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Article

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The CFTR Cl^- channel controls salt and water transport across epithelial tissues. Previously, we showed that CFTR-mediated Cl^- currents in the *Xenopus* oocyte expression system are inhibited by syntaxin 1A, a component of the membrane trafficking machinery. This negative modulation of CFTR function can be reversed by soluble syntaxin 1A peptides and by the syntaxin 1A binding protein, Munc-18. In the present study, we determined whether syntaxin 1A is expressed in native epithelial tissues that normally express CFTR and whether it modulates CFTR currents in these tissues. Using immunoblotting and immunofluorescence, we observed syntaxin 1A in native gut and airway epithelial tissues and showed that epithelial cells from these tissues express syntaxin 1A at >10-fold molar excess over CFTR. Syntaxin 1A is seen near the apical cell surfaces of human bronchial airway epithelium. Reagents that disrupt the CFTR-syntaxin 1A interaction, including soluble syntaxin 1A cytosolic domain and recombinant Munc-18, augmented cAMP-dependent CFTR Cl^- currents by more than 2- to 4-fold in mouse tracheal epithelial cells and cells derived from human nasal polyps, but these reagents did not affect CaMK II–activated Cl^- currents in these cells.

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Introduction

The cystic fibrosis transmembrane conductance regulator (CFTR) is a cAMP-activated Cl^- channel that is localized to the apical membranes of epithelial cells lining the airway, gut, and exocrine glands (1). CFTR is causal in 2 major human diseases: cystic fibrosis (CF) and secretory diarrhea. CF is caused by mutations in the CFTR gene that reduce the synthesis or the functional activity of the CFTR Cl^- channel. This autosomal recessive disorder affects approximately 1 in 2,500 Caucasians in the United States (1). The severest forms of the disease are associated with early onset of *Pseudomonas aeruginosa* infection in airway epithelia and premature death (1). Currently, lung transplantation is the only effective therapy in CF. Other symptoms include pancreatic insufficiency, meconium ileus, and infertility. Whereas reduced CFTR activity causes CF, excessive CFTR activity is implicated in cases of toxin-induced secretory diarrhea (e.g., by cholera toxin and heat stable *Escherichia coli* enterotoxin) that stimulate cAMP or cGMP production in the gut. It is estimated that approximately 12,600 children from Asia, Africa, and Latin America die each day as a result of diarrhea and that the majority of these cases are caused by enterotoxigenic *E. coli* (2).

Recently, we demonstrated that CFTR-mediated Cl^- currents in *Xenopus* oocytes can be inhibited by recombinant syntaxin 1A (3, 4). Syntaxin 1A is highly expressed in the brain, where it regulates synaptic vesicle fusion (5) possibly in concert with Munc-18, a syntaxin 1A–binding protein (6, 7). Syntaxin 1A is also expressed in certain CFTR-expressing colonic carcinoma cell lines (3), although the expression of syntaxin 1A protein in native gut or airway epithelia has not been reported. In addition to regulating vesicle fusion at the synapse, syntaxin 1A has been observed to bind directly to presynaptic Ca^{2+} channels and to modulate the gating of these channels (8). It has been proposed that the binding of syntaxin 1A to Ca^{2+} channels may spatially and temporally couple the exocytotic machinery

