Molecular Biology of the Cell
Vol. 21, 1178–1187, April 1, 2010

Syntaxin 6 and CAL Mediate the Degradation of the Cystic Fibrosis Transmembrane Conductance Regulator

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Submitted March 20, 2009; Revised January 21, 2010; Accepted January 27, 2010
Monitoring Editor: Adam Linstedt

The PDZ domain–containing protein CAL mediates lysosomal trafficking and degradation of CFTR. Here we demonstrate the involvement of a CAL-binding SNARE protein syntaxin 6 (STX6) in this process. Overexpression of STX6, which colocalizes and coimmunoprecipitates with CAL, dramatically reduces the steady-state level and stability of CFTR. Conversely, overexpression of a STX6 dominant-negative mutant increases CFTR. Silencing endogenous STX6 increases CFTR but has no effect on ΔTRL-CFTR, which cannot bind to CAL. Silencing CAL eliminates the effect of STX6 on CFTR. Both results suggest a dependence of CAL on STX6 function. Consistent with its Golgi localization, STX6 does not bind to ER-localized ΔF508-CFTR. Silencing STX6 has no effect on ΔF508-CFTR expression. However, overexpression of STX6 coimmunoprecipitates with and reduces temperature-rescued ΔF508-CFTR that escapes ER degradation. Conversely, silencing STX6 enhances the effect of low temperature in rescuing ΔF508-CFTR. Finally, in human bronchial epithelial cells, silencing endogenous STX6 leads to increases in protein levels and Cl^- currents of both wild-type and temperature-rescued CFTR. We have identified STX6 as a new component of the CAL complex that regulates the abundance and function of CFTR at the post-ER level. Our results suggest a therapeutic role of STX6 in enhancing rescued ΔF508-CFTR.

INTRODUCTION

The cystic fibrosis transmembrane conductance regulator (CFTR) is a member of the ATP-binding cassette transporter superfamily that functions as a cAMP-dependent Cl^- channel and as a regulator for Cl^-, Na^+, HCO^-, and water transport across the apical membrane of epithelial cells (Kerem *et al.*, 1989; Riordan *et al.*, 1989; Schwiebert *et al.*, 1999). Loss of CFTR function, expression, or apical localization leads to CF, characterized by abnormal electrolyte homeostasis in multiple epithelial tissues such as lung and pancreatic ducts (Pilewski and Frizzell, 1999; Boucher, 2007). The C-terminus of CFTR contains a type I PDZ-binding motif whose interaction with PDZ proteins plays an important role in its function, localization, and protein expression (Guggino, 2004; Lamprecht and Seidler, 2006).

Four PDZ domain proteins (NHERF-1, NHERF-2, PDZK1, and CAL) interact with the carboxyl-tail of CFTR (Short *et al.*, 1998; Wang *et al.*, 1998; Sun *et al.*, 2000; Wang *et al.*, 2000; Cheng *et al.*, 2002). Among those four proteins, NHERF-1 is the most thoroughly characterized. It anchors CFTR to the actin cytoskeleton via the ERM (ezrin, radixin, and merlin) domain and influences the mobility of CFTR at the plasma membrane (Short *et al.*, 1998; Haggie *et al.*, 2004). NHERF-1 acts as a scaffold protein linking CFTR to the β2-adrenergic receptor (Naren *et al.*, 2003), SLC26T (Ko *et al.*, 2004), and ROMK (Yoo *et al.*, 2004). PDZK1 links CFTR to the multidrug resistance protein 4 (MRP4), which transports cAMP (Li *et al.*, 2007). In addition, both recombinant NHERF-1 and PDZK1 modulate CFTR channel activity (Wang *et al.*, 2000; Raghuram *et al.*, 2001). Interaction of CFTR with PDZ domain proteins may also play a role in the polarization of CFTR in epithelial cells (Moyer *et al.*, 1999; Benharouga *et al.*, 2003), in multimerization of CFTR (Ramjeesingh *et al.*, 2003; Li *et al.*, 2004), and in cytokine secretion (Estell *et al.*, 2003).

CAL is the only CFTR-binding PDZ protein that is localized at the Golgi and in Golgi-derived vesicles. It is known to reduce cell surface CFTR, retain CFTR in the cell, and promote CFTR degradation in the lysosome (Cheng *et al.*, 2002, 2004). CAL also plays a role in the degradation of CLC-3B (Gentzsch *et al.*, 2003) and in inhibiting β1-adrenergic receptor surface expression (He *et al.*, 2004) and mGluR1a activity (Zhang *et al.*, 2008). When CAL is over expressed, it reduces the surface expression of CFTR (Cheng *et al.*, 2002). The CAL-induced reduction in CFTR expression at the plasma member can be reversed by NHERF-1 overexpression in a dose-dependent manner, suggesting that NHERF-1 competes with CAL for binding to the same PDZ motif (Cheng *et al.*, 2002). Recently, silencing of CAL or overexpression of NHERF-1 has been shown to restore function to mutant ΔF508-CFTR (Guerra *et al.*, 2005; Wolde *et al.*, 2007). Interestingly, the carboxyl terminal PDZ-binding motif of CFTR plays a role in the sorting of rapidly endocytosed CFTR either back to the cell surface or alternatively to the lysosome (Swiatecka-Urban *et al.*, 2002). TC10, a Rho family small GTPase, binds to CAL. The constitutively active form of TC10 dramatically increases both total levels and the plasma membrane expression of CFTR by keeping CFTR away from lysosomal degradation (Cheng *et al.*, 2005). One hypothesis is that CAL is an adaptor protein that couples CFTR to the trafficking machinery that routes CFTR either to

This article was published online ahead of print in *MBoC in Press* (http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E09–03–0229) on February 3, 2010.

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Abbreviations used: CAL, CFTR-associated ligand; CFTR, cystic fibrosis transmembrane conductance regulator; CHC, clathrin heavy chain; ER, endoplasmic reticulum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescent protein; GLUT4, glucose transporter 4; HA, hemagglutinin; SNARE, *N*-ethylmaleimide–sensitive factor attachment protein receptor; STX6, 8, and 16, syntaxins 6, 8, and 16; siRNA, short interfering RNA.

the plasma membrane or to the lysosome, with the final post-ER processing of CFTR conferred ultimately by CAL's binding partners.

In this study, we investigate the role of another CAL-interacting protein STX6 in CFTR trafficking (Charest *et al.*, 2001). Syntaxin 6 (STX6) is a member of the Q_{bc}-type *N*-ethylmaleimide–sensitive factor attachment protein receptor (Q_{bc}-SNARE) protein family (Wendler and Tooze, 2001). The *trans*-Golgi network (TGN) and endosomally localized STX6 plays an important role in protein trafficking between the TGN and the endosomal system (Chao *et al.*, 1999; Mallard *et al.*, 2002; Perera *et al.*, 2003; Kuliawat *et al.*, 2004). It is involved in a membrane-trafficking step that sequesters Glut4 away from the plasma membrane (Perera *et al.*, 2003). STX6 is required for sorting of proteins from endosomes toward either the TGN or lysosomes (Kuliawat *et al.*, 2004). Recently, STX6 was also implicated in caveolar endocytosis and the recycling of insulin-responsive amino-peptidase (IRAP) from the plasma membrane back to the insulin-responsive compartment (Choudhury *et al.*, 2006; Watson *et al.*, 2008). The question raised here is whether STX6 by binding to CAL can also influence the post-ER fate of CFTR.

In this article, we show that STX6 colocalizes with CAL at perinuclear compartments and coimmunoprecipitates with CAL and CFTR. Manipulation of STX6 abundance and function by multiple methods, including overexpression, a dominant-negative mutant, and small interfering RNA (siRNA) silencing in multiple cell lines including HEK293, Hela, and the CF bronchial epithelial cell line CFBE14o$^-$ stably expressing either wild-type (wt)-CFTR or ΔF508-CFTR defined its role as a negative regulator of the stability and function of wt-CFTR and of the temperature-rescued ΔF508-CFTR.

