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CFTR channel in oocytes from *Xenopus laevis* and its regulation by xShroom1 protein

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Abstract Shroom is a family of related proteins linked to the actin cytoskeleton. xShroom1 is constitutively expressed in *Xenopus laevis* oocytes, and it is required for the expression of the epithelial sodium channel (ENaC). As there is a close relationship between ENaC and the cystic fibrosis transmembrane regulator (CFTR), we examined the action of xShroom1 on CFTR expression and activity. Biotinylation was used to measure CFTR surface expression, and currents were registered with voltage clamp when stimulated with forskolin and 3-isobutyl-1-methylxanthine. Oocytes were coinjected with CFTR complementary RNAs (cRNAs) and xShroom1 sense or antisense oligonucleotides. We observed an increment in CFTR currents and CFTR surface expression in oocytes coinjected with CFTR and xShroom1 antisense oligonucleotides. MG-132, a proteasome inhibitor, did not prevent the increment in currents when xShroom1 was suppressed by antisense oligonucleotides. In addition, we inhibited the delivery of newly synthesized proteins to the plasma membrane with BFA and we found that the half-life of plasma membrane CFTR was prolonged when coinjected with the xShroom1 antisense oligonucleotides. Chloroquine, an inhibitor of the late endosome/lysosome, did not significantly increase CFTR currents when xShroom1 expression was inhibited. The higher expression of CFTR when xShroom1 is suppressed is in concordance with the functional studies suggesting that the suppression of the xShroom1 protein resulted in an increment in CFTR currents by promoting the increase of the half-life of CFTR in the plasma membrane. The role of xShroom1 in regulating CFTR expression could be relevant in the understanding of the channel malfunction in several diseases.

Keywords CFTR · Shroom · Oocytes · Ion currents

Introduction

The cystic fibrosis transmembrane regulator (CFTR) is activated by the increase of intracellular cAMP levels and protein kinase C (PKC) and protein kinase A (PKA)-mediated phosphorylation. Other additional factors influence the amount, stability, half-life and activity of CFTR at the surface membrane [see 7 for references]. F-actin is essential for the regulation of the activity and expression of CFTR at the plasma membrane [9], and this observation was further extended by Chasan et al. [12] who showed by atomic force microscopy a direct interaction between actin and CFTR, which may explain the regulatory role of the cytoskeleton in ion channel function. Thus, cytoskeletal proteins form a dynamic interaction with CFTR, participating in the regulation of CFTR-dependent ion transport, CFTR trafficking and degradation [18, 32–34]. To date, it is unclear how all these interactions modulate the cell-surface expression of CFTR, although the available data indicate that CFTR is present in cell-surface and intracellular compartments and undergoes constitutive rapid endocytosis coupled with highly efficient recycling [1, 26].

Shroom is a family of proteins involved in the regulation and maintenance of cytoskeletal architecture by binding to actin. It has an important role in the morphogenesis of several different embryonic epithelial tissues and neuronal growth [17, 22, 25]. Hagens et al. [21] classified four different proteins (Shroom1 to Shroom4) in this family, showing similarity

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in their domains with a PDZ domain and two other domains named ASD1 and ASD2 (Apx/Shrm domains). The ASD2 domain seems to be the common denominator among family members.

Of particular interest for us is xShroom1 (formerly known as APX), a large protein formed by 1420 amino acids (160 kDa), which lacks the PDZ domain and is constitutively expressed in *Xenopus laevis* oocytes, and most important, it is necessary for the expression and activity of the epithelial sodium channel ENaC in oocytes [2, 42]. There is a marked reduction in ENaC currents in oocytes coinjected with ENaC and xShroom1 antisense oligonucleotides, and in addition, fluorescence positive staining of plasma membrane ENaC in oocytes expressing α, β and γ mENaC and xShroom1 sense oligonucleotides was observed, but not in oocytes coinjected with ENaC cRNA and xShroom1 antisense oligonucleotides.

