 60 crypts/4 mice) was not significantly different in crypts taken from uninfected and infected animals (P < 0.01, Student’s t-test; Fig. 2B) A few cells within the upper reaches of the crypt were labeled in both instances. Mucosal inflammation within the gut mucosa is characterized by excessive colonocyte apoptosis (19).

Our results confirmed that similar conditions were absent at day 12 after Citrobacter infection. The lack of counterbalancing programmed cell death in the presence of elevated rates of mitosis within the crypt therefore provides a mechanism for mucosal hyperplasia in Citrobacter-infected mice.

**Fig. 1.** Citrobacter-induced hyperplasia. Normal (N) and day 12 Citrobacter-infected (H) mucosa showing thin (5 µm) cryosections of mouse distal colon fixed and incubated with hematoxylin and eosin stain magnified at ×4 (A) and higher magnification view (×20) of purified isolated crypts (described in METHODS) from similar tissues (B). Mucosal overgrowth in whole sections of Citrobacter-infected mucosa correlated with a dramatic increase in isolated crypt length. Bars *1 and *2 = 500 µm and 250 µm, respectively.

**Fig. 2.** Measurement of crypt cell dynamics. Proliferative index (PI; A) and apoptotic index (AI; B) for distal colonic crypts isolated from normal (N) and day 12 post-Citrobacter-infected (H) mice. PI (see METHODS) was elevated more than eightfold during Citrobacter infection, with higher number of mitotic cells being found within lower regions of elongated crypts. AI (see METHODS) did not change during this period. Values are means ± SD from 3 independent experiments.

Mucosal Hyperproliferation Affects cAMP-Mediated Cl⁻ Secretion

To examine the effect of colonic mucosal hyperproliferation on CFTR-dependent fluid secretion, we measured Cl⁻ secretion in response to elevated cellular cAMP levels in both normal and day 12 post-Citrobacter-inoculated mice. Short-circuited secretory currents (Isec) were recorded across the four 1.5-cm colonic regions encompassing the cecal (region 1) through rectal (region 4) colonic boundaries.

Bilateral addition of the cAMP-generating agonist forskolin (10 µM) to normal mucosa elicited between +14 and +30 µA/cm² of Isec (region 1 to region 4, respectively; n = 6; Fig. 3). This current was abolished by the removal of bath Cl⁻ (n = 6; Table 1). In contrast, forskolin addition to Citrobacter-infected mouse colonic segments elicited a significantly larger Isec (P < 0.001) that averaged +67 ± 17 µA/cm² within distal regions 3 and 4 (n = 6; Fig. 3). In some mice (3 out of 6), a smaller increase in Isec in region 2 was observed. However, none
Hyperplasia affects CAMP-dependent Cl⁻ secretion. Ion transport measurements made across normal (open bars) and day 12 post-Citrobacter-infected hyperplastic (hatched bars) mouse colonic mucosal sheets. Bilateral addition of forskolin (10 µM) elicited changes in short-circuited Cl⁻ secretory current (Isc) in all colonic segments, which encompassed proximal (region 1) to distal (region 4) colonic boundaries. Mucosal hyperplasia was associated with dramatically enhanced short-circuit current Isc responses to forskolin within distal colon segments 3 and 4. All currents were inhibited by serosal addition of furosemide (300 µM) or removal of bath Cl⁻. Values are means ± SD from 6 animals.

Table 1. Effects of crypt hyperproliferation on transmucosal electrical parameters