MATERIALS AND METHODS

Cell Culture and Chemicals

Human embryonic kidney cells (HEK293, obtained from American Type Tissue Culture, Manassas, VA) were maintained in DMEM:F12 (1:1), 20 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 5% fetal calf serum. Hela cells stably expressing ΔF508-CFTR (Hela-ΔFCFTR, gift from Dr. J. P. Clancy, University of Alabama at Birmingham) were maintained in DMEM, 20 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 μg/ml puromycin, and 10% fetal calf serum. CFBE41o$^-$ cells stably expressing ΔF508-CFTR (CFBE-ΔF508CFTR, gift from Dr. J. P. Clancy) were maintained in MEM, 20 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 μg/ml puromycin, and 10% fetal calf serum. CFBE41o$^-$ cells stably expressing wt-CFTR (CFBE-CFTR; gift from Dr. J. P. Clancy) were maintained in MEM, 20 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 μg/ml puromycin, and 10% fetal calf serum. Media and other components were purchased from Invitrogen (Carlsbad, CA). HEK293 and Hela-ΔFCFTR cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Unless indicated, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO). E-64 was purchased from Roche Applied Science USA (Indianapolis, IN).

Plasmids and Plasmid Constructions

The hemagglutinin (HA)-tagged STX6 (HA-STX6) was made by PCR amplification of human lung cDNA and subcloning into SalI/NotI sites of the HA-tagged pRK5 vector (pRK5- HA; gift from Drs. A. A. Lanahan and P. F. Worley, The Johns Hopkins University). The dominant-negative form of STX6 (HA-STX6ΔC) was constructed with the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Full-length human syntaxin 8 and 16 (STX8 and STX16) in pCMV-SPORT6 was obtained from the Open Biosystems (Huntsville, AL). Vti1b cDNA was obtained from Origene (Rockville, MD). HA-tagged constructs were made by PCR amplification of CDNA and subcloning into SalI/NotI sites of the HA-tagged PRK5 vectors. pCMV-CFTR was a gift from Dr. G. R. Cutting (The Johns Hopkins University). All of the constructs were sequence-verified in both directions by automated fluorescent sequencing (The Johns Hopkins University Biosynthesis and Sequencing Facility). The other plasmid constructs used, GFP-CFTR, GFP-CFTRΔF508, GFPCFTRΔTRL, and myc-CAL, were described previously (Cheng *et al.*, 2002).

siRNA Silencing

Annealed, double-stranded siRNA was transfected into HEK293, CFBE-CFTR, and CFBE-ΔF508CFTR cells using HiPerFect transfection reagent (Qiagen, Valencia, CA) using protocol that was previously described (Wolde *et al.*, 2007). CAL siRNA, Hs_GOPC_3_HP siRNA; STX6 siRNA, Hs_STX6_2_HP siRNA (similar effects were observed with Hs_STX6_6_HP siRNA and Hs_STX6_10_HP siRNA); STX8 siRNA, Hs_STX8_3_HP siRNA (similar effects were observed with Hs_STX8_4_HP siRNA and Hs_STX8_6_HP siRNA); STX16 siRNA, Hs_STX16_6 HP siRNA (similar effects were observed with Hs_STX16_7_HP siRNA and Hs_STX16_8_HP siRNA), and negative control siRNA, AllStars Neg. control siRNA were purchased from Qiagen. Twenty-four hours after siRNA transfection, DNA plasmids were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Immunoblotting

The cells were harvested and processed as described previously (Cheng *et al.*, 2002). Briefly, cells were solubilized in lysis buffer (50 mM NaCl, 150 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, and Complete protease inhibitor; Roche Applied Science). The cell lysates were spun at 14,000 rpm for 15 min at 4°C in a microcentrifuge (Eppendorf, Fremont, CA) to pellet insoluble material. The protein concentrations of the supernatants were quantified with a BCA protein assay kit (Thermo Scientific, Rockford, IL). The normalized supernatants were subjected to SDS-PAGE on 5% or 4–15% Ready-gel (Bio-Rad, Hercules, CA) and Western blotting, followed by ECL (Amersham Biosciences, Pittsburgh, PA). The chemiluminescence signal on the polyvinylidene difluoride membrane was directly captured by a FujiFilm LAS-1000 plus system (Stamford, CT) with a 1,300,000-pixel cooled CCD camera having a linearity of 3.7 orders of magnitude. Quantification was carried out within the linear range using the Image Gauge version 3.2 software (FujiFilm). STX6 was detected with a mouse mAb (1:1000, BD Transduction Laboratories, Lexington, KY) or a rabbit polyclonal antibody (1:2000, Synaptic Systems, Göttingen, Germany). STX16 was detected with a rabbit polyclonal antibody (1:1000, Synaptic Systems). STX8 was detected with a mouse mAb (1:1000, BD Transduction Laboratories). CFTR was detected with mouse mAb M3A7 (1:1000, Upstate Biotechnology, Lake Placid, NY) or 217 (1:2000, University of North Carolina, Chapel Hill, NC). Green fluorescent protein (GFP)-tagged proteins were detected with a rabbit polyclonal anti-GFP antibody (1:1000; BD Biosciences, Boston, MA). HA-tagged proteins were detected with a mouse monoclonal HA antibody (1:2000; Roche Applied Science) or a rabbit polyclonal HA antibody (1:500; Roche Applied Science). Myc-tagged proteins were detected with a mouse monoclonal Myc antibody (1:2000; Roche Applied Science). Tubulin was detected with a mouse monoclonal tubulin antibody (1:1000; Sigma). GAPDH was detected with a mouse mAb (1:5000; United States Biological, Swampscott, MA). Clathrin heavy chain was detected with a mouse monoclonal tubulin antibody (1:2000; BD Biosciences).

Confocal Microscope

Cells were placed on glass coverslips 1 d before transfection. Cells were fixed in 4% paraformaldehyde and permeabilized in 0.2% Nonidet P-40 1 d after transfection. Nonspecific binding sites were blocked with 5% normal goat serum. The cells were stained with primary antibodies in 5% normal goat serum. STX6 was detected with a mouse mAb (1:500, BD Transduction Laboratories). CFTR was detected with mouse mAb M3A7 (1:500, Upstate Biotechnology). CAL was detected with a rabbit polyclonal antibody (1:1000). Cells were then washed with 1% bovine serum albumin and incubated with goat Cy3-conjugated secondary antibodies (1:200; Jackson ImmunoResearch, West Grove, PA) or Alex488-conjugated secondary antibodies (1:200, Invitrogen) in 1% normal goat serum. DAPI (Invitrogen) was used to stain the cell nuclei. The specimens were washed, mounted, and viewed on LSM510-Meta laser confocal microscope (Zeiss, Thornwood, NY).

Short-Circuit Current Measurements

Short-circuit current (I_{sc}) measurements were performed in six-channel Easy-Mount chambers system (Physiologic Instruments, San Diego, CA) that accepts Snapwell filters (Corning Costar, Acton, MA; 3407). I_{sc} was measured with a VCCMC6 multichannel voltage-current clamp amplifier (Physiologic Instruments) in the voltage-clamp mode. Data were acquired on a 1.71-GHz PC running Windows XP (Microsoft, Redmond, WA) equipped with DI-720 (DATAQ Instruments, Akron, OH) with software Acquire and Analyze version 2.3.159 (Physiologic Instruments). Cells were cultured to confluence on Snapwell filters before measurement. The cell monolayers were bathed on both sides with a solution containing (in mM): 115 NaCl, 25 Na-gluconate, 5 K-gluconate, 1.2 $MgCl_2$, 1.2 $CaCl_2$, 10 D-glucose, and 10 HEPES (pH 7.4 with NaOH). The mucosal side was replaced with a low Cl^- solution containing (in mM): 139 Na-gluconate, 1.2 NaCl, 5 K-gluconate, 1.2 $MgCl_2$, 1.2 $CaCl_2$, 10 D-glucose, and 10 HEPES (pH 7.4 with NaOH). The solution was constantly circulated, maintained at 37°C, and bubbled gently with air. Amiloride (10 μM) was added to the mucosal solution, and after stabilization, forskolin or genistein was added to the serosal chamber followed by the CFTR channel inhibitor $CFTR_{inh}$-172 (1 μM).

