Syntaxin 8 impairs trafficking of cystic fibrosis transmembrane conductance regulator (CFTR) and inhibits its channel activity

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Summary

The cystic fibrosis transmembrane conductance regulator (CFTR) is a cyclic AMP-dependent chloride channel that mediates electrolyte transport across the luminal surface of epithelial cells. In this paper, we describe the CFTR regulation by syntaxin 8, a t-SNARE protein (target soluble N-ethylmaleimide-sensitive factor attachment protein receptor) involved in the SNARE endosomal complex. Syntaxin family members are key molecules implicated in diverse vesicle docking and membrane fusion events. We found that syntaxin 8 physically interacts with CFTR: recombinant syntaxin 8 binds CFTR in vitro and both proteins co-immunoprecipitate in HT29 cells. Syntaxin 8 regulates CFTR-mediated currents in chinese hamster ovary (CHO) cells stably expressing CFTR and syntaxin 8. Iodide efflux and whole-cell patch-clamp experiments on these cells indicate a strong inhibition of CFTR chloride current by syntaxin 8 overexpression. At the cellular level, we observed that syntaxin 8 overexpression disturbs CFTR trafficking. Confocal microscopy shows a dramatic decrease in green fluorescent protein-tagged CFTR plasma membrane staining, when syntaxin 8 is coexpressed in COS-7 cells. Using antibodies against Lamp-1, TfR or Rab11 we determined by immunofluorescence assays that both proteins are mainly accumulated in recycling endosomes. Our results evidence that syntaxin 8 contributes to the regulation of CFTR trafficking and chloride channel activity by the SNARE machinery.

Key words: CFTR, Syntaxin 8, SNARE, Recycling endosome, Trafficking

Introduction

Cystic fibrosis is the broadest autosomal recessive disorder among Caucasian populations. It is characterized by mutations in the *CFTR* (cystic fibrosis transmembrane conductance regulator) gene that encodes a cAMP-activated chloride channel expressed at the apical membrane of epithelial cells (Rommens et al., 1989; Riordan et al., 1989). The CFTR protein is an ATP-binding cassette (ABC) transporter with a specific cytoplasmic regulator domain (R) whose phosphorylation by protein kinase A (PKA) activates the CFTR chloride conductance (reviewed by Ostedgaard et al., 2001).

In cystic fibrosis patients, dysfunction in the chloride channel activity of CFTR alone seems insufficient to elicit the deep disorders in the ionic equilibrium observed at the surface of epithelial tissues. Indeed, CFTR expression has been linked to the regulation of numerous ionic channels and it is now assumed that CFTR is a key member of a complex network of ionic channels (Gabriel et al., 1993; Stutts et al., 1995; Sugita et al., 1998; Ji et al., 2000) and cytoplasmic proteins in epithelia. For example, CFTR interacts with several proteins containing PDZ (PSD-95/Dlg/ZO-1) domains. Most of these proteins are localized in a subplasma membrane region (Short et al., 1998; Wang et al., 1998; Wang et al., 2000; Raghuram et al., 2001), and one was found in the Golgi apparatus (Cheng et al., 2002). Interactions were also detected between CFTR and proteins involved in the intracellular vesicle trafficking. Notably, the C-terminus of CFTR binds to the endocytic clathrin adaptator complex AP-2 (Weixel and Bradbury, 2000), presumably during the internalization of CFTR from the plasma membrane. Several studies are now focusing on the relationships between CFTR and proteins of the SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) family. These proteins are involved in the membrane fusion events happening during the different steps of vesicle trafficking in eukaryotic cells (reviewed by Chen and Scheller, 2001).

Syntaxins belong to a subfamily of the t-SNAREs, whose C-terminal half contains the t-SNARE homology domain (Weimbs et al., 1997) followed by a short hydrophobic tail that anchors the protein in its resident membrane. The region of syntaxins upstream of this transmembrane domain protrudes at the cytoplasmic side of vesicle or plasma membrane. Remarkably, a direct interaction between the SNARE homology domain of syntaxin 1A and the N-terminal cytoplasmic tail of CFTR is thought to be responsible for

inhibiting CFTR chloride channel activity (Naren et al., 1997; Naren et al., 1998; Naren et al., 2000; Cormet-Boyaka et al., 2002).

We have previously described the cloning of human syntaxin 8 cDNA (*STX8* gene), using the R domain of CFTR as a bait in the two-hybrid screening of a human fetal lung library (Thoreau et al., 1999). Subsequently, syntaxin 8 has been localized in different endosomal compartments: it has been involved in trafficking from early endosome (EE) to late endosome (LE), or in homotypic fusion events in LE (Prekeris et al., 1999; Subramaniam et al., 2000; Antonin et al., 2000). In the present study, we establish that syntaxin 8 overexpression inhibits CFTR channel activity and trafficking to the plasma membrane. A direct regulation can also be suggested as we show evidence for the existence of a physical interaction between CFTR and syntaxin 8, either directly or within a protein complex. Thus, we propose that syntaxin 8 is a member of the CFTR interaction network. Our results strengthen the idea that the SNARE machinery directly regulates CFTR trafficking and activity.

Materials and Methods

Constructs and antibodies

cDNAs encoding human syntaxin 8, syntaxin 1A and syntaxin 3 were obtained by RT-PCR on total RNA isolated from the human colonic epithelial cell line HT29-CL19A. Full-length *STX8* cDNA was cloned into *Eco*RI and *Bam*HI sites of the mammalian expression vector pcDNA3.1/Zeo(–) (Invitrogen). Full-length *STX1A* or *STX3* cDNAs were cloned into *Not*I and *Xho*I sites of pcDNA3.1/Zeo(–). Using directed mutagenesis (QuikChange™ Site-Directed Mutagenesis Kit, Stratagene), we have constructed a cytosoluble syntaxin 8 with a stop codon at position 211, noted Syn8ΔTM, a protein that lacks its hydrophobic transmembrane anchor. Three different recombinant syntaxin 8 in GST (glutathione-S-transferase)-fusion were generated inserting PCR fragments into *Bam*HI and *Sal*I sites of the bacterial expression vector pGEX-5X-3 (Pharmacia): Syn8ΔTM (i.e. Syn8 [aa 1-209]), Syn8 [aa 1-99] and Syn8 [aa 99-209]. GST-Syn1AΔTM bacterial expression vector was a gift from A. P. Naren (University of Alabama, Birmingham, AL). GST-VAMP8ΔTM (aa 1-74) and GST-Syn7ΔTM (aa 1-236) bacterial expression vectors were obtained from W. Antonin (Max-Planck-Institute for Biophysical Chemistry, Göttingen, Germany). GST-vti1bΔTM vector (aa 1-207) was a gift from G. F. von Mollard (Georg-August-Universität, Göttingen, Germany). Expression of CFTR tagged with GFP (green fluorescent protein) was analyzed using pS65T/EGFP-C1/WT-CFTR construct, a gift from K. H. Karlson (Dartmouth College, Hanover, NH).

The following antibodies were used in this study: rabbit polyclonal antibody against syntaxin 3, a gift from A. LeBivic (Delgrossi et al., 1997), monoclonal antibody against syntaxin 1A clone 78.3 (Synaptic Systems, Germany), monoclonal antibody against syntaxin 8 clone 48 (Transduction Laboratories); antibodies against CFTR, monoclonal antibody clone M3A7 with epitope at amino acid 1370-1380 (Chemicon International), used for immunoblotting, and monoclonal antibody clone 24-1 with epitope at amino acid 1377-1480 (R&D Systems), used for immunoprecipitation; antibody against GFP, monoclonal antibody clone GEP-20 (Sigma-Aldrich). For immunofluorescence experiments, anti-syntaxin 8 antibody was prepared by immunization of a rabbit with bacterially expressed GST-Syn8ΔTM. Polyclonal antibody was then affinity purified from rabbit antiserum using CNBr-activated sepharose beads (Amersham Pharmacia Biotech). Monoclonal anti-TfR antibody was purchased from Sigma-Aldrich (clone DF1513), monoclonal anti-Lamp 1 was obtained from Pharmingen, rabbit polyclonal anti-Rab11 antibody was a gift from B. Goud and J. Salamero (Wilcke et al., 2000) and rabbit polyclonal anti-NCX2 was provided by the intermediary of C. Cognard (LBSC, Poitiers, France). All fluorescent secondary antibodies were obtained from Jackson Immunoresearch Laboratories. Secondary antibody for western blot was obtained from Amersham Pharmacia Biotech: horseradish peroxidase-conjugated goat anti-mouse IgG.

Cell culture

All cell lines were cultured at 37°C in 5% CO_2 in a medium containing 100 IU/ml penicillin (Panpharma SA, France) and 100 μg/ml streptomycin (Solvay Pharma, France). CHO cells transfected with *CFTR* cDNA (CHO-CFTR), established by J. R. Riordan and X. B. Chang (Tabcharani et al., 1991), were grown in αMEM medium with Glutamax-I (Life Technologies) containing 100 μM methotrexate (Sigma-Aldrich) and supplemented with 7% fetal calf serum (Life Technologies). COS-7 and HT29-CL19A cells were cultured in DMEM with Glutamax-I supplemented with 10% fetal calf serum.