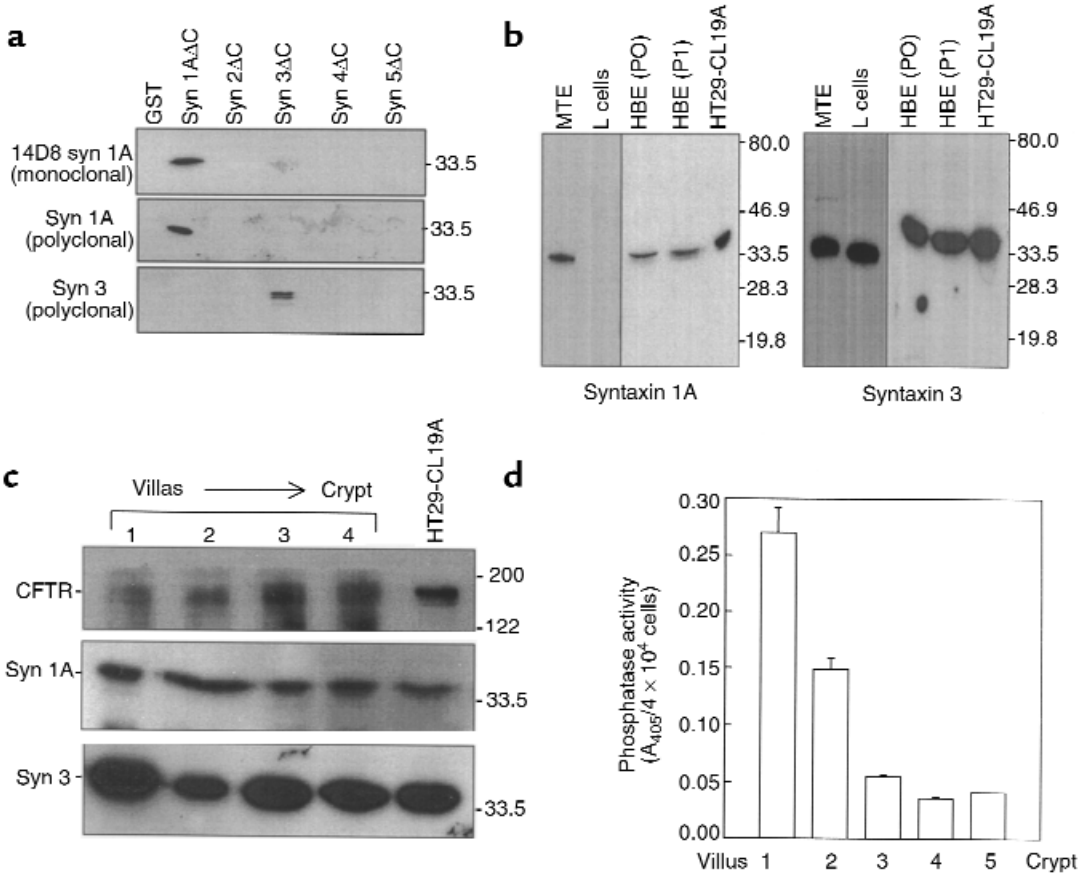

Figure 1

Syntaxin 1A is expressed in epithelial cells that normally express CFTR. (**a**) Isoform specificity of syntaxin 1A and syntaxin 3 antibodies (each lane: 50 ng fusion protein cleaved free of GST by thrombin; ref. 4). (**b**) Expression of syntaxin 1A and syntaxin 3 proteins in MTE cells and HBE cells. The original isolate (P0) and primary culture (P1) of HBE cells were both analyzed for syntaxin protein expression. HT29-CL19A colonic carcinoma cells and mouse L fibroblasts were also analyzed for syntaxin protein expression. Antibodies for **a** and **b** were as follows: 14D8 syntaxin 1A mAb (0.1 μg/mL) and affinity-purified syntaxin 3 polyclonal (0.1 μg/mL). (**c**) Expression of CFTR protein, syntaxin 1A protein, and syntaxin 3 protein in freshly isolated MIE cells. MIE cells were isolated and fractionated as described in the methods. CFTR protein was detected by immunoprecipitation from cell lysate (200 μg protein) as described in Methods. Syntaxin 1A and 3 were detected by immunoblotting cell lysates (50 μg protein each) using 14D8 monoclonal and syntaxin 3 affinity-purified polyclonal antibodies. (**d**) Profile of alkaline phosphatase activity (villus marker) in corresponding fractions.

to Ca^{2+} influx at the synapse (9). Recent evidence that syntaxins may also regulate the activities of epithelial Na^+ channels (ENaC) and plant K^+ and Cl^- channels implies that syntaxins may be relatively general regulators of ion channel activity (10, 11).

The regulation of CFTR Cl^- channels by recombinant syntaxin 1A in *Xenopus* oocytes appears to be mediated in part by a direct protein-protein interaction. The inhibition of CFTR activity and modulation of Ca^{2+} channel gating each requires the membrane anchor of syntaxin 1A (4, 8). Soluble syntaxin 1A peptides and recombinant Munc-18 can reverse the inhibition of CFTR activity by syntaxin 1A in oocytes (3), apparently by blocking the physical interaction between CFTR and membrane-anchored syntaxin 1A. It has been reported recently that syntaxin 1A inhibits CFTR Cl^- channel activity in *Xenopus* oocytes by altering CFTR trafficking to the cell surface (12). However, syntaxin 1A directly binds to the NH_2-terminal tail of CFTR, which is a region that modulates CFTR gating (13). Thus, it is conceivable that this interaction can influence CFTR function at multiple levels, i.e., channel gating and intracellular traffic.

The broad goal of the present study was to determine whether syntaxin 1A is expressed in native epithelial tissues and whether syntaxin 1A regulates CFTR activity in these tissues. Syntaxin 1A has been argued to be a neural-specific protein (14, 15); whether it is expressed in normal gastrointestinal or airway epithelial cells is unknown. Moreover, nearly all the functional data regarding the regulation of CFTR activity by syntaxin 1A are derived from studies in heterologous expression systems such as *Xenopus* oocytes. In this study, we establish that syntaxin 1A is expressed in native epithelial tissues, where it appears to limit the functional activity of the CFTR Cl^- channel.

Methods

Isolation of epithelial cells from mouse trachea and intestine. Mouse intestinal epithelium (MIE) and mouse tracheal epithelium (MTE) cells were isolated from C57

BL/6 mice aged 8–12 weeks. Intestinal epithelial cells from villi tips and crypt portions of mouse intestine were isolated from fragments of small intestine as described previously (16). The villi tip or crypt origin of epithelial cells in each fraction was determined by analysis of alkaline phosphatase activity, which is enriched in villus (Figure 1d). For this purpose, 1.9 mM *p*-nitrophenylphosphate (Sigma Chemical Co., Saint Louis, Missouri, USA) in 50 mM carbonate buffer was added to 5×10^4 cells, and the absorbance was measured for 10 minutes at 405 nm (16).

For the isolation of tracheal cells for Western blotting, MTE were longitudinally opened and subjected to 5 sequential incubations (10 minutes at 37°C) in F12 media. Supernatants were pooled and layered onto discontinuous Percoll gradients made of 75%, 55%, 40%, and 20% Percoll layers. The epithelial cells were collected at the 20-40% Percoll layer interface (16).

Seeding MTE cells and human nasal polyps for patch clamp studies. Primary cells from MTE were prepared and cultured for 2–3 days as described previously (17). Cells were split into 35-mm dishes and cultured 4–6 days before whole-cell patch clamp studies. Nasal polyp specimens were obtained from 2 subjects according to an Institutional Review Board–approved protocol. The methods for obtaining and enzymatically dispersing human airway epithelial cells were adapted from Coleman et al. (18). Dissociated nasal polyp cells were cultured for 6–7 days on 35-mm dishes before patch clamp analysis.

Bronchial airway epithelial cell culture. Primary human bronchial epithelial (HBE) cell cultures were generated as described previously (19) from human bronchial tis-