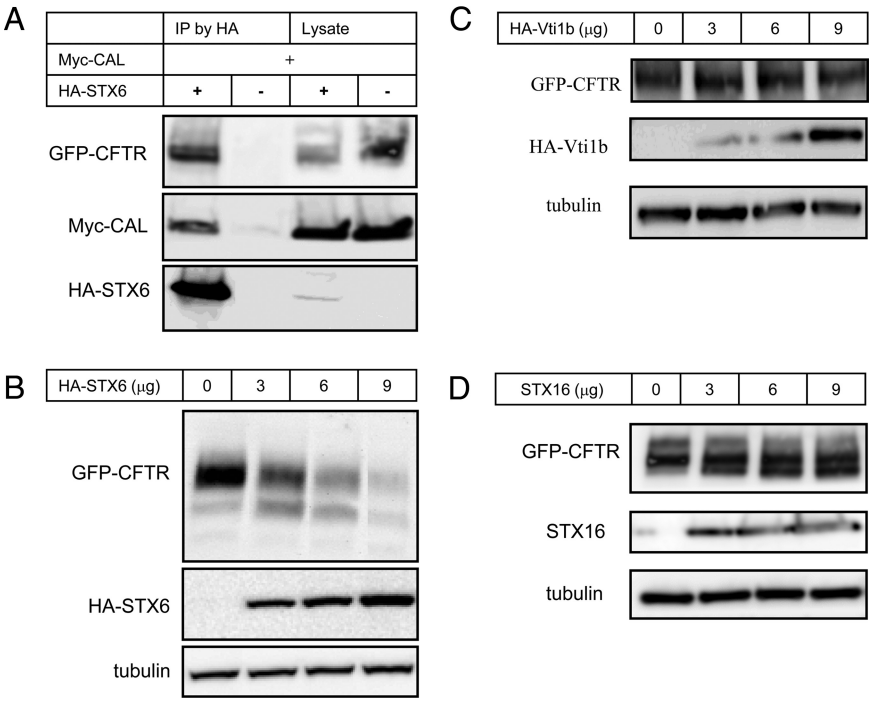

Figure 1. STX6 interacts with CAL and promotes CFTR degradation. (A) HEK293 cells were cotransfected with 3 μg myc-CAL and 3 μg GFP-CFTR with or without 6 μg HA-STX6 as indicated. Forty-eight hours after transfection, cell lysates were harvested and immunoprecipitated by HA-affinity matrix. HA-STX6 interacted with both myc-CAL and GFP-CFTR. (B) HEK293 cells were cotransfected with 3 μg GFP-CFTR and increasing amount of HA-STX6 (3, 6, and 9 μg as indicated). Forty-eight hours after transfection, cell lysates were harvested and subjected to Western blotting analysis. HA-STX6 in a dose-dependent manner reduced GFP-CFTR protein expression. (C) HEK293 cells were cotransfected with 3 μg GFP-CFTR and HA-Vti1b (3, 6, and 9 μg as indicated). Forty-eight hours after transfection, cell lysates were harvested and subjected to Western blotting analysis. HA-Vti1b had no effect on GFP-CFTR protein expression. (D) HEK293 cells were cotransfected with 3 μg GFP-CFTR and STX16 (3, 6, and 9 μg as indicated). At 48 h after transfection, STX16 had no effect on GFP-CFTR protein expression. In these and all subsequent experiments, data shown are representative of at least three independent experiments.

Statistical Analysis

The data are presented as mean ± SE. Statistical significance was determined by Student's *t* test. We assigned significance at $p < 0.05$.

RESULTS

STX6 Promotes CFTR Degradation

As a first step, we tested the interaction of STX6 with CAL and CFTR by transient transfection experiments in HEK293 cells. Cells were triple-transfected with HA-STX6, myc-CAL, and GFP-CFTR. Immunoprecipitation of HA-STX6 by an HA-affinity matrix brought down both myc-CAL and GFP-CFTR (Figure 1A, lane 1). As a control, neither myc-CAL nor GFP-CFTR was brought down by the HA-affinity matrix in the absence of HA-STX6 (Figure 1A, lane 2). In addition, immunoprecipitation CFTR brought down HA-STX6 (see Figure 7B) but not HA-Vti1b (Figure S1A). In these same experiments we observed that coexpression of HA-STX6 and myc-CAL reduced the steady state levels of GFP-CFTR protein (Figure 1A, lane 3 vs. lane 4). Because overexpression of myc-CAL alone is already known to reduce the steady-state levels of CFTR protein (Cheng *et al.*, 2004), we next examined whether the overexpression of STX6 by itself is capable of reducing the amount of CFTR protein. As shown in Figure 1B, HA-STX6 led to a dose-dependent reduction of GFP-CFTR protein in cells cotransfected with both HA-STX6 and GFP-CFTR. Likewise, cell surface GFP-CFTR as assessed by cell surface biotinylation was also reduced by the coexpression of HA-STX6 (data not shown). To rule out any possible effect of GFP on GFP-CFTR, we cotransfected HA-STX6 with an untagged wt-CFTR plasmid and observed a similar reduction of CFTR by HA-STX6 (Figure S1B). STX6 is a Q_{bc} family SNARE protein (Wendler and Tooze, 2001). Overexpression of another HA-tagged Q_{bc}-SNARE HA-Vti1b had no effect on steady-state levels of GFP-CFTR protein (Figure 1C). STX6 is known to colocalize with STX16 at the TGN (Simonsen *et al.*, 1998; Mallard *et al.*, 2002). Both STX16 and STX6 regulate GLUT4 trafficking (Perera *et al.*, 2003; Shewan *et al.*, 2003; Proctor *et al.*, 2006). Overexpression of STX16 had no effect on GFP-CFTR expression (Figure 1D). Thus, the abundance of CFTR is specifically down-regulated by overexpression of STX6, a Golgi-localized CFTR-interacting Q_{bc} SNARE protein.

To investigate the role of endogenous STX6 in controlling the abundance of CFTR, we tested the effects of disrupting the endogenous STX6 activity by dominant-negative interaction and of knocking down the endogenous STX6 protein level by siRNA silencing. HA-STX6ΔC (deletion of the last 21 amino acid residues), lacking the C-terminal, membrane anchoring domain, is a well-established dominant-negative STX6 construct (Perera *et al.*, 2003; Kuliawat *et al.*, 2004). Transfecting HA-STX6ΔC dramatically increased the steady-state levels of GFP-CFTR protein (Figure 2A). All overexpression experiments of wt and dominant-negative mutants were carefully controlled with equal total DNA, identical transfecting reagents and procedures, and equal total protein loading.

