Functional vacuolar ATPase (V-ATPase) proton pumps traffic to the enterocyte brush border membrane and require CFTR

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Collaco AM, Geibel P, Lee BS, Geibel JP, Ameen NA. Functional vacuolar ATPase (V-ATPase) proton pumps traffic to the enterocyte brush border membrane and require CFTR. Am J Physiol Cell Physiol 305: C981–C996, 2013. First published August 28, 2013; doi:10.1152/ajpcell.00067.2013.—Vacuolar ATPases (V-ATPases) are highly conserved proton pumps that regulate organelle pHi. Epithelial luminal pH is also regulated by cAMP-dependent transport of specific subunits of the V-ATPase complex from endosomes into the apical membrane. In the intestine, cAMP-dependent traffic of cystic fibrosis transmembrane conductance regulator (CFTR) channels and the sodium hydrogen exchanger (NHE3) in the brush border regulate luminal pH. V-ATPase was found to colocalize with CFTR in intestinal CFTR high expresser (CHE) cells recently. Moreover, apical traffic of V-ATPase and CFTR in rat Brunner’s glands was shown to be dependent on cAMP/PKA. These observations support a functional relationship between V-ATPase and CFTR in the intestine. The current study examined V-ATPase and CFTR distribution in intestines from wild-type, CFTR intestinal CFTR high expresser (CHE) cells. Immunoprecipitation studies examined V-ATPase interaction with CFTR. The pH-sensitive dye BCECF determined proton efflux and its dependence on V-ATPase/CFTR in intestinal cells. cAMP increased V-ATPase/CFTR colocalization in the apical domain of intestinal cells and redistributed the V-ATPase V₀a₁ and V₀a₂ trafficking subunits from the basolateral membrane to the brush border membrane. V₀a₁ and V₀a₂ subunits were localized to endosomes beneath the terminal web in untreated CFTR intestinal CFTR and redistributed the subapical cytoplasm following cAMP treatment. Inhibition of CFTR or V-ATPase significantly decreased pH in cells, confirming their functional interdependence. These data establish that V-ATPase traffics into the brush border membrane to regulate proton efflux and this activity is dependent on CFTR in the intestine.

CFTR; V-ATPase; cAMP-regulated traffic; intestine

THE VACUOLAR H⁺ ATPASE (V-ATPase) pump is ubiquitously expressed, highly conserved, and necessary for eukaryotic cells. V-ATPases mediate a variety of essential cellular functions including receptor-mediated endocytosis, membrane and intracellular trafficking, and fusion of organelles with the plasma membrane (47, 61). V-ATPases play a critical role in regulation, and function of V-ATPase at the plasma membrane of epithelial cells are complex. In kidney epithelial cells, V-ATPase pumps regulate acid/base homeostasis (18, 89), and in the epididymis, the pumps maintain low luminal pH that is necessary for maturation and storage of sperm (79). The bicarbonate-secreting epithelium of salivary ducts responds to changes in the acid-base balance by redistribution of H⁺ and HCO₃⁻ transporters to increase the buffering capacity of saliva. Acidosis triggers V-ATPase traffic towards the apical membrane of salivary gland and that requires PKA phosphorylation and the Rab GTPase Rab 11b (62, 63). In these epithelial tissues, changes in cellular environments and cAMP second messenger activation can modulate extracellular proton efflux by recruitment of V-ATPase to the plasma membrane. In addition to its role in proton efflux, specific subunits have been shown to play a role in apical sorting of intestinal transport proteins (84).

Like the kidney, the intestine is a major ion transporting epithelium responsible for fluid and luminal pH homeostasis. In the mammalian intestine, members of the sodium hydrogen exchanger (NHE) family are important regulators of apical proton secretion where they function together with the cystic fibrosis transmembrane conductance regulator (CFTR) chloride channels and chloride bicarbonate exchangers to regulate luminal pH and homeostasis (15, 32). In the small intestine, traffic of NHE3 into and out of the enterocyte brush border (BB) membrane (BBM) is functionally linked to CFTR (46). Emerging studies in mammalian intestine identified another H⁺ pump, V-ATPase in the apical BBM of enterocytes (24, 45) and Rab 4 and Rab 5b positive early and recycling endosomes (88); however, the function of the V-ATPase proton pump in mammalian intestine is unknown. In Caenorhabditis elegans (C. elegans), the cysticidal V₀a₁ subunit and the plasma membrane-associated apical V-ATPase V₀a₂ subunits play important functional roles in the intestine. Mutants lacking the vha-8 gene encoding the V-ATPase E subunit demonstrate developmental arrest, improper intestinal functions, and...
larval lethality (48, 53). Furthermore, loss of vha-6 gene that encodes the proton-translocating V_o subunit on the apical plasma membrane prevents normal acidification of the intestinal lumen that is required following defecation (3). In addition, vacuolar-type H^+-ATPase was identified in the apical membrane of marine fish intestine enterocytes where it sequesters acid and facilitates Cl^-/HCO_3^- exchange (38).

V-ATPase proton pumps have been proposed to function in parallel with members of the CLC family and the CFTR chloride channels to regulate intraorganelle pH and thus contribute to disease pathophysiology (82, 91). However, the role of CFTR in organelle acidification remains controversial (2, 9, 21). At the plasma membrane, CFTR and ClC5 modulate V-ATPase function in kidney proximal epithelial tubules (19). Furthermore, in cystic fibrosis (CF), genetic mutations lead to defective traffic of CFTR to the apical plasma membrane that is associated with defects in luminal alkalization in the intestine (74, 92). These findings align with our published observations that luminal pH in the intestine is modulated by traffic of CFTR channels into the apical membrane of enterocytes (6, 7, 44).