ENaC-CFTR interactions were demonstrated in various heterologous expression systems including *Xenopus* oocytes co-expressing both channels with a reduction of amiloride-sensitive Na^+ current by cAMP-stimulated CFTR [31, 38]. In addition, CFTR and ENaC co-immunoprecipitate in oocytes from *X. laevis* [20], and a direct interaction is seen between them by fluorescence resonance energy transfer in human embryonic kidney cells [5]. This suggests that they can be functionally linked, although the exact mechanism by which CFTR regulates ENaC remains unknown, see [13, 15, 19].

Based on the available data on the effects of xShroom1 on ENaC function and the close relationship between ENaC and CFTR, we examined the action of xShroom1 on CFTR currents and CFTR expression in the plasma membrane of oocytes. We wanted to know if xShroom1 is a molecule that regulates ENaC channels or is a component that extends its role to other ion channels present in the cell. From our experiments, we suggest that the latter possibility may be correct because the suppression of xShroom1 enhances the function of CFTR by promoting the increase of the half-life of plasma membrane CFTR.

Material and methods

X. laevis oocytes

Adult female *X. laevis* frogs were anesthetized with 0.3 % tricaine (MS-222), and the oocytes were surgically removed from the abdominal incision. Oocytes were defolliculated by incubation with 1 mg/ml type IV collagenase for 40 min. The oocytes were placed in ND96 medium containing (in mM) NaCl 96, KCl 2, $CaCl_2$ 1.8 and HEPES 5 (pH 7.4) supplemented with 1 μg/ml gentamicin.

We synthesized complementary RNAs (cRNAs) for human wild type CFTR using the T7 mMessagemMachine kit (Ambion, Austin, TX). We used synthetic oligodeoxynucleotides complementary to nucleotides +455 to +479 of xShroom1 [42] (sense, 5′-GCA TTA AGC AGA ATC GCC CTA ACC AC-3′; antisense, 5′-GTG GTT AGG GCG ATT CTG CTT ATG C-3′, Integrated DNA Technologies, Biodynamics SRL). Oocytes were injected with a Drummond injector (Drummond, Broomall, PA) with 4 ng of CFTR cRNA and 25 ng of xShroom1 sense or antisense oligonucleotides (total volume 50 nl) or 50 nl of H_2O alone in some cases.

Reagents

The reagents were forskolin 10 μM (Alomone Labs, Jerusalem, Israel), IBMX 1 mM (Sigma-Aldrich, St. Louis, USA), DPC (Sigma-Aldrich, St. Louis, USA), brefeldin A 5 μM (Sigma-Aldrich, St. Louis, USA), MG-132 10 μM (Sigma-Aldrich, St. Louis, USA) and chloroquine 50 μM (HLB Pharma).

Electrophysiology

A standard two-electrode voltage clamp was performed using a Warner Oocyte Clamp OC 725C (Warner Instruments, Hamden, CT) with a bath probe circuit. We acquired data through Clampex 8.0 (Axon Instruments, Union City, CA) using a DigiData 1220A interface at 1 kHz. Micropipettes had resistances of 1–3 MΩ when filled with 3 M KCl. We clamped the bath with two chloride silver wires through 3 % agar bridges in 3 M KCl and positioned close to the oocyte. In the well with the oocyte, we estimated the bath-fluid resistance as the resistance between both electrodes (about 100–200 Ω). Without the bath probe, this value is increased by a factor of 10 or 20. Thus, all the experiments were done using the bath probe circuit to keep this resistance in series with the membrane and between electrodes as low as possible.

We perfused the oocyte chamber (0.6 ml/min) with a peristaltic pump (Dynamax RP-1; Rainin Instruments, Woburn, MA) and the solution ejected by a needle placed on top of the well containing the oocyte. Following the insertion of both microelectrodes, we waited for 5 min before starting the experiment. We ran two sets of records with a delay of 5 min to be sure that the currents were stable. Then we applied 10 μM forskolin + 1 mM IBMX, and the currents were recorded at 15 min of incubation, enough time to have a stable channel activation effect.

For the current-voltage (I-V) relationships, we applied a series of 500 ms voltage steps from −160 to +40 mV in 20 mV increments. The currents were measured after 400 ms at a clamp potential of 0 mV. CFTR-mediated Cl^- currents were defined as the current difference measured in the absence versus the presence of forskolin + IBMX in the bath solution [30, 35].

