Syntaxin 3 is necessary for cAMP and cGMP-regulated exocytosis of CFTR: implications for enterotoxigenic diarrhea

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Abstract

Enterotoxins elaborated by Vibrio cholera and E. Coli cannot elicit fluid secretion in the absence of functional cystic fibrosis transmembrane conductance regulator (CFTR) chloride channels. Following enterotoxin exposure, CFTR channels are rapidly recruited from endosomes and undergo exocytic insertion into the apical plasma membrane of enterocytes to increase the number of channels on the cell surface by at least four-fold. But the molecular machinery that orchestrates exocytic insertion of CFTR into the plasma membrane is largely unknown. The current study used immunofluorescence, immunoblotting, surface biotinylation, GST-pull down assays and immunoprecipitation to identify components of the exocytic SNARE (soluble N-ethylmaleimide (NEM)-sensitive factor attachment receptor) vesicle fusion machinery in cyclic nucleotide activated exocytosis of CFTR in rat jejunum and polarized intestinal CaCo-2Bbe cells. Syntaxin 3, an intestine specific SNARE co-localized with CFTR on the apical domain of enterocytes in rat jejunum and polarized CaCo-2Bbe cells. Co-immunoprecipitation and GST binding studies confirmed that syntaxin 3 interacts with CFTR in vivo. Moreover, Heat Stable Enterotoxin (STa) activated exocytosis of both CFTR and syntaxin 3 to the surface of rat jejunum. Silencing of syntaxin 3 by shRNA interference abrogated cyclic nucleotide stimulated exocytosis of CFTR in cells. These observations reveal a new and important role for syntaxin 3 in the pathophysiology of enterotoxin-elicited diarrhea.

Key words: Cystic Fibrosis Transmembrane Conductance Regulator, intestine, exocytosis, secretory diarrhea, soluble N-ethylmaleimide (NEM)-sensitive factor attachment protein receptor (SNARE), syntaxin 3
**Introduction:**

Despite widespread use of oral rehydration therapies, infectious diarrhea remains a major socioeconomic and global health problem that claims the lives of more than 2.5 million children each year (41). Rapid advances in the pace of biomedical research continue to benefit diseases that affect the developed world. But sadly, this is not the case for underdeveloped countries. As a result, there is currently no effective drug therapy available for the treatment of secretory diarrhea (18).

*Vibrio cholera* and *Escherichia Coli*, the two major bacteria responsible for enterotoxigenic diarrhea, target the proximal small intestine where they release enterotoxins. Once released, enterotoxins bind to receptors on the apical membrane of enterocytes and signal intracellular cAMP and cGMP-dependent pathways that converge to activate cystic fibrosis transmembrane conductance regulator (CFTR) chloride channels on the apical membrane and mediate anion secretion (8, 28). The importance of CFTR to intestinal fluid secretion is underscored by the complete inability of enterotoxigenic bacteria to elicit fluid secretion in individuals lacking functional CFTR channels as observed in the genetic disease Cystic Fibrosis (23).

We previously demonstrated that cAMP/protein kinase A (PKA) and cGMP/protein kinase G (PKG) elicited fluid secretion requires recruitment and insertion of CFTR from endosomes to the surface of enterocytes in rat small intestine (3, 4). Fluid secretion elicited following E. Coli Heat Stable Enterotoxin (STa) in the jejunum was observed following PKG dependent increase in surface CFTR by at least four-fold. This scenario is possible because most (> than 50%) of the CFTR in enterocytes reside on membranes of subapical endosomes at steady state, a distribution required for agonist-stimulated exocytic recruitment in regulating fluid secretion (5).

Constitutive endocytic (clathrin-mediated) recycling to and from the plasma membrane efficiently controls the number of channels on the cell surface under steady state because CFTR has a long half-life (>20hr) in intestinal epithelial cells (12, 44, 48)(55). Endocytic recycling and regulated exocytosis directly modulate CFTR ion transport in the intestine in a cell-type specific manner in
endogenous CFTR-expressing epithelial cells and tissues (9, 27). For example, the rab GTPase rab 11a regulates apical recycling of CFTR in airway cells, but rab11b (not rab11a) regulates its apical recycling in the intestine (48, 50). Elucidating the proteins that direct CFTR endocytosis and exocytosis in the intestine is critical for identifying therapeutic targets to treat secretory diarrhea and CF because both pathways are implicated in intestinal fluid secretion (2, 25).

Establishment of apical polarity requires precise targeting of proteins from the Golgi to the plasma membrane. This function is carried out by specific SNARE proteins (soluble N-ethylmaleimide (NEM)-sensitive factor attachment receptor). SNARE membrane fusion machinery is essential for all membrane trafficking pathways. Vesicular cargo (v-SNAREs) interact with target membrane (t-SNARE) to mediate membrane fusion (30, 35). In epithelial cells, target SNARES of the syntaxin family localize to distinct compartments where they direct trafficking of apical or basolateral proteins. It is this property that enables the cell to exert its specific function in epithelial tissues (31, 34, 46)

Syntaxin 3 is involved in apical recycling and in biosynthetic traffic from the trans-Golgi network (TGN) to the apical surface of epithelial cells and its distribution in the small intestine resembles that of CFTR (34, 47) (1, 21, 45). In gastric parietal cells of the stomach, syntaxin 3 regulates acid secretion by recruitment of H^+K^+ ATPase proton pumps from subapical vesicles to the apical membrane (6, 38, 47, 51). The behavior of syntaxin 3 in gastric parietal cells and its localization in the intestine suggested that it could play a similar role in second messenger-regulated exocytosis of CFTR to the surface of enterocytes.

In the current study, we examined whether syntaxin 3 is necessary for regulated exocytosis of CFTR in the small intestine. The data are consistent with a requirement for syntaxin 3 in second messenger-dependent recruitment of CFTR to the cell surface and a role in the pathophysiology of enterotoxin-mediated diarrhea. We also characterized a polarized cell model of differentiated enterocytes (CaCo-2_BBe) to examine apical recycling and regulated trafficking of CFTR in villus enterocytes of the small intestine. This was important because villus enterocytes are increasingly recognized as important sites for CFTR anion secretion and regulated trafficking (24, 53) but there
are no validated intestinal cell models to study endocytic recycling and regulated exocytosis of endogenous CFTR in this compartment (25). We recently demonstrated that CaCo-2<sub>BBe</sub> cells are a useful model for studies of clathrin-mediated endocytosis of CFTR (16). Here, we extend the characterization of this cell model and show that CaCo-2<sub>BBe</sub> cells express the machinery to regulate apical recycling and regulated recruitment of CFTR to the cell surface similar to its behavior in villus enterocytes of rat jejunum.