V-ATPase and CFTR share a number of features in epithelial cells: both transporters are present on the apical plasma membrane of ion transporting epithelia, are regulated in a cell- and tissue-specific manner and exhibit cAMP-dependent traffic from endosomal stores into the plasma membrane. We recently reported that unlike villus enterocytes in the small intestine, villus CFTR high expresser (CHE) cells lack expression for the proton exchanger NHE3 on the enterocyte BBM but express high levels of V-ATPase (45). In other studies of Brunner’s glands in the rat proximal duodenum, we observed high levels of endogenous V-ATPase (24) and CFTR (46). Moreover, cAMP-stimulated traffic of V-ATPase and CFTR from subapical endosomes into the apical membrane of Brunner’s gland acinar cells was dependent on PKA (24). The functional implication of V-ATPase association with CFTR was not examined in those studies due to inaccessibility of the gland and lack of suitable probes to investigate the function of CHE cells. However, those observations suggested that CFTR Cl^- channels are functionally linked to the V-ATPase proton pump in the intestine. Since V-ATPase traffic at the epithelial plasma membrane is associated with specific subunits of the complex, we used antibodies against specific V-ATPase subunits to examine cAMP/PKA trafficking in native wild-type (WT) and CFTR ^--/- mouse intestine and cultured human intestinal cells. The biological interaction, localization, and functional dependence of V-ATPase on CFTR were examined. The results of the current study reveal that V-ATPase pumps traffic to the enterocyte BB following cAMP/PKA activation. Moreover, specific V-ATPase subunits (V_o1, V_o2, and V_E) associate with CFTR and participate in cAMP-regulated traffic in the intestine. Finally, V-ATPase proton efflux requires CFTR channels in the enterocyte BBM.

MATERIALS AND METHODS

Reagents and antibodies. Three anti-CFTR antibodies were used in this study. AME4991 is an affinity-purified, rabbit polyclonal antibody raised against the carboxy-terminal peptide of rat CFTR (5). The M3A7 monoclonal mouse antibody raised against the COOH terminus of human CFTR was purchased from Chemicon International (Temecula, CA), and the monoclonal antibody to human CFTR 217 was obtained from Dr. John Riordan (University of North Carolina-Chapel Hill and Cystic Fibrosis Foundation Therapeutics). The V-ATPase subunit V_oE, chicken IgY polyclonal antibody (anti-ATP6V1E1), and β-actin monoclonal antibody were purchased from Sigma-Aldrich (St. Louis, MO). The V_o2 antibody was a gift from Dr. Fred Gorelick (Yale University School of Medicine). Antibodies against the V-ATPase V_o1 and V_o3 subunits were generated by Dr. Beth Lee (Ohio State University). Alkaline phosphatase (ALP) polyclonal antibody was purchased from Accurate Chemicals (Westbury, NY), and gaelict 4 antibody was purchased from RD Systems (Minneapolis, MN). Antibodies raised against the electrogenic sodium-coupled bi-carbonate cotransporter 1 (NBCe1, K1A) antibody were provided by Dr. Walter Boron (Case Western University). Fluorescent-tagged secondary antibodies were purchased from Invitrogen (Carlsbad, CA) and Jackson ImmunoResearch Laboratories (West Grove, PA). Horseradish peroxidase-conjugated secondary antibodies were purchased from BD Biosciences (San Jose, CA). The PKA inhibitor H-89 (dilhydrochloride) was purchased from Calbiochem/EMD Millipore (Billerica, MA). All other drugs, reagents, and chemicals were purchased from Sigma unless otherwise stated.

Human intestinal CaCo-2^neuro cells. CaCo-2^neuro cells were purchased from American Type Culture Collection (ATCC, Manassas, VA) and maintained as previously described (23). Cells were grown on Transwell permeable supports (Corning, Corning, NY) for 1–3 wk to reach confluency and transepithelial resistance (TER) >300 Ω/cm^2 before experimentation. Confluent monolayers of CaCo-2^neuro cells were used for Western blot analysis, communoprecipitation, and immunofluorescence labeling. Proton efflux and pH assays were conducted on fully confluent cells grown on Cel-Tek (BD Biosciences)-coated glass coverslips.

Animals. All rodent studies were approved by the Institutional Animal Care and Use Committee of Yale University School of Medicine. Adult male Sprague-Dawley rats (250–300 g; Charles River Laboratories, Wilmington, MA) were fasted overnight with access to water. Rats were anesthetized with Inactin (120 mg/kg) administered by intraperitoneal injection. The abdomen was opened, and the jejunum was identified, removed, and flushed with cold phosphate buffer solution. Mucosal scrapings from jejunum were used for Western blot analyses and preparation of brush border membrane vesicles (BBMV). Animals were euthanized at the end of the experiments with an overdose of Inactin.

WT (C57/B6L) and CFTR ^--/- (BP.129P2-Cftr^tm1Unc) mice were provided by Dr. Marie Egan (Yale University School of Medicine). The abdomen was opened, jejunum loops were created with sutures, and loops were filled with 0.5 ml of warm (37°C) normal saline or N^e2',O'-dibutyltyrosyladenosine 3',5'-cylic monophosphate sodium salt (dBcAMP; 1 mM) for 30 min. Body temperature was maintained during procedures by keeping animals on a heating pad at 37°C. Animals were euthanized at the end of the experiments. Tissues were used for Western blot analyses, preparation of BBMV, and immunofluorescence microscopy.

Western blot analysis. Preparation of cell lysates and Western blot analysis were performed as described previously (5, 23). Briefly, CaCo-2^neuro cells and intestinal mucosal scrapings were homogenized in freshly prepared TGH buffer containing protease inhibitors. Samples were vortexed and centrifuged, and supernatants were collected. Protein concentration was determined with Coomassie Protein Assay (Pierce, Rockford, IL). Thirty micrograms of protein were resolved on SDS-polyacrylamide gels and transferred onto Immoblot PVDF membranes (Bio-Rad Laboratories, Hercules, CA). Membranes were probed with the appropriate primary antibodies (CFTR, V-ATPase V_oE subunit and V_o1–3 subunit isoforms, β-actin, gaelict 4, ALP, or NBCe1), and proteins were detected by chemiluminescence (Pierce Biotechnology) using X-ray film (Kodak Biomax Light film, VWR).