Next, we performed siRNA silencing to knockdown the expression of endogenous STX6. Silencing of STX6 with 20 nM Hs_STX6_2_HP siRNA in HEK293 cells significantly increased the protein level of transiently transfected GFP-CFTR (176.0 ± 47.1%, $p < 0.05$, $n = 3$; Figure 2B). As controls, silencing of STX16 or STX8 had no effect on the protein levels of transiently transfected GFP-CFTR and/or untagged CFTR (Figure 2C, Figure S1, C and D). Silencing of STX6 was confirmed by Western blotting (Figure 2B) and by immunostaining (Figure 2D). CAL colocalizes with STX6 at the perinuclear region (Figure 2D, top panels). The subcellular localization of CAL was unchanged (Figure 2D, bottom panels). Increases in GFP-CFTR protein expression was also observed by confocal microscopy (Figure 2E). To further rule out the potential off-target effects of siRNA, a rescue experiment with siRNA-resistant plasmid construct was performed. The expression of HA-STX6, which only contains the coding region of STX6, was not affected by Hs_STX6_2_HP siRNA, which targets to the 3′ untranslated

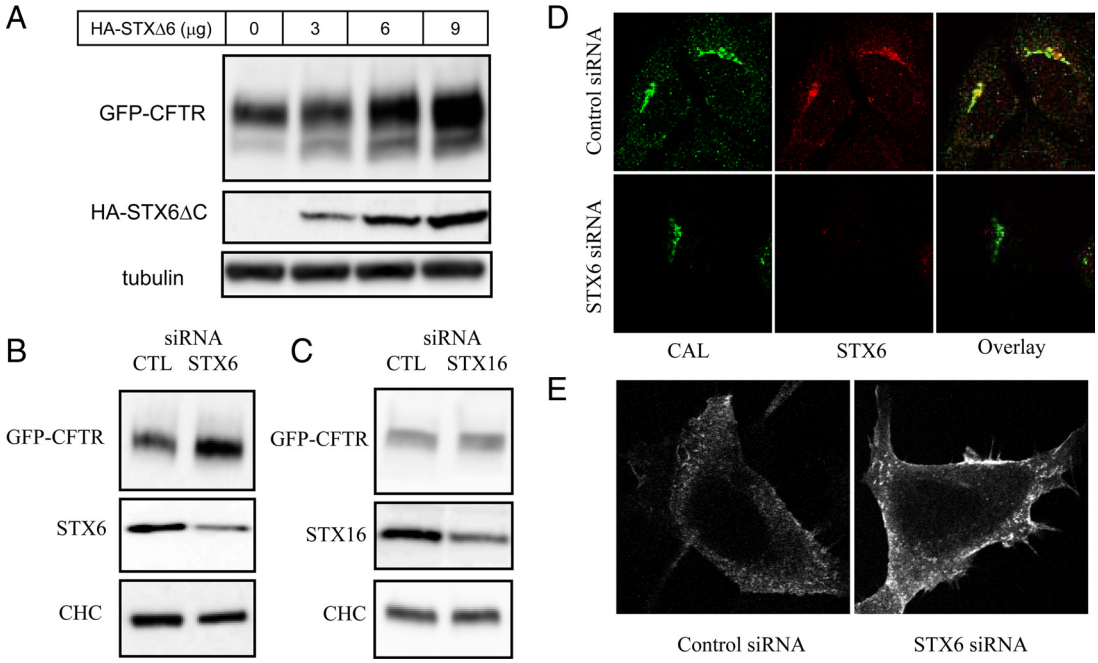

Figure 2. Dominant-negative interaction and silencing of endogenous SXT6 increases GFP-CFTR (A) HEK293 cells were cotransfected with 3 μg GFP-CFTR and increasing amount of HA-STX6ΔC (3, 6, and 9 μg as indicated). At 48 h after transfection, cell lysates were harvested and subjected to Western blotting analysis. HA-STX6ΔC dose-dependently increases GFP-CFTR protein expression. (B) HEK293 cells were sequentially transfected with 20 nM STX6 siRNA (Hs_STX6_6_HP) and 3 μg GFP-CFTR plasmid (see *Materials and Methods*). Forty-eight hours after GFP-CFTR transfection, cell lysates were harvested and subjected to Western blotting analysis. Silencing of STX6 increased GFP-CFTR protein expression. (C) HEK293 cells were sequentially transfected with 20 nM STX16 siRNA and 3 μg GFP-CFTR. Forty-eight hours after GFP-CFTR transfection, cell lysates were subjected to Western blotting analysis. Silencing of STX16 had no effect on GFP-CFTR protein expression. (D) HEK293 cells grown on coverslips were sequentially transfected with 20 nM STX6 siRNA and 3 μg GFP-CFTR as in B. Forty-eight hours after GFP-CFTR transfection, cells were fixed and subjected to indirect fluorescence immunocytochemical staining with a STX6 mAb and a CAL polyclonal antibody. CAL is pseudocolored in green, and STX6 is pseudocolored in red (E), as described in D except CFTR was detected with a mouse mAb.

region. Transfection of HA-STX6 reversed the effect of Hs_STX6_2_HP siRNA (Figure S2).

Taken together, these results demonstrate clearly the role of endogenous STX6 protein in regulating the abundance of CFTR, with overexpression of STX6 causing a down-regulation of the detectable amounts of total and surface-localized CFTR and reduced STX6 levels by having the opposite effect.

STX6-mediated CFTR Degradation Is Lysosome-dependent and CAL-dependent

To understand the mechanisms of STX6 on CFTR, we determined the effect of STX6 on the degradation of CFTR in the absence of new protein synthesis. GFP-CFTR was transiently transfected into HEK293 cells with or without HA-STX6. Twenty-four hours after transfection, new protein synthesis was terminated by cycloheximide (100 μg/ml), and the remaining GFP-CFTR was quantified by Western blot analysis. As shown is Figure 3, A and B, GFP-CFTR was relatively stable in the presence of cycloheximide, whereas overexpression of HA-STX6 dramatically reduced the stability of GFP-CFTR. The GFP-CFTR remaining 3 h after cycloheximide treatment was 82.0 ± 2.8% in the absence of HA-STX6 and 65.0 ± 6.2% in the presence of HA-STX6. As shown in Figure 3C, bafilomycin A1, which inhibits lysosome activity, largely reversed the effect of HA-STX6 (83.0 ± 3.2% of control level 3 h after cycloheximide treatment). Similarly, E-64, an inhibitor of lysosomal cysteine proteinases, also reversed the effect of HA-STX6 (88.7 ± 9.6% of control level; Figure 3D). In contrast, the proteasome inhibitor MG132 was without effect (data not shown). These results indicated that STX6 not only binds to CAL but also promotes the lysosome-dependent degradation of CFTR as CAL does (Cheng *et al.*, 2004).

A hint that STX6 may require the presence of CAL to promote CFTR degradation came from a C-terminal deletion mutant. GFP-CFTRΔTRL is a mutant CFTR lacking the C-terminal PDZ motif that does not interact with CAL (Cheng *et al.*, 2002). Although silencing of STX6 dramatically increased GFP-CFTR expression, it had no effect on GFP-CFTRΔTRL expression (Figure 4A). Curiously, although the level of CAL expression is equal, the expression of CFTRΔTRL is much higher than wt GFP-CFTR, presumably because of the lack of interaction with CAL (Figure 4A). To test this directly, endogenous CAL was silenced with siRNA, and indeed, cellular GFP-CFTR was substantially increased (Figure 4B, lane 1 vs. lane 2). More importantly, silencing of endogenous CAL eliminates the inhibitory effect of STX6 overexpression on GFP-CFTR (Figure 4B, lane 2 vs. lane 3). Thus CAL is required for the STX6-mediated, lysosome-dependent degradation of cellular CFTR.