<table>
<thead>
<tr>
<th>Region</th>
<th>1</th>
<th>2</th>
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<tr>
<td>Basal</td>
<td>30.4 ± 3.0</td>
<td>28.3 ± 3.1</td>
<td>34.0 ± 3.6</td>
<td>34.3 ± 3.0</td>
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<tr>
<td>G</td>
<td>10.7 ± 2.0</td>
<td>9.7 ± 1.5</td>
<td>9.2 ± 1.3</td>
<td>8.3 ± 1.6</td>
<td>6</td>
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<tr>
<td>FSK</td>
<td>44.5 ± 3.0</td>
<td>44.4 ± 3.0</td>
<td>59.0 ± 8.0</td>
<td>64.3 ± 5.3</td>
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<tr>
<td>G</td>
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<td>9.8 ± 1.1</td>
<td>9.3 ± 1.05</td>
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<tr>
<td>ΔIsc</td>
<td>14.1 ± 3.0</td>
<td>15.9 ± 3.0</td>
<td>25.0 ± 10.0</td>
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<td>6</td>
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<td>31.7 ± 5.0</td>
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<tr>
<td>G</td>
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<tr>
<td>Basal + 0 [Cl⁻]</td>
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Values are means ± SD; n = no. of animals. Unstripped colonic sheets of colons from age-matched mice unexposed to (Normal) and 12 days after infection with (Hyperproliferating) Citrobacter freundii are shown. Basal current measurements were made 5–10 min after mounting intestine in Ussing chambers. Isc, short-circuit current expressed as µA/cm²; G, tissue conductance expressed as mS/cm²; FSK, bilateral addition of 10 µM forskolin. ΔIsc, change in Isc between basal and FSK-stimulated conditions; FSK + furosemide, bilateral FSK with 300 µM furosemide. + 0 [Cl⁻], bilateral replacement of Cl⁻ with gluconate.

Fig. 3. Hyperplasia affects CAMP-dependent Cl⁻ secretion. Ion transport measurements made across normal (open bars) and day 12 post-Citrobacter-infected hyperplastic (hatched bars) mouse colonic mucosal sheets. Bilateral addition of forskolin (10 µM) elicited changes in short-circuited Cl⁻ secretory current (Isc) in all colonic segments, which encompassed proximal (region 1) to distal (region 4) colonic boundaries. Mucosal hyperplasia was associated with dramatically enhanced short-circuit current Isc responses to forskolin within distal colon segments 3 and 4. All currents were inhibited by serosal addition of furosemide (300 µM) or removal of bath Cl⁻. Values are means ± SD from 6 animals.
The intensity of the 6.5-kb CFTR band was normalized by stripping and reprobing the blots for GAPDH. Normalization to the housekeeping mRNA demonstrated that purified colonic crypts undergoing higher rates of cell turnover exhibited higher average cellular CFTR message levels.

Cellular CFTR protein levels. To test whether the changes in mRNA are also reflected at the protein level, Western blot analysis was carried out in normal and day 12–15 post-Citrobacter-infected mice utilizing whole distal colon tissue extracts from colonic segments 3 and 4. For this purpose, polyclonal anti-CFTR antibody made against the COOH-terminal 13-amino acid cytoplasmic tail of purified bovine CFTR with nearly complete homology to murine CFTR was used as a probe (a kind gift from Dr. W. Dubinsky). When CFTR was immunoblotted for these extracts, which were run with purified bovine CFTR as positive control (Fig. 4B), an increase in cellular CFTR protein expression normalized to β-actin was recorded (2.4 ± 0.2-fold compared with normally proliferating mucosa). Antibody specificity was demonstrated with molar excess of antigenic peptide from which the antibody was raised (Fig. 4B, left, b, bovine tracheal extract run on a separate gel). Because the rabbit polyclonal antibody failed to detect a broad band of fully glycosylated CFTR protein in any sample (Fig. 4B, left), this finding was independently confirmed by Western blotting with TAM18 murine anti-human whole molecule CFTR monoclonal antibody (Fig. 5).

When utilizing the murine-derived IgM anti-human CFTR monoclonal antibody, three immunoreactive bands were seen (Fig. 5). Band C, corresponding to fully glycosylated forms of the protein, was present in both normal and hyperproliferative crypts. Core glycosylated protein (band A) and partially glycosylated protein (band B) (20) were not clearly evident in normal crypt extracts but were present in hyperproliferating crypts, although the latter band was fainter. These differences in expression may simply reflect the higher levels of fully glycosylated CFTR protein in both samples. Mucosal crypt hyperproliferation was therefore associated with increases in the cellular abundance of mature CFTR protein, which did not match elevations in steady-state CFTR poly(A)± mRNA levels.