**Materials and Methods**

**Reagents and Antibodies**

The following antibodies were used in this study: CFTR (M3A7) (Chemicon International Temecula, CA), CFTR 217 (Cystic Fibrosis Foundation Therapeutics, Bethesda, MD), AME 4991 CFTR (2) alkaline phosphatase (BYA1191) (Accurate Chemicals, Westbury, NY), syntaxin 3 (Alomone Labs, Jerusalem, Israel), rme-1 (gift from Barth Grant, Rutgers University), NKCC1 (gift from Dr Chris Lytle, UC Riverside, CA), EEA-1 (BD Transduction, San Jose, CA), myosin VI (Sigma Aldrich Chemicals, St. Louis, MO), SNAP-23 (United States Biological, Swampscott, MA), munc-18, rab11 (BD Transduction) β-actin (Sigma), syntaxin 2 (Synaptic Systems, Germany), mouse and rabbit IgG (Sigma). Rhodamine labeled phalloidin and all other fluorescent secondary antibodies were purchased from Molecular Probes Inc. (Eugene, OR). Horseradish peroxidase conjugated goat-anti-rabbit IgG and goat-anti-mouse IgG was obtained from BD Biosciences. EZ-Link Sulfo-NHS-SS-biotin and Immunopure Immobilized Streptavidin Agarose were obtained from Pierce Biotechnology (Rockford, IL). Heat Stable Enterotoxin (STa) and N<sup>6</sup>, 2′-O-Dibutyryladenosine 3′, 5′-cyclic monophosphate sodium salt (dbcAMP) were purchased from Sigma.

**Animal Preparation:**

Rodent studies were performed with the approval of Institutional Animal Care and Use Committee of the University Of Pittsburgh and Yale University School of Medicine. Sprague-Dawley rats (250–300
grams, Charles River Laboratories) were anesthetized with Inactin (120 mg/kg) by intraperitoneal injection. The abdomen was opened, segments of jejunum were identified, loops were created using sutures and filled with 0.5mL of freshly prepared warm (37°C) STa (0.5µM) or saline (NS). The abdomen was closed and animals were kept warm on a heating pad at 37°C. Jejunal loops were excised 30 min later and prepared for surface biotinylation or immunofluorescence microscopy. Adult male Balb C mice were anesthetized using Avertin (2,2,2, Tribromoethanol, Sigma) (180mg/kg) administered by intraperitoneal injection. Intestinal tissues were removed and rats were euthanized using an overdose of Inactin.

**Cells**

Human embryonic kidney (HEK-293) cells were a gift from Dr Neil Bradbury (Chicago Medical School). Cells were grown in DMEM with 10% fetal bovine serum (FBS, Gibco, Glasgow, Scotland) on 35 mm dishes prior to Western Blot analysis. CaCo-2 BBe cells were obtained from ATCC (Manassas, VA) and grown at 37°C in 5% CO₂/90% air atmosphere in high glucose with L-glutamine Dulbecco Modified Eagle Medium (Gibco), supplemented with 10% FBS, 10µg/ml apo-transferrin (Chemicon International Temecula, CA), 1mM/ml sodium pyruvate (Sigma St. Louis, MO), 1% penicillin-streptomycin (Gibco), 1µg/ml Fungizone (Gibco) and 5µg/ml Plasmocin (Invitrogen, San Diego CA). CaCo-2 BBe cells were seeded at 1x10⁵ cells per cm² on 100mm cell culture dishes (Corning Incorporated, Corning NY) and passaged when 70% confluent onto a 75mm Transwell (Costar Inc. Cambridge, MA). Confluent monolayers of CaCo-2 BBe cells were used for immunofluorescence labeling, Western Blot analysis and surface biotinylation experiments. Surface biotinylation was performed on CaCo-2 BBe cells after 120 hours in culture in all shRNA induced silencing studies.

**Immunofluorescence Microscopy of Cells**

Confluent monolayers of polarized CaCo-2 BBe cells were treated with 1mM dbcAMP or PBS for 30 min prior to fixation. CaCo-2 BBe cells were fixed in 2% PFA-PBS for 10 min at room temperature.
Cells were permeabilized with 0.1% Triton X-100 in PBS for 10 min followed by incubation with 0.5%BSA and 0.15% Glycine in PBS for 45 min to block non-specific binding. Primary antibodies were prepared in blocking solution containing 0.1%Triton X-100 and applied overnight at 4°C. Cells were washed, incubated with the appropriate secondary antibodies for 1 hr at room temperature and nuclear stain (Draq 5 or Dapi) was applied. Filter-grown CaCo-2 BBm labeled cells were mounted with Slow Fade medium and/or embedded in OCT and sectioned in the vertical plane prior to examination by confocal microscopy.

**Western Blot Analysis**

CaCo-2 BBm, HEK-293 cells or intestinal mucosal scrapings were homogenized in TGH lysis buffer (25mM HEPES, 10% [v/v] glycerol, 1% [v/v] Triton-X 100 pH 7.4) containing protease inhibitor cocktail (10nM iodoacetamide, 1mM phenylmethylsulfonyl fluoride, and 2 μg/ml leupeptin) for 30 min on ice. Homogenates were centrifuged at 14,000 rpm for 15 min at 4°C and supernatants recovered. Protein concentration of the supernatant was determined using Coomassie Protein Assay Reagent (Pierce, Rockville, IL). Samples were prepared in 2X SDS sample buffer and boiled for 5 min except when detecting CFTR; in which case the samples were warmed at 37°C for 5 min. Proteins (20 μg) were resolved by SDS-PAGE. Gels were transferred onto Immun-Blot PVDF membranes (Bio-Rad Laboratories Inc, Hercules, CA) and incubated with primary antibodies diluted in blocking buffer (5% dry fat milk, 0.1% Tween 20, Tris-buffered saline, pH 7.5) for 1 hr at room temperature. Membranes were incubated with secondary antibodies diluted in blocking buffer for 1 hr at room temperature and proteins were detected by chemiluminescence (Pierce Biotechnology Inc., Rockford, IL) using Kodak Biomax Light Film (VWR).