The simplest explanation is that STX6 may depend on CAL to bind to CFTR. Alternatively, STX6 might be capable of binding to CFTR but require CAL for its activity. Figure 4C showed that HA-STX6 coimmunoprecipitated with GFP-CFTRΔTRL, which lacks the CAL-binding PDZ motif. To confirm this unexpected finding, three additional independent approaches were taken to further characterize the in-

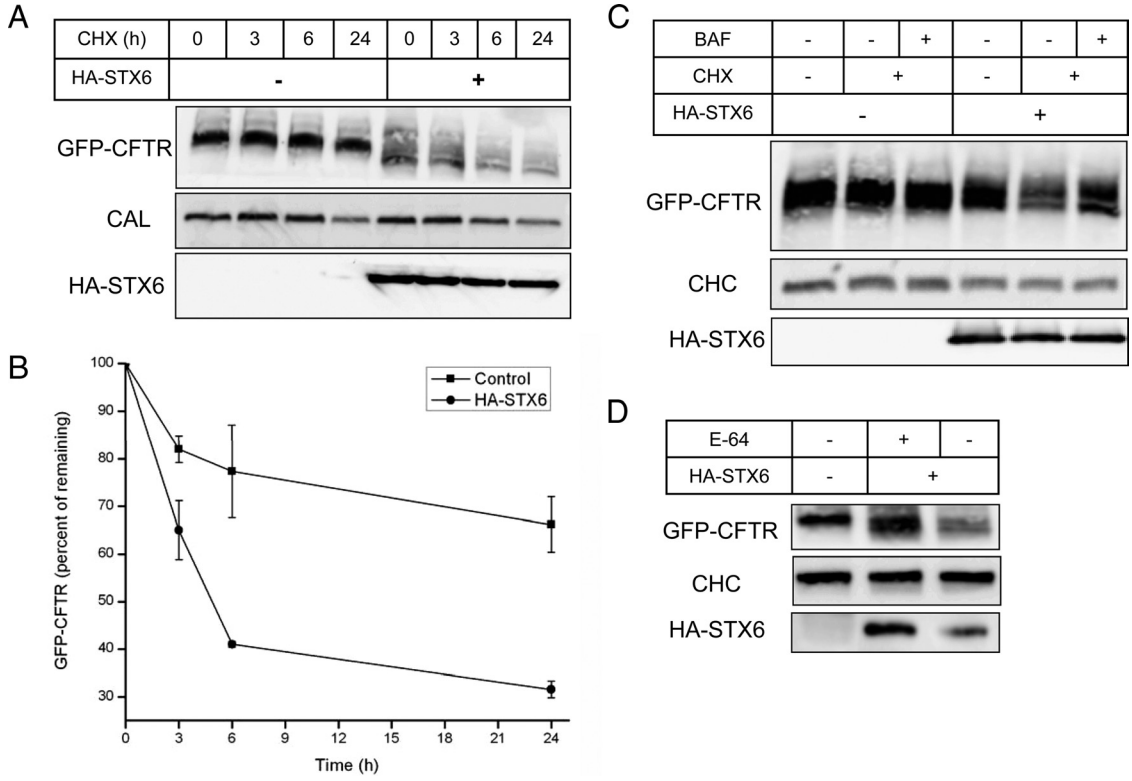

Figure 3. STX6-mediated CFTR degradation (A) HEK293 cells were transfected with 3 μg GFP-CFTR with or without 6 μg HA-STX6. Twenty-four hours after transfection, the cell culture media was replaced with serum-free media supplemented with 100 μg/ml cycloheximide. Cell lysates were collected at indicated times and subjected to Western blot analysis. (B) Percent of remaining GFP-CFTR relative to the time immediately before cycloheximide treatment. Data are the average of three independent experiments. Error bars, SDs. (C) Cells were transfected as in (A) and treated with 100 ng/ml bafilomycin A1 (BAF) and 100 μg/ml cycloheximide as indicated for 3 h. Cell lysates were collected and subjected to Western blot analysis. (D) Cells were transfected as in (A) and treated with 10 μg/ml E-64 as indicated for 16 h. Cell lysates were collected and subjected to Western blot analysis.

teraction of STX6 with CFTR. First, we tested the interaction of STX6 and CFTR when CAL was reduced by siRNA silencing (Figure 5A). HA-STX6 was shown to coimmunoprecipitate with GFP-CFTR (Figure 5B). CAL knockdown led to increased GFP-CFTR protein that complicated the interpretation of experiments. Therefore, we took a second approach where GFP-CFTR was kept constant, whereas CAL protein was eliminated by immunodepletion. Cells lysates expressing GFP-CFTR and HA-STX6 were harvested and subjected to immunoprecipitation with CAL antibody. Western blot analysis confirmed that all the endogenous CAL was depleted (Figure 5C). CAL depleted lysates were then subject to immunoprecipitation with an HA-affinity matrix. Figure 5D clearly showed the binding of

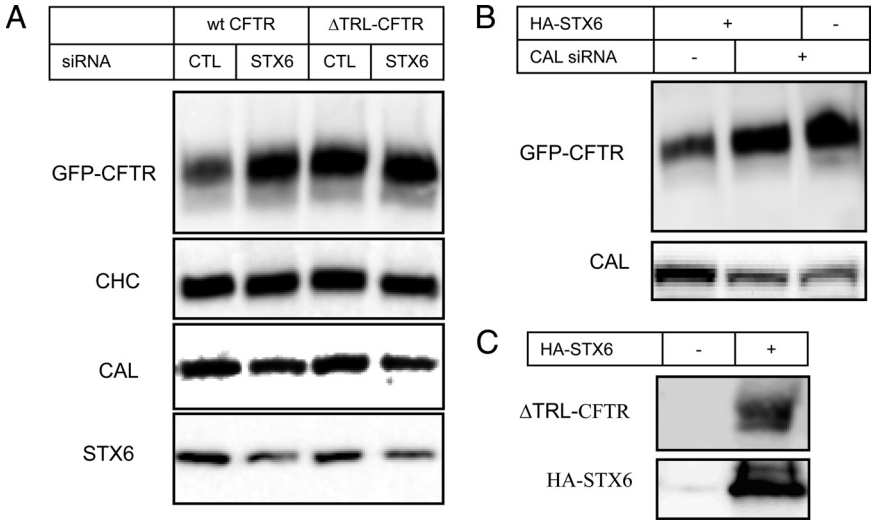

Figure 4. The dependence of STX6 activity on the carboxyl PDZ biding motif and CAL. (A) HEK293 cells were sequentially transfected with 20 nM STX6 siRNA and 3 μg GFP-CFTR or 3 μg GFP-CFTRΔTRL as indicated. Forty-eight hours after the second transfection, cell lysates were collected and subjected to Western blot analysis. (B) HEK293 cells were sequentially transfected with 20 nM CAL siRNA and 3 μg GFP-CFTR with or without 6 μg HA-STX6 as indicated. Forty-eight hours after the second transfection, cell lysates were collected and subjected to SDS-PAGE and Western blotting analysis. (C) HEK293 cells were cotransfected with 3 μg GFP-CFTRΔTRL with or without 3 μg HA-STX6 as indicated. Forty-eight hours after transfection, cell lysates were harvested and immunoprecipitated by HA-affinity matrix. HA-STX6 interacted with GFP-CFTRΔTRL.

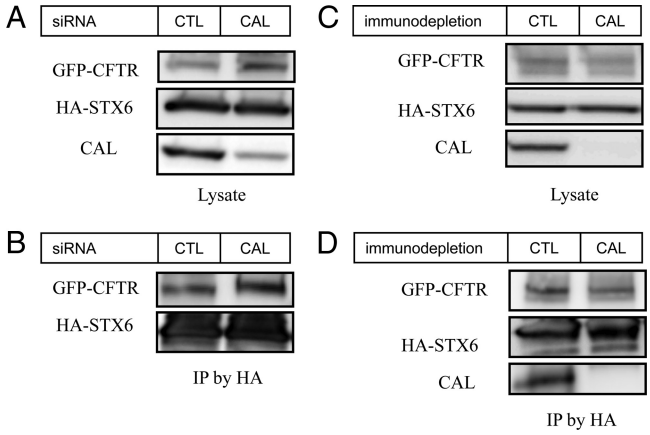

Figure 5. The independence of STX6 on CAL in binding to CFTR. (A) HEK293 cells were sequentially transfected with 20 nM CAL siRNA and 3 μg GFP-CFTR as indicated. Forty-eight hours after the second transfection, cell lysates were collected and subjected to Western blot analysis. (B) Cell lysates in A were subjected to immunoprecipitation with HA-affinity matrix. HA-STX6 interacted with GFP-CFTR when CAL was silenced with siRNA. (C) HEK293 cells were cotransfected with 3 μg GFP-CFTR with or without 3 μg HA-STX6 as indicated. Forty-eight hours after transfection, cell lysates were harvested and immunoprecipitated by CAL antibody and protein A/G beads. The supernatant after CAL immunoprecipitation was subjected to Western blot analysis to show the complete depletion of CAL. (D) CAL-depleted lysate in C was subjected to immunoprecipitation with HA-affinity matrix. HA-STX6 interacted with GFP-CFTR even when CAL was depleted.