The horseradish peroxidase-conjugated goat antimouse IgM secondary antibody failed to detect endogenous levels of tissue-specific IgM (no heavy- or light-chain IgM bands were seen on the CFTR Western blot; Fig. 5). The murine anti-CFTR monoclonal antibody was used extensively by us in the following studies for immunolocating CFTR within the mouse crypt epithelium.

Increases in both CFTR-expressing cell number and subcellular CFTR content occur in hyperproliferating crypts. Although both molecular and biochemical analysis revealed that average values of cellular CFTR expression increase in hyperproliferating crypts, they do not directly address whether this epithelial cell-specific induction of anion channel protein represents either overexpression within specific regions of the crypt normally expressing CFTR or new CFTR expression in crypt regions normally devoid of or expressing low levels of this protein. To address this concern, we performed immunofluorescence localization studies in formalin- and methanol-fixed crypts (see METHODS) isolated from the distal colon of both normal and TMCH mice (Figs. 6–8 and 10–12).

CFTR immunoreactive protein was initially detected in isolated crypts from day 12 post-Citrobacter-infected mouse distal colon using the TAM18 anti-CFTR monoclonal antibody. Images were collected with the CLSM (Fig. 6).

Figure 6A is a representative example of CFTR immunostaining in a 0.4-μm (midcrypt) z-axis fluorescent light section obtained from segment 4 of the day 12 TMCH mouse colon. A similar staining pattern has been reproduced in 18 separate animals at this time point. CFTR immunoreactivity accumulated at or below the cellular apical membrane (defined by us as the cellular apical pole) and was extended throughout the longitudinal cellular axis of the crypt (base-to-neck region). Staining within the cellular subapical pole also extended internally toward the apical-lateral junction, more clearly seen at higher spatial resolution as a subapical circumferential net (Fig. 10B). CFTR was
also found to accumulate in perinuclear but apically oriented intracellular compartments throughout individual cells within the crypt. Only background levels of CFTR basolateral signal were detected in hyperproliferative crypts (Fig. 6A). Simultaneously processed crypts from the same animal but controlled by omission of the anti-CFTR primary antibody from the staining protocol gave barely detectable diffuse levels of nonspecific immunostaining (Fig. 6B), whereas replacement of the anti-CFTR primary antibody with a matched anti-CD15 panlymphocyte-specific IgM monoclonal antibody revealed that cellular CFTR immunostaining was specific (Fig. 6C). Confirming the antigenic specificity of the secondary antibody, which in this instance is being used to detect a murine primary antibody in murine tissue, we also immunolocated native IgM in the same crypt samples using a separate goat anti-mouse IgM primary polyclonal antibody and FITC-conjugated rabbit anti-goat secondary antibody pair (Fig. 6D). (Note that the goat anti-mouse IgM isoform-specific secondary antibody used for these studies, already preabsorbed against human IgA and IgG but not their murine counterparts, did not detect significant levels of these endogenous immunoglobulins.) Endogenous levels of IgM were found to be predominantly basolateral and mainly within the upper reaches of the longitudinal crypt axis (crypt neck region). Clearly, high cellular levels of CFTR were detected throughout the crypt axis of hyperproliferating crypts, and CFTR immunostaining at the cellular apical pole in hyperproliferating crypts was immunospecific. For comparison, CFTR was also immunolocalized in simultaneously processed normal crypts (Fig. 7). Immunofluorescent light was collected from these crypts using the same signal gains applied in Fig. 6. No postimage capture contrast enhancement was performed. Because of the linear nature of the digital CLSM detection system (captured 16-bit images contained 65,536 grey levels), images in Figs. 6 and 7 were directly comparable. The fluorescent light CFTR immunostaining pattern within the midcrypt (0.4 µm) z-axis from matched (segment 4) normal mouse colon exhibited a very different staining pattern (Fig. 7A). Although apical pole immunoreactivity was recorded in cells within the lower (basal) regions of the longitudinal
crypt axis (Fig. 7A), overall levels were quantitatively much lower. The mean digital signal collected from a 10-µm² sample window was 23 ± 4% of that recorded in protocol-matched hyperproliferating crypts and was significantly less (P < 0.001; n = 16 individual observations). However, unlike hyperproliferating crypts, immunoreactivity was also recorded at or below the basal plasma membrane in all cells within this structure. This staining pattern was most prominent in the crypt neck region, where it extended into the cellular basolateral membrane. Sample and protocol-matched control crypts in which the anti-CFTR primary antibody was omitted (Fig. 7B) or replaced with a CD15 panlymphocyte-specific IgM monoclonal antibody (Fig. 7C) failed to reproduce the apical or basolateral staining pattern. The goat anti-murine IgM control performed at the same time in the same crypt samples revealed that endogenous IgM levels were higher when compared with hyperproliferating crypts (Fig. 7D), with immunoreactivity being localized almost exclusively to the cellular basolateral membrane. Thus cells within the basal region of normal crypts exhibited weak apical pole CFTR staining and, additionally, a distinct basal cellular pole staining pattern that was most prominent in cells within the crypt neck region. This result has been replicated in normal distal colonic crypts from six animals.