BBMV preparation. Enterocytes from rat and WT mouse small intestine were scraped into PBS containing protease inhibitors, resus-
pended, and centrifuged as described previously (23). Supernatants were discarded; pellets were resuspended and homogenized in NET buffer (0.13 mM NaCl, 5 mM EDTA, and 10 mM Tris base, pH 7.4) containing protease inhibitors. Samples were centrifuged, pellets were washed, and protein/antibody complex pellets were washed, 30 min). Cell supernatants were precleared with protein G beads, the PKA inhibitor (H-89; 10 μM) was added, and loaded with 10 μM of pH-sensitive BCECF dye (Invitrogen) for 30 min in HEPES-Ringer solution. The chamber was then mounted on an inverted microscope where it remained for the duration of the experiment. Following incubation of cells with the BCECF dye, HEPES buffer (37°C) was perfused for 5 min to remove any residual extracellular dye. Areas of interest were chosen, and data were collected every 15 s at a ratio of 490 nm/440 nm at an emission of 535 nm as previously described (43). The resultant ratio intensity data were converted to intracellular pH using the nigericin and high K calibration method. Slo (15 mM) was added to the initial perfusate (HEPES buffer) and in all treated conditions. Functional CFTR was confirmed using the inhibitor CF-inh172 (10 μM) in the perfusate. Inhibition of V-ATPase was achieved using ConA (200 μM). Second messenger stimulation of proton efflux was examined in cells following treatment with DBcAMP (1 mM). Data were analyzed using Graphpad Software and ANOVA analysis with P < 0.05 considered significant. Results are expressed as the mean of ΔpHi/min ± SE.

**Tissue preparation for fluorescence microscopy.** Intestinal tissues from mouse jejunum (2–3 cm length) were fixed in 2% (wt/vol) paraformaldehyde for 1 h at room temperature, cryoprotected in 30% sucrose in PBS overnight at 4°C, embedded in OCT medium (Miles Laboratory, Elkhart, IN), and frozen in liquid nitrogen precooled isopentane, and tissue blocks were stored at −20°C until sectioning. Cryostat sections (5 μm) were collected on a Leica 1900 microscope, placed on coated slides (Fisher Scientific), and stored at −20°C.

**Immunofluorescence microscopy of tissue sections.** Immunofluorescence labeling on tissues was performed as described previously (5, 23). Briefly, cryostat sections were immobilized with V-ATPase V1a/E subunit (dilution of 1:500), Vα1 isoform (dilution of 1:250), Vα2 isoform (dilution of 1:250), or Vα3 isoform (dilution of 1:250) and β-actin (dilution 1:5,000) antibodies overnight at 4°C. The following day, tissues were washed and incubated with appropriate secondary antibody. Sections were stained with 1% Hoechst nuclear stain. The filters were mounted with Slow Fade medium, and immunolabeled sections were examined on a Zeiss LSM 510-META laser scanning confocal microscope using ×20 or ×63 objectives. Image deconvolution was performed with Huygens Essential Version 4.2 deconvolution software using the company’s parameters (Scientific Volume Imaging, Hilversum, The Netherlands). The Z-stack images were converted to Tiff files using LSM Image Browser and ImageJ software.

**Quantification of CFTR/V-ATPase colocalization.** At least five independent confocal images were processed and analyzed from three separate experiments. Treated cells were fixed and double-labeled to detect Vα1/CFTR, Vα2/CFTR, Vα3/CFTR, and V1/E/CFTR. Double-labeled images were split into red and green channels and converted into a single image. The position of the microvillar tip in image stacks was set as 0 μm, and the microvillar base at −1 μm and XY and XZ images were taken for quantification. Background corrected fluorescence intensity (FI) values were measured by selecting regions of interest over the apical membrane for V-ATPase and CFTR. Densitometric analysis was performed using the “Analysis and Record Measurements” features of Adobe Photoshop CS4 Extended. The Record Measurement tools provided intensities (mean gray values) of the selected areas. Areas of colocalization were identified in white by ImageJ colocalization plug in (http://rsbweb.nih.gov/ij/plugins/colocalization.html). Quantification data show the normalized apical CFTR, Vα1, Vα2, Vα3, and V1/E FI as well as the apical percentage of CFTR colocalized with Vα1, Vα2, Vα3, and V1/E in PBS and treated cells. Data are represented as means ± SE and were further analyzed using Graphpad Software. Significance in mean value was determined by a Student’s t-test and ANOVA analysis. P < 0.05 was considered significant.

**RESULTS**

**Western blot analysis of endogenous V-ATPase in CaCo2BBe intestinal cells and rodent jejunum.** To determine whether endogenous V-ATPase was present in the intestine, immunoblot analysis of mucosal scrapings from CaCo2BBe intestinal cells and rodent jejunum was performed (Fig. 1). Analysis (30 μg of protein) using antibodies raised against subunits (transmembrane Vα1 and cytosolic V1/E) associated with V-ATPase trafficking to the plasma membrane (35, 39, 68, 76, 86) confirmed that Vα1, Vα2, and V1/E were present in both native epithelium and cultured intestinal cells (Fig. 1). Although characterization of the antibodies raised against the Vα1 subunit isoform has not been published, our results are consistent with findings in the literature (57, 67, 76, 77). In rodent tissue and cell lysates, the Vα1 antibody recognized a band of the expected size between 100 and 120 kDa and a band at 50 kDa in mouse and rat jejunum. The Vα2 antibody detected a band of the predicted size of 100 kDa in the CaCo2BBe lysate and 110–115 kDa in mouse lysates. Differences in band sizes may be due to further processing of Vα1, such as glycosylation or splice variants in human and rodent. Our finding that Vα3 (~105 kDa) was not abundant in the intestine (data not shown) is not surprising. This subunit is abundant in osteoclasts (87) with lower levels of mRNA expression identified in nonintes-
Further identification of specific V-ATPase subunits associated with the enterocyte BBM, rodent BBM preparations and antibodies against V-ATPase $V_{o}a1$, $V_{o}a2$, and $V_{i}E$ were employed. Immunoblot confirmed that membrane $V_{o}a1$ and $V_{o}a2$ and cytoplasmic $V_{i}E$ were indeed present in BBMV (Fig. 2A). Antibodies to $V_{o}a3$ subunit detected very low levels of protein.

Fig. 1. Western blot analysis of endogenous vacuolar ATPase (V-ATPase) $V_{o}a$ isoforms and $V_{i}E$ subunit in polarized intestinal cells and rodent jejunum. Lysates were prepared from CaCo-2BBE cells and rat and wild-type (WT) mouse jejunum (30 μg of protein). Proteins were separated by SDS-PAGE and detected by Western blot. Bands reveal patterns of endogenous $V_{o}a1$ or $V_{o}a2$ subunit isoforms and $V_{i}E$ subunit in lysates. β-Actin loading controls are shown. Molecular mass standards (kDa) are indicated.