HA-STX6 to GFP-CFTR in the absence of CAL. In a third approach, we tested the interaction of HA-STX6 with a truncation mutant of CFTR. HA- STX6 was shown to bind to GFP-TNR, which comprises mainly the N-terminal half of CFTR (Figure S1E).

Taken together, STX6 and CAL bind to separate sites in CFTR (N-terminus for STX6 and C-terminus for CAL). However, STX6 requires the presence of CAL in promoting lysosome-mediated degradation of CFTR.

Silencing of STX6 Increased CFTR Protein Expression and Function in Airway Epithelial Cells

The role of STX6 on the expression and function of CFTR in CFBE-CFTR cells was tested by siRNA silencing. Consistent with the observations in HEK293 cells, silencing of STX6 (5–20 nM STX6 siRNA) led to dose-dependent increases in CFTR protein expression (Figure 6A). Twenty nanomolar STX6 siRNA treatment almost tripled the CFTR protein levels (284.6 $\pm$ 71.7% of the control, $p < 0.05$, $n = 3$). Two additional STX6 siRNAs similarly increased CFTR expression (Figure S2A). The level of the expression of CAL or GAPDH was unchanged (Figure 6A). Indirect immunofluorescence confocal microscopy confirmed the increases in CFTR as well as the silencing of endogenous STX6 (Figure 6B). As a control, silencing of STX8 has no effect on CFTR expression in CFBE-CFTR cells (Figure S1D).

Because CFBE-CFTR forms high-resistance epithelial layers on permeable supports, we measured CFTR-mediated Cl^- transport activity in STX6 siRNA-treated cells. Subconfluent CFBE-CFTR cells were transfected with either control siRNA or STX6 siRNA. Cells were allowed to grow to full confluence on permeable supports. Three days after transfection, the permeable supports were mounted in Ussing chambers to measure the I_{sc}s (average resistance ~1200

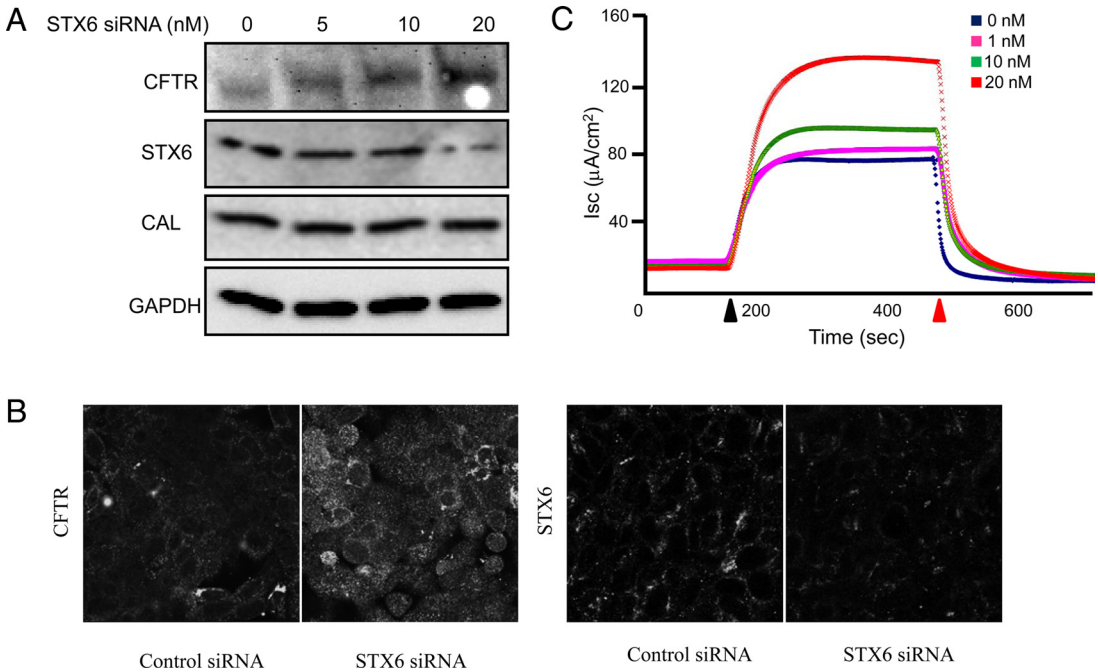

Figure 6. Silencing of STX6 in CF epithelial cell line CFBE-CFTR. (A) CFBE-CFTR cells were transfected with 20 nM STX6 siRNA as indicated. Seventy-two hours after the transfection, cell lysates were collected and subjected to Western blot analysis by indicated antibodies; GAPDH was used as the loading control. (B) CFBE-CFTR cells were transfected with 20 nM STX6 siRNA or 20 nM control siRNA. Seventy-two hours after the cells were fixed and subjected to indirect fluorescence immunocytochemical staining with a CFTR mAb (left panels) or STX6 mAb (right panels). (C) CFBE-CFTR cells grown on permeable supports were transfected with 1, 10, or 20 nM STX6 siRNA or 20 nM control siRNA as indicated. Seventy-two hours after transfection, cells were mounted on the Ussing chamber setup where the short-circuit current was measured as described in *Materials and Methods.* In the graph, 10 μM forskolin was added at the first arrow (black), and 1 μM $CFTR_{inh}$-172 was added at the second arrow (red).

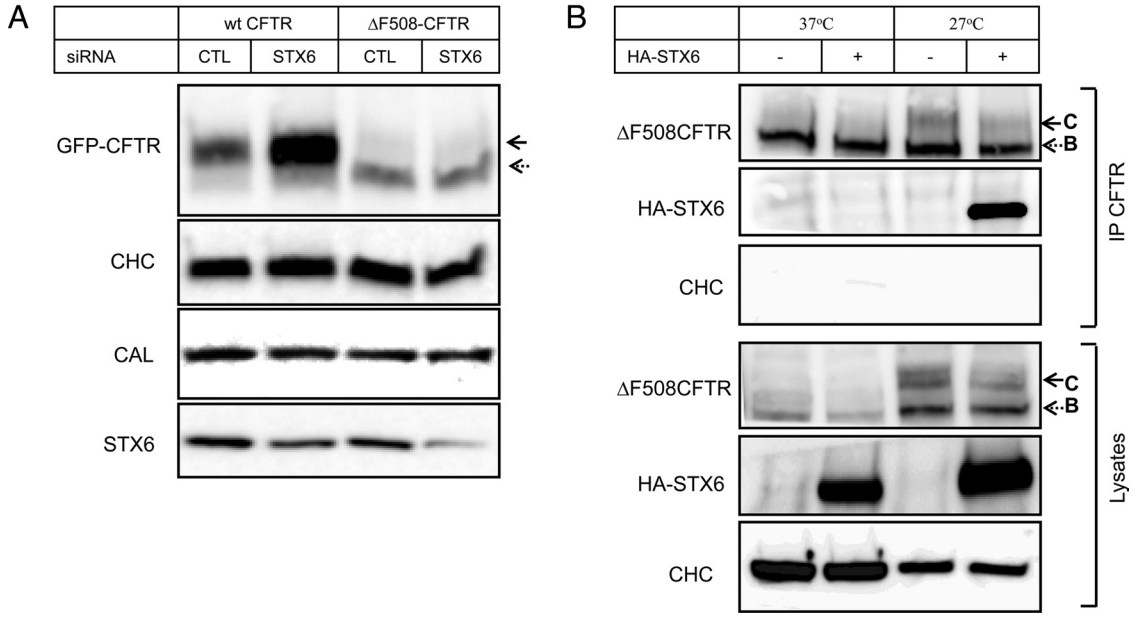

Figure 7. STX6 interacts with CFTR in post-ER subcellular compartments. (A) HEK293 cells were transfected with 20 nM STX6 siRNA and 3 μg GFP-CFTR or 3 μg GFP-ΔF508CFTR. Forty-eight hours after the second transfection, cells lysates were collected and subjected to Western blot analysis; CHC was used as a loading control and as an immunoprecipitation control. (B) Hela-ΔF508CFTR cells were transfected with HA-STX6 as indicated and, the next day, cells were either moved to a 27°C incubator or kept at 37°C as indicated. Forty-eight hours after transfection, cell lysates were collected and immunoprecipitated with the CFTR mAb and protein A/G beads. The immunoprecipitated material and cell lysates were subjected Western blot analysis; CHC was used as a loading control and as an immunoprecipitation control. Arrows with solid line point to C bands. Arrows with dashed line point to B bands.