**Brush Border Membrane (BBMV) Isolation and Preparation**

Enterocytes from rat jejunum and polarized intestinal cells were isolated in NET buffer (0.13m NaCl, 5mM EDTA, 10mM Tris base, pH 7.4) containing protease inhibitors and centrifuged at 500gx10 min.
Cell pellets were resuspended in Buffer 1 (0.298M Mannitol, 12mM Tris, 1.5M Na Azide, pH 7.4 containing protease inhibitors), and sonicated to break vesicular cell membranes on ice. Samples were incubated with 10mM MgCl₂ at 4°C for 15 min, centrifuged at 5,000rpm at 4°C. The pellets were discarded and the supernatants were centrifuged again at 13,000 rpm at 4°C, and the cycle was repeated until pure BBMV preparations were obtained and verified by microscopic evidence of enriched microvillar profiles with few cytoplasmic contaminants. Pellets were stored at -80°C overnight and resuspended in 200µl of Buffer 1, transferred to 1.5 mL eppendorf tubes and centrifuged at maximum speed for 30 minutes. Pellets were lysed in TGH buffer containing protease inhibitors on ice and samples analyzed by Western Blot.

**Immunofluorescence Microscopy of Tissue Sections and Image Quantification**

Segments of jejunum from rat or mice were embedded in Tissue-Tek O.C.T embedding medium (Miles Laboratory, Elkhardt, IN) and frozen in isopentene pre-cooled with liquid nitrogen. Cryosections were prepared as previously described (2). Briefly, frozen tissue sections were fixed with 2% paraformaldehyde for 10 min, rehydrated with PBS and non-specific labeling was blocked with normal goat serum (1:20) for 45 min. Primary antibodies were applied for 1-2 hours at room temperature or overnight at 4°C. Sections were washed with PBS containing 0.15% glycine and secondary antibodies were applied for 1 hr. Following secondary antibody application, Rhodamine phalloidin (Invitrogen) was applied for 20 min to stain F-actin. Nuclei were stained using Draq 5 (Biostatus, UK) or Hoechst. Immunofluorescence images were acquired on Olympus Fluoview 500 confocal microscope using either 60X oil or 100X oil objectives. Images of NS and STa-treated rat jejunum were taken at 60X magnification with constant settings for laser, PMT, gain and offset at medium scan rate. Fluorescence intensities of CFTR and syntaxin 3 labeling were determined in images taken from fields containing an average of 6-8 crypts and villi. Fluorescence intensities were obtained in a region within a 1.5µm distance beneath the apical border facing the lumen and the pixel intensity
within the region was normalized to area (µm²). Analysis was performed using Metamorph software (UIC, Downington, PA) as before (2). Data are expressed as +SEM and significance in mean values was determined by two-tailed Student’s t-test. P<0.05 was considered significant.

**Surface Biotinylation in Rat Jejunum and Cells**

Surface biotinylation was performed as previously described (2, 3). Thirty minutes following enterotoxin treatment of jejunum, intestinal loops were excised, immediately placed on ice, and the lumen was gently flushed with cold saline and ends were re-secured with ligatures. Freshly prepared Sulfo-NH-SS biotin (1mg/ml) in PBS-CM (PBS containing 0.1mM CaCl₂ and 1.0mM MgCl₂) was introduced into the lumen and loops were incubated in the cold for 30 min (25). In separate experiments, PBS and 1mM dbcAMP treated CaCo-2 BBe cells were incubated with NHS-SS Biotin for 30 min on ice. Following surface biotinylation labeling, cells or mucosal scrapings were lysed in TGH buffer containing protease inhibitors. Equivalent amounts of protein were incubated with Immunopure Immobilized Streptavidin Agarose overnight at 4°C on a rotator. The following day, the biotinylated proteins were dissociated from Streptavidin agarose by 2X SDS sample buffer. Cell lysates (20µg protein) and biotinylated samples were resolved by SDS-PAGE to detect CFTR and/or syntaxin 3 by Western Blot analysis. Quantification of surface labeled proteins was performed using a Biorad Fluor S-Multi-imager and Quantity one Image Analysis Software. Data are expressed as +SEM significance in mean values was determined by two-tailed Student’s t-test. P<0.05 was considered significant.

**Immunoprecipitation**

Mucosal scrapings from rat jejunum were lysed in TGH buffer containing protease inhibitors and samples were spun at 15,000 rpm for 15 min at 4°C. Supernatants were pre-cleared by incubation with 25µL of Protein A beads for 20 min on ice. Samples were centrifuged for 30 sec at maximum speed, supernatants were incubated with 1µg of specific antibody (anti-CFTR) or IgG control antibody on ice for 90 minutes, and then incubated with 20µL of 50% protein A beads and rocked for one hour
at 4°C. Following centrifugation (14,000 rpm), protein/antibody/bead complexes were washed with 1X RIPA (500mM Hepes, 150mM NaCl, 1%Triton-X, and 1mM EDTA) buffer and samples were eluted with 5X SDS sample buffer prior to Western Blot analysis (2, 25).

**DNA Constructs**

Glutathione S-transferase (GST) was provided by Dr. Linton Traub (University of Pittsburgh, Department of Cell Biology). GST-full length human syntaxin 3 (GST-STX 3, 1-290aa)(Open Biosystems) was cloned into pGEX4T-1 using EcoRI and Xho1 sites and clones were verified by sequencing.