Fig. 2. Western blot analysis of V-ATPase $V_{o}a$ isoforms and $V_{i}E$ subunit, cystic fibrosis transmembrane conductance regulator (CFTR), alkaline phosphatase (ALP), and sodium-coupled bicarbonate cotransporter 1 (NBCe1) in brush border membrane vesicle (BBMV) preparations from rat and WT mouse jejunum. Proteins from rat, WT mouse lysates, and BBMV (30 μg of protein) were resolved by SDS-PAGE and detected with antibodies against $V_{o}a1$, $V_{o}a2$, $V_{i}E$, CFTR, ALP, galectin 4, and NBCe1. A: bands consistent with $V_{o}a1$, $V_{o}a2$, $V_{i}E$, CFTR, ALP, and galectin 4 are detected in BBMV preparations from rat and WT mouse jejunum. B: NBCe1 protein expression is detected in rat and WT mouse jejunum lysates but absent in rat and WT mouse BBMV preparations. Molecular mass standards (kDa) are indicated.
in BBMV (data not shown). The presence of abundant CFTR, the BB ALP, and galectin 4 confirmed the purity of the intestinal BBM preparation (Fig. 2A). Immunoblot analysis of rodent tissue lysates confirmed expression for the basolateral protein NBCe1 that was absent in the BBMV preparations. These results confirmed the specificity of V-ATPase expression in the apical BBM (Fig. 2B).

V-ATPase is in a biological complex with CFTR in the intestine. The observation that CFTR and V-ATPase Vo, a1, a2, and E subunits are present in the same BBMV preparation suggested that V-ATPase could be in a biological complex with CFTR in the intestinal epithelium. To determine whether CFTR physically interacts with V-ATPase, coimmunoprecipitation experiments were conducted using PBS- or DBcAMP (1 mM)-treated polarized CaCo-2BBe cells (Fig. 3).

To confirm that V-ATPase subunits, CFTR and galectin 4 in CaCo-2BBe cells treated with PBS, PKA inhibitor (H-89; 10 μM) before DBcAMP confirmed equivalent protein expression for Vo, a1, a2, and E, respectively. These results confirmed the specificity of V-ATPase expression in the apical BBM (Fig. 2B).

cAMP-induced trafficking of V-ATPase to the apical surface of polarized intestinal cells. Polarized human intestinal CaCo-2BBe cells resemble mature villus enterocytes of the small intestine and possess a well-defined BB (69, 70). In this cell model, endogenous CFTR undergoes endocytic recycling and cAMP-regulated exocytosis at the apical domain (22, 23). Both CFTR and V-ATPase are identified in subapical trafficking compartments but in different epithelia (13, 25, 40, 46, 71). Previous studies identified V-ATPase in early and recycling endosomes similar to CFTR (75, 88). In proton-secreting epithelia such as the kidney and epididymis, cAMP-regulated traffic of the cytosolic V1, E subunit of V-ATPase from subapical vesicles to the BBM is associated with functional V-ATPase at the membrane (35, 64, 65). Our findings that V-ATPase and CFTR colocalize and undergo cAMP-regulated trafficking into the apical membrane in Brunner’s glands (24), coupled with the observations that CAMP regulates trafficking and insertion of CFTR from subapical endosomes into the apical membrane of polarized CaCo-2BBe cells and native intestinal epithelium (6, 7, 23, 24, 34, 44), suggest that both transporters share a common trafficking pathway in intestinal epithelial tissues.

Localization and immunoblot analysis indicated that V-ATPase is present and associates with CFTR in rodent intestine and polarized human intestinal cells. To determine whether endogenous V-ATPase traffic behavior resembles CFTR in the intestine, we examined the subcellular distribution of both transporters in polarized CaCo-2BBe cells following cAMP stimulation. Polarized filter-grown cells were stimulated with PBS or DBcAMP (1 mM) for 30 min and placed on ice to halt protein traffic. Cells were fixed and double-labeled with antibodies against CFTR and V1, E or V1, a1, a2, and a3 and examined by confocal microscopy (Fig. 4). XZ views of images show apical CFTR and apical and cytoplasmic V-ATPase labeled punctate structures; increased fluorescence intensity of apical CFTR, V1, a1, a2, and V1, E labeling; and increased colocalization of CFTR and V1, E, a1, or a2 (Fig. 4, A–C, and E). DBcAMP-treated cells revealed a 2.23-fold increase in apical CFTR FI and 1.84-, 1.65-, and 1.91-fold increase in apical V1, a1, a2, and V1, E FI, respectively. Quantification of
colocalization of V-o1, V-o2, or V-E with CFTR in PBS vs. DBcAMP-treated cells revealed 1.92-, 2.58-, and 2.63-fold increases, respectively. Weak V-o3 staining was detected at the apical domain of PBS and DBcAMP-treated cells (Fig. 4, D and E), with a 1.33-fold increase in V-o3 FI. These results suggest that the V-o3 subunit does not play a major role in cAMP-regulated trafficking. The observed cAMP-induced trafficking of CFTR to the apical surface is consistent with our previous observations in this intestinal cell model (23).

Apical trafficking of V-ATPase/CFTR in polarized CaCo-2BBe cells requires PKA. Apical traffic of V-ATPase into the plasma membrane of salivary duct requires cAMP/PKA/cAMP response element binding protein (CREB) signaling (63). Apical CFTR traffic in the intestine is also dependent on cAMP/PKA signaling (23).
The results of immunoprecipitation studies indicated that CFTR association with V-ATPase is dependent on PKA in polarized CaCo-2Be cells. To provide an independent approach to further confirm a role for PKA in CFTR/V-ATPase association, cAMP/PKA signaling of CFTR and V-ATPase apical trafficking was investigated using confocal microscopy. Confluent monolayers of cells were treated for 30 min with PBS, DBcAMP (1 mM), or pretreated with the PKA inhibitor (H-89; 10 μM) before DBcAMP (1 mM; Fig. 5). Cells were fixed, double-labeled using CFTR and V-ATPase antibodies (V₁E), and examined by confocal microscopy. XZ views show sections of CFTR and V-ATPase labeling in control and treated cells, with apical and cytoplasmic labeling for CFTR and V₁E. En face views of images taken at the level of the BBM revealed punctate label for CFTR and V-ATPase (V₁E) in control (PBS) and treated cells with areas of V₁E/CFTR colocalization (Fig. 5). Consistent with the data shown in Fig. 4A, examination of DBcAMP-treated cells revealed an increase in CFTR and V₁E-labeled punctuate structures at the apical domain (Fig. 5B). Quantification analysis of DBcAMP-treated cells revealed a 2.41- and 1.73-fold increase in the FI of apical CFTR and V₁E label (Fig. 5, B and E) and a 2.98-fold increase in V₁E/CFTR colocalization at the apical surface (Fig. 5E). Treatment with H-89 increased apical CFTR FI by 1.19-fold and decreased apical V₁E FI by 28% (Fig. 5, C and E); however, the changes were not statistically significant. CFTR/V-ATPase colocalization was higher in PBS cells compared with H-89-treated cells, but the difference was not statistically significant.