Transfection

CHO-CFTR cells were transfected with 1 μg of pcDNA3.1/Zeo(–) plasmids encoding syntaxins, using 3 μl of FuGene6™ transfection reagent (Roche Diagnostics Corp.), according to the manufacturer's protocol. After 24 to 48 hours incubation 40 μg/ml zeocin (Invitrogen) was added to the culture medium. Resistant cells were cultured with 20 μg/ml zeocin and were tested by western blot

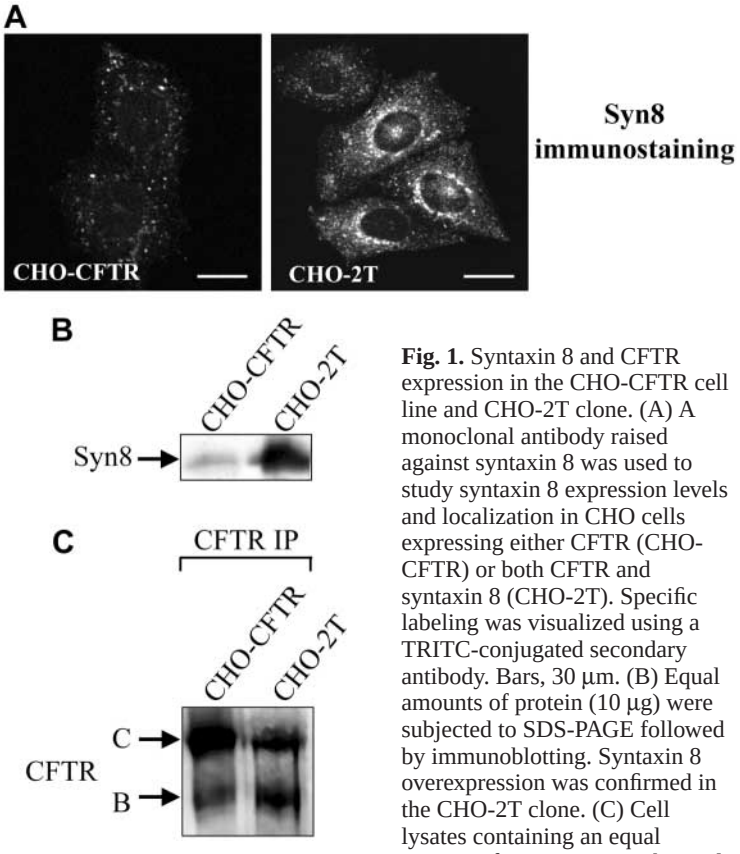


Fig. 1. Syntaxin 8 and CFTR expression in the CHO-CFTR cell line and CHO-2T clone. (A) A monoclonal antibody raised against syntaxin 8 was used to study syntaxin 8 expression levels and localization in CHO cells expressing either CFTR (CHO-CFTR) or both CFTR and syntaxin 8 (CHO-2T). Specific labeling was visualized using a TRITC-conjugated secondary antibody. Bars, 30 μm. (B) Equal amounts of protein (10 μg) were subjected to SDS-PAGE followed by immunoblotting. Syntaxin 8 overexpression was confirmed in the CHO-2T clone. (C) Cell lysates containing an equal amount of protein were subjected to immunoprecipitation with CFTR antibody. The presence of CFTR in CHO-CFTR and in CHO-2T cells was revealed by immunoblotting.

and immunofluorescence. To isolate clones overexpressing Syn8 or Syn8ΔTM, cells were diluted and cultured in 24-well plates.

For the electroporation of COS-7 cells, 2×10^6 cells at 90% confluence were trypsinized and resuspended in 800 μl of PBS with 10 μg of each plasmid. After 5 minutes incubation on ice, cells were transferred into an electroporation cuvette (4 mm electrode gap, EquiBio Ltd) and electroporated with one shock at 300 V, 450 μF, using an EasyjecT Plus multipurpose electroporation system (EquiBio Ltd). The cells were then incubated on ice for 10 minutes in 10 ml of culture medium, plated on glass coverslips, grown for 48 hours and treated subsequently for immunofluorescence analysis.

Iodide efflux experiments

CFTR chloride channel activity was assayed by measuring the rate of iodide (^{125}I) efflux from transfected CHO cells as previously described (Dérand et al., 2001). All experiments were performed at 37°C. The loss of intracellular ^{125}I was determined by removing the medium with efflux buffer every 1 minute for up to 10 minutes. The fraction of initial intracellular ^{125}I lost during each interval time was determined and time-dependent rates of ^{125}I efflux were calculated from $\ln(^{125}I_{t1} / {}^{125}I_{t2})/(t_1 - t_2)$ where $^{125}I_t$ is the intracellular ^{125}I at time t, and t_1 and t_2 are successive time-points (Venglarik et al., 1990).

Patch-clamp experiments

Whole-cell chloride currents were recorded from CHO cells at room temperature with a List EPC-7 patch-clamp amplifier. I-V relationships were built by clamping the membrane potential to –40 mV and by pulses from –100 mV to +100 mV by 20 mV increments. Mediums generating a chloride gradient of 151 mM external concentration and 28 mM internal concentration were used. The pipette solution contained: 113 mM L-aspartic acid, 113 mM CsOH, 27 mM CsCl, 1 mM NaCl, 1 mM $MgCl_2$, 1 mM EGTA, 10 mM TES, 285 mOsm (pH 7.2). MgATP (3 mM) was added just before patch-clamp experiments were started. The external solution consisted of 145 mM NaCl, 4 mM CsCl, 1 mM $CaCl_2$, 5 mM glucose, 10 mM TES, 340 mOsm (pH 7.4). Results were analyzed with the pCLAMP6 package software (pCLAMP, Axon Instruments). Cells were stimulated with forskolin (Sigma-Aldrich) or by the CFTR activator MPB-91 (5-butyl-6-hydroxy-10-chlorobenzo[c]quinolizinium) synthesized as previously described (Dérand et al., 2001).

Statistics

Results are expressed as means ± s.e.m. of n observations. To compare sets of data, we used either an analysis of variance (ANOVA) or Student's t test. Differences were considered statistically significant when $P<0.05$. All statistical tests were performed using GraphPad Prism version 3.0 for Windows (Graphpad Software).

Immunofluorescence

Cells grown on glass coverslips were washed with PBS^{++} (PBS buffer supplemented with 1 mM $CaCl_2$ and 1 mM $MgCl_2$) and fixed for 10 minutes in 3% paraformaldehyde in PBS and permeabilized for 10 minutes in saponin buffer (0.5% BSA and 0.02% saponin in PBS^{++}). Cells were incubated in PBS^{++} containing 0.5% BSA for 45 minutes at room temperature to block unspecific antigen sites. Then, coverslips were labeled for 1 hour with primary antibody (1:100 in blocking buffer). The cells were washed three times in PBS^{++} containing 0.5% BSA and labeled with conjugated antibody (1:100) for 1 hour. Coverslips were mounted with VectaShield fluorescence medium H1000 (Vector Lab).

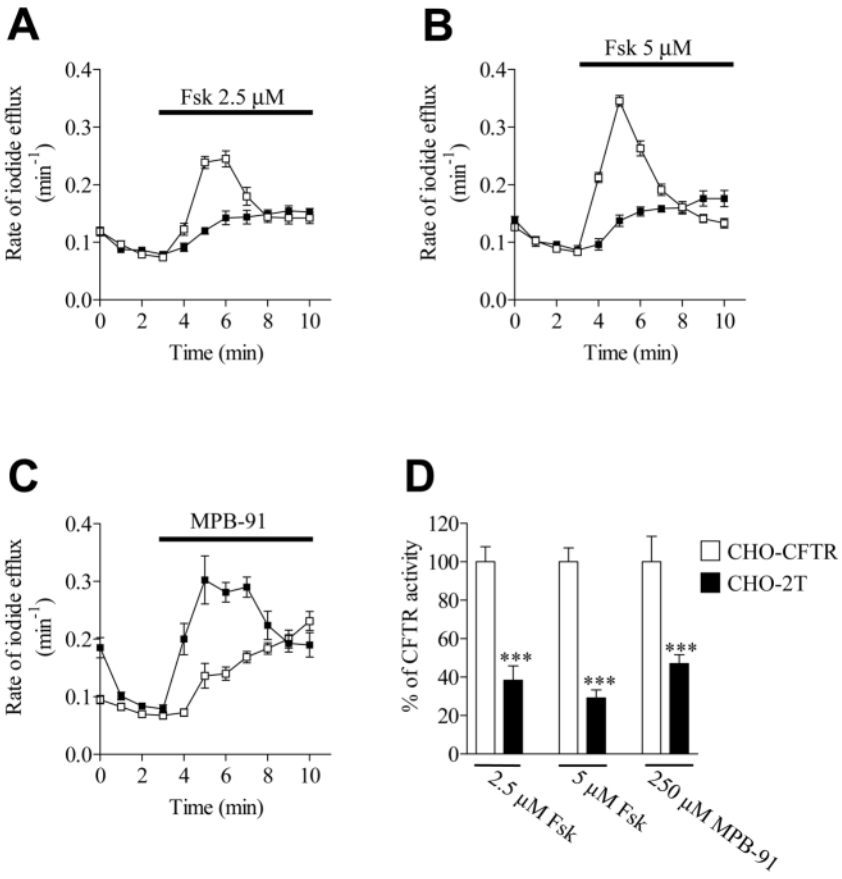


Fig. 2. Analysis by iodide efflux assay of CFTR chloride channel activity in the presence of syntaxin 8. (A-C) Iodide effluxes were measured using CHO-CFTR cells (□) or CFTR and Syn8 transfected CHO-2T cells (■). Cells were treated with 2.5 μM forskolin (A), 5 μM forskolin (B) or 250 μM MPB-91 (C) during the interval time indicated by the bar at the top of each panel. (D) Summary of the data collected from 8-16 different experiments using CHO-CFTR cells (empty columns) or CHO-2T cells (filled columns). Data are expressed as a percentage of maximal activity in the presence of the corresponding agent in CHO-CFTR cells. ***$P<0.0001$.