**Protein and Peptide Preparation**

Expression and purification of GST fusion proteins were performed as described (16, 29, 36). GST and GST- STX 3 fusion proteins produced in *Escherichia coli* BL21 cells were grown in 20mL LB Broth (Invitrogen) plus Ampicillin (200mg/ml; Fisher Scientific) overnight at 37°C with constant shaking to create a starter culture. From the starter culture, bacteria was diluted 1:50 in the LB plus Ampicillin (200mg/mL), grown to an Absorbance of 0.6 at 37°C with constant shaking and induced by adding isopropyl-1-thio-β-D-galactopyranoside (100uM). After 3-5 hr with constant shaking at room temperature or Absorbance of 1.3-1.4, the bacteria were recovered by centrifugation at 15,000 rpm (JA-14 rotor) at 4°C for 15 min and pellets stored at -80°C. Pellets were resuspended in bacterial lysis sonification buffer (50mM Tris-HCl, pH8.0, 300mM NaCl, 0.2% Triton-X100 and 100mM 2-mercaptoethanol) containing 100mM PMSF, sonicated and centrifuged at 14,000 rpm (JA-20 rotor) at 4°C for 15 min. Supernatants were incubated with 1mL of 75% glutathione Sepharose 4B bead slurry (Amhersham, Piscataway, NJ). Beads were collected by centrifugation at 500xg for 5 min at 4°C. GST fusion proteins bound to beads were washed in PBS and proteins were eluted with glutathione elution buffer (25mM Tris-HCl, pH8.0, 200mM NaCl and 10mM Glutathione) with 1mM DTT on ice. Eluted fusion proteins were pooled, dialyzed in PBS and stored at -80°C prior to performing binding assays.
**Binding assays**

Binding assays were performed as described (37). Mucosal scrapings from rat jejunum were collected using a glass slide and homogenized in freshly prepared in TGH buffer containing protease inhibitors. GST and GST–STX 3 (200µg) were bound to lysate supernatants and 1mL PBS and incubated at 4°C for 1 hr while continuously rotating. After 1hr, 75µl of glutathione Sepharose 4B beads were added to the complex of bound proteins/supernatants and were incubated at 4°C overnight with continuous rotation. The GST fusion proteins and bead complex were centrifuged at 10,000 rpm at 4°C for 1 min and supernatant was discarded. The GST fusion protein, and bead complex were washed with cold PBS, and pellets were resuspended in 30µl of 5X SDS sample buffer. Gels were stained with Coomassie Blue (Biorad) and samples were separated by SDS-PAGE and analyzed by Western Blot.

**Syntaxin 3 silencing in CaCo-2 BBe cells**

Syntaxin 3 mRNA was targeted using shRNA delivered by a lentiviral system based on a pLKO.1-puro vector. Cells were transduced to stably express scrambled, or syntaxin3 shRNA. Syntaxin3-targeting shRNA (5`-ggaacaaactgaagagcat-3`) was designed using AsiDesigner online tool (http://sysbio.kribb.re.kr:8080/AsiDesigner/menuDesigner.jsf) and subcloned into Agel and EcoRI restriction sites of pLKO.1-TRC vector (Addgene # 10878), immediately downstream from the U6 promoter. Scrambled shRNA in the pLKO.1-Puro vector was obtained from Addgene (# 1864). HEK 293 t/17 (ATCC) packaging cell line was transfected with aforementioned constructs using FuGENE 6 transfection reagent (Roche Diagnostics) according to Addgene’s protocol. Cells were plated in 25cc flasks at 1.5x10^5 cells/ml, transfected the following day, at about 60-70% confluence with 1 ug of shRNA-containing vector, 0.75 ug of packaging plasmid (psPAX2, Addgene # 12260) and 0.25 ug of envelop plasmid (pMD2.G, Addgene # 12259). Medium was replaced after 15 hours at 37°C. Virus-containing medium was collected 24 and 48 hours post-transfection and titrated for optimal multiplicity of infection (MOI). CaCo-2BBe cells seeded at 4x10^5 were transduced at 60-70% confluency using
Polybrene (Millipore) at 5ug/ml final concentration. Following transduction, cells were selected with 6ug/ml Puromycin (Sigma). Efficiency of shRNA silencing was determined by Western Blot. Surface biotinylation was performed on cells as described above.

**Cell Viability**

Controls (non-transduced or scrambled shRNA transduced) and syntaxin 3 shRNA transduced CaCo-2BBe cells were grown in 60mm tissue culture dishes. Cells were scraped into 500\( \mu \)l of complete medium without serum at a concentration of 1X100 cells per ml. 100\( \mu \)l of 0.4\% Trypan Blue Stain (Invitrogen) was added to the cell suspension, mixed and incubated at room temperature for 5 minutes. Samples (10-20\( \mu \)L) were added to a hemocytometer and viewed under a microscope to observe non-viable (stained) and viable (unstained) cells. Cell viability as determined by trypan blue exclusion was >95\%. No difference in viability was detected between controls or syntaxin3 shRNA transduced cells.

**Surface CFTR detection in CaCo-2BBe cells following silencing of syntaxin 3**

Control (non-transduced and scrambled shRNA transduced) or syntaxin 3 shRNA transduced CaCo-2BBe cells were grown in 60 mm tissue culture dishes. Five days following transduction, cells were divided into two groups and treated with either PBS or 1mM dbcAMP for 10 min at 37°C. Following treatment, cells were immediately placed on ice and surface biotinylation assays performed. Western Blots were analyzed using CFTR, syntaxin 3 and \( \beta \)-actin antibodies.

**Statistical Analysis**

Data are expressed as mean ±SEM. The significance of differences in mean values was determined by the two tailed Student’s T-test. P values <0.05 was considered significant.
RESULTS

Polarized CaCo-2 \textsubscript{BB}e cells express the machinery to regulate endocytic recycling and CFTR anion transport