**Fig. 5.** H-89 inhibits cAMP-mediated trafficking of CFTR and V-ATPase V₁E subunit in polarized CaCo-2Be cells. Confluent monolayers of CaCo-2Be cells were treated for 30 min with PBS (A), DBcAMP (B; 1 mM), and PKA inhibitor (C; H-89, 10 μM). Cells were also pretreated with H-89 (D; 10 μM, 30 min) followed by DBcAMP (1 mM, 30 min). Cells were fixed, immunolabeled with antibodies against CFTR and V-ATPase (V₁E), and examined by confocal microscopy as described in MATERIALS AND METHODS. A–D: vertical XZ sections and en face views of immunolabeled PBS and treated cells reveal the distribution of CFTR (green) or V₁E (red). Merged images show areas of colocalization (yellow). A higher magnification image taken from the inset shows areas of colocalization indicated in white. En face views of images taken at the level of the BBM reveal punctate label for CFTR and V-ATPase (V₁E) in control (PBS) and treated cells with areas of V₁E/CFTR colocalization (Fig. 5). Consistent with the data shown in Fig. 4A, examination of DBcAMP-treated cells revealed an increase in CFTR and V₁E-labeled punctuate structures at the apical domain (Fig. 5B). Quantification analysis of DBcAMP-treated cells revealed a 2.41- and 1.73-fold increase in the FI of apical CFTR and V₁E label (Fig. 5, B and E) and a 2.98-fold increase in V₁E/CFTR colocalization at the apical surface (Fig. 5E). Treatment with H-89 increased apical CFTR FI by 1.19-fold and decreased apical V₁E FI by 28% (Fig. 5, C and E); however, the changes were not statistically significant. CFTR/V-ATPase colocalization was higher in PBS cells compared with H-89-treated cells, but the difference was not statistically significant.
Apical CFTR/V-ATPase colocalization was reduced in DBcAMP-stimulated cells pretreated with H-89 compared with cAMP-treated cells without H-89 (2.72 vs. 1.73-fold; Fig. 5, B–E). The results indicate some dependence on PKA for CFTR/V-ATPase apical traffic.

Apical traffic of CFTR and V-ATPase are interdependent. To determine whether the apical traffic of CFTR and V-ATPase are interdependent, the distribution of each transporter was examined in the presence of CFTR or V-ATPase inhibitors in polarized intestinal CaCo-2bBe cells. The CFTR inhibitor CF-inh172 has been widely used to examine CFTR channel function in epithelial tissues (36, 52, 54), but its role in CFTR traffic is less clear. The highly specific V-ATPase inhibitor ConA binds to the V_o domain of V-ATPase where it alters...
intracellular protein trafficking (81) and alkalinizes the secretory pathway (29). Confluent monolayers of CaCo-2BBbe cells were treated for 30 min in Fig. 6 as follows: A: PBS; B: Slo (15 nM), C: Slo plus DBCAMP (1 mM), D: Slo plus CF-inh172 (10 μM), and F: Slo plus ConA (200 μM). Cells were also treated for 30 min with Slo plus CF-inh172 (E; 10 μM) or Slo plus ConA (G; 200 μM) followed by treatment for 30 min with DBCAMP (1 mM). Cells were fixed, double-labeled with antibodies against CFTR and V-ATPase (V1E), and examined by confocal microscopy (Fig. 6). XZ views of images show apical sections of CFTR and V-ATPase labeling in control and treated cells. En face views of images taken at the level of the BBM revealed punctate label for CFTR and V1E in PBS-treated cells with some V1E/CFTR colocalization (Fig. 6, A and H). Although apical localization and FI of CFTR and V1E were similar in Slo-treated and PBS cells, treatment with Slo resulted in a slight decrease in apical CFTR and V1E FI by 11% and 15% respectively (Fig. 6, B and H). Examination of DBCAMP-treated cells revealed a 2.80-fold significant increase in CFTR FI compared with PBS cells, a 2.48-fold increase in V1E FI labeling at the apical domain, and significantly increased CFTR/V1E colocalization at the apical surface (2.63-fold; Fig. 6, C and H), despite the reduction of apical CFTR and V1E FI in Slo-treated cells. Comparable results for CFTR and V1E FI and colocalization levels were observed in cells treated with DBCAMP alone (Fig. 5, B and E) and cells treated with Slo plus DBCAMP (Fig. 6, C and H), indicating that treatment with Slo does not significantly impact cAMP-regulated localization of CFTR and V-ATPase. Apical and punctuate labeling for CFTR and V1E was observed in cells treated with Slo, Slo plus CF-inh172 or Slo plus ConA, with levels of CFTR FI, V-ATPase FI and CFTR/V-ATPase colocalization similar to PBS-treated cells (Fig. 6, A, B, D, F and H). Apical CFTR and V-ATPase FI and CFTR/V-ATPase colocalization increased in cells pretreated with CF-inh172 or ConA before DBCAMP (Fig. 6, E, G, and H), but the increase was not as high compared with DBCAMP treatment alone (2.80- vs. 1.63-fold; Fig. 6H) consistent with some blunting but not complete abrogation of apical traffic by CF-inh172 and ConA.