Confocal imaging

The labeled samples were examined by confocal laser scanning microscopy using a BioRad MRC 1024. The confocal unit was attached to an inverted microscope (Olympus IX70). Maximal resolution was obtained with Olympus plan apo ×60 water, 1.3 numerical aperture objective lens. Fluorescence signal collection, image construction and scaling were performed through the control software (Lasersharp 3.2, BioRad). For colocalization study of CFTR, syntaxin 8 and endosomal markers, bleed-through was avoided during acquisition with red, green and far red fluorescence: images were collected sequentially and then merged as an RGB colored image. Lasersharp 3.2 software permitted analysis of the degree of colocalization in the same focal plane. For each pair of color

combinations, a two-dimensional fluorogram displayed the intensity and distribution of different colored pixels within a merged image as a scattergram. It was possible to select a subset of pixels from the scattergram that have significant intensity in both colors (as shown by colored rectangle) to visualize on the cell image the localization of the selected pixels.

SDS-PAGE and immunoblot

Cells were homogenized by several passes through a 23-gauge syringe needle in RIPA buffer (50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 100 mM NaCl, 1% Triton X-100) containing protease inhibitors (20 μM leupeptin, 0.8 μM aprotinin, 10 μM pepstatin and 1.25 mM phenylmethylsulfonyl fluoride). Cell lysates were incubated on ice for 30 minutes and clarified by centrifugation at 15,000 ***g*** for 5 minutes at 4°C. Total proteins were quantified using the BCA protein assay reagent (Pierce), and 10-50 μg of proteins were loaded onto an SDS-PAGE apparatus. SDS-PAGE was performed according to Laëmmli and Favre (Laëmmli and Favre, 1973). Extracts were separated on 15% polyacrylamide gels to study syntaxin 8, and 7% for CFTR. For western blots, proteins were transferred from gels to a 0.20 μm pore nitrocellulose membrane (Sartorius, Germany) using a Miniprotean III electroblotter (BioRad Laboratories). Immunoblots were washed in PBS containing 0.1% Tween-20 (PBS-Tween) and then probed overnight at 4°C with primary antibody in PBS-Tween. Membranes were washed and incubated for 1 hour at 4°C with the secondary antibody. Bound antibodies were detected using enhanced luminol and oxidizing reagents as specified by the manufacturer (ECL, Amersham Pharmacia Biotech).

Immunoprecipitation

Cell lysates were prepared as described above with 1 ml of lysis buffer per 100 mm culture dish. In these experiments, 3 ml of cell lysate supplemented with 9 ml of NET buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 0.05% Nonidet P-40) were incubated overnight at 4°C with 2 μg of CFTR or Syn8 antibodies. To precipitate immune complexes, an incubation with 3 μg of protein G-sepharose (Amersham Pharmacia Biotech) was conducted for 1 hour at 4°C. Bead-bound complexes were washed three times with cold NET buffer and denatured in Laëmmli buffer for 15 minutes at room temperature. Samples were separated on SDS-PAGE and analyzed by western blot.

Pull-down assays

GST-fusion proteins were produced in *E. coli* (BL21 strain). Cells transformed with pGEX (Amersham Pharmacia Biotech) constructs were grown and lysed according to the manufacturer's protocol.

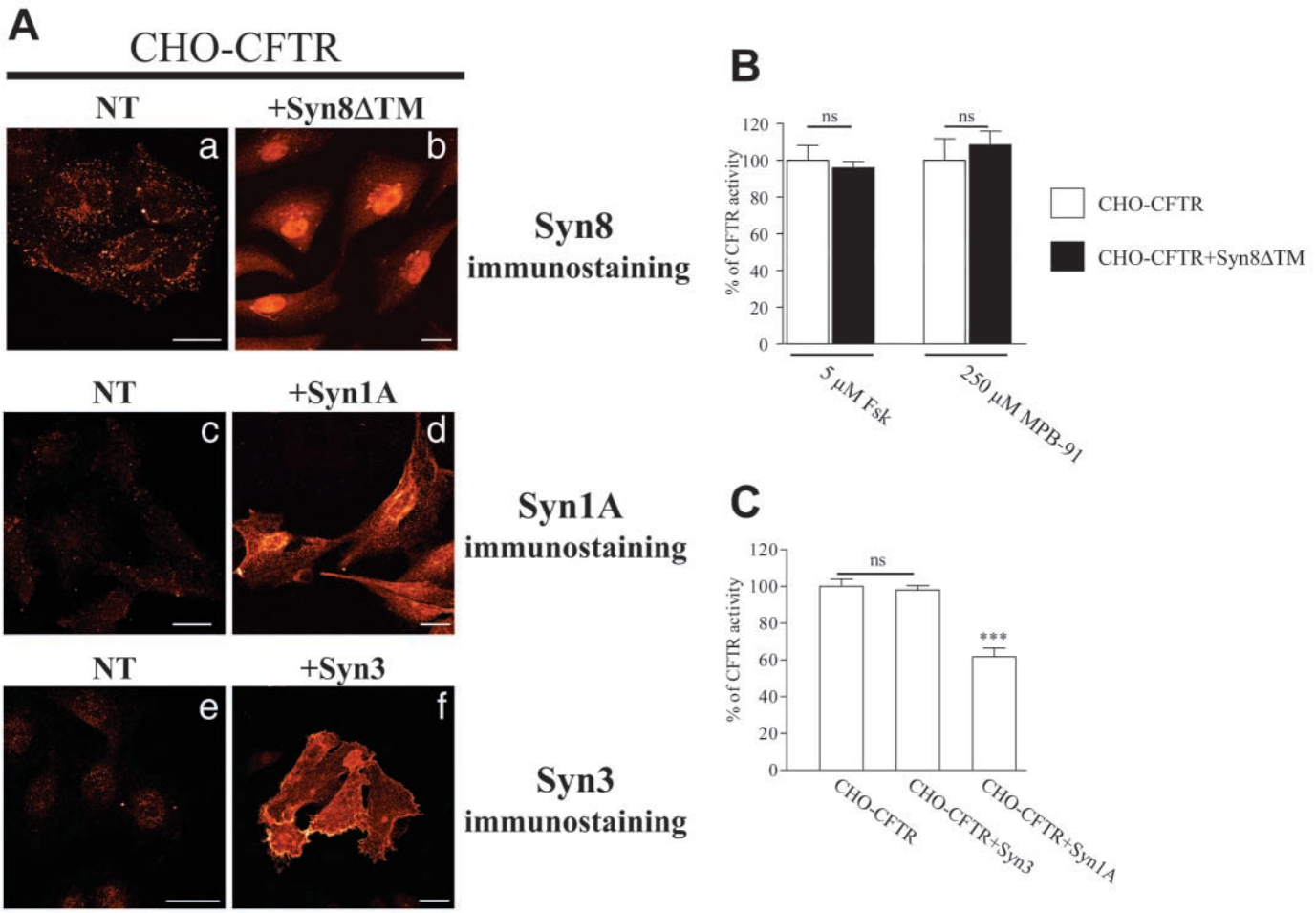


Fig. 3. Analysis of CFTR channel activity in the presence of different syntaxins. (A) Expression profile analysis of different syntaxins before (NT) or after plasmid transfection in CHO-CFTR cells (Syn8ΔTM, Syn1A and Syn3). Bars, 20 μm. (B) Summary of data collected from four independent iodide efflux experiments using CHO-CFTR cells (empty columns) or CHO+CFTR+Syn8ΔTM clone (filled columns). (C) Summary of data obtained from 12-16 independent iodide efflux experiments using CHO-CFTR, CHO-CFTR+Syn3 and CHO-CFTR+Syn1A stimulated by 5 μM forskolin. Data are expressed as a percentage of CFTR maximal activity. ***P<0.0001; ns, not significant.

Recombinant proteins contained in lysates were purified by incubation for 30 minutes at 4°C with 500 µl of glutathione-sepharose beads (Amersham Pharmacia Biotech) followed by three washes with PBS, 1% Triton X-100. GST-fusion protein yield was controlled by SDS-PAGE followed by Coomassie blue gel staining. Then, 8×10^6 COS-7 cells stably transfected with GFP-CFTR were lysed as described above. Lysates were mixed with 200 µg of recombinant proteins bound to glutathione-sepharose beads. Incubation was conducted overnight at 4°C. Bead-bound complexes were washed three times in lysis buffer, denatured in Laëmmli buffer for 15 minutes at room temperature, loaded on a 7% SDS-PAGE and analyzed by western blot using GFP antibody.

Results

Inhibition of CFTR channel activity by syntaxin 8 overexpression in CHO cells

In the first part of our study, we addressed the functional interactions of syntaxin 8 with CFTR. CHO-CFTR cells were transfected with the plasmid pcDNA3.1/Zeo+STX8. After transfection, about 50% of the cells were found to overexpress syntaxin 8, as determined by immunofluorescence analysis (data not shown). At this step, we observed by iodide efflux experiments a significant inhibition (~40%) of CFTR channel activity in CHO-CFTR+Syn8 cells as compared with control CHO-CFTR cells (data not shown). For further experiments we selected one clone, named CHO-2T (doubly transfected), highly overexpressing syntaxin 8 (Fig. 1A,B).

The immunostaining of endogenous syntaxin 8 in CHO-CFTR cells presented a vesicle-like pattern. The intensity of this staining was increased after *STX8* cDNA transfection. Moreover, a strong perinuclear staining appeared in the doubly transfected CHO-2T clone (Fig. 1A). Overexpression of syntaxin 8 was also assessed by immunoblot (Fig. 1B).

To verify that CFTR expression in CHO-2T clone was not affected compared with CHO-CFTR cells, we controlled the *CFTR* mRNA level by quantitative RT-PCR and found no significant difference between the two cell lines (data not shown). Moreover, we performed immunoprecipitation to assess the presence of CFTR protein in each cell line (Fig. 1C). CFTR could be immunoprecipitated with comparable efficiency in CHO-CFTR and CHO-2T cell lysates. Anti-CFTR immunoblotting revealed the characteristic mature (band C) and immature (band B) of CFTR in each CHO cell lines.