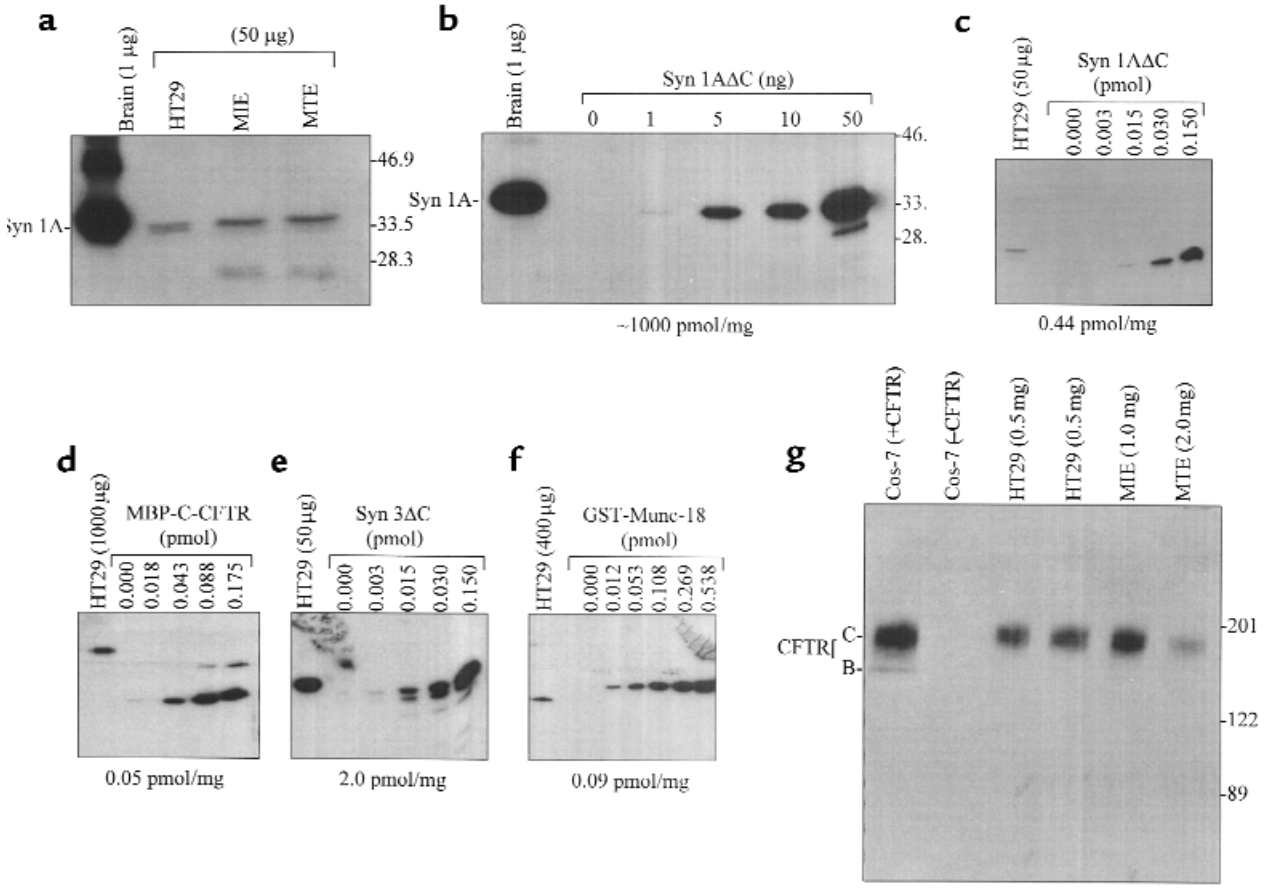

Figure 2

Syntaxin 1A protein is expressed in epithelial cells to a much lesser extent than in brain, but at large mole excess over CFTR. (**a**) Comparison of syntaxin 1A protein expression in rat brain, HT 29-CL19A cells, native MIE cells, and native MTE cells. Monoclonal syntaxin 1A antibody (14D8) was used at 0.1 µg/mL. MIE cells were pooled across Percoll gradient fractions. (**b**) Quantitation of syntaxin 1A protein in rat brain lysate (14D8 mAb) determined from a standard curve generated using the indicated amounts of syn 1AΔC protein cleaved free of GST. (**c**) Quantitation of syntaxin 1A in HT29-CL19A cells. Syn1AΔC was used as a standard and 14D8 mAb for detection. (**d**) Quantitation of CFTR protein in HT29-CL19A cells. MBP-C-CFTR was used as standard (see Methods). Genzyme C-CFTR monoclonal was used at 0.25 µg/mL dilution. (**e**) Quantitation of syntaxin 3 protein in HT29-CL19A cells. Syn 3ΔC (cleaved to remove GST) was used as standard. Affinity-purified syntaxin 3 polyclonal antibody was used for blotting (0.1 µg/mL). (**f**) Quantitation of Munc-18 protein in HT29-CL19A cells. GST-Munc-18 was used as standard, and affinity-purified Munc-18 monoclonal antibody was used for blotting at 0.25 µg/mL. (**g**) Relative amounts of CFTR protein in MIE cells (IP from 1,000 µg lysate), MTE cells (IP from 2,000 µg lysate), and HT29-CL19A cells (IP from 1,000 µg lysate). Cos-7 cells transiently expressing or not expressing recombinant CFTR (3, 4) served as controls (IP from ~0.5 mg total protein). Genzyme C-CFTR mAb was used both for immunoprecipitation and for detection by immunoblotting.

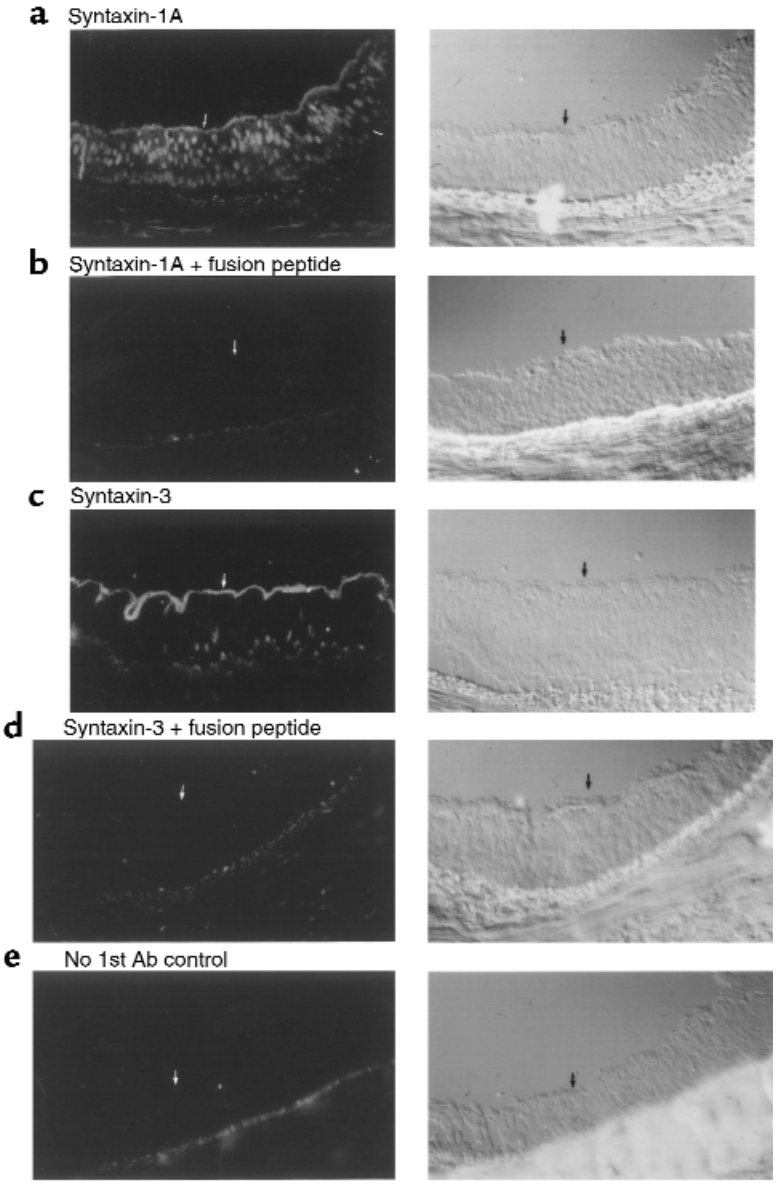

Figure 3
Immunofluorescence localization of syntaxin 1A (**a**) and 3 (**c**) in human bronchus. Nonimmune control lacking primary antibody (**e**) and inhibition of staining by fusion peptide competition (**b** and **d**) are also shown. Arrows represent the apical pole of the bronchial epithelium. Affinity-purified syntaxin 1A and 3 polyclonal antibodies were used at 5 μg/mL. Transmitted light micrographs were generated using Nomarski optics.