**CFTR regulates V-ATPase function in the intestine.** To determine whether CFTR mediates V-ATPase activity in intestinal CaCo-2BBbe cells, changes in pH were examined in the presence/absence of chloride or sodium. Following an NH4Cl− preincubate to acidify the cells, Na+ was removed from the media (to inhibit Na-H exchange and further acidify the cells). The cells spontaneously recovered to pH 7.0, demonstrating the presence of a Na-independent acid extrusion pathway (Fig. 7A). Figure 7B shows a tracing from a cell that received the same NH4Cl acidification prepulse, but the perfused buffer was then exchanged for a Na and Cl− free perfusate. The cell did not recover under these conditions, demonstrating that the Na-independent acid extrusion pathway requires Cl−. When cells were acidified with NH4Cl in the presence of the selective V-ATPase inhibitor ConA, (Fig. 7, C and D, 5th bar), they failed to recover from the acid load. These results confirm that this Na-independent acid extrusion pathway acts through V-ATPase. Figure 7D shows data indicating that the CaCo-2BBbe cells possess a chloride dependent V-ATPase. Slo (15 nM; used to increase permeability of CF-inh172) does not affect the proton extrusion rate (Fig. 7D, 2nd bar). Further, the chloride dependent hydrogen efflux is mediated by CFTR because treatment with the CFTR inhibitor CF-inh172 resulted in minimal pH recovery (Fig. 7D, 4th bar). In addition, DBCAMP stimulation significantly increased proton movement across the membrane (Fig. 7D, 6th bar), consistent with increased apical traffic of CFTR and V-ATPase into the BBM.

**cAMP-regulated trafficking of V-ATPase into the enterocyte BBM is dependent on CFTR.** The data in polarized cells and native tissues indicated that V-ATPase undergoes cAMP-regulated trafficking and colocalizes with CFTR in the BBM in the intestine. To provide more robust evidence that apical V-ATPase traffic requires CFTR in the native epithelium, further studies investigated changes in V-ATPase distribution in DBCAMP-stimulated intestine using a CF mouse model lacking CFTR expression. WT and CFTR−/− mice were anesthetized and ligated loops of small intestine (jejunum) were treated with normal saline (NS) or DBCAMP (1 mM) for 30 min. Following treatment, tissues were fixed, cryosectioned, immunoabeled, and examined by confocal microscopy (Fig. 8). Immunolabeling of WT tissues with antibodies against the V-ATPase trafficking subunits Voα1 and Voα2 revealed that DBCAMP redistributed V-ATPase from the basolateral domain to the BBM and subapical region of enterocytes (Fig. 8, A and C). This was in marked contrast to DBCAMP treatment of CFTR−/− intestine. In untreated CFTR−/− intestine, the label for Voα1 and Voα2 were prominent in the terminal web rather than the basolateral domain of enterocytes as observed in WT. In DBCAMP-

**Fig. 6.** Concansamycin A (ConA) and CFTR-inh172 inhibit cAMP-mediated trafficking of CFTR and V-ATPase V1E subunit in polarized CaCo-2BBbe cells. Confluent monolayers were treated for 30 min with PBS (A), Streptolysin O (Slo; B; 15 nM), DBCAMP (C; 1 mM), Slo plus CFTR inhibitor, CF-inh172 (D; 10 μM), or Slo plus the V-ATPase inhibitor ConA (E; 200 μM). Cells were also treated with CF-inh172 (E; 10 μM, 30 min) or ConA (G; 200 μM) before treatment with DBCAMP (1 mM, 30 min). Cells were fixed and labeled with antibodies against CFTR and V1E and examined by confocal microscopy as described in the MATERIALS AND METHODS. Vertical (XZ) merged images of CFTR (green) with V1E (red) show areas of colocalization (yellow) on the apical membrane. En face views of immunoabeled cells show the distribution of CFTR (green), V-ATPase V1E (red), and merged images (yellow). A higher magnification image taken from the inset shows areas of colocalization indicated in white. H: graphs show quantification of normalized CFTR and V1E FI and percentage of CFTR colocalized with V1E at the base of the microvilli. White arrowhead in XZ images indicates section level taken for enface views and quantification. Data represent means ±SE (n = 3). Normalized V1E FI graph: *P < 0.05 for significant increase compared with PBS, Slo, or Slo plus CF-inh172. Slo plus ConA, and Slo plus ConA and DBCAMP-treated cells. **P < 0.05 for significant increase compared with H-89-treated cells. Normalized CFTR FI graph: *P < 0.05 for significant increase compared with PBS, Slo, or Slo plus ConA- and Slo plus ConA and DBCAMP-treated cells. *P < 0.05 for significant increase compared with H-89-treated cells. Percentage of V1E/CFTR colocalization graph: *P < 0.05 for significant increase compared with PBS, Slo, and Slo plus ConA-treated cells. Scale bar = 10 or 100 μm.

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stimulated CFTR−/− tissues, in contrast to the WT response of insertion into the BBM, label for V_{o1} and V_{o2} extended below the terminal web to subapical compartments deeper in the cytoplasm. Basolateral staining in untreated CFTR−/− tissues was very weak compared with WT and increased slightly in the stimulated condition (Fig. 8, B and D). This distribution resembled the pattern of V_{o1} and V_{o2} label in PBS-treated CaCo-2BBe cells (Fig. 4). A low level of V_{o3} labeling was detected throughout the cytoplasm in WT jejunum, and weak staining was detected in the terminal web region of CFTR−/− in NS- and DBcAMP-treated jejunum (Fig. 8, E and F). Labeling with antibodies against V_{1}E revealed diffuse cytoplasmic staining in NS-treated tissues that redistributed to the enterocyte BBM following DBcAMP treatment (Fig. 8, G and H). In the NS- and DBcAMP-treated CFTR−/− tissues, the distribution pattern of V_{1}E was similar to that of V_{o1} and V_{o2}.

Redistribution of V-ATPase in CFTR−/− enterocytes is not due to changes in protein expression. To confirm that the observed redistribution of V_{o1}, V_{o2}, and V_{o3} in WT and CFTR−/− mouse intestine was not due to altered levels of V-ATPase protein in the absence of CFTR, Western blot analyses of equivalent protein loads from WT and CFTR−/− tissue lysates (jejunum) were performed (Fig. 9). Immuno-
blots confirmed that levels of $V_{o1}$, $V_{o2}$, $V_{o3}$, and $V1\alpha$ remain unchanged in WT and CFTR$^{-/-}$ tissues.