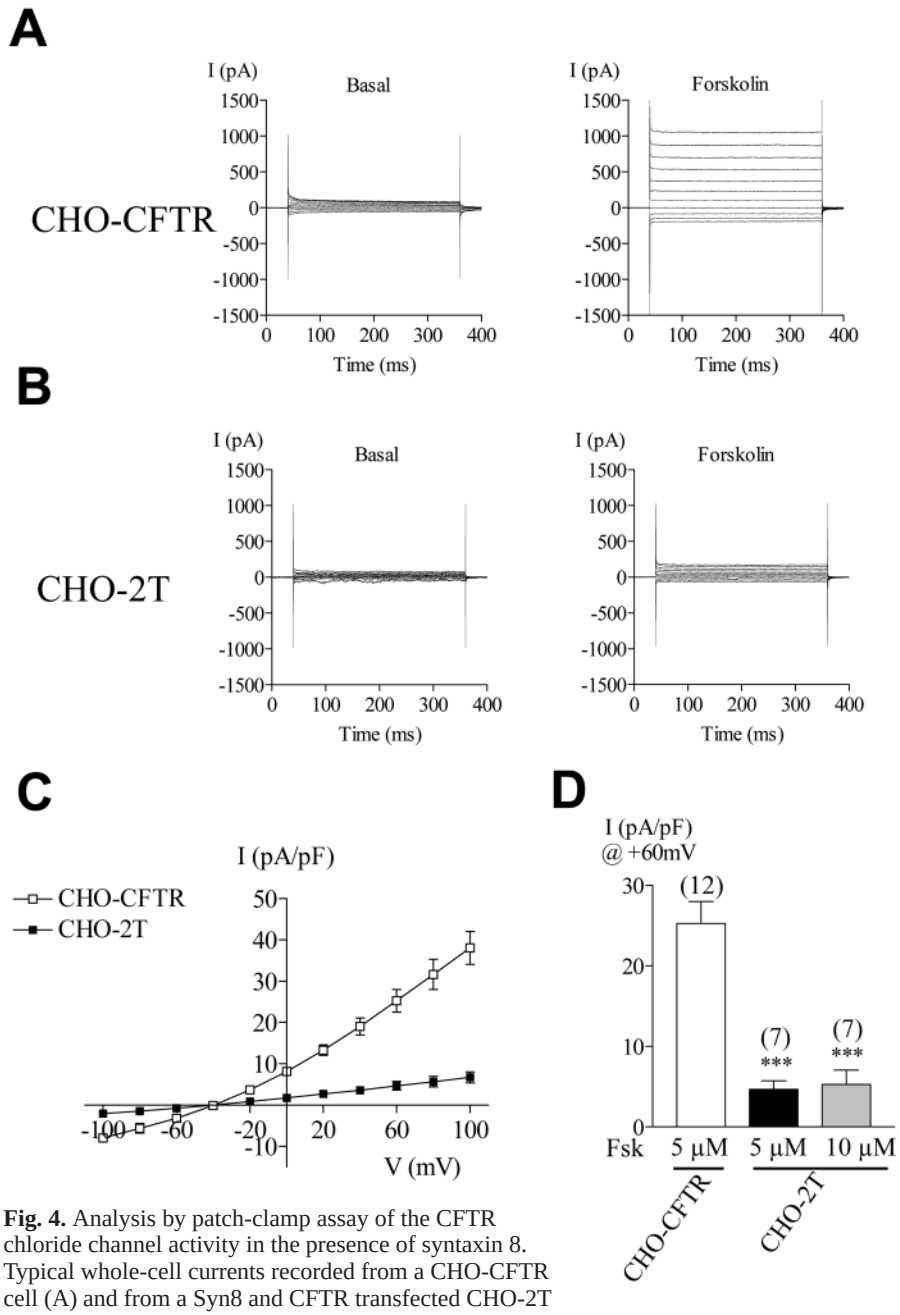


Fig. 4. Analysis by patch-clamp assay of the CFTR chloride channel activity in the presence of syntaxin 8. Typical whole-cell currents recorded from a CHO-CFTR cell (A) and from a Syn8 and CFTR transfected CHO-2T cell (B) in the absence or presence of 5 µM forskolin in the bath. Cells capacitances are 30 pS and 27 pS in A and B, respectively. (C) Averaged current-voltage relationships from 12 CHO-CFTR cells and seven CHO-2T cells in the presence of 5 µM forskolin. (D) Histograms showing current densities measured at +60 mV for the different experimental conditions indicated at the bottom of each column. The number of experiments is indicated on the graph. ***P<0.0001.

The consequences of syntaxin 8 overexpression on CFTR channel activity were studied using iodide efflux and whole-cell patch-clamp experiments in CHO-CFTR and CHO-2T cells. Control experiments were performed on CHO-CFTR cells transfected with a pcDNA3.1/Zeo(–) empty vector. Resulting data exhibited no significant difference in CFTR channel activity between mock transfected and control CHO-CFTR cells (data not shown). Fig. 2 shows the results from iodide efflux experiments. In control CHO-CFTR cells, CFTR

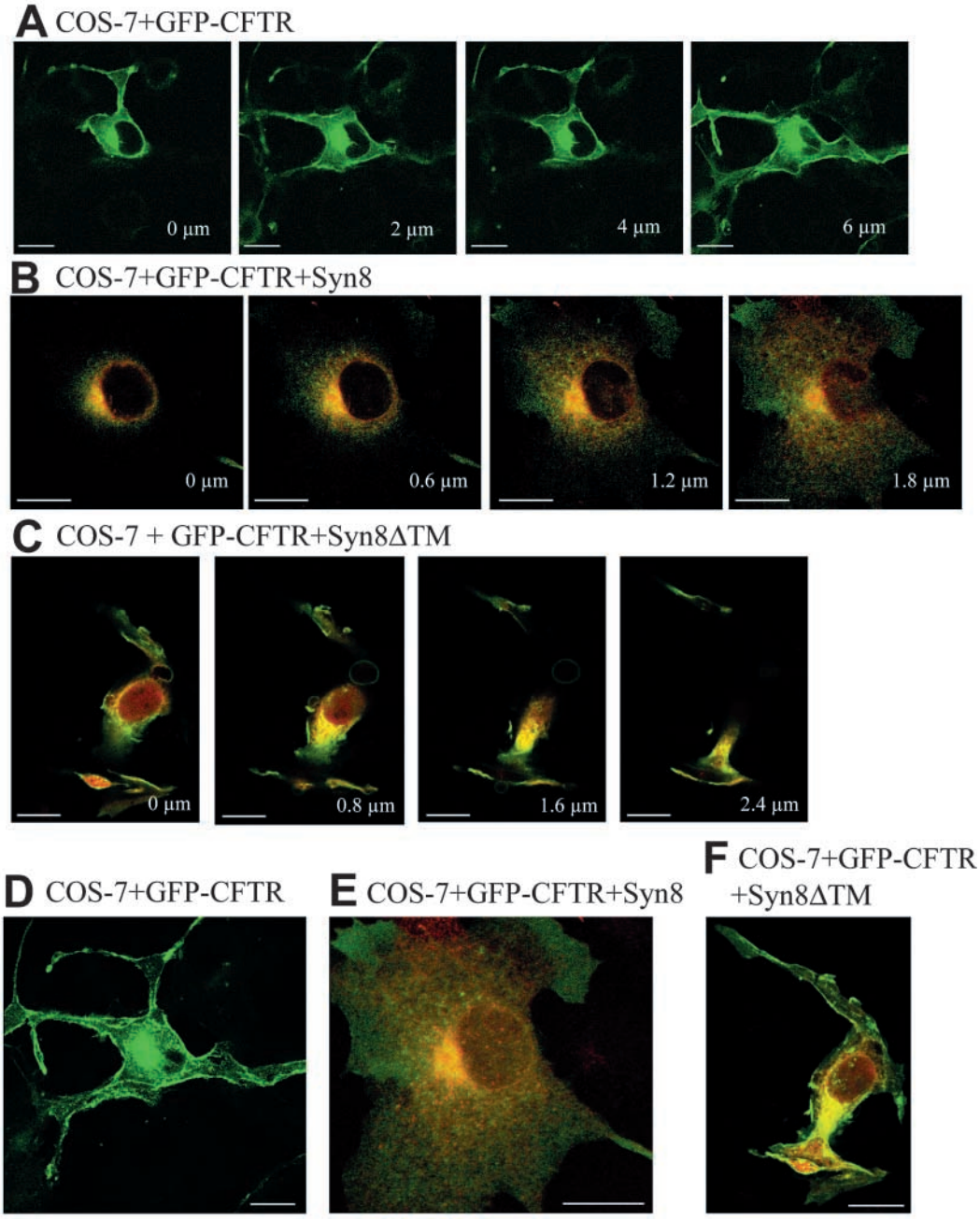
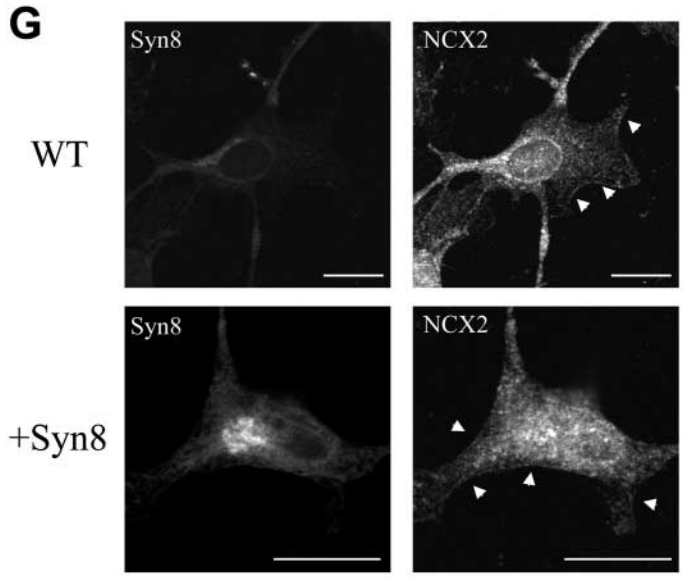