sue acquired at the time of lung transplantation. Briefly, surface airway epithelial cells were isolated from bronchial samples by protease XIV digestion and cultured in hormonally defined BEGM media for less than 1 week (Clonetics Inc., San Diego, California, USA). Freshly isolated (P0) and primary cultures (P1) of bronchial surface airway epithelial cells were analyzed for syntaxin 1A and 3 protein by Western blotting.

Antibodies. Syntaxin 1A– and syntaxin 3–specific antibodies were generated by immunizing rabbits with purified GST-syntaxin fusion proteins containing the respective cytoplasmic domains (amino acids 1–266). The antibodies were first subjected to Protein A affinity chromatography and further purified by passing them through a column containing the respective syntaxin fusion protein lacking GST that was immobilized on CNBr-activated Sepharose. The monoclonal syntaxin 1A antibody (14D8) was developed as described earlier (20). Syntaxin 1A is highly conserved across species and shares approximately 93% amino acid identity between mouse and human; therefore, the syntaxin 1A antibodies (both 14D8 and affinity-purified polyclonal) recognize mouse as well as human syntaxin 1A. mAb's to the COOH-terminal tail of CFTR (C-CFTR) were procured from Genzyme Pharmaceuticals (Cambridge, Massachusetts, USA). This antibody recognizes the last 4 amino acids of human CFTR (i.e., DTRL). Murine CFTR varies slightly at the COOH-terminal tail (ETRL), but it is still efficiently immunoprecipitated by this antibody (Figure 1c). Rabbit polyclonal C-CFTR antibody was raised against a synthetic peptide mimicking the last 14 amino acids of human CFTR (1467–1480). Munc-18 antibodies were procured from Transduction Laboratories (Lexington, Kentucky, USA). Horseradish peroxidase–conjugated (HRP-conjugated) secondary antibodies (anti-rabbit and anti-mouse) were from Pierce Chemical Co. (Rockford, Illinois, USA).

Immunoprecipitation and Western blotting. CFTR immunoprecipitations (IPs) were performed in cell lysates (PBS containing 1% Triton X-100 and protease inhibitors: 1 mM PMSF, 1 μg/mL leupeptin, 1 μg/mL pepstatin, and 1 μg/mL aprotinin) using 0.5 μg C-CFTR mAb. Nonimmune mouse IgG (Santa Cruz Biotechnology Inc., Santa Cruz, California, USA) was used as a control. The IgG was cross-linked to protein A/G agarose using 10 mM dimethylpimelimidate (DMP; Pierce Chemical Co.) for 30 minutes at 22°C. Western blotting was performed on proteins transferred to PVDF membrane (BioRad Laboratories, Inc., Hercules, California, USA) and blocked with 5% dry milk. The primary antibody dilution depended on the antibody being used (e.g., anti–C-CFTR monoclonal: 0.25 μg/mL; anti–syntaxin 1A monoclonal: 0.1 μg/mL), and the secondary antibody HRP conjugate was used at 0.02 μg/mL. An enhanced chemiluminescence detection system was used (NEN Life Science Products Inc., Boston, Massachusetts, USA). Signals were quantified by densitometry using recombinant fusion proteins as standards (see later here).

Quantitation of syntaxin, Munc-18, and CFTR protein levels. Syntaxin 1A and 3 protein levels in HT29-CL19A cells (3) were quantitated by immunoblotting 50 μg of

lysate (PBS-1% Triton X-100) and comparing signals to the respective syntaxin standards; i.e., GST-fusion proteins. Purified fusion proteins lacking the COOH-terminal membrane anchor (syn 1AΔC and syn 3ΔC: where ΔC refers to deletion of the 21–23 amino acid membrane anchor) were used over a wide range of concentrations to quantitate the particular syntaxin present in the lysate. To quantitate Munc-18, 10 μg of GST-syn 1AΔC was added to cell lysates containing 200 μg total protein. The complex (GST-syn 1AΔC-Munc-18) was then pulled down using excess glutathione agarose, eluted in SDS-sample buffer, and immunoblotted after SDS-PAGE. We were able to deplete the lysates of Munc 18 by more than 90% by syntaxin 1A pulldown (data not shown), as expected given the high affinity of this interaction (21). Recombinant GST-Munc-18a was used as a standard for quantifying Munc-18 protein levels. CFTR was quantitated by immunoprecipitation from 1 mg of cell lysate (PBS-1% Triton-X-100) using the monoclonal C-CFTR antibody. Under the conditions used, this antibody immunodepletes more than 95% of CFTR (data not shown) from cell lysates. Maltose binding protein-C-CFTR (MBP-C-CFTR; residues 1387–1480) was used as standard. The CFTR protein was run on a 10% gel so that the mature CFTR band could be detected as a sharp band, which could be quantitated by densitometry.

Immunofluorescence localization of syntaxin 1A and 3 in native human bronchus. Immunofluorescence localization of syntaxin 1A and 3 was performed on fresh frozen sections (5 μm) of native human bronchus. Bronchial samples were harvested at the time of lung transplantation and frozen directly in OCT media using liquid nitrogen. Three independent samples were evaluated from nonsmokers undergoing transplantation for chronic obstructive pulmonary disease (COPD). Sections were fixed in –20°C methanol for 30 minutes and post-fixed in 3% paraformaldehyde/PBS for 15 minutes followed by quenching in 50 mM NH_4Cl/PBS for 10 minutes. Slides were blocked in 3% BSA/PBS for 45 minutes at 22°C and incubated with 5 μg/mL of affinity-purified rabbit syntaxin 1A and syntaxin 3 antibodies in 1% BSA/PBS overnight at 4°C. Slides were then blocked in 5% donkey serum/PBS for 45 minutes at 22°C before incubation with FITC-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories Inc., West Grove, Pennsylvania, USA) at a concentration of 25 μg/mL for 45 minutes at 22°C. Competition experiments were performed by preabsorption of each syntaxin antibody with 5 μg/mL of the relevant syntaxin fusion protein before application to tissue section.