Because cAMP and cGMP can regulate exocytic insertion of CFTR to the surface of villus enterocytes and fluid secretion, we characterized an intestinal cell model that resembles villus enterocytes of the small intestine for the current studies of syntaxin 3 and regulated trafficking of CFTR to the cell surface. CaCo-2 \textsubscript{BB}e cells consist of differentiated enterocytes possessing a well-developed brush border (39, 40). We recently used this model to examine endocytic adaptors in clathrin-mediated endocytosis of CFTR in the intestine (15). Immunoblots of CaCo-2 \textsubscript{BB}e cell lysates (Figure 1) revealed endogenous expression of endocytic (EEA-1, myosin VI) apical recycling (rme-1, rab 11), exocytic proteins involved in vesicle fusion (SNAP 23, munc-18, syntaxin 3) and basolateral chloride entry transport (NKCC1) and apical exit (CFTR) proteins necessary for endocytic recycling, exocytosis and CFTR-mediated ion transport. Western Blot analysis of brush border membranes from cultured T84 cells, rat jejunum and HEK-293 CFTR expressing cells also confirmed expression of CFTR, syntaxin 3 and SNAP 23 (Figure 3). Syntaxin 2, another Q-SNARE family member, is expressed apically in polarized pancreatic acinar and MDCK II cells. We compared the distribution of syntaxin 2 and syntaxin 3 to determine which syntaxin predominates in the apical domain of polarized CaCo-2 \textsubscript{BB}e cells. Immunofluorescence labeling of CaCo-2 \textsubscript{BB}e cell monolayers (Figure 2,A) reveals apical staining for syntaxin 3, while syntaxin 2 is predominantly confined to the basolateral membranes. Immunofluorescence labeling of cryosections from mouse jejunum corroborated the observations in CaCo-2 \textsubscript{BB}e cells, that the distribution of syntaxin 3 was apical and mirrored that of CFTR in native epithelial tissues (Figure 2, B).
Distribution of apical recycling and exocytic vesicle SNARE machinery in polarized CaCo-2 BBe cells

Using confocal microscopy, we examined the distribution of components of the apical recycling, and syntaxin 3 SNARE machinery to understand their relationship to endogenous CFTR in polarized monolayers of CaCo-2 BBe cells. Images of views from en face and vertical sections of immunolabeled cells are shown (Figure 4). The distribution of syntaxin 3 mirrored that of CFTR in the apical domain as evidenced by their co-localization (Figure 4, A, F, arrowhead). Although less abundant than syntaxin 3, the plasma membrane-associated SNARE protein SNAP 23 co-localized with CFTR on the apical membrane (Figure 4B, F), consistent with its role as a syntaxin 3 associated t-SNARE in polarized cells (56). The recycling marker, rme-1 also localized to the apical domain and co-localized with CFTR (Figure 4, C, F). Labeling for the brush border hydrolase, alkaline phosphatase (ALP) confirmed its localization on microvillar membranes (Figure 4D, F) where it co-localizes with CFTR. Control labeling with the relevant IgG confirmed specificity of antibody staining (Figure 4, E).

cAMP regulates the recruitment of CFTR and syntaxin 3 to the surface of polarized CaCo-2 BBe cells

To characterize the CaCo-2 BBe cell model for studies of agonist-stimulated trafficking of CFTR in the villus compartment, we used two independent approaches to examine changes in the distribution of endogenous CFTR and syntaxin 3 in the apical domain following cAMP stimulation. Cells were treated with PBS or a cAMP agonist for thirty minutes, then placed on ice, fixed and immunolabeled and examined by confocal microscopy. Surface proteins were analyzed by cell surface biotinylation and Western Blot. The distribution of syntaxin 3 and CFTR fluorescence are shown in Figure 5 (A, B). En face views of cells labeled for syntaxin 3, CFTR and merged images of the apical surface of PBS or cAMP-treated cells (Figure 5A), reveal increased intensity of labeling for both proteins on the
surface following cAMP treatment. Views of vertical sections (Figure 5B) confirmed the increased labeling intensity and co-localization of syntaxin 3 and CFTR following cAMP treatment. The apical recruitment of CFTR following cAMP resembled its behavior in villus enterocytes of rat jejunum (25). Interestingly, apical labeling for syntaxin 3 also increased following cAMP treatment (Figure 5, A, B). Surface biotinylation confirmed cAMP stimulated trafficking of CFTR to the cell surface in CaCo-2 BBé cells (Figure 5C), similar to what we observed in native intestinal tissues (3, 25).

**Heat Stable Enterotoxin (STa) increases syntaxin 3 and CFTR in the apical domain of rat jejunum**

The cGMP agonist, Heat Stable Enterotoxin (STa) elicits massive fluid secretion and robust exocytosis of CFTR to the surface of rat jejunum (25, 54). To determine whether syntaxin 3 or the apical recycling protein rab 11 were involved in STa- dependent exocytosis of CFTR, we immunolabeled sections of rat jejunum following STa (0.5µM) or saline treatment as before and examined the sections by confocal microscopy to detect changes in their distribution in vivo. As predicted, STa treatment resulted in increased CFTR fluorescence in the apical domain (Figure 6, A, B, arrows). The distribution of the apical recycling marker rab 11 did not appear to be altered following STa (Figure 6, A-D), consistent with its role in constitutive apical recycling. However, similar to what we observed following cAMP treatment of CaCo-2 BBé cells, STa treatment resulted in a 50% increase in syntaxin 3 fluorescence labeling intensity in the apical domain of rat jejunum (Figure 7, A, C). These results indicate that stimulation with CFTR second messengers simultaneously recruits syntaxin 3 to the apical domain in the intestine.

**Heat Stable Enterotoxin (STa) increases surface expression of syntaxin 3 in rat jejunum**

The increase in syntaxin 3 fluorescence in the apical domain of rat jejunum following STa treatment (Figure 6, 7) was surprising because unlike CFTR, syntaxin 3 or members of the SNARE family are not regulated by PKA or PKG (49). To confirm that the observed increase in syntaxin 3 labeling in the
apical domain reflected increased abundance of syntaxin 3 on the surface of enterocytes, we
performed cell surface biotinylation and analyzed syntaxin 3 or CFTR surface expression by Western
Blot (Figure 7, D). Consistent with our previous results, STa stimulated a robust increase in surface
CFTR expression in rat jejunum (25). Surface biotinylation also confirmed that indeed STa also
increased syntaxin 3 surface expression by approximately 50% (Figure 7,E), consistent with the
increase in apical fluorescence intensities (Figure 7,A, B). The observation that the CFTR agonist
STa recruited both syntaxin 3 and CFTR to the cell surface and that both co-localized in subapical
vesicles strongly suggested that syntaxin 3 and CFTR share a common endosomal compartment that
undergoes agonist stimulated recruitment to the plasma membrane. To determine whether both
proteins physically interact in vivo, we performed co-immunoprecipitation experiments in rat jejunum
following saline or STa treatment using equivalent amounts of protein (6mg) from each condition and
a rat specific anti-CFTR antibody. Immunoprecipitates from control and STa-treated jejunum were
analyzed by Western Blot to detect syntaxin 3. CFTR co-immunoprecipitated with syntaxin 3 in both
control and STa treated tissues, but there was a 1.6 fold increase in syntaxin 3 detected in
immunoprecipitates from STa treated samples (Figure 7,F). These results suggest that both proteins
traffic together in their exocytic route to the apical membrane in the intestine.