Cell-surface biotinylation

Cell-surface expression levels of the CFTR protein were examined using the membrane-impermeant biotinylation reagent (Pierce Chemical, Rockford, IL, USA). Groups of 20–40 injected oocytes were incubated with forskolin (10 μM) plus IBMX (1 mM) for 15 min. The medium was removed, and the oocytes were washed twice with forskolin + IBMX. Each group of oocytes was incubated with 1 ml of sulfo-NHS-SS-biotin (0.5 mg/ml in ND96; Pierce) and gently agitated for 60 min at 4 °C. The reagent was freshly prepared for incubation. After biotinylation, each group was washed and incubated with 1 ml of quenching solution (192 mM glycine, 25 mM Tris-Cl [pH 7.4]) for 5 min on ice to ensure complete quenching of the unreacted sulfo-NHS-SS-biotin. Oocytes were incubated in 1 ml of homogenization buffer (150 mM NaCl, 2 mM $CaCl_2$, 20 mM Tris, 2 % Nonidet P-40) with 1 % protease inhibitor cocktail and were lysed on ice by passing sequentially through a 23-gauge needle and then through a 27-gauge needle and then vortexed. The homogenate was centrifuged at 4000 rpm for 10 min at 4 °C. Biotinylated proteins were precipitated with NeutrAvidin-agarose beads (100 μl, Pierce Chemical). These were added to the supernatant to isolate cell-surface proteins and incubated overnight in an end-over-end mixing rotator. Plasma membrane proteins were dissolved in loading buffer with 50 mM DTT. CFTR was detected by polyacrylamide gel electrophoresis and immunoblotting as it is described below with a mouse monoclonal CFTR antibody directed against the R domain (R&D Systems). The immunoblots were quantified by densitometric analysis. The intensities of each band were normalized using staining with ponceau red to estimate the relative amounts of proteins. The calculated values for CFTR in sense treatment were set as 1. Values were plotted as intensity of CFTR with respect to protein loading (mean percent change ± SE). Each experiment was repeated five times.

Immunoblotting

For immunoblot studies, proteins were resolved on 6 % polyacrylamide gel and electro transferred onto nitrocellulose membranes (Hybond ECL; Amersham, GE Healthcare, Little Chalfont, UK). Membranes were blocked for 1 h with 2 % (*w*/*v*) defatted milk in Tris-buffered saline-Tween 0.1 % (T-TBS) at room temperature and incubated overnight with the CFTR antibody in T-TBS buffer supplemented with 0.5 % (*w*/*v*) bovine serum albumin (BSA, Sigma). To detect CFTR protein, we used a specific antibody described above at a dilution of 1:1000 overnight. Membranes were washed with T-TBS and incubated for 1 h at room temperature with goat anti-mouse secondary antibody conjugated to horseradish peroxidase (1:5000) (Vector Lab, Burlingame, CA). Filters were washed, and immunoreactivity was detected using the ECL Western Blotting Analysis System (Amersham, GE Healthcare). The chemoluminescence reaction was visualized on AGFA Medical X-Ray films (Agfa-Gevaert). The relative level of each protein was obtained using the ImageJ 1.37v densitometric software.

Reverse transcription polymerase chain reaction

Total RNA from ten oocytes per treatment was purified using Trizol Reagent (Molecular Research Center, Inc.). First-strand cDNA was synthesized as described before [32] using 5 μg of RNA as template. PCR started at 94 °C for 3 min, and then 28 cycles were performed at 94 °C for 30 s, 58 °C for 30 s and 72 °C for 45 s, followed by a final extension of 20 min at 72 °C. The reaction was carried out using specific oligonucleotide primers coding for nucleotides 4131–4152 and 4527–4546, respectively, of xShroom1 sequence (TCT GGAGAAAGTGGTGAGCCTG (forward), TCATTTG TAGCGGGTGGACG (reverse)) and ornithine decarboxylase (ODC; internal control) ((5′ GCAAGGAATCACCCGAATG-3′ (forward), 5′ GCAACATAGTATCTCCCAGGCTC-3′ (reverse)) [42].