Transient expression of membrane-anchored syntaxin 1A in L cells. cRNA coding for full-length, membrane-anchored syntaxin 1A was microinjected into individual attached, nonconfluent L cells expressing CFTR (22) using an Eppendorf 5242 microinjection system (Eppendorf, Hamburg, Germany). Commercially available sterile, RNAse free Femtotips (Eppendorf) were filled with the cRNA solution and manipulated under microscopic observation using an Eppendorf 5171 micromanipulator under remote control. Cells were injected with 0.5% Texas Red diluted with DEPC-treated water for later identification or with dye plus 1 ng/μL syntaxin 1A cRNA approximately 24 hours before use in the electrophysiological studies. Injected cells were readily observable under fluorescent magnification.

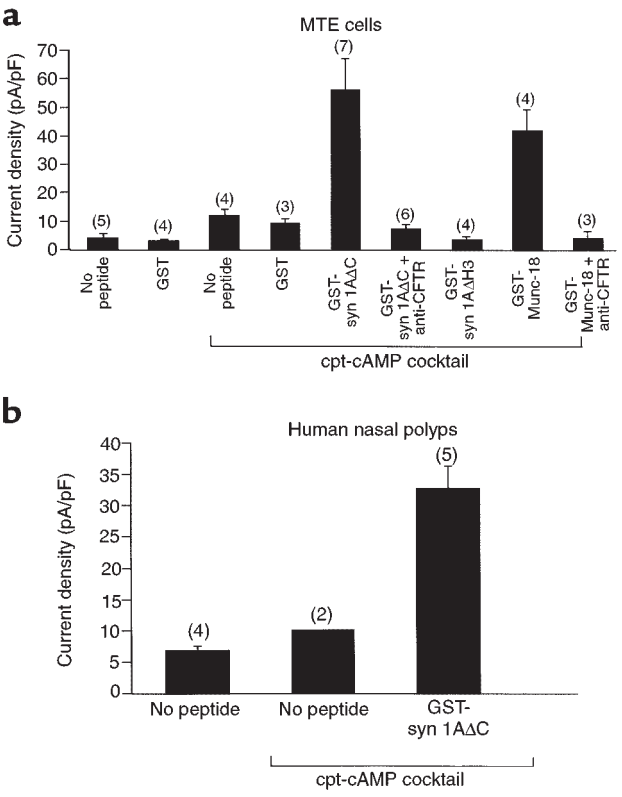

Figure 4
GST-syn 1AΔC and GST-Munc-18 potentiate cAMP-dependent Cl^- currents in MTE cells and human nasal polyps. (**a**) GST-syn 1AΔC (350 nM), GST-Munc-18a (200 nM), and GST alone (750 nM) were included in the patch pipette in the absence or presence of a cAMP-cocktail (50 μM forskolin, 50 μM IBMX, 100 μM cpt-cAMP). The cAMP cocktail was added to the bath 5–10 minutes after seal formation. In some cases, the patch pipette also contained a CFTR neutralizing antibody (24). GST-syn 1AΔH3 (350 nM), which lacks H3 domain of syntaxin 1A (amino acids 194–266) required for CFTR binding (4), had no effect on Cl^- currents. (**b**) GST-syn 1AΔC (350 nM) also potentiates cAMP-mediated Cl^- currents in human nasal polyps. Numbers of experiments are indicated in parentheses. Error bars are SEMs. Holding potential: +110 mV. Currents (pA) are normalized to cell capacitance (pF).

Electrophysiology. Whole-cell patch clamping and data acquisition were carried out according to methods described previously (3, 4, 23). CFTR currents were blocked using a site-directed antibody (0.1 μg/mL) against a synthetic peptide, corresponding to amino acids 505–511 of CFTR, as described previously (24).

Results

Syntaxin 1A is expressed in native airway and intestinal epithelial cells. Multiple isoforms of syntaxin have been described, 2 of which are reportedly brain specific (syn-

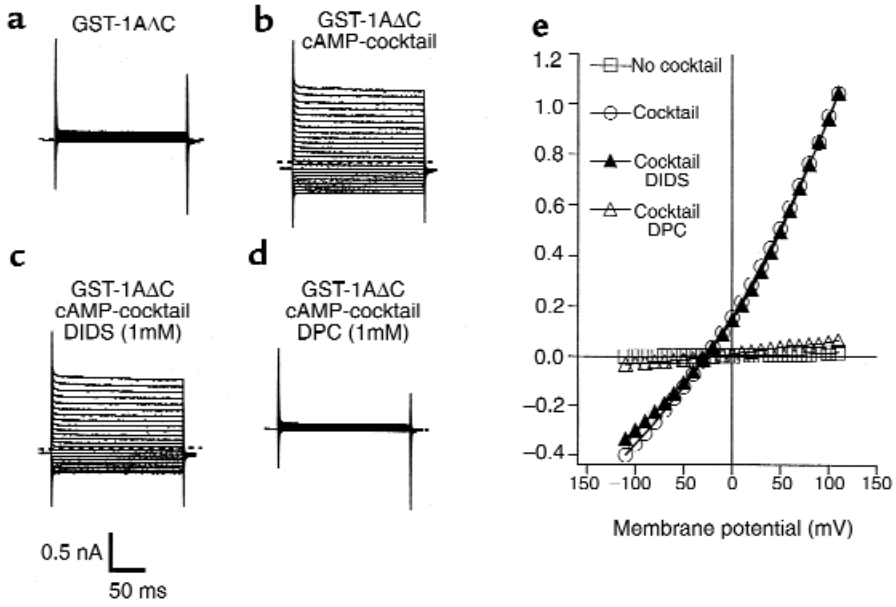

Figure 5
Pharmacology and I–V behavior of Cl^- currents in tracheal epithelial cells potentiated by GST-syn 1AΔC. (**a**) Basal Cl^- currents in presence of GST-syn 1AΔC (350 nM). (**b**) Large Cl^- currents in the presence of GST-syn 1AΔC and cAMP cocktail. (**c**) No effect of DIDS (1 mM) on Cl^- currents in the presence of GST-syn 1AΔC. (**d**) Complete block of GST-syn 1AΔC-potentiated Cl^- currents by DPC (1 mM). (**e**) Current-voltage relationships for **a–d**. All data are for the same cell.