Ω/cm^2 for both control siRNA and STX6 siRNA-transfected cells). As shown in Figure 6C, silencing of STX6 led to dose-dependent increases in forskolin-activated I_{sc}s that was completely inhibited by the CFTR-specific channel blocker $CFTR_{inh}$-172. At 20 nM STX6 siRNA, this I_{sc} is 193.6 ± 27.8% ($p < 0.05$, $n = 4$) of that of the control treatment. Therefore, STX6 not only modulates transfected GFP-CFTR in HEK293 cells, it also modulates the CFTR protein level and function in bronchial epithelial cells.

STX6 Exerts Its Effect in Post-ER Compartments

The subcellular localization of STX6 suggests its role in trafficking of CFTR in the post-ER compartments. To test this, we took advantage of the ER-localized CF mutant ΔF508CFTR. Although silencing of endogenous STX6 dramatically increased wt GFP-CFTR protein, it had no effect on the protein level of GFP-ΔF508CFTR (Figure 7A). Unlike wt GFP-CFTR, ΔF508CFTR did not bind to STX6 (Figure 7B, lane 2). ΔF508CFTR is a temperature-sensitive mutant that escapes ER quality control and traffics to post-ER compartments when cells are incubated at low temperature (Denning *et al.*, 1992). The mature C band appeared after incubating cells at 27°C for 24 h (Figure 7B, cf. lanes 1 and 3). Treatment at 27°C also caused ΔF508CFTR to bind to STX6 (Figure 6B, cf. lanes 2 and 4). In addition, overexpression of HA-STX6 reduced the rescued C band of ΔF508CFTR (Figure 7B, cf. lanes 3 and 4). Thus, consistent with its subcellular localization, STX6 interacts with and promotes degradation of post-ER CFTR but not ER-localized CFTR.

Silencing of STX6 Further Increased Mature CFTR Protein Expression and Function in Temperature-rescued ΔF508-CFTR from Airway Epithelial Cells

Approximately 70% of CF patients are homozygous for the ΔF508 mutation (http://www.genet.sickkids.on.ca/cftr/). Understanding how to rescue the ΔF508-CFTR expression and activity is a key step in CF therapy. To this end, we assessed the effect of silencing STX6 on ΔF508-CFTR bronchial epithelial cells (CFBE-ΔF508CFTR). Similar to CFBE-CFTR cells, CFBE-ΔF508CFTR maintains characteristics of CF epithelial tissue but lacks CFTR-mediated function. Cells were first transfected with 20 nM STX6 siRNA or 20 nM control siRNA. Two days after transfection, cells were moved to a 27°C incubator for an additional 24 h. The low temperature rescued the ER export of ΔF508-CFTR, as evident by the appearance of the mature C band (Figure 8A, lanes 1 and 2). Twenty nanomolar STX6 siRNA treatment led to further increases in rescued CFTR levels (Figure 8A, lanes 3 and 4, 178.3 ± 26.7% of the control, $p < 0.03$, $n = 3$). Similar increases in I_{sc}s were observed in the Ussing chamber assay. At 20 nM STX6 siRNA, CFTR-mediated I_{sc} is 232.3 ± 27.8% ($p < 0.05$, $n = 4$) of that of the control treatment (Figure 8B). STX6 is thus a potential therapeutic target to further enhance the expression and function of ΔF508-CFTR rescued by other mechanisms.

DISCUSSION

Intracellular vesicular trafficking is accomplished by continuous budding of donor membrane compartments, the transporting of vesicles, the tethering of vesicles to the target membrane, and fusion of the vesicles to the acceptor membrane compartments (Cai *et al.*, 2007). SNARE proteins play important roles in the fusion of donor and acceptor membrane compartments (Jahn and Scheller, 2006). In humans, 36 SNAREs have been identified that distribute throughout the intracellular membranes (Jahn and Scheller, 2006). Two SNAREs, syntaxin 1A (STX1A) and STX8, were found to interact with CFTR and modulate its intracellular trafficking.

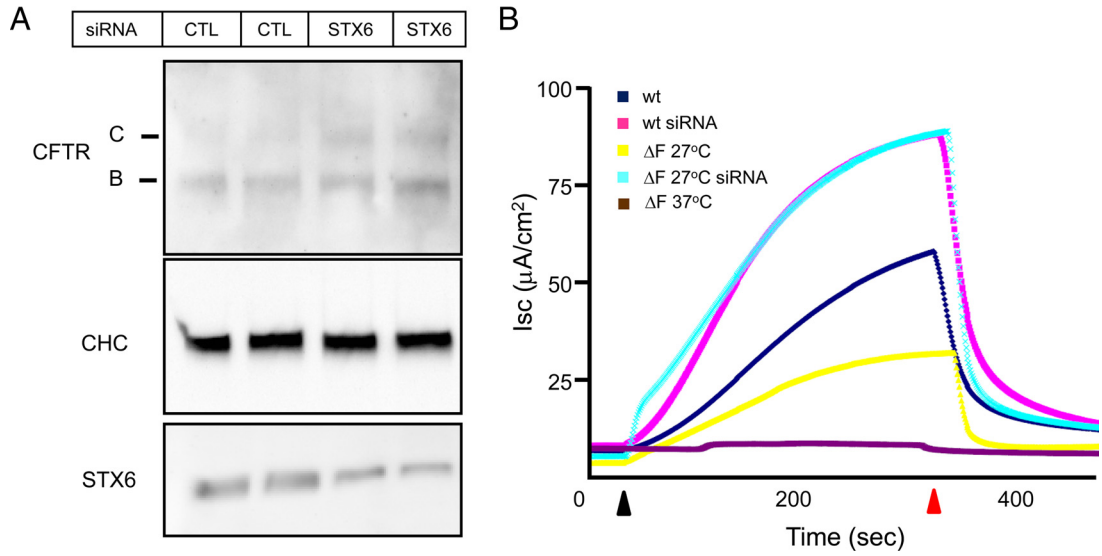

Figure 8. Silencing of STX6 in CF epithelial cell line CFBE-ΔF508CFTR. (A) CFBE-ΔF508CFTR cells were transfected with 20 nM STX6 siRNA or 20 nM control siRNA as indicated. Seventy-two hours after the transfection, cell lysates were collected and subjected to Western blot analysis by indicated antibodies. (B) CFBE-CFTR cells and CFBE-ΔF508CFTR cells grown on permeable supports were transfected with 20 nM STX6 siRNA or 20 nM control siRNA as indicated. Seventy-two hours after transfection, cells were mounted on the Ussing chamber set-up and the short-circuit current was measured as described in *Materials and Methods*. In the graph, 50 μM genistein was added at the first arrow (black), and 1 μM $CFTR_{inh}$-172 was added at the second arrow (red). Brown line, CFBE-ΔF508CFTR grown at 37°C; yellow line, CFBE-ΔF508CFTR grown at 27°C; light blue line, CFBE-ΔF508CFTR grown at 27°C and transfected with STX6 siRNA; dark blue line, CFBE-CFTR grown at 37°C; and pink line, CFBE-CFTR grown at 37°C and transfected with STX6 siRNA.