Although the appearance of CFTR immunoreactivity within the apical cellular pole confirmed the observations made in cultured colonocytes in vitro (14), our finding of a basolateral signal was unexpected. One of the problems of detecting CFTR in native gastrointestinal epithelia has been the question of specificity with regards to both primary and secondary antibodies. As shown in Figs. 6D and 7D, the basal plasma membrane region of mature epithelial crypt neck cells in particular is a site at which both IgM (shown) and IgA (not shown) accumulate. These molecules, complexed with secretory component, then translocate across the epithelium, where they act as the primary host defense mechanism (5). Even though no immunoreactive endogenous IgM protein was detected in the apical plasma membrane in any instance (n = 47 crypt preparations), and the anti-IgM isotype specific secondary antibody did not detect significant levels of endogenous IgM (see Fig. 5), we measured total cellular levels of both IgM and IgA in isolated crypts from both sources (Fig. 8).

We found that both IgM and IgA levels were slightly reduced in hyperproliferating distal colonic crypt samples, a finding that corroborates earlier studies demonstrating that a reduction in both secretory component and immunoglobulin immunoreactivity closely parallels loss of differentiation within crypts in both benign and malignant neoplastic disorders (10). However, the magnitude of this change is unlikely to account for the normal crypt cellular basolateral signal because the present studies have ruled out nonspecificity as a problem for the secondary antibody and, quantitatively, the increase in cellular basolateral signal is much larger than any change in IgA/IgM immunoreactivity recorded by either immunohistochemistry (Fig. 6D vs. Fig. 7D) or Western blotting (Fig. 8). We therefore cannot exclude the possibility that this signal is either real (related to CFTR antigenicity) or represents a protocol artifact that is selectively recorded in normal, but not hyperproliferative, crypts. Because

Fig. 7. CLSM images made in age-matched distal colonic crypts from normal mice at high spatial resolution (×400). Single 0.4-µm midcrypt z-axis planes of TAM18 antibody-dependent CFTR immunoreactive staining within apical subcellular pole of crypt basal region cells and subcellular basal pole staining in crypt neck region cells are shown in A. Similarly processed crypts lacking primary antibody displayed low background levels (B). Crypts in which anti-CFTR primary antibody was replaced with CD15 pan-lymphocyte specific antibody exhibited nonspecific staining (C). Staining obtained with goat anti-mouse IgM primary antibody detecting endogenous levels of this immunoglobulin within cellular basolateral pole are shown in D. Higher endogenous levels of this immunoglobulin were detected throughout crypt axis (n = 6 crypt preparations from 6 normal mice). Images were collected using same image capture setting as Fig. 6 and were not differentially enhanced.

Fig. 8. Western blot of endogenous IgA and IgM levels in normal (N) and TMCH (H) crypt extracts. In both instances, crypt hyperproliferation caused a slight reduction in total amount of detectable protein. hc, Immunoglobulin heavy chain; lc, immunoglobulin light chain.
this antibody failed to reproduce the basolateral staining pattern in human colonic cell lines (HT-29 Cl.19A, data not shown) we believe this staining to be nonspecific.