**DISCUSSION**

Our recent studies in rat intestinal CHE cells and Brunner’s gland provided the first evidence that V-ATPase proton pumps colocalize with CFTR chloride channels in the mammalian intestine (24, 45). Moreover, data supporting a functional link between CFTR and V-ATPase pumps in cAMP-regulated traffic in epithelial cells have not been published. The current study examined the distribution of V-ATPase proton pumps following cAMP activation in native intestinal tissues and cultured polarized human intestinal cells expressing endogenous CFTR. The results of this study demonstrate that V-ATPase proton pumps are present in cytoplasmic vesicles and the apical domain in the intestine and undergo robust traffic into the BBM following cAMP activation. Enterocyte V-ATPase associates with and is dependent on CFTR for proper proton efflux and its traffic into the enterocyte BBM. Moreover, the current study identified specific transmembrane ($V_{o1}$ and $V_{o2}$) and cytosolic ($V1\alpha$) subunits of the pump that are associated with V-ATPase traffic into the BBM in the intestine. The data presented in the current study strongly suggest that

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**Fig. 8.** cAMP-induced traffic of V-ATPase $V_{o1}$ and $V1\alpha$ subunits in WT and CFTR$^{-/-}$ mouse jejunum. Intestinal loops from WT and CFTR$^{-/-}$ mouse jejunum were treated with normal saline (NS) or DBcAMP (1 mM). Tissues were sectioned and double-labeled with antibodies against $V_{o1}$, $V_{o2}$, $V_{o3}$, $V1\alpha$, and F-actin and examined by confocal microscopy as described in MATERIALS AND METHODS. A–H: single and merged images show distribution of $V_{o1}$, $V_{o2}$, $V_{o3}$, or $V1\alpha$ in enterocytes from villus sections in NS and DBcAMP-treated WT (A, C, E, and G) and CFTR$^{-/-}$ (B, D, F, and H) jejunum. Arrowheads indicate staining on the lateral membranes, and empty arrowheads indicate subapical staining in villus enterocytes. Hoechst stain labels nuclei blue. Scale bar = 10 um.
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Fig. 9. Western blot analysis of V-ATPase $V_o a$ isoforms and $V_i E$ subunit in WT and CFTR $^{-/-}$ mouse jejunum. Tissue lysates (30 µg of protein) from WT and CFTR $^{-/-}$ mouse jejunum were separated by SDS-PAGE. Proteins were detected using CFTR, $V_o a1$, $V_o a2$, $V_o a3$, or $V_i E$, and β-actin antibodies. Bands consistent with $V_o a1$ and $V_o a2$ isoforms, and $V_i E$ isoform in WT and CFTR $^{-/-}$ mouse jejunum are shown. Low levels of $V_o a3$ protein were detected. β-Actin loading controls are shown. Molecular mass standards (kDa) are indicated.

CFTR is necessary for V-ATPase localization and function in polarized intestinal cells (Fig. 10).

Localization of V-ATPase subunits in intestine. In C. elegans the VHA-6 gene encodes for $V_o a$ subunit and VHA-8 encodes for the $V_i E$ subunit of mammalian V-ATPase. VHA-6 localizes to the apical membrane in C. elegans intestine where it is required for luminal acidification following defecation. Similarly, disruption of VHA-8 leads to larval lethality likely due to defective acidification in the intestine (3, 48, 53). These observations are consistent with the localization of transmembrane $V_o a1$, $V_o a2$, and $V_i E$ subunits of V-ATPase in the apical and basolateral membrane of rodent and human intestine as demonstrated in the current study. Proton secretion from the BBM in the intestine is largely a function attributed to NHE Na$^+$/H$^+$ exchanger family members (28, 93). Uptake of di- and tripeptides utilizes electrical and pH differences across the enterocyte BBM as driving forces that are coupled to H$^+$. The pH difference may be entirely due to the contribution of NHE2 and NHE3 activity on the BBM of mature enterocytes. However, inhibition of the Na$^+$/H$^+$ exchangers in rat intestine either by removal of Na$^+$ or pharmacologic blockade with amiloride failed to eliminate luminal acidification, suggesting the presence of an alternate route for H$^+$ secretion. Previous attempts to identify a vacuolar H$^+$-ATPase in rat enterocyte BBM proved negative, leading to conclusions that vacuolar H$^+$-ATPase cannot contribute to luminal H$^+$ secretion in the intestine (75). It is possible that detection of V-ATPase was not possible in that study due to lack of affinity of antibodies since similar approaches were used to isolate rat BBMV in the current study. In the small intestine, NHE3 regulates Na absorption by a process of Na$^+$/H$^+$ exchange on the apical membrane of enterocytes. In disease states such as secretory diarrhea, cAMP elevation leads to simultaneous upregulation of CFTR anion secretion by exocytic traffic into the BBM and inhibition of Na absorption by increased endocytosis or internalization of NHE3 (6, 44, 50, 58). Previous observations that cAMP increases intracellular pH in intestinal cells (85) and the findings of the current study and our recent study on CHE cells (45) suggest that under conditions of elevated cAMP levels V-ATPase pumps may provide an alternate source of protons necessary to maintain intestinal luminal equilibrium with anions (Cl$^-$ and HCO$_3^-$) in the absence of NHE3.

CAMP/PKA-regulated traffic of V-ATPase and CFTR to the BBM in the intestine. The observation in the current study that CAMP/PKA induces the recruitment of V-ATPase and CFTR to the enterocyte BBM is new but not totally surprising. The data from this study are consistent with our recent findings that V-ATPase and CFTR undergo CAMP/PKA regulated traffic to the apical domain of rat Brunner’s gland (24). Moreover, although CAMP activated traffic of V-ATPase was not demonstrated in villus CHE cells, these enterocytes express high levels of V-ATPase in the apical domain and possess a prominent pool of subapical endosomes that traffic CFTR into the BBM following CAMP/PKA (7, 45). The observations that PKA inhibition by H-89 reduced apical traffic while CAMP increases CFTR/V-ATPase localization and function on the BBM support PKA signaling in CFTR/V-ATPase traffic and function in the intestine. The incomplete abrogation of apical CFTR traffic by H-89 (Fig. 5) in cultured intestinal CaCo-2BBe cells may be due to incomplete apical delivery of the drug due to mucus accumulation that is observed in this cell line.