Fig. 5. The impact of syntaxin 8 overexpression on GFP-CFTR cellular localization in COS-7 cells. (A-C) Thick optical sections acquired with 2, 0.6 or 0.8 μm steps (from top to bottom). (D-F) Projections of entire Z series. Cells were transfected with GFP-CFTR alone (A,D), cotransfected with GFP-CFTR and Syn8 (B,E) and cotransfected with GFP-CFTR and the cytosoluble form of syntaxin 8: Syn8ΔTM (C,F). Green fluorescence resulting from GFP-CFTR expression shows strong and continuous localization of GFP-CFTR concentrated in plasma membrane of control cells (A,D). In doubly transfected cells, overexpression of Syn8 (as revealed by red fluorescence) is associated with a strong reduction of GFP-CFTR plasma membrane localization, while a partial colocalization is visualized in a juxtanuclear region as revealed by yellow fluorescence. By contrast, experiments using Syn8ΔTM exhibit a large cytosolic staining of syntaxin 8 soluble form and a plasma membrane staining for GFP-CFTR (C,F). (G) Control experiments studying NCX2 localization in wild-type or Syn8-transfected COS-7 cells. Arrowheads indicate plasma membrane localization of NCX2 in both cases. Bars, 20 μm. Each image is representative of six independent experiments.

activity was stimulated by 2.5 μM or 5 μM forskolin (Fig. 2A,B,D) and by the CFTR channel activator MPB-91 (Fig. 2C,D) (Dérand et al., 2001). The peak rates were 0.245±0.014 (n=8), 0.350±0.009 (n=16) and 0.300±0.042 (n=8) in the presence of 2.5 μM forskolin, 5 μM forskolin and 250 μM MPB-91, respectively. In CHO-2T cells, the corresponding peak rates were reduced (P<0.0001) by more than 60% to 0.142±0.012 (n=8), 0.150±0.06 (n=8) and 0.130±0.021 (n=8), respectively as shown in Fig. 2A-C. These results, summarized in Fig. 2D, showed that the expression of syntaxin 8 altered the activation process of CFTR (100±7.85 (n=8) to 38.31±7.34 (n=8); 100±7.25 (n=16) to 29.15±4.18 (n=8) and 100±13.28 (n=8) to 47.05±7.64 (n=8), respectively, expressed as a percentage of CFTR maximal activity).

To determine whether the cytosolic domain (90% of the entire protein) of syntaxin 8 could modulate CFTR channel activity, we used a soluble form of this protein (deleted of its transmembrane domain, noted Syn8ΔTM). CHO-CFTR cells were transfected with the appropriate vector as described above. One clone was isolated and characterized by immunofluorescence. This clone exhibited a diffuse cytosolic and a nuclear staining (Fig. 3Ab), representative of Syn8ΔTM overexpression pattern observed in noncloned cells (data not shown). This staining was clearly distinct from endogenous syntaxin 8 expression profile, which presented an organized vesicular-like structure in CHO-CFTR cells (Fig. 3Aa). Iodide efflux experiments (Fig. 3B) using 5 μM forskolin or 250 μM MPB-91 stimulations activated CFTR channel with no significant difference between control CHO-CFTR (100±8.027 (n=4) and 100±11.67 (n=4), respectively, expressed as



percentage of CFTR maximal activity) or Syn8ΔTM expressing cloned cells (95.836±3.40 (*n*=4) and 108.33±7.5 (*n*=4), respectively, expressed as a percentage of CFTR activity). Similar results were obtained with the noncloned CHO-CFTR cells overexpressing Syn8ΔTM (data not shown).

At this stage, we agreed that CFTR inhibition was not due to overexpression of any SNARE proteins. Syntaxin 3, unlike syntaxin 1A, is known not to physically interact with CFTR (Naren et al., 1997). So, we have assayed CFTR channel modulation under overexpression of each of these syntaxins in the CHO-CFTR model. Stably transfected cells were characterized by fluorescent immunostaining (Fig. 3Ac-f). We noticed that syntaxin 1A as well as syntaxin 3 antibodies were unable to detect endogenous proteins (Fig. 3Ac,e). As expected, cells overexpressing syntaxin 1A or syntaxin 3 presented plasma membrane localization (Fig. 3Ad,f) of these proteins with a predominant perinuclear staining observed in the case of syntaxin 1A (Fig. 3Ad). In each case, more than 40% of cells exhibited high level expression (data not shown). We measured CFTR response by iodide efflux experiments under 5 μM forskolin stimulation and we found no significant difference between control and Syn3-transfected CHO-CFTR cells, whereas syntaxin 1A overexpression induced a 40% inhibition of CFTR channel activity (100±3.978 (*n*=12), 98.06±2.254 (*n*=16) and 61.787±4.63 (*n*=12), respectively, expressed as a percentage of CFTR maximal activity) (Fig. 3C).

To confirm our iodide efflux results, we performed whole-cell patch-clamp recordings and compared the properties of CFTR chloride currents in the presence or absence of syntaxin 8. In control CHO-CFTR cells, the activity of CFTR Cl^- current was stimulated by 5 μM forskolin and the current measured at +60 mV was 25.25±2.74 pA/pF (*n*=12, Fig. 4A,C,D). The expression of syntaxin 8 in CHO-2T cells resulted in a dramatic reduction in the CFTR chloride current, with a current density, measured at +60 mV, of only 4.65±1.05 pA/pF and 5.27±1.77 pA/pF (*n*=7 for each concentration, Fig. 4B-D) with 5 μM and 10 μM forskolin, respectively. These observations confirmed the flux study and showed that the chloride channel activity of CFTR is strongly affected by syntaxin 8.

GFP-tagged CFTR trafficking is impaired by syntaxin 8 overexpression in COS-7 cells

To understand how syntaxin 8 overexpression induces an inhibition of the CFTR chloride current, we investigated the impact of syntaxin 8 or cytosoluble syntaxin 8 expression on the cellular distribution of a GFP-tagged CFTR in COS-7 cells. GFP-CFTR is known to be functional and the GFP tag does not affect its trafficking and localization (Moyer et al., 1998). Moreover, we have observed, in COS-7 cells, that two cystic fibrosis widespread mutants, GFP-(F508del)CFTR and GFP-(G551D)CFTR, exhibited a pharmacological response and a trafficking consistent with the physiological data (data not shown). Immunofluorescence study showed that in both cases (endogenous or overexpressed), syntaxin 8 was concentrated in a structure close to nuclear envelope, and a cytoplasm punctuated staining indicated the presence of syntaxin 8 in vesicle-like structures.

For the following experiments, we have chosen a photomultiplier sensitivity allowing us to visualize overexpressed syntaxin 8 without fluorescence saturation. At this sensitivity, endogenous syntaxin 8 was not detected. Most of the cells overexpressing GFP-CFTR exhibited a strong plasma membrane staining with weaker intracellular staining (Fig. 5A,D). When COS-7 cells were cotransfected with syntaxin 8 and GFP-CFTR, we observed a strong decrease or a disappearance of GFP-CFTR plasma membrane staining (Fig. 5B,E). GFP-CFTR appeared in cytosolic or punctuated pattern as shown in Fig. 5B with observation at different Z positions. Moreover, cotransfection with the empty pcDNA3.1/Zeo(–) vector did not affect GFP-CFTR localization at the plasma membrane (data not shown).

Control experiments using Syn8ΔTM exhibited a large cytosolic staining that clearly differed from the wild-type syntaxin 8 immunostaining pattern. Cells overexpressing Syn8ΔTM presented a plasma membrane staining of GFP-CFTR in focal planes (Fig. 5C) and in the projection of all XY sections (Fig. 5F), with an intensity level comparable to GFP-CFTR overexpressing cells. This result evidenced that double transfection did not affect the expression level of exogenous proteins such Syn8ΔTM or GFP-CFTR as compared with cells transfected with only one of these cDNAs. Thus, we could discount the possibility that the syntaxin 8 inhibitory effect on CFTR trafficking was simply due to an excess of protein biosynthesis in the cell.

Cells overexpressing wild-type syntaxin 8 exhibited a colocalization with GFP-CFTR only in the juxtanuclear region (Fig. 5B,E and Fig. 6A). However, accumulation of GFP-CFTR or syntaxin 8 in this region did not seem to be linked to the coexpression of both proteins, as GFP-CFTR or syntaxin 8 alone also had a strong staining of this organelle near the nucleus. In the cytoplasm, syntaxin 8 often appeared as dot-like structures corresponding presumably to endosome/lysosome structures, whereas GFP-CFTR appeared more homogenous in the entire cytosol. So, apart from juxtanuclear organelles, wild-type syntaxin 8 and GFP-CFTR did not seem to be colocalized in the distal part of cytosol. By contrast, the Syn8ΔTM immunostaining pattern colocalized with GFP-CFTR not only in the juxtanuclear area but also in a large part of the cytosol. A colocalization between the two proteins was also observed at the plasma membrane in several focal planes (Fig. 5C,F and Fig. 6B).