**CFTR interacts with syntaxin 3 in the intestine**

The results of our immunoprecipitation, morphologic and surface biotinylation experiments strongly
suggested that CFTR interacts with syntaxin 3 to regulate apical exocytosis in the native intestine.
Since second messenger-stimulated recruitment of CFTR and syntaxin 3 could be replicated in
tissues and polarized CaCo-2\(_{BBE}\) cells, we used independent approaches of GST binding assays and
lysates from polarized monolayers of CaCo-2\(_{BBE}\) cells to determine whether endogenous CFTR is a
binding partner for syntaxin 3 (Figure 8). The Coomassie stained gel (Figure 8 A) shows bands of
either purified GST or GST-syntaxin 3 fusion proteins. GST or GST-syntaxin 3 were allowed to
interact with cell supernatents and protein complexes were analyzed by Western Blot to detect the
CFTR-syntaxin 3 interaction (Figure 8, B). These results indicated that approximately 14% CFTR interacts with syntaxin 3 in the intestine.

**Introduction of syntaxin 3-shRNA interrupts cAMP-dependent recruitment of CFTR to the cell surface**

Finally, to determine whether syntaxin 3 was necessary for agonist-stimulated exocytosis of CFTR to the plasma membrane, surface CFTR was examined in CaCo-2 BE cells lacking syntaxin 3. Controls (non-transduced (NT) or transduced with scrambled shRNA (SC)) and cells transduced with syntaxin 3 shRNA (S3) were subjected to surface biotinylation thirty minutes following treatment with PBS or cAMP. Immunoblot analysis of cell lysates revealed that the total amount of CFTR in PBS or cAMP treated cells remained unchanged (Figure 9, A). Immunoblot analysis of PBS or cAMP treated control cells showed similar levels of syntaxin 3, while cells transduced with syntaxin 3 shRNA (S3) efficiently reduced (~100%) its expression in PBS or cAMP treated cells. cAMP increased surface levels of CFTR 3.5-fold in control cells. In cells lacking syntaxin 3, treatment with cAMP reduced surface CFTR by about 30% to levels similar PBS treated control (Figure 9C). These results indicate a requirement for syntaxin 3 in the exocytosis of CFTR.
**Discussion**

We previously identified cAMP/PKA and cGMP/PKG-regulated recruitment of CFTR from subapical vesicles to the surface of enterocytes as a critical step in enterotoxin-elicited fluid secretion in the small intestine (25). But the proteins regulating recruitment of CFTR from endosomes to the cell surface were unknown. In this study we identified apical SNARE (syntaxin 3, SNAP 23, munc-18) and recycling proteins (rme-1 and rab11) in CFTR-positive endosomes in the apical domain of enterocytes, indicating a close association of the apical recycling and regulated exocytic trafficking machinery *in vivo*. Using an antibody that recognizes both α and β isoforms of rab 11, apical staining remained unchanged following cGMP stimulation with STa, suggesting that rab 11 is not involved in cGMP-regulated recruitment in the native intestine. Rme-1, a basolateral recycling protein in the *C. elegans* intestine, regulates CFTR recycling in non-polarized cells, but prior to the current study there was no evidence for apical localization of rme-1 in native CFTR-expressing epithelia (26, 42). The identification of rme-1 in subapical endosomes supports its role in apical recycling of CFTR in higher species. Furthermore, the distribution and expression of the proteins examined here are consistent with their role in apical trafficking and transport in epithelial cells (7, 32, 45).

We focused on syntaxin 3 because of its importance in apical targeting, recycling and vesicle fusion in intestinal cells (13, 21, 47). Importantly, syntaxin 3 was shown to regulate second messenger stimulated apical recruitment and function of proton transport in gastric epithelial cells of the stomach (6, 38). Gastric parietal cells resemble the subpopulation of CFTR High Expresser cells (CHE) that we first used to demonstrate regulated trafficking of CFTR to the apical domain of the intestine. Like parietal cells, CHE cells also possess a prominent subapical endosomal pool of CFTR-positive vesicles. But more is known about the proteins that regulate apical recycling and traffic in gastric parietal cells compared to CHE cells (3, 32). Although the subapical endosomal pool is not as prominent in crypt and villus enterocytes, regulated trafficking of CFTR is observed in both cell types.
In the gastrointestinal tract, syntaxin 2 and 3 are implicated in apical exocytosis of zymogen granules in pancreatic acinar cells (43). In cultured intestinal epithelial cells, agonist dependent trafficking of the adenosine 2b receptor to the apical domain was shown to involve the SNARE machinery vesicle associated membrane protein VAMP-2 and SNAP-23, suggesting that syntaxin 3 may be involved, but it was not examined in that study (56). Surprisingly, a recent study in guinea pig duodenal Brunner’s gland identified syntaxin 4 and SNAP-23 in cholinergic stimulated apical exocytosis and dilation of the ductal lumen (17). In the rat and mouse duodenum, high levels of CFTR are in the apical domain of Brunner’s Glands acinar cells but the expression and function of specific syntaxins in CFTR function in the Brunner’s Gland has not been identified (1, 5).

The role of syntaxins (including syntaxin 3) in CFTR trafficking and transport has been examined, but this is the first evidence demonstrating the involvement of syntaxin 3 in CFTR trafficking. Studies of native CFTR-expressing airway epithelia identified endogenous syntaxin 3 and syntaxin 1A in the apical domain. However, in contrast to the intestine, where syntaxin 3 binds to CFTR and is recruited to the cell surface following CFTR agonists, in the airway CFTR binds to syntaxin 1A (but not syntaxin 3) and rather than regulating trafficking, syntaxin1A interacts with CFTR to modulate its gating (14, 37). These findings and those of the current study are in agreement with the absence of second messenger-regulated trafficking of CFTR in native airway epithelial cells (9). Other studies identified interactions of the endosomal SNARE proteins syntaxin 7 and 8 (but not syntaxin 3) with CFTR that modulated trafficking and function (10, 11). The failure to identify a CFTR-syntaxin 3 interaction and a role for syntaxin 3 trafficking in airway epithelial cells further supports the notion that apical trafficking directs CFTR function in native epithelia through its interaction with cell-specific proteins in the same manner that syntaxins display isoform specificity in its regulation of exocytosis in epithelial cells (33) (27) (48, 50).