When the base region of normal crypts was imaged at even higher magnification (×800), fine subcellular detail of the anti-CFTR immunoreactive staining pattern was recorded (Fig. 9). CFTR accumulated in the subapical pole of colonocytes in punctate vesicular structures, and occasionally CFTR immunoreactivity extended throughout the cell (Fig. 9B). Vertically reconstructed sections at three locations separated by 50 µm along the longitudinal (x-y) crypt axis (Fig. 9C) clearly showed that CFTR immunoreactivity was concentrated at the apical pole in cells within the basal crypt region (Fig. 9C, iii). In cells nearer the midcrypt region, a more intense perinuclear intracellularly localized CFTR staining pattern existed, with no staining being seen in either the nuclear (Fig. 9, B and C) or cellular apical pole (Fig. 9C, i and ii).

Midcrypt z-axis planes from hyperproliferating crypts revealed even more detail to the cellular and subcellular CFTR staining profile (Fig. 10). Because the tubular crypts adhere to the coverslip at different points along their length, undulation of the crypt created, at this optical resolution, areas within the midaxis plane where individual cells were bisected both across their apical plasma membrane and, in some cases, just below this structure. A subapical circumferential staining pattern for CFTR was seen in these later instances (Fig. 10B), coinciding with the cellular plane of the zonula adherens. Intense apical pole staining within cells of the crypt base (Fig. 10B, iv and v) was replaced by less intense but still present apical pole staining in crypt neck cells (Fig. 10B, ii). In contrast, apically oriented perinuclear staining increased along the longitudinal x-y crypt axis (base to neck). This gradient was much more intense than that recorded in normal crypts.

Further investigation into the appearance of subcellular CFTR immunoreactivity revealed that elongated crypts accumulated more signal within the apically oriented perinuclear region than their shorter counterparts (Fig. 11). CFTR immunoreactivity was nearly exclusively localized to the cellular apical pole throughout the longitudinal crypt axis of short crypts (<200 µm in length), which on average made up only a small fraction (<10%) of the total number harvested from day 12 post-Citrobacter-infected mice (n = 48 animals; Fig. 11A). In the major portion of crypts isolated at this time point, which range in length between 400–500 µm (68% of all crypts), both base-to-neck perinuclear and neck-to-base cellular apical pole labeling coexisted (Fig. 11B). In longer crypts, characterized by lengths >500 µm (~18% of population), subcellular apically oriented perinuclear labeling predominated over apical pole staining (Fig. 11C). Thus the subcellular CFTR staining pattern in hyperproliferating crypts appeared to be modulated by crypt length and, by inference, the timing of hyperproliferatory change within this structure.

Lastly, the murine monoclonal anti-human CFTR antibody was used extensively for these studies because it was much more efficient at discriminating CFTR at both the cellular apical plasma membrane and within subcellular structures (staining was ~8-fold brighter than that obtained with the rabbit anti-bovine CFTR polyclonal used for Western blotting in Fig. 4). When normal and day 12 post-Citrobacter-infected hyperproliferating crypts were simultaneously probed with the peptide-based anti-CFTR polyclonal antibody

Fig. 9. CLSM high-power (×800) image of an isolated crypt from normal mouse distal colon. Bright field (A) and CFTR immunoreactive (B) signals from a single 0.4-µm midcrypt z-axis fluorescence light plane detected with TAM18 monoclonal antibody. At this resolution, punctate vesicular staining within cellular apical pole was seen in crypt base colonocytes, with occasional cells exhibiting extensive cytoplasmic staining (*). C: cross-sectional reconstructions of 100 individual x-z (75 µm × 0.4 µm) axial planes at 3 50-µm intervals between mid- (C, i) and basal (C, ii and iii) crypt axes showing immunoreactive CFTR signal within cellular subapical pole of crypt region colonocytes. Nuclear region, devoid of immunoreactivity, is marked (arrow in B and C).
and FITC-conjugated goat anti-rabbit secondary pair, the following signals were detected (Fig. 12). Immunofluorescent light was collected at matched signal gains. Apical cellular pole CFTR staining was recorded in cells within normal crypt base (Fig. 12A). Clear increases in apical plasma membrane and cellular CFTR immunoreactivity were recorded in hyperproliferating crypts, with staining extending throughout the crypt axis in a pattern very similar to that recorded with the TAM18 anti-CFTR monoclonal antibody (Figs. 7, 10, and 11). Staining was, however, less intense (Fig. 12B).