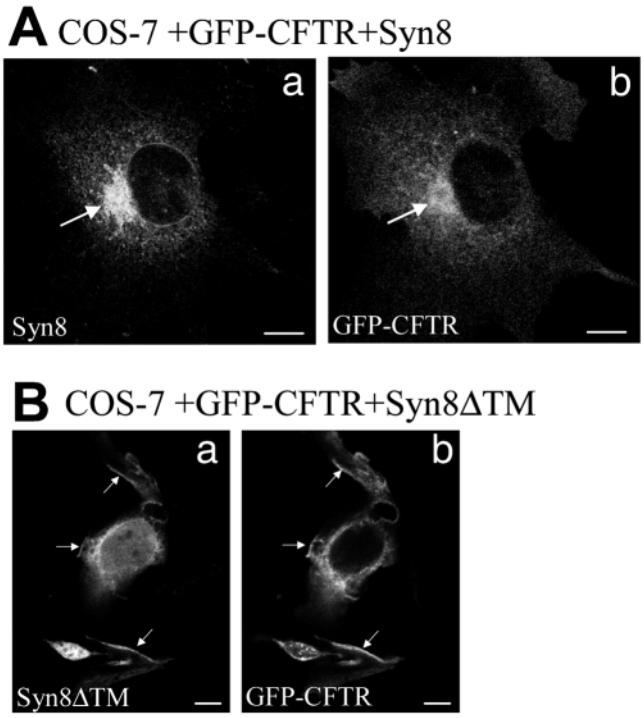


Fig. 6. Syntaxin 8 must be anchored in the membrane to inhibit GFP-CFTR plasma membrane localization. We compared the intracellular localization of both proteins in COS-7 cells cotransfected with GFP-CFTR and Syn8 (A) or by GFP-CFTR and Syn8ΔTM (B). Overexpressed (Aa) syntaxin 8 shows a predominantly perinuclear staining and also appears in a cytoplasm punctuated pattern. GFP-CFTR presents no plasma membrane localization (Ab), but is accumulated and colocalizes with syntaxin 8 only in the perinuclear region (arrows). (B) Soluble syntaxin 8 (Syn8ΔTM) displays a continuous staining within the cell, but is predominantly accumulated in compartments where GFP-CFTR is present, notably at the plasma membrane. Bars, 10 μm.

These results showed that overexpressing wild-type syntaxin 8, which is anchored on the vesicle membrane, disturbed CFTR trafficking, whereas cytosoluble syntaxin 8 had no or little effect on CFTR plasma membrane localization. As a control, we analyzed the distribution of a plasma membrane Na^+/Ca^{2+} exchanger isoform (NCX2) (Li et al., 1994), which is not known to interact with SNARE proteins. As expected, the surface localization of NCX2 in COS-7 cells was not affected by syntaxin 8 overexpression (Fig. 5G).

GFP-CFTR and syntaxin 8 colocalize and are accumulated in recycling endosomes (RE) in COS-7 transfected cells

To identify the subcellular compartments in which GFP-CFTR and syntaxin 8 colocalize, we performed immunofluorescence studies with three well characterized markers of the endosomal and lysosomal compartments on wild-type and doubly transfected COS-7 cells. As expected, cells overexpressing GFP-CFTR and syntaxin 8 exhibited a strong staining for both proteins in a juxtanuclear area, a compartment where the two proteins highly colocalize (Fig. 7Ad,Bd,Cd). Each fluorogram showed that most syntaxin 8 and CFTR fluorescent plots were placed on the bisector line (Fig. 7Ae,Be,Ce). This indicated a high degree of colocalization of both proteins. In each immunostaining experiment, the highest intensity of merged fluorescence corresponded to the juxtanuclear region (data not shown). Syntaxin 8 immunostaining, whether using the rabbit polyclonal antibody (Fig. 7Aa,Ba) or the mouse monoclonal antibody (Fig. 7Ca), gave a classical tubular/vesicular-like pattern. GFP-CFTR, as described above, was not localized on plasma membrane but on punctuated and tubular-like structures in the cytosol, and a strong signal was seen on the juxtanuclear area (Fig. 7Ab,Bb,Cb). Lamp-1 (an LE/lysosomal marker) staining was observed as a strong vesicular and perinuclear pattern (Fig. 7Ac), but its distribution did not colocalize with Syn8 and GFP-CFTR cellular staining (Fig. 7Ad). These data were confirmed by fluorograms showing that Lamp-1 fluorescence distribution was clearly separated from CFTR and Syn8 fluorescences (Fig. 7Af,g). By contrast, TfR immunostaining (endocytosis-recycling pathway) revealed a dot-like vesicular pattern more extensive in the cytosol than Lamp-1 staining (Fig. 7Bc). Intracellular distribution between TfR and CFTR on the one hand (noted TfR/CFTR) and between TfR and Syn8 on the other hand (noted TfR/Syn8) was mostly in distinct compartments within the cell (Fig. 7Bd). These results were supported by data from fluorograms, which showed two distinct fluorescences in each case. Several plots with high intensity of overlapping fluorescence suggested a colocalization between TfR and CFTR on the one hand, and between TfR and Syn8 on the other, in vesicular structures near the nucleus (Fig. 7Bf',g'). When we compared the TfR/CFTR and TfR/Syn8 vesicular pattern in this region (insert), we observed many similarities suggesting that TfR, CFTR and Syn8 were present in the same vesicles. As the juxtanuclear colocalization between CFTR and Syn8 partially matched to TfR localization, we could identify these vesicles as recycling or post-Golgi endosomes. To test our hypothesis, we have used a rabbit polyclonal antibody against Rab11, a protein that has been found associated with trans-Golgi network (TGN) membranes, post-Golgi vesicles and recycling endosomes (Urbe et al., 1993; Ullrich et al., 1996). In COS-7 cells, endogenous Rab11 displayed a punctuated vesicular pattern scattered throughout the cytoplasm and seemed to accumulate in a tubulo-vesicular structure near the nucleus (Fig. 7Cc). In wild-type cells, a partial colocalization was found between Rab11 and TfR stainings (Fig. 7Db), but no overlap was detected between Rab11 and Lamp-1 stainings (Fig. 7Da). Finally, in COS-7 cells transfected with GFP-CFTR and Syn8, the Rab11 immunostaining profile matched exactly with CFTR and Syn8 stainings (Fig. 7Ca-d). These results were sustained by fluorogram data showing Rab11/Syn8 and Rab11/CFTR fluorescent plots only on bisector line (Fig. 7Ce-g). This clearly showed that these proteins had the same localization. In conclusion, when syntaxin 8 inhibits CFTR trafficking in COS-7 transfected cells, CFTR appears to be restricted to a compartment including at least the recycling endosomes and possibly other post-Golgi vesicles.

Syntaxin 8 physically interacts with CFTR

To test the physical interaction between syntaxin 8 and CFTR, we performed immunoprecipitation experiments in CHO-2T and HT29-CL19A cell lines expressing both syntaxin 8 and