Because syntaxin 3 is critical to apical polarity, attempts to silence its expression in polarized CaCo-2BBe cells proved challenging in our hands. These cells acquire apical and basolateral polarity following at least two weeks of growth on permeable membrane supports. However, shRNA induced
silencing effectively (~100%) reduced syntaxin 3 expression in CaCo-2BBe cells at 120 hours. Control
cells grown under these conditions demonstrated a modest increase in surface CFTR following cAMP
stimulation (~3-fold), compared to the almost five fold response observed in native tissues.
Nevertheless, cells lacking syntaxin 3 demonstrated reduced exocytic response to cAMP and lower
surface CFTR levels compared to controls. These data confirm an important role for syntaxin 3 in
regulated trafficking and exocytosis of CFTR to the apical membrane of intestinal cells.
Investigators have emphasized CFTR's role in anion secretion in crypt cells since its transport activity
is high in this intestinal compartment. Importantly CFTR behavior in the crypt is replicated
phenotypically and functionally in the T84 cell model (22). Villus enterocytes possess distinct
phenotypic and functional characteristics compared to their immature crypt cell counterpart. Villus
enterocytes are the major sites of CFTR-mediated bicarbonate secretion and participate in regulated
trafficking of CFTR to the cell surface (25, 53). But there is no validated polarized intestinal cell model
available that replicates CFTR behavior in villus enterocytes in the small intestine. We recently
validated CaCo2BBe as a model for studies of clathrin-mediated endocytosis of endogenous CFTR in
the intestine. The current study extends this characterization by demonstrating the usefulness of this
model for studies of CFTR apical recycling and regulated trafficking to the plasma membrane.
Although the current study did not examine CFTR ion transport in CaCo2BBe cells, we observed
CFTR currents in this model when mounted in Ussing chambers. The identification of syntaxin 3
SNARE and associated apical recycling machinery in regulated exocytosis of CFTR is an important
step towards elucidating the role of CFTR and villus cells in the pathogenesis of secretory diarrhea.
These studies are important because prior to identification of the CFTR gene, investigators had long
noted that both cholera toxin and Heat Stable Enterotoxin (STa) from E. Coli target villus enterocytes
in the jejunum (19, 20). This suggests that molecular based studies using this cell model could prove
vital in further elucidating villus specific-pathways of CFTR regulation.

The recent surge in global health awareness has stimulated a burst of philanthropic efforts and
investment in research to develop compounds to treat enterotoxic-elicited diarrhea. Some of these
compounds target CFTR channels on the surface of the intestine (52). But these approaches are
designed to inhibit CFTR channels that are already resident on the plasma membrane rather than
exocytic-based mechanisms that increase CFTR on the cell surface to elicit fluid secretion. The
findings reported here suggest that identification of physiologically relevant targets of apical
endocytosis and exocytosis into the plasma membrane of the intestine will prove vital for effective
drug therapies to treat enterotoxin-elicited diarrheal disease.

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Abbreviations: CFTR, cystic fibrosis transmembrane conductance regulator; PKA, protein kinase A;
PKG, protein kinase G; STa, Heat Stable Enterotoxin, SNARE, soluble N-ethylmaleimide (NEM)-
sensitive factor attachment protein receptor, cAMP, N6, 2'-O-Dibutyryladenosine 3', 5'-cyclic
monophosphate
Figure Legends

**Figure 1: Western Blot analysis of endogenous CFTR, apical recycling, exocytic and ion transport proteins in polarized CaCo-2 BBe cells.**

CaCo-2 BBe cell lysates (20µg) were resolved by SDS-PAGE and proteins were detected by Western Blot. Bands show endogenous expression of endocytic (EEA-1, myosin VI) apical recycling (rme-1, rab 11), exocytic proteins involved in vesicle fusion (SNAP 23, munc-18, syntaxin 3) basolateral chloride entry transport (NKCC1) and apical exit (CFTR) proteins. Control immunoblots probed with relevant IgG and β-actin loading are shown. Molecular weight standards (kDa) are indicated.

**Figure 2: Polarized distribution of syntaxin 2 and 3 in the intestine**

Confluent polarized CaCo-2 BBe cells (Panel A) and cryostat sections of mouse jejunum (Panel B) were fixed and immunofluorescence performed as described in the methods. A) Enface image taken at the level of the brush border shows the apical distribution of syntaxin 3 (Syn3, open arrowhead). Image taken at basal level shows distribution of syntaxin 2 (Syn2, closed arrowhead). B) Staining for CFTR (green) and syntaxin 3 (green) in the apical domain of crypt and villus sections of mouse jejunum. Control label of cells (Panel A) and tissue sections (Panel B) with rabbit IgG (Rab IgG) antibody is shown. Hoechst nuclear stain labels the nuclei (blue) V= villus. Scale bar = 10µm.

**Figure 3: Western Blot analysis of SNARE proteins and CFTR in cultured cells and rat jejunum.**

Brush border membrane preparations and cell lysates (10-40µg protein) were resolved by SDS-PAGE and proteins were detected by immunoblotting. A) Bands show endogenous expression of CFTR, syntaxin 3 (syn 3) and SNAP-23 in T84, CaCo-2 BBe, HEK-293 cells and rat jejunum. B)
Control immunoblot probed with relevant IgG antibodies and β-actin loading controls are shown. C) Bands show CFTR, syntaxin 3 (syn3) and SNAP-23 detected in brush border membrane vesicle (BBMV) preparations from rat jejunum. Molecular weight standards (kDa) are indicated.