Similar results have been previously reported in abstract form by us (18). This independent finding with a different peptide-based polyclonal anti-CFTR antibody clearly confirmed both the specificity of the cellular apical pole CFTR immunoreactive signal and the fact that both the number of cells expressing this anion channel as well as the overall cellular level of CFTR increased during crypt hyperproliferation. In no instance was a basolateral signal recorded in either sample with this latter antibody (crypts isolated from 16 normal and 8 day 12 post-Citrobacter-infected animals). Thus, although in this instance the lower signal-to-noise ratio afforded by the polyclonal anti-CFTR antibody may have possibly masked a basolateral staining pattern, it is more likely that cellular basal pole CFTR immunoreactivity detected in normal crypts with the TAM18 monoclonal antibody is nonspecific.

DISCUSSION

In Vivo Effects of Hyperproliferation on CFTR Abundance and Function

Previously, we and others (Ref. 13, reviewed in Refs. 7 and 12) have demonstrated that CFTR mRNA and protein expression in colonic cell lines does not correlate with cAMP-dependent Cl\(^-\) anion transport. Both CFTR message and protein levels remain unaltered in transformed colonocytes regardless of whether they were proliferating or undergoing contact-induced growth cessation. CFTR-dependent anion transport was, however, dependent on differentiation-specific changes in cytoplasmic polarization and apical plasma membrane CFTR targeting (13, 14). To address whether cellular proliferatory status likewise failed to affect native cell CFTR expression while inhibiting in vivo CFTR-dependent Cl\(^-\) secretion, we utilized the TMCH model of mucosal hyperplasia (Fig. 1). In this model increases in proliferating colonocyte number were seen: elongated crypts contained a smaller percentage of mature goblet cells and exhibited packing of nonvacuolated cells within the middle to lower crypt regions (Fig. 1), and BrdU labeling was found throughout the crypt axis. The fact that apoptosis was unchanged explained our reported eightfold increase in proliferatory index (Fig. 2). TMCH-dependent increases in
transmucosal CFTR-dependent Cl\(^{-}\) current generation (Fig. 3) established that proliferatory conditions within the epithelium promoted rather than inhibited secretory function at the tissue level. Native colonocytes even at the base of the crypt possess tight junctions, are cytoarchitecturally polarized (8), and are thus, by in vitro standards, differentiated. Thus there were clearly important differences between transformed cell lines and native colonocytes.

To begin to address the nature of these differences, we tested the hypothesis that the enhanced Cl\(^{-}\) secretory response of TMCH mucosa was due to either an increase in CFTR-containing cell number within the crypt unit and/or an increase in CFTR anion channel expression in individual cells within the crypt. In fact, we found that normalized cellular levels of both CFTR mRNA and protein were higher in hyperproliferating crypt cells (Fig. 4). However, whereas cellular poly(A)\(^{+}\) mRNA expression increased 8-fold, only a 2.4-fold increase in cellular protein was recorded. Colonocytes are estimated to take 16–18 h to traverse, 200-µm-long normal crypts (see reviews in Refs. 12 and 20), whereas CFTR protein turnover rate (production and degradation) has been estimated in vitro to be on the order of 7–12 h. Thus we concluded that either colonocytes fail to remain within the crypt unit long enough to attain maximal levels of CFTR protein or that elevated endogenous poly(A)\(^{+}\) CFTR message was inefficiently translated. Given that CFTR transcript levels are not characterized as being abundant (6, 11, 15), it seemed unlikely that the cellular biosynthetic capacity for CFTR had been reached. Rather, our studies suggested that posttranslational modes of CFTR regulation were present within native colonocytes that protect the cell from the pathophysiological consequences of excessive anion channel expression.