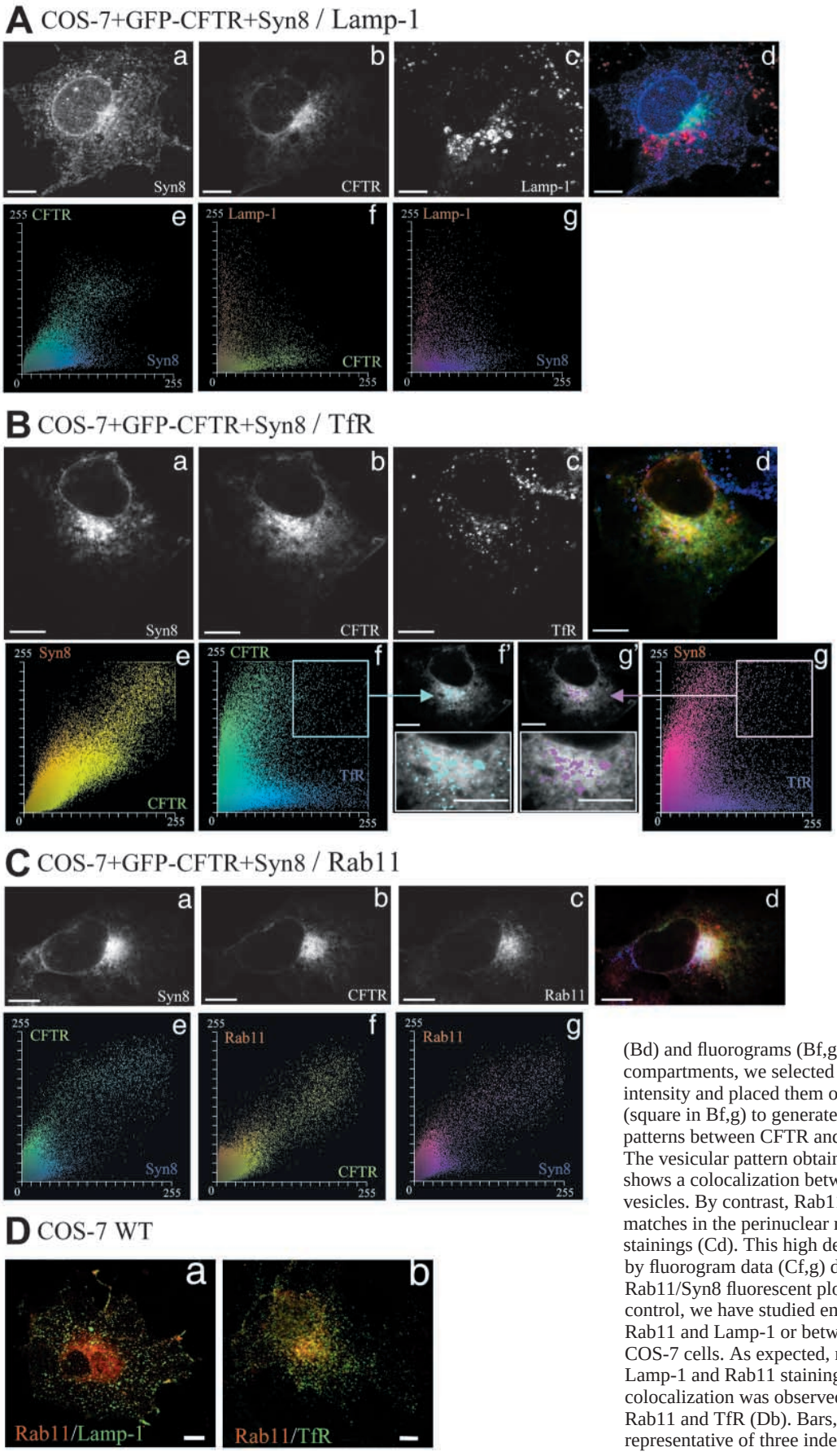


Fig. 7. GFP-CFTR and syntaxin 8 colocalize at least in the recycling endosome complexes in doubly transfected COS-7 cells. In each experiment (except D), COS-7 cells were cotransfected with GFP-CFTR and Syn8, then costained with anti-syntaxin 8 antibody and anti-Lamp-1 (A), anti-TfR (B) or anti-Rab11 (C) antibodies. All cell images present the projection of the entire Z series sections acquired by fluorescent confocal microscopy. Fluorograms were obtained as described in Materials and Methods. In each experiment, protein colocalizations were analyzed with three fluorograms. Syntaxin 8 (a in A-C) and GFP-CFTR (b in A-C) exhibit perinuclear staining as expected. These two proteins colocalize in the perinuclear region as shown in the merged images (d in A-C) and fluorograms (e in A-C). Lamp-1 immunostaining pattern (Ac) exhibits no colocalization with GFP-CFTR and syntaxin 8 both on merged image (Ad) and fluorograms (Af,g), whereas the immunostaining profile obtained with TfR (Bc) presents a partial colocalization on merged picture (Bd) and fluorograms (Bf,g). To further analyze colocalization compartments, we selected plots with high fluorescence intensity and placed them on the fluorogram bisector line (square in Bf,g) to generate images showing the colocalization patterns between CFTR and TfR (Bf') or Syn8 and TfR (Bg'). The vesicular pattern obtained in each case is very similar and shows a colocalization between the three proteins within these vesicles. By contrast, Rab11 immunostaining (Cc) closely matches in the perinuclear region with CFTR and syntaxin 8 stainings (Cd). This high degree of colocalization is sustained by fluorogram data (Cf,g) displaying Rab11/CFTR or Rab11/Syn8 fluorescent plots placed on the bisector line. As a control, we have studied endogenous localization between Rab11 and Lamp-1 or between Rab11 and TfR in wild type COS-7 cells. As expected, no overlap was found between Lamp-1 and Rab11 stainings (Da), whereas a partial colocalization was observed in the perinuclear region between Rab11 and TfR (Db). Bars, 20 μm. Each analysis is representative of three independent experiments.

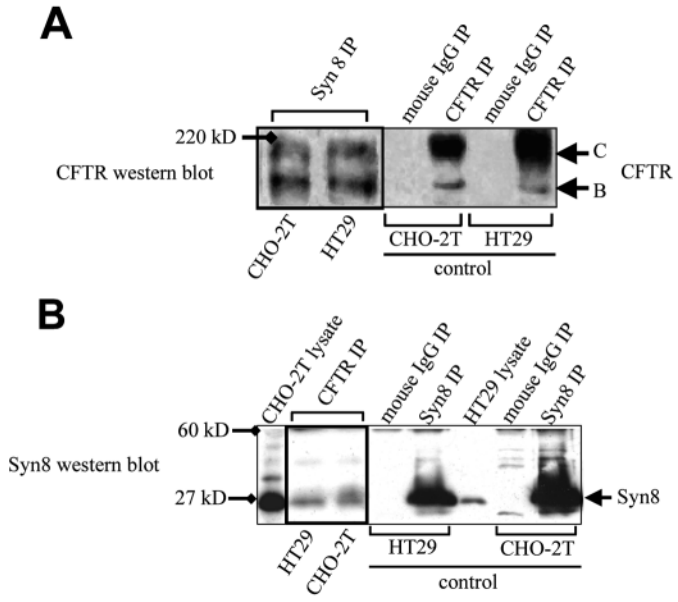


Fig. 8. CFTR and syntaxin 8 belong to the same protein complex. (A) Co-immunoprecipitation of CFTR with Syn8 in CHO-2T and HT29-CL19A cell lysates. Immunoprecipitated proteins were revealed after western blot with antibodies against CFTR. Bands B and C represent immature and mature forms of CFTR, respectively. CFTR antibody and nonimmune mouse IgG were used as positive and negative immunoprecipitation controls, respectively. (B) Co-immunoprecipitation of Syn8 with CFTR in CHO-2T and HT29-CL19A cell lysates. Immunoprecipitated proteins were revealed after western blot with antibodies against Syn8. CHO-2T and HT29 cells lysates were used as a positive control for western blot. Syn 8 antibody and nonimmune mouse IgG were used as positive and negative immunoprecipitation controls, respectively.

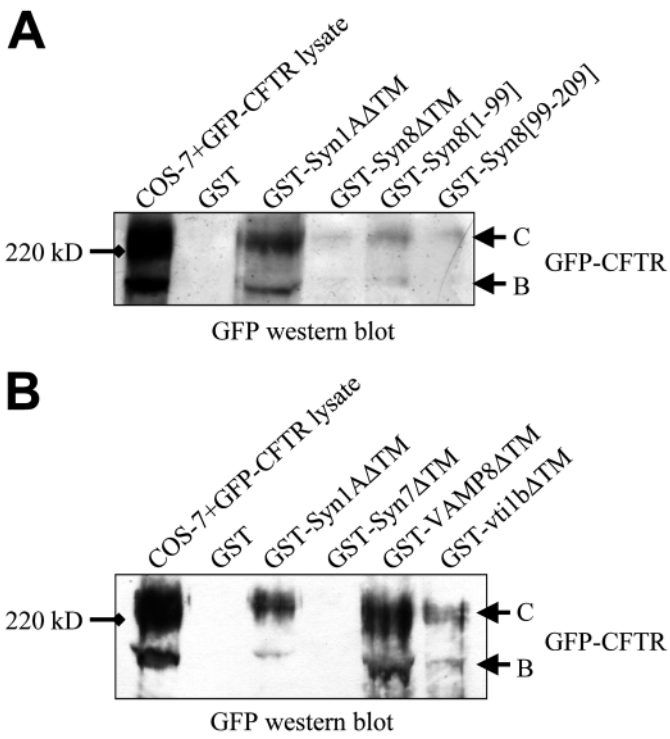


Fig. 9. In vitro interactions between CFTR and endosomal SNARE proteins. (A) In vitro binding of GFP-CFTR with different recombinant cytosolic domains of syntaxin 8: GST-Syn8ΔTM, GST-Syn8[1-99] and GST-Syn8[99-209]. (B) In vitro binding of GFP-CFTR with different recombinant endosomal SNARE proteins: GST-Syn7ΔTM, GST-VAMP8ΔTM and GST-vti1bΔTM. (A,B) Glutathione-sepharose-immobilized GST or GST-fusion proteins were incubated with COS-7+GFP-CFTR cell extracts. Bound proteins were studied by anti-GFP immunoblot. COS-7+GFP-CFTR lysate was used as a GFP-CFTR control; GST-Syn1AΔTM and GST alone were used, respectively, as positive and negative pull-down controls.

CFTR. Using lysates from both cell lines, we purified syntaxin 8 or CFTR complexes using monoclonal syntaxin 8 or CFTR antibodies, respectively. As expected, syntaxin 8 and CFTR immunoprecipitated with high efficiency (Fig. 8A,B). In these syntaxin 8 complexes, anti-CFTR western blot revealed the presence of the mature (band C) and immature (band B) forms of CFTR in each cell line (Fig. 8A). Reversibly, in CFTR complexes, anti-syntaxin 8 western blot revealed a significant amount of the protein (Fig. 8B). Notably, the existence of such an interaction in untransfected HT29-CL19A cells suggests the physiological relevance of this result.

We also investigated the interaction between CFTR and syntaxin 8 by an in vitro affinity binding assay using four recombinant GST-fusion proteins: as a positive control, the cytosolic domain of syntaxin 1A (Syn1AΔTM), the cytosolic domain of syntaxin 8 (Syn8ΔTM), and two truncated domains of syntaxin 8 (Syn8[1-99] and Syn8[99-209]). Recombinant GST protein was used as a negative control. Bead-bound recombinant proteins were incubated with COS-7+GFP-CFTR cell lysate, and retained proteins were analyzed by western blot using an anti-GFP antibody (Fig. 9A). A COS-7+GFP-CFTR cell lysate was used as a GFP-CFTR control. The N-terminal tagging of CFTR by GFP did not affect the in vitro binding of syntaxin 1A, which has been shown to interact with the N-terminus of CFTR (Naren et al., 1998). GFP-CFTR was pulled down with syntaxin 8 either with the full-length cytosolic domain of syntaxin 8 or with each of its truncated domain but predominantly with its N-terminal half upstream of its t-SNARE domain. However, this interaction was far weaker compared with GFP-CFTR binding to syntaxin 1A. Nevertheless, these results were significant as CFTR was not retained by GST alone. Because pull-down experiments revealed a weak physical interaction between syntaxin 8 and CFTR, we hypothesized that intermediary proteins could be involved in CFTR/syntaxin 8 complexes. The best candidates were the other components of the endosomal SNARE complex: VAMP8, vti1b and syntaxin 7. Similarly, we produced these recombinant SNARE proteins to test their interaction with CFTR. GFP-CFTR was pulled down either with VAMP8 or vti1b but not with syntaxin 7 (Fig. 9B). These data suggested that endosomal SNARE proteins VAMP8 or vti1b bound CFTR in vitro with a comparable efficiency to syntaxin 1A (Fig. 9B).