Both STX1A and STX8 inhibit the trafficking of CFTR to the plasma membrane (Peters *et al.*, 1999; Bilan *et al.*, 2004). Several lines of evidence suggest that STX1A inhibits the insertion of CFTR into the plasma membrane while STX8 blocks the recycling of CFTR.

In this article, we found a third SNARE, STX6, that is involved in modulating intracellular CFTR trafficking. STX6 is a TGN and endosomal localized syntaxin subfamily Q-SNARE that plays a role in TGN to endosome trafficking. STX6 is involved in endosomal trafficking of a number of membrane proteins, notably keeping Glut4 sequestered in the cell away from the plasma membrane (Perera *et al.*, 2003). It is required for sorting of proteins from endosomes toward either the TGN or lysosomes (Kuliawat *et al.*, 2004). What prompted us to investigate the role of STX6 in the trafficking and degradation of CFTR was its interaction with CAL (Charest *et al.*, 2001; Figure 1A), which itself binds to the PDZ motif at the carboxy terminus of CFTR and promotes lysosomal degradation of CFTR (Cheng *et al.*, 2002, 2004). Consistent with its known functions, STX6 may act on endosomes to facilitate lysosomal sorting of CFTR.

Three lines of evidence suggested that STX6 similarly promotes degradation of mature CFTR. 1) Overexpression of STX6 dramatically reduced the steady-state protein levels of CFTR (Figure 1B). 2) A STX6 dominant negative mutant increased CFTR protein expression (Figure 2A). 3) Silencing of endogenous STX6 also increased CFTR protein expression (Figure 2B). The degradation of CFTR by STX6 can be measured by the reduction of the stability of CFTR (Figure 3, A and B). The degradation is consistent with a lysosome-dependent degradation that was reversed by bafilomycin A1 and E-64 treatments (Figure 3, C and D). Additional data suggested that STX6 requires CAL in promoting the degradation of mature CFTR: 1) Silencing of STX6 has no effect on the expression of GFP-CFTRΔTRL, which is incapable of binding to CAL (Figure 4A). 2) Silencing of CAL abolished the effect of STX6 in the reduction of wt GFP-CFTR expression (Figure 4B). We have found that STX6 binds to CAL but also can bind to CFTR in the absence of CAL (Figure 5). CAL is known to bind to the C-terminus of CFTR (Cheng *et al.*, 2002), so it is an interesting finding that STX6 binds to the N-terminal half of CFTR (Figure S1E). The functional requirement of CAL suggested a model that the CAL/STX6/CFTR complex directs CFTR trafficking to the lysosome. The observation that the role of STX6 in directing CFTR to the lysosome is abolished in the CFTRΔTRL mutant, which is incapable of binding to CAL, suggests that the binding of STX6 to CAL is functionally important in this process, whereas the binding of STX6 to CFTR does not have the same functional role.

The CFTR/CAL/CFTR complex functions oppositely to what we have noted previously for the CFTR/CAL/TC10 interaction. TC10, a small Rho family GTPase binds to CAL and CFTR and directs CFTR trafficking to the plasma membrane away from the degradation pathway (Cheng *et al.*, 2005). On the basis of these observations, we hypothesized that the interaction of CFTR/CAL with different trafficking molecules such as TC10 and STX6 determine whether CFTR will traffic to the plasma membrane or the lysosome.

There are some interesting parallels between post-ER trafficking of CFTR and that of the glucose transporter Glut4. In both cases, the constitutively active form of a Rho family GTPase TC10 and dominant-negative form of STX6 cause their translocation to the plasma membrane (Chiang *et al.*, 2001, 2005; Perera *et al.*, 2003). In adipocytes, STX6 sequesters Glut4 away from the plasma membrane (Perera *et al.*, 2003). In epithelial cells, overexpression of STX6 causes decreases in cell surface expression and the degradation of CFTR. Because TC10 and STX6 participate in the insulin-stimulated translocation of Glut4 to the plasma, it is tempting to speculate a similar role of TC10 and STX6 in the regulated trafficking of CFTR and other plasma membrane proteins, through signal transduction pathways active in the epithelial cells that function through the CAL complex. Fur-

thermore, CFTR has much higher maturation efficiency in epithelial cells than in fibroblast cells (Varga *et al.*, 2004). The molecular mechanism is poorly defined. CAL complex has the potential to contribute to this process by differentially affecting the stability of mature CFTR in a tissue specific manner.

The role of STX6 in epithelial cells was confirmed in CFBE-CFTR and CFBE-ΔF508CFTR expressing wt CFTR and ΔF508 CFTR, respectively, by immunoblotting, immunocytochemistry and I_{sc} functional assay. Silencing of the endogenous STX6 by siRNA increased the levels of CFTR in CFBE-CFTR cells as well as the forskolin-stimulated Cl^- currents (Figure 6). The stimulated currents are specific to CFTR, as they were completely eliminated by the CFTR inhibitor, $CFTR_{inh}$-172 (Figure 6C). In CF cell lines such as ΔF508 CFTR, nearly all newly synthesized ΔF508-CFTR is misfolded, fails to pass the ER quality control system, and is eliminated by ER-associated degradation (ERAD; Jensen *et al.*, 1995; Ward *et al.*, 1995; Turnbull *et al.*, 2007). Treatment that increases the efficiency of ER export and subsequent cell surface expression of ΔF508-CFTR can therefore "rescue" the trafficking of ΔF508-CFTR to the cell surface, where it can be functional. Low temperature is frequently used in the laboratory to "rescue" ΔF508-CFTR (Denning *et al.*, 1992). Consistent with its TGN localization, STX6 did not interact with ER-localized ΔF508-CFTR (Figure 7B), nor did silencing of STX6 have an effect on the protein expression of ΔF508CFTR (Figure 7A). However, after ΔF508CFTR was rescued by growing cells at 27°C, STX6 associated with ΔF508-CFTR, and overexpression of HA-STX6 reduced the level of rescued ΔF508CFTR (Figure 7B). Silencing of SXT6 approximately doubled the I_{sc}s of temperature-"rescued" ΔF508-CFTR in CFBE-ΔF508CFTR cells (Figure 8B).

Recently, high-throughput screenings have yielded novel small molecules that rescue ΔF508-CFTR from ERAD (CFTR correctors; Pedemonte *et al.*, 2005; Van *et al.*, 2006). However current generation of correctors obtained to bypass ERAD are inefficient in fully correcting CFTR (Pedemonte *et al.*, 2005; Van *et al.*, 2006; Wang *et al.*, 2007). Although silencing STX6 does not promote ER exit of ΔF508-CFTR, it can enhance temperature rescue of ΔF508-CFTR. It is possible that manipulating the function of endogenous STX6 could be a way of increasing further the potency of small-molecule correctors to restore ΔF508CFTR function at the plasma membrane.

The question then is why regulate the post-ER processing of CFTR by having the adaptor protein CAL binding to two regulatory proteins: TC10, whose GTP bound form promotes CFTR processing to the plasma membrane, and STX6, which promotes its degradation in the lysosomes? We would posit that in mucosal membranes where CFTR function is essential two major regulatory mechanisms can control CFTR function: one that modulates channel activity via cAMP and a second that can relatively rapidly either enhance (TC10) or reduce (STX6) the amount of CFTR at the plasma membrane. In organs that depend on CFTR for proper hydration, proper fluid balance essential. Too little fluid (cystic fibrosis) or too much fluid (diarrhea or pneumonia) is pathological. Thus having two mechanisms may guarantee that the function of one regulatory pathway could be either enhanced or could override the other.

ACKNOWLEDGMENTS

The author thanks Dr. Kristina Krasnov for the critical reading of the manuscript. This work was supported by The Cystic Fibrosis Foundation Research Development Programs (W.B.G.) and National Institutes of Health Grant HL-47122 (W.B.G.) and P01DK072084 (W.B.G.).

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