**Figure 4: Subcellular distribution of endogenous CFTR, apical recycling and exocytic proteins in polarized CaCo-2 BBe cells.**

Confluent polarized CaCo-2 BBe cells were fixed and double label immunofluorescence performed and cells were examined by confocal microscopy A-D) Enface images taken just above the level of the brush border show the distribution of syntaxin 3 (syn 3, A, red), SNAP-23 (B, red), rme-1 (C, red), alkaline phosphatase (ALP, D, red), and CFTR (A, B, C, and D, green). Merged images show co-localization (merge, yellow, arrowhead). E) Control labeling with rabbit (Rab) and/or mouse (Mse) IgG antibodies. F) Images of XZ vertical sections of cells. Hoechst nuclear stain labels nuclei blue. Scale bar = 10μm.

**Figure 5: cAMP-stimulates apical recruitment of syntaxin 3 and CFTR in CaCo-2 BBe cells.**

Confluent monolayers of CaCo-2 BBe cells were treated with PBS or 1mM cAMP. Cells were fixed, immunolabeled and examined by confocal microscopy. A) Enface views show the distribution of syntaxin 3 (syn 3, red) CFTR (green) and merged images show areas of co-localization (yellow) in PBS (top panel) or cAMP (bottom panel) treated cells. Control staining with relevant IgG antibodies is shown. B) Images of vertical XZ sections of immunolabeled cells show CFTR (green), syn3 (red) and merged images (yellow) show co-localization (arrowhead). Scale bar=10μm. C) Following PBS or cAMP treatment surface proteins were detected by sulfo-NHS-SS Biotin labeling and analyzed by Western Blot as described in the methods. Cell lysates (Lys, 20μg protein) and equivalent loads of surface biotinylated (+Biotin) proteins were resolved by SDS-PAGE and immunoblotted to detect CFTR. Molecular weight standards (kDa) are indicated.
Figure 6: Apical distribution of CFTR, syntaxin 3 and rab11 in rat jejunum following Heat Stable Enterotoxin (STa).

Cryostat sections of rat jejunum treated with normal saline (NS) or STa were immunolabeled and examined by confocal microscopy as described in the methods. Low and high magnification images of crypt sections are shown. Images of immunolabeled sections from rat jejunum following normal saline (NS) (A and C) or STa (B and D) show the distribution of CFTR (A and B, green, arrow), F-actin (A-D, red), rab11 (A-D, blue), and syntaxin 3 (C and D, syn3, green, arrow). Merged images show areas of co-localization (white; arrowhead). Differential interference contrast (DIC). Scale bar = 10µm and 100µm.

Figure 7: Heat Stable Enterotoxin (STa) stimulates recruitment of CFTR and syntaxin3 to the surface of rat jejunum.

Cryostat sections of normal saline (NS or STa) treated rat jejunum were immunolabeled and examined by confocal microscopy. High magnification images of crypt from NS (A) or STa (B) treated tissues show the apical distribution of syntaxin 3 (syn 3, red), CFTR (green) and merge (yellow). Increased apical staining for syntaxin 3 (B, arrowhead) and CFTR (green, arrowhead) following STa. Merged images show areas of co-localization of CFTR and syntaxin 3 in NS (Figure 7A, arrow) and STa treated (Figure 7B, arrowhead) jejunum. Differential interference contrast (DIC). Nuclei are stained with Hoechst stain (blue), Scale bar = 10µm. C) Quantification of fluorescence intensity (FI) of syn 3 and CFTR label in the apical domain of NS and STa treated rat jejunum. Data represent + SEM (n>10), * p<0.01. D) Following NS or STa treatment, surface proteins were labeled with sulfo-NHS-SS biotin in vivo, cells were lysed and proteins bound to Streptavidin Agarose. Equivalent (20µg) loads of protein from cell lysates (lys) and surface biotinylated (+Biotin) samples were resolved by SDS-PAGE and immunoblotted to detect CFTR, syntaxin 3 (syn 3) and alkaline phosphatase (ALP). E) Quantification (mean pixel values) of surface labeled (B+) syntaxin 3 (Syn 3)
in NS and STa-treated enterocytes. Data represent +SEM (n>4),* P=0.05. F) Western Blot of CFTR co-immunoprecipitates from NS and STa treated jejunum detects increased syntaxin 3 in STa treated jejunum. Equivalent starting protein from tissue lysates were immunoprecipitated using either anti-CFTR or control rabbit IgG antibodies, bound to protein A beads, samples were separated by SDS-PAGE and immunoblotted with anti-syntaxin 3 (Syn3) antibodies. Molecular weight standards (kDa) are indicated.

**Figure 8: GST-pull down assay detects CFTR interaction with syntaxin 3 in rat jejunum.**

200μg of GST or GST+syntaxin 3 (GST-STX 3) were bound to Glutathione Sepharose beads and incubated with rat jejunum lysates. Lysate (Lys) and GST samples were resolved on SDS-PAGE gels and stained with Coomassie Blue (A) or transferred to PDVF nitrocellulose (B) and immunoblotted with anti-CFTR antibody. Molecular mass standards (kDa) are indicated.

**Figure 9. shRNA silencing of syntaxin 3 reduces surface CFTR in cAMP stimulated CaCo-2 BBe cells.**

A) Immunoblot analysis of CaCo-2 BBe cell lysates from control (non-transduced (NT) or scrambled shRNA (SC)) or following transduction with lentiviral particles containing syntaxin 3 (S3). Cells were treated with PBS or 1mMdbcAMP (cAMP) for 30 minutes. Lysates (20μg) were separated on SDS-PAGE gels and analyzed by Western blot using CFTR, syntaxin 3 (Syn3) or β-actin antibodies. Syntaxin3 shRNA reduced syntaxin 3 expression by ~100%. β-actin loading control bands are shown.

B) Surface biotinylated CFTR (+ Biotin) in PBS or cAMP stimulated cells transduced with syntaxin 3 shRNA. Following PBS or 1mMdbcAMP (cAMP) treatment, surface proteins were labeled with sulfo-NHS-SS biotin. Cells were lysed and proteins bound to Streptavidin Agarose. Equivalent biotinylated protein samples (+Biotin) were resolved by SDS-PAGE and immunoblotted to detect CFTR. C) Quantification (mean pixel values) of surface CFTR in control and syntaxin-3 shRNA (S3) transduced
CaCo-2_BBe cells. Data represent +SEM (n>3), * P=0.05. Molecular weight standards (kDa) are indicated.

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Figure 1
Figure 3
Figure 5
Figure 9