CAMP regulates luminal pH in epithelial cells of the kidney, salivary gland, and epididymis by recruiting V-ATPase proton pumps from endosomal compartments into the BBM (62, 63, 65, 66). Moreover, CAMP/PKA and Rab 11b regulate V-ATPase traffic into the apical BB of salivary gland duct cells, an epithelial tissue that possesses high levels of endosomal and apical CFTR (62, 63, 90). In the intestine, CAMP/PKA and Rab 11b regulate fluid secretion by trafficking and insertion of CFTR chloride channels from subapical endosomes into the...
apical BBM (7, 80). The findings here are consistent with published observations that PKA phosphorylation regulates CFTR traffic into the BBM in the intestine (6, 34) and traffic of the V-ATPase V1 cytosolic domain from the cytoplasm to the apical membrane in rat kidney intercalated cells and cultured rabbit kidney collecting duct cells (4, 35).

The V-ATPase specific inhibitor ConA has been widely used to examine V-ATPase pump function and its role in mediated intracellular organelles (29, 41, 42). Whether ConA modulates transporter traffic is unknown. Our results suggest that treatment with ConA reduces cAMP-regulated trafficking of CFTR and V-ATPase to the apical domain of polarized intestinal cells, albeit to a minor degree. In addition to its effects on luminal pH, ConA also alters endocytosis of apical proteins (1, 81), perturbs the recruitment of scaffolding proteins to endosomal membranes, blocks early to late endosomal trafficking (40), and disrupts interactions of V0 subunits with the soluble N-ethylmaleimide-sensitive fusion attachment protein receptor (SNARE) proteins (12, 39, 81). CFTR-inh172 is commonly used as a selective inhibitor of CFTR chloride channel function, but whether the compound modulates trafficking is unknown. Treatment with CFTR-inh172 did not perturb apical localization of CFTR and V-ATPase in intestinal cells, supporting the notion that the CFTR inhibitor does not directly affect the cellular distribution of CFTR.

**V-ATPase proton efflux is CFTR dependent.** The role of CFTR in regulating H+ -ATPase activity in intracellular organelles has been highly controversial and inconclusive (10, 11, 14, 21, 26, 27, 55, 73, 78). Studies have begun to examine the modulation of plasma membrane H+ -ATPase activity by chloride channels in renal tubules (19, 30). With the use of similar pH cell recovery assays employed in the current study, plasma membrane V-ATPase activity was shown to be dependent on CFTR and CiC5 in rat kidney (19). Our findings that intestinal cells possess functional V-ATPase pumps at the apical membrane and that V-ATPase proton efflux from the plasma membrane was dependent on CFTR are new. Inhibition of either V-ATPase or CFTR resulted in a significant decrease in intracellular pH, supporting a functional link between both transporters in the membrane.

**BBM traffic of V-ATPase requires CFTR in vivo.** Confocal microscopy revealed that V-ATPase redistributed from a basolateral location in native enterocytes to colocalize with CFTR on the BBM following cAMP activation. Furthermore,

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**Fig. 10.** Schematic diagram summarizing effects of agonists and inhibitors on CFTR and V-ATPase function and regulated trafficking in polarized intestinal CaCo-2Be cells. A: schematic diagram illustrates proton movement across the apical membrane of cells. CF-inh172 and/or ConA inhibit proton movement, whereas DBcAMP stimulates proton secretion across the membrane. B: schematic drawing illustrates cellular localization and apical trafficking patterns of CFTR and V-ATPase in intestinal cells. At steady state, CFTR is localized to the apical membrane and subapical endosomes, while V-ATPase is distributed throughout the cytoplasm and on the apical or basolateral domains. CAMP recruits both CFTR and V-ATPase to the apical membrane. The distribution of CFTR and V-ATPase in H-89 (PKA inhibitor), CF-inh172, and ConA-treated cells is similar to the localization patterns in steady-state conditions. In contrast, treatment with H-89, CF-inh172, and ConA followed by DBcAMP induces trafficking of CFTR and V-ATPase to the apical membrane of the cells, indicating that CFTR is tightly linked to V-ATPase.
coimmunoprecipitation confirmed that CFTR is in a biological complex with V-ATPase and examination of H+/ATPase proton efflux in intestinal cells indicated its dependence on CFTR. These observations prompted further studies to determine whether indeed CFTR is necessary for cAMP-dependent traffic of V-ATPase into the enterocyte BBM in the native intestine in vivo. In contrast to WT intestine where V_o α1, V_o α2, and V_i E subunits were recruited into the enterocyte BBM following cAMP treatment, the same V-ATPase subunits were internalized and accumulated in the subapical region just below the terminal web in CFTR−/− jejunum. These data provide strong in vivo confirmation that CFTR is necessary for V-ATPase traffic into the enterocyte BBM in vivo. These findings raise the possibility that chloride channels, such as CFTR, may be required for V-ATPase pump movement and cellular distribution within the enterocyte. Limited studies have suggested that WT CFTR is necessary for the localization of proteins in epithelial models. Na+/H+ exchanger regulatory factor (NHERF-1), which regulates CFTR trafficking to the apical membrane, was localized to apical membrane in normal cultured human bronchial epithelial cells (16HBE14o−). Although these results were reported in cell models, they raise the possibility that the mechanisms for protein localization maybe different in epithelial cells expressing CFTR vs. those deficient in CFTR that should be explored further.

The observation that CFTR is in a biological complex and colocalizes with and is necessary for functional V-ATPase activity in vivo is not surprising. CFTR and V-ATPase have been identified in the same intracellular trafficking compartments within the endocytic and exocytic pathways. Moreover, both transporters interact with similar binding partners (AP-2 and SNARE; Refs. 8, 12, 22, 23, 33, 59) but in a tissue-specific manner. The findings in the current study that 1) endogenous V-ATPase pumps undergo cAMP-regulated traffic to the BBM that is dependent on CFTR in vivo, 2) PKA signaling mediates the trafficking of CFTR and V-ATPase, 3) the inhibition of CFTR or V-ATPase alters the cAMP-induced trafficking of V-ATPase and CFTR to the apical membrane, and 4) V-ATPase proton pump function requires CFTR in the intestine are new. Further studies will be necessary to elucidate the role of V-ATPase and CFTR in the pathophysiology of intestinal diseases. These studies are likely to modify the existing paradigm for understanding intestinal transport and luminal homeostasis in health and disease states.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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