Discussion

We have previously described the molecular cloning of human syntaxin 8 cDNA by the two-hybrid system using the R domain of CFTR as a bait to screen a human fetal lung cDNA library

(Thoreau et al., 1999). In the present report, we establish the functional relevance of an interaction between these two proteins.

Regulation of CFTR channel activity by syntaxin 8

We have observed that syntaxin 8 overexpression is responsible for a strong inhibition of CFTR channel, activated either by the cAMP pathway (forskolin stimulation) or by a cAMP-independent pathway through the CFTR activator MPB-91 (Dérand et al., 2001; Dormer et al., 2001). Such regulation of ionic channels by SNARE proteins has already been described. For example, physical interactions have been assessed between syntaxin 1A and P/Q-type calcium channels, N-type calcium channels (Bezprozvanny et al., 1995; Sheng et al., 1996; Jarvis et al., 2000) and amiloride-sensitive epithelial sodium channels (Saxena et al., 1999). Interestingly, syntaxin 1A inhibits CFTR chloride current by direct protein-protein interaction between the t-SNARE homology domain of syntaxin 1A and the N-terminal cytoplasmic tail of CFTR (Naren et al., 1997; Naren et al., 1998). In another study (Peters et al., 1999), cAMP-induced delivery to plasma membrane of CFTR present in peripheral vesicles was inhibited by syntaxin 1A in *Xenopus* oocytes. As in the case of syntaxin 1A, our electrophysiological data indeed showed an inhibition of the CFTR activity due to syntaxin 8 overexpression. This regulation is protein specific, as when we performed the same experiment with syntaxin 3, a SNARE protein that does not bind CFTR, we obtained a normal CFTR activation. Moreover, CFTR modulation required syntaxin 8 to be anchored in membrane, as we showed that its soluble form has no effect on CFTR activity. Thus, CFTR may directly be regulated by a protein-protein interaction, or alternatively, syntaxin 8 overexpression may disturb the vesicular transport machinery. This could affect CFTR recycling or cellular targeting towards the plasma membrane and consequently inhibit the chloride channel activity measured at the cell level.

Physical interactions between syntaxin 8 and CFTR

We have shown a direct protein-protein interaction, given that CFTR was pulled down using immobilized GST-syntaxin 8 recombinant proteins. Moreover, CFTR and syntaxin 8 could be co-immunoprecipitated with each other from CHO-2T or HT29-CL19A cell extracts. These data suggest that syntaxin 8 interacts with CFTR either directly, or as a component of a CFTR-containing complex. However, this interaction is different from the one observed between syntaxin 1A and CFTR. Recombinant GST-syntaxin 1A binds CFTR with a high affinity (Naren et al., 1997), whereas the interaction between syntaxin 8 and CFTR appears to be weaker. Moreover, unlike syntaxin 1A in which only the t-SNARE domain interacts with CFTR, each moiety of syntaxin 8 binds CFTR. Despite the fact that it will be necessary to test the in vitro binding between the different domains of CFTR and syntaxin 8 to ascertain a direct physical interaction, the immunoprecipitation results confirm that syntaxin 8 and CFTR are part of the same complex. As we have precipitated both proteins with the same efficiency in CHO transfected cells and in HT29-CL19A cells (endogenously expressing both CFTR and syntaxin 8), we conclude that this interaction was not induced solely by overexpression. As the affinity between syntaxin 8 and CFTR is weak, syntaxin 8 may not be the major partner of CFTR. Tierce proteins may be necessary to establish a protein complex containing syntaxin 8 and CFTR. This hypothesis is consistent with the pull-down and immunoprecipitation experiments, and could explain the small amount of CFTR retained by GST-syntaxin 8. Possible candidates partner are the different components of the heterotetrameric endosomal SNARE core complex: vti1b, syntaxin 7 and VAMP8 (Antonin et al., 2000; Antonin et al., 2002). Our pull-down experiments suggest that VAMP8 and vti1b, which strongly and directly interact with CFTR, could be partner proteins involved in a CFTR/syntaxin 8 multimeric protein complex. This result strengthens the idea that an entire SNARE complex could regulate ion channel delivery/insertion, retrieval or recycling to the plasma membrane.

Syntaxin 8 is involved in CFTR trafficking

Syntaxin 8 physical binding and functional regulation of CFTR prompted us to investigate, at the cellular level, the effect of syntaxin 8 overexpression on CFTR localization. GFP fluorescence analysis on COS-7+GFP-CFTR cells showed a strong plasma membrane staining characteristic of mature CFTR cellular localization. However, when syntaxin 8 was cotransfected with GFP-CFTR, a dramatic decrease or a disappearance of CFTR plasma membrane staining was observed and both proteins colocalized in a perinuclear region. Syntaxin 8 impact on CFTR trafficking does not apply to any channel as, when we overexpressed syntaxin 8, the plasma membrane localization of endogenous Na^+/Ca^{2+} exchanger was not affected.

Moreover, coexpression of GFP-CFTR and Syn8ΔTM, a cytosoluble form of syntaxin 8 (depleted of its transmembrane domain), led to a normal trafficking of GFP-CFTR to the plasma membrane. Furthermore, a plasma membrane colocalization between Syn8ΔTM and GFP-CFTR was observed and, interestingly, Syn8ΔTM was seen in the plasma membrane only if CFTR was present too. More generally, Syn8ΔTM is located mainly in cellular structures where GFP-CFTR is abundant. So, when syntaxin 8 is soluble, it may physically bind CFTR and follow its trafficking, but when syntaxin 8 is anchored in vesicle membrane, it binds CFTR too, but only in the cellular compartment where syntaxin 8 assumes its function (i.e. endosome compartments). As Syn8ΔTM had no or little effect on CFTR trafficking, inhibition of CFTR trafficking by syntaxin 8 overexpression could not be related to an excess of protein synthesis in endoplasmic reticulum. Moreover, syntaxin 8 must be anchored in vesicle membranes to impair CFTR trafficking, which means that the t-SNARE function of syntaxin 8 can be engaged. These results reinforce the physiological relevance of a putative regulator role of syntaxin 8 on CFTR channel activity.

Syntaxin 8 is involved in CFTR recycling pathway

The precise identification of the colocalization region of CFTR and syntaxin 8 was determined using fluorescent confocal microscopy and three well characterized antibodies against Lamp-1, an LE/lysosomal marker protein, TfR (transferrin

receptor), an EE/RE marker protein, and Rab11, which stains RE and TGN membranes. Our results exhibited no colocalization between Lamp-1 staining and the juxtanuclear compartment where CFTR and syntaxin 8 were accumulated. TfR vesicular pattern partially matched with this region, and CFTR, syntaxin 8 and TfR seemed to be located together in thin perinuclear vesicles. By contrast, Rab11 staining was highly similar to syntaxin 8 and CFTR juxtanuclear staining and we observed a high degree of colocalization both on fluorograms and on the merged image. These results indicate that when syntaxin 8 is overexpressed with GFP-CFTR, both proteins are mainly localized in the same compartment, which could be identified as recycling endosomes and/or TGN membranes. Because we have colocalized CFTR and syntaxin 8 with TfR and Rab11 proteins, we favour the recycling endosome hypothesis.

Syntaxin 8 is a t-SNARE protein that belongs at least to the endosomal SNARE complex (Antonin et al., 2000), and is consequently involved in the endosomal pathway like syntaxins 7, 11 and 12/13 (Prekeris et al., 1998; Tang et al., 1998; Wong et al., 1998; Valdez et al., 1999; Mullock et al., 2000). Its cellular localization was determined mainly in the early endosome, but syntaxin 8 was also detected in the late endosome and lysosome organelles. The early endosome is a dynamic compartment displaying a highly complex and pleiomorphic organization that contains clearly two functionally distinct subcompartments without real physical boundaries: plasma membrane proteins are recycled via the recycling endosomes or degraded via the endosomal carrier vesicles/multivesicular bodies of the degradation pathway (reviewed by Gruenberg, 2001). In some cases, syntaxin 8 was also observed at the plasma membrane (Subramaniam et al., 2000; Kasai and Akagawa, 2001), suggesting a vesicular cycling of syntaxin 8.

The mechanism of CFTR recycling is still poorly understood, but CFTR has previously been detected in recycling endosomes using fluorescence microscopy in human bronchial epithelial cells (Poschet et al., 2002). Thus, physical binding between CFTR and syntaxin 8 may occur in recycling endosomes, a cellular compartment which is required for CFTR recycling pathway and where syntaxin 8, probably assembled with other SNARE proteins (i.e. endosomal SNARE complex), is implicated in its vesicular trafficking function. Syntaxin 8 overexpression is likely to disrupt the vesicular transport step in which it is involved, as it has been previously observed for several SNARE proteins (Dascher and Balch, 1996; Low et al., 1998; Peters et al., 1999). This could explain CFTR accumulation in recycling endosomes in our experiments. So our results show that syntaxin 8 appears as an important regulatory protein directly implicated in CFTR recycling pathway.

In conclusion, SNARE proteins, and particularly syntaxin 8, participate actively in CFTR trafficking, recycling and/or in direct regulation of CFTR activity. Here, we describe a relationship between CFTR and syntaxin 8 in the recycling endosome. A recent paper showed that in cystic fibrosis cells a dysfunction in the recycling endosome pathway may have many repercussions on endocytic and plasma membrane processes (Poschet et al., 2002). So elucidating the recycling pathway of CFTR could be important in understanding the physiopathology of cystic fibrosis.

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