taxin 1A and 1B; ref. 14). In the present study, we observed by immunoblotting that syntaxin 1A is also expressed in native airway and gut epithelial cells. Two syntaxin 1A–specific antibodies were used; an mAb (14D8) and an affinity-purified rabbit polyclonal antibody (see Methods for details). These 2 syntaxin 1A antibodies do not cross react with other syntaxins that we have tested, as shown in Figure 1a (14D8 mAb has been shown not to cross react with syntaxin 1B as well; ref. 20). A syntaxin 3–specific polyclonal antibody (Figure 1a) was also used to verify the expression of this broadly distributed syntaxin isoform in epithelial cells. Syntaxin 1A protein was detected in freshly isolated MTE cells, native HBE cells, and HT29-CL19A colonic carcinoma cells using 14D8 mAb (Figure 1b). The expression of syntaxin 1A protein in these tissues and cells was also detected using the affinity-purified polyclonal antibody (data not shown). Conversely, syntaxin 1A protein was not detected in lysates of mouse fibroblasts (L cells). However, these cells and all others we have tested do express syntaxin 3. Intestinal epithelial cells freshly isolated from mouse small intestine (MIE cells) were also observed to express both syntaxin 1A and 3 protein. Neither syntaxin isoform exhibited a detectable gradient of expression from villus to crypt (Figure 1c) when the isolated intestinal cells were fractionated by Percoll gradient centrifugation and assayed for alkaline phosphatase activity, which is enriched in villus (Figure 1d; ref. 16). Thus, by using 2 different isoform specific antibodies, we were able to detect expression of syntaxin 1A in native airway and intestinal epithelial cells.

Syntaxin 1A is expressed in epithelial cells at much lower levels than in brain but is in molar excess over CFTR. At first glance, these results seem inconsistent with initial reports that syntaxin 1A is highly neural specific (14, 15). To resolve this apparent discrepancy, we quantitated the expression of syntaxin 1A in epithelial cells relative to brain, and then directly compared the expression of syntaxin 1A to that of CFTR in the same epithelial cell type. Syntaxin 1A is expressed abundantly in the brain when compared with MIE or MTE cells (Figure 2a). In rat brain lysate (SDS-detergent extract), syntaxin 1A constitutes approximately 1% (wt/wt) of the total detergent extractable protein (Figure 2b), as determined by quantitative immunoblotting using rat syntaxin 1A fusion protein as a standard. On the other hand, syntaxin 1A comprises only about 0.002% of total detergent-extractable cell protein (wt/wt) in HT29-CL19A colonic epithelial cells (Figure 2c), MIE cells, and MTE cells (data not shown). The MTE lysates tested negative for synaptophysin, a synaptic marker, which indicates that these cells were not contaminated with nerve endings in the process of isolation (data not shown).

Whereas syntaxin 1A is expressed at low levels in epithelial cells relative to brain, it is nonetheless at considerable excess over CFTR in epithelial cells (Figure 2, c–f). With the use of quantitative immunoblotting, we determined that the amounts of syntaxin 1A and CFTR in HT29-CL19A cells correspond to approximately 0.44 pmol/mg and 0.05 pmol/mg of total protein, respectively (Figure 2, c and d). Syntaxin 3 and Munc-18 protein levels in HT 29-CL19A cells were similarly quantitated (Figure 2, d and e). Syntaxin 3 is considerably more abundant than syntaxin 1A in HT29-CL19A cells as well as in T84 colonic epithelial cells and native MIE cells (data not shown). We also attempted to estimate the relative amounts of syntaxin 1A and CFTR in MTE cells. We were able to detect syntaxin 1A protein in freshly isolated MTE cells, where the protein amount was comparable to that found in MIE cells (Figure 2a). However, the CFTR protein amount was considerably lower in the MTE cells compared with the MIE cells (Figure 2g). This is consistent with previous reports that CFTR is more abundant in gut than in airway epithelial cells (25) and indicates that syntaxin 1A is at an even greater excess over CFTR in MTE cells.

Syntaxin 1A resides at the apical poles of airway epithelial cells. To analyze the distribution of syntaxin 1A and 3 in epithelial cells, we immunolocalized these isoforms in sections of native human bronchus using affinity-purified syntaxin 1A and syntaxin 3 antibodies. Specific syntaxin 1A staining was observed at or near the apical cell surface as well as in numerous intracellular puncta (Figure 3). Syntaxin 3 staining was more intense, and the distribution was almost exclusively at or near the apical cell surface. Nonimmune controls were negative for staining. Importantly, syntaxin 1A staining at the apical pole could be blocked by competition with excess GST-syn 1AΔC fusion protein (Figure 3), but not by GST-syn 3ΔC fusion protein (data not shown). Conversely, syntaxin 3 staining could be blocked by excess GST-syn 3ΔC, but not by GST-syn 1AΔC. We also observed a punctate pattern of staining for syntaxin 1A at the apical surfaces of HT29-CL19A cells (results not shown). These results confirm that syntaxins 1A and 3 are expressed in HBE cells and that they have partially overlapping distributions in the vicinity of the apical plasma membrane.

Syntaxin 1A modulates CFTR function in airway epithelial cells. Given that native epithelial cells express syntaxin 1A in addition to CFTR, we determined whether CFTR activity is modulated by syntaxin 1A in airway epithelial cells. Toward this end, we used 2 reagents that block the physical interaction between CFTR and syntaxin 1A and were previously documented to rescue CFTR from syntaxin 1A inhibition in *Xenopus* oocytes (3, 4); namely, the cytosolic domain of syntaxin 1A as a GST fusion protein (GST-syn 1AΔC) and the syntaxin 1A binding protein, Munc-18 (GST-Munc 18a). Both reagents markedly potentiated cAMP-dependent chloride currents in primary cultures of MTE cells when introduced into the cells through whole-cell patch pipettes (Figure 4a), as expected if CFTR activity is limited by its interaction with native syntaxin 1A. The addition of a cAMP cocktail increased the current amplitude at +110 mV by about 2-fold in the absence of peptide and by 6- to 10-fold in the presence of either GST-syn 1AΔC or GST-Munc-18. Basal currents in the absence of cAMP were not affected by either reagent. In addition, control peptides that do not bind to CFTR or affect CFTR currents in *Xenopus* oocytes (GST and GST-syn 1AΔH3, which lacks the CFTR binding domain [H3]; ref. 4) had no effect on cAMP-dependent current amplitude. The large cAMP-dependent currents that were potentiated by GST-syn 1AΔC or GST-Munc-18 reached a maximum in 5–10 minutes after exposure of the cell to cAMP cocktail (see Figure 4 legend). The potentiation of Cl^- currents by GST-syn 1AΔC and GST-Munc-18 could be completely inhibited by a CFTR-neutralizing antibody (Figure 4a). In a limited number of experiments, we also observed that GST-syn 1AΔC could stimulate cAMP-dependent Cl^- currents in human nasal polyp cells (Figure 4b). These cAMP-mediated currents in nasal polyp cells could also be completely blocked by CFTR-neutralizing antibody (data not shown).