A corollary of our above hypothesis was that CFTR anion channel protein expression was predicted to extend into the neck and surface regions of hyperproliferating crypts. [This was theorized on the basis of the short transit time for cell movement along the crypt axis, the long half-life of cellular CFTR protein turnover, and the fact that normally only a small proportion of cells within the crypt express detectable CFTR mRNA levels (6).] To test this hypothesis, we quantitatively measured CFTR immunoreactivity in paired crypt preparations from normal and TMCH mice using two methods of fluorescent light microscopy. We found that CFTR expression was indeed extended into neck and surface regions of hyperproliferating crypts (Fig. 11). Subcellular location of CFTR appeared to be dependent on either time of onset or duration of cellular hyperproliferative signal.

Fig. 11. Composite of single CLSM 0.4-µm midcrypt z-axis fluorescence light planes taken at ×400 magnification showing TAM18 monoclonal antibody-specific CFTR immunostaining pattern in 3 crypts of different lengths from same TMCH mouse distal colon sample. CFTR was found predominantly within cellular apical pole of short crypts (<200 µm; A), whereas it accumulated within apically oriented perinuclear locations in correspondingly longer crypts (B and C). Subcellular location of CFTR appeared to be dependent on either time of onset or duration of cellular hyperproliferative signal.

Fig. 12. CFTR immunofluorescence staining pattern detected in age-matched normally proliferating (A) and day 12 post-Citrobacter infected hyperproliferating (B) crypts probed with rabbit anti-bovine CFTR peptide-based polyclonal antibody. Both images were collected at ×400 using same capture gains and were not differentially enhanced. Confirming results obtained with TAM18 anti-CFTR monoclonal antibody, cellular apical pole CFTR immunoreactivity greatly increased during crypt hyperproliferation (n = 22 animals; see RESULTS).
regions of the hyperproliferating crypt (Figs. 6A, 10B, 11, and 12). Furthermore, we found that total subcellular levels of immunoreactive CFTR protein were higher in hyperproliferating crypt colonocytes than their normal crypt counterparts (Fig. 6A vs. Fig. 7A; see results). Thus elevated native mucosal proliferation promoted increased cellular CFTR protein levels both within areas in which CFTR was normally detected and in regions in which CFTR was undetectable under normal conditions. Hyperproliferating colonocytes, although structurally more polarized than their cell line counterparts, therefore differ in an important respect: their CFTR message levels are dramatically altered by their proliferatory status (13).

The second major finding of this study was that CFTR accumulated in apically oriented perinuclear structures in hyperproliferating crypts to a much larger extent (Fig. 6A and Fig. 10, B and C) than that observed in normal crypts (Fig. 7A and Fig. 9, B and C). We found that accumulation within this structure was dependent on crypt length (Fig. 11), suggesting that either the onset or duration of the hyperproliferatory signal was important. The fact that short hyperproliferating crypts exhibited nearly exclusive apical pole CFTR labeling (Fig. 11A), whereas elongated hyperproliferating crypts exhibited mainly perinuclear labeling (Fig. 11C), allowed us to theorize where in the cell this pool of CFTR had originated from. The glycosylation pattern of CFTR Western blotted with TAM 18 antibody demonstrated that mature (post-Golgi-processed) protein was overproduced by hyperproliferating colonocytes (Fig. 5). We suggest that anion channel retrieval from the apical pole or late stages of the biosynthetic pathway, rather than inhibition of nascent channel movement from within early (endoplasmic reticulum) compartments, may explain this phenomenon.

Subcellular biochemical and structural studies are currently underway to test the apical plasma membrane CFTR retrieval hypothesis, which we believe may serve as an important physiological defense mechanism against cellular CFTR overexpression in vivo. This could explain why CFTR-mediated current generation across TMCH distal colon did not greatly exceed the theorized twofold increase in mucosal surface area predicted by a twofold elongation in crypt length (Fig. 1). These findings highlight an important new aspect of CFTR regulation in vivo.

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