To verify further that the large currents in MTE cells that were induced by GST-syn 1AΔC were CFTR mediated, we characterized the I–V relationship and inhibitor sensitivity of these currents (Figure 5). The large Cl^- currents in the presence of GST-syn 1AΔC were not affected by DIDS, which blocks a number of Cl^- channels other than CFTR (26). Conversely, diphenylamine carboxylate (DPC), which does block CFTR Cl^- channels (27), completely inhibited the currents that were potentiated by GST-syn 1AΔC (Figure 5). The I–V relationship in the presence of either reagent was also approximately linear, which is a hallmark of CFTR Cl^- channels (28). In combination with the blocking effect of the CFTR-neutralizing antibody on the syntaxin 1A–modulated currents (Figure 4a), these results confirm that the large cAMP-dependent currents in MTE cells that are potentiated by GST-syn 1AΔC are indeed mediated by CFTR.

We next determined whether the functional effects of the syntaxin 1A peptide on CFTR currents in MTE cells could be explained by the disruption of an inter-

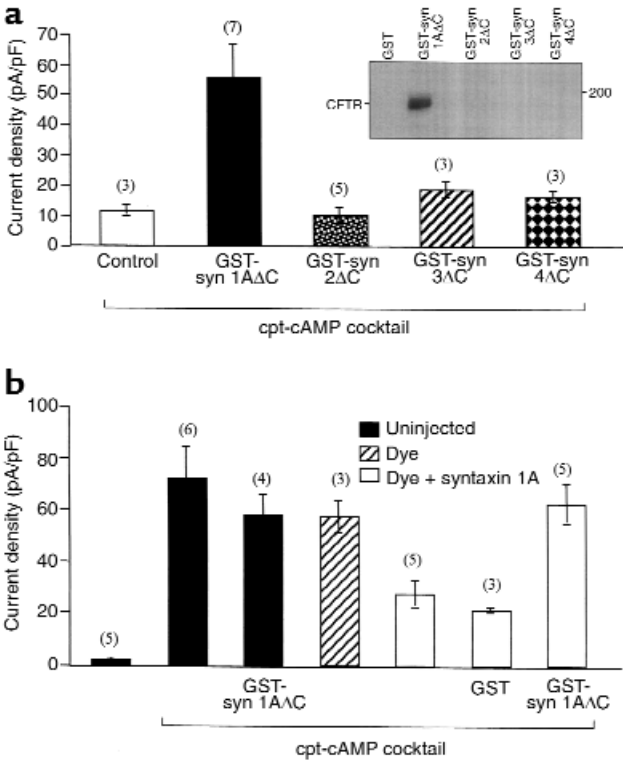

Figure 6
Mouse CFTR Cl^- channels physically and functionally interact with syntaxin 1A in an isoform-specific manner. (**a**) CFTR Cl^- current activity in MTE cells is potentiated by GST-syn 1AΔC but not by other syntaxin fusion proteins (350 nM). Inset shows the binding of mouse CFTR to mouse syn 1AΔC in a pulldown assay performed as described elsewhere (3, 4). (**b**) GST-syn 1AΔC (350 nM) does not potentiate CFTR Cl^- current activity in L cells stably expressing recombinant CFTR (black bars). Transient expression of membrane-anchored syntaxin 1A reduces CFTR Cl^- current, which can be reversed by GST-syn 1AΔC fusion protein but not GST alone (750 nM). Numbers of experiments are indicated in parentheses. Holding potential: + 110 mV.

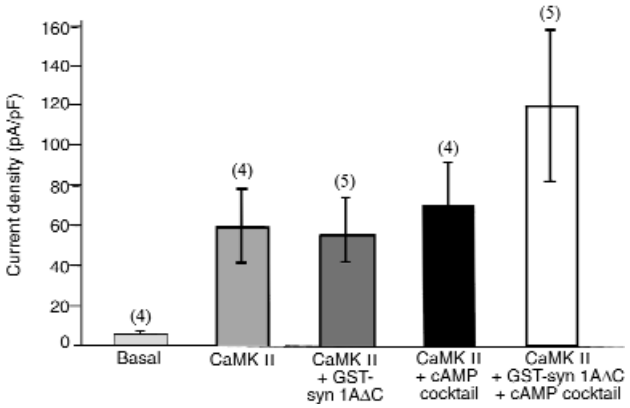

Figure 7
Syntaxin 1A does not influence Cl^- currents in MTE cells induced by CaMK II. Cl^- currents were activated by inclusion of 50 ng CaMK II in the pipette in the presence or absence of 350 nM GST-syn 1AΔC (refer to bars). In some cells, currents were activated with cAMP cocktail with or without CAMK II and GST-syn 1AΔC. Numbers of experiments are indicated in parentheses. Holding potential: +110 mV.

action between CFTR and native syntaxin 1A. In this regard, the physical interaction between human CFTR and syntaxin 1A was previously determined to be syntaxin 1A–isoform specific (3), unlike most other syntaxin binding interactions that do not discriminate between isoforms (29). Figure 6a shows the results of a pulldown assay that indicate that mouse CFTR like human CFTR also binds to syntaxin 1A in a highly isoform-specific manner (Figure 6, inset). In addition, of the 4 syntaxin peptides that were tested for CFTR binding and for effects on CFTR currents, only the syntaxin 1A peptide substantially potentiated CFTR activity in MTE. Moreover, Figure 6b shows that the syntaxin 1A peptide had no effect on the Cl^- currents mediated by recombinant CFTR in mouse L cells that do not express syntaxin 1A (see Figure 1b). However, this syntaxin 1A peptide did stimulate CFTR-mediated chloride currents in L cells that were microinjected with recombinant cRNA coding for full-length syntaxin 1A. Thus, the simplest interpretation of our peptide data is that GST-syn 1AΔC stimulates CFTR-mediated Cl^- currents in MTE cells by competing with native, membrane-anchored syntaxin 1A for binding to CFTR.

Syntaxin 1A does not regulate CaM Kinase II–activated Cl^- currents in airway epithelial cells. Epithelial cells also exhibit a calcium-calmodulin–dependent protein kinase II–regulated (CaMK II–regulated) chloride conductance that is not mediated by CFTR. To determine the specificity of the effects of the syntaxin 1A peptide on Cl^- currents in airway epithelial cells, we also tested the effects of this reagent on CaMK II–activated Cl^- currents (Figure 7). CaMK II, when introduced into the cell via the patch pipette, increased the Cl^- current amplitude in MTE cells similar to that previously reported for colonic epithelial cells (30). The CaMK II–activated currents were not affected by inclusion of GST-syn1AΔC in the pipette. However, the currents did tend toward higher levels in the presence of GST-syn 1AΔC when the cells were activated with CaMK II plus the cAMP cocktail, as expected if CaMK II and cAMP activate parallel Cl^- conductances. These results indicate that the effects of GST-syn 1AΔC in airway epithelial cells are specific for CFTR-mediated chloride currents.

Discussion

Previous studies have shown that syntaxin 1A regulates CFTR chloride channels and epithelial sodium channels (ENaCs) when these proteins are expressed in *Xenopus* oocytes (3, 4, 10, 12). Although those studies have provided evidence that syntaxin 1A has the capacity to regulate CFTR and ENaC under certain conditions, they do not speak to the issue of whether syntaxin 1A modulates ion channel function in native epithelial tissues that are targets for diseases such as cystic fibrosis (CFTR), secretory diarrhea (CFTR), and Liddle's syndrome (ENaC). This issue is particularly significant given that syntaxin 1A was originally reported to be neural specific (14, 15). The present results indicate that syntaxin 1A protein is expressed in cells that normally express CFTR; namely, native MTE cells, MIE cells, and HBE cells. The amount of syntaxin 1A protein expressed in these native epithelial cells is much lower than in brain, which probably explains why some groups have difficulty in detecting this protein by immunoblotting or by immunofluorescence in colonic epithelial cells lines such as CaCo-2 (ref. 31; although we have detected syntaxin 1A expression in CaCo-2 cells as well [data not shown]). We have confidence in these data as we have used at least 6 different antibodies to detect syntaxin 1A protein in epithelial cells, of which the 2 described in this study are highly isoform specific (Figure 1a). Importantly, although syntaxin 1A is present in relatively low abundance in the gut and trachea compared with in brain, it is nonetheless considerably more abundant than CFTR in epithelial cells. In particular, gut and airway epithelial cells express syntaxin 1A protein at a greater than 10-fold molar excess over CFTR.

Our immunofluorescence results place syntaxin 1A at the apical poles of HBE cells, although this protein was also detected in multiple compartments within the cell. Syntaxin 1A is also rather broadly distributed in neurons, where it has been observed along the axon and in endosomes and synaptic vesicles (32). CFTR protein has also been localized to multiple intracellular compartments including endosomes as well as the apical membrane (33). The intracellular localization of syntaxin 1A and CFTR raises the possibility that these proteins could interact in intracellular compartments as well as at the apical surface. In contrast to syntaxin 1A, syntaxin 3 protein was more precisely localized to the apical cell surface. A very similar pattern has been reported for syntaxin 3 in other epithelial cells such as MDCK cells (34) and CaCo2 colonic epithelial cells (35), although

there is 1 report that this syntaxin isoform is localized to the basolateral surfaces of renal collecting duct cells (36). Interestingly, syntaxin 3 does not appear to directly interact with CFTR or to regulate CFTR Cl^- currents (Figure 6a), in spite of the fact that it is more abundant than syntaxin 1A and is more precisely localized to the apical membranes of airway and gut epithelial cells.

The results of our functional studies indicate that syntaxin 1A downregulates CFTR activity in airway epithelial cells. CFTR-mediated chloride currents were stimulated by 2 reagents that rescue CFTR from inhibition by membrane anchored syntaxin 1A in *Xenopus* oocytes (3, 4) and mouse L fibroblasts (Figure 6b). These reagents perturb the interaction between CFTR and membrane-anchored syntaxin 1A by different mechanisms. Munc-18 blocks the regulation of CFTR currents by binding to native syntaxin 1A and inhibiting the physical interaction between these proteins. GST-syn 1AΔC appears to have a dominant negative effect on this interaction by competing with native syntaxin 1A for CFTR. The potentiation of CFTR-mediated chloride currents in mouse tracheal epithelial cells by the syntaxin 1A peptide was highly isoform specific, as was the binding of this peptide to mouse CFTR (Figure 6a). This agrees with our earlier biochemical evidence for an isoform-specific binding interaction between syntaxin 1A and the NH_2-terminal cytoplasmic tail of human CFTR (ref. 3; the NH_2-terminal tails of mouse and human CFTR are 80% identical). In contrast, syntaxins generally interact with broad specificity with other components of the membrane traffic machinery (29); thus, it seems unlikely that the specific effect of the syntaxin 1A peptide on CFTR activity occurred through some generic effect on membrane trafficking. However, this does not exclude the possibility that the intracellular traffic of CFTR is more specifically influenced by its physical interaction with syntaxin 1A. For example, Peters et al. (12) have recently argued that syntaxin 1A (but not syntaxin 3) inhibits CFTR delivery to the plasma membranes of *Xenopus* oocytes. To what extent syntaxin 1A regulates CFTR activity in epithelial cells by this mechanism or by effects on channel gating is unknown.

In addition to regulating CFTR, syntaxin 1A also appears to regulate presynaptic calcium channels and the physical coupling of these channels to the exocytotic machinery. A plant syntaxin has also been identified on the basis of its ability to regulate K+ and Cl^- channels in guard cells (11). Moreover, syntaxin 1A reportedly inhibits ENaC currents in *Xenopus* oocytes, possibly by affecting the gating properties of this sodium channel (10). On the other hand, it is clear from the present results and from previous oocyte expression studies that there are numerous other ion channels that are not affected by syntaxin 1A (3, 10, 12). For example, we observed no effect of GST-syn1AΔC on the CaMK II–activated Cl^- currents in mouse tracheocytes. Conceivably, there exists a subset of ion channels that physically and functionally interact with syntaxins as a means to coordinate the regulation of ion transport and membrane traffic in certain tissues.

The present results set the stage for determining whether syntaxin 1A regulates the activity of wild-type CFTR or disease-associated mutants in intact animals. Cystic fibrosis can be caused by 1 of more than 800 different CFTR mutations, many of which lead to partial-loss-of-function phenotypes. Conceivably, the functional activity of certain disease-associated mutants could be augmented by reagents that block their interaction with syntaxin 1A in epithelial cells. The results of the present study indicate that the development of cell permeant reagents that could block the CFTR-syntaxin 1A interaction in vivo is worth considering as an alternative approach to stimulating mutant CFTR activity.

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