Statistical analysis

Data were expressed as mean values ± standard error (SE) (n = number of cells and repetitions). Statistical analysis for differences between experimental groups was performed using Graphpad Prism software, applying unpaired Student's t test. Differences were considered statistically significant when $p < 0.05$.

Results

Expression of CFTR

The main factors regulating CFTR channel gating are phosphorylation by PKA and interaction with ATP, both acting on intracellular domains. In most intact cell studies, CFTR is activated by extracellular application of membrane penetrating forskolin, which activates the adenylate cyclase, and hence PKA, eventually resulting in an increase in CFTR whole-cell conductance [23].

Solutions containing a CFTR activator cocktail with 10 μM forskolin and 1 mM 3-isobutyl-1-methylxanthine (IBMX; a phosphodiesterase inhibitor) were used. Their full effect took 10–15 min; therefore, the currents were allowed to stabilize before measurements were made. The difference between recordings with and without this cocktail was attributed to CFTR [35].

After 24–36 h of CFTR cRNA injection, we obtained steady CFTR currents (I_{CFTR}) in the range of microamperes

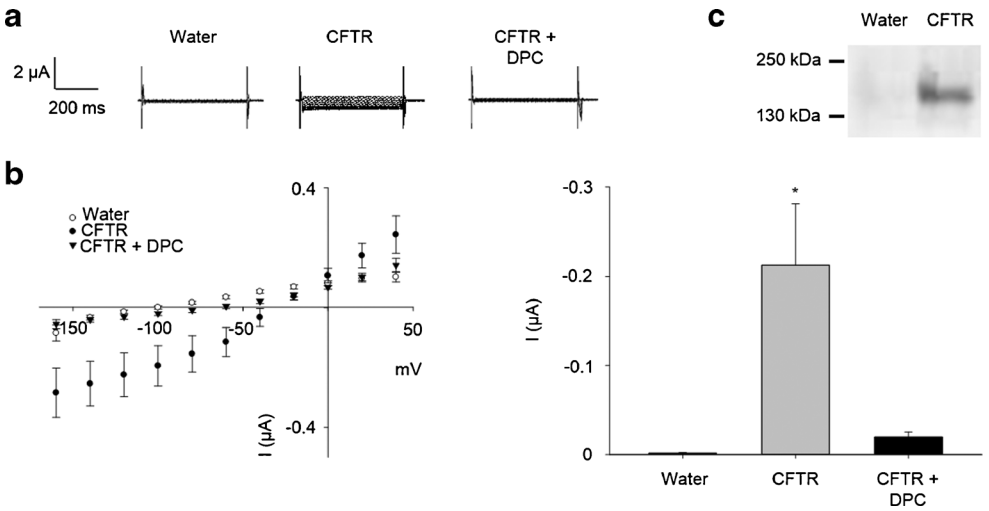

Fig. 1 Currents in H_2O or CFTR cRNA-injected oocytes and the effect of DPC. **a** Current traces in which the Vm was held at 0 mV and jumped to values of between −160 and +40 mV for 500 ms in H_2O or CFTR cRNA-injected oocytes. Inward currents (*downward deflections*) and outward currents were higher in oocytes injected with CFTR cRNA than the currents observed in oocytes injected with H_2O ($p < 0.05$) and were abolished by 1 mM DPC (CFTR + DPC). **b** *Left panel* shows the I-V plot from these experiments in **a**, in oocytes injected with H_2O (*open circles*, $n = 10$) or CFTR cRNA (*filled circles*, $n = 11$) in control solution or in the presence of DPC (CFTR + DPC, *triangles*). The mean values obtained at −100 mV in all conditions are depicted in the *right panel*. **c** CFTR presence was measured by monitoring the protein expression at the cell plasma membrane with a surface protein biotinylation technique. We used a specific CFTR antibody directed against the regulatory R domain. CFTR expression was detected in oocytes injected with CFTR cRNA but not observed in control oocytes injected with H_2O. The band corresponding to CFTR was found in the range of 160–180 kDa. The *asterisk* indicates a significant difference in the currents in oocytes injected with CFTR cRNA with respect to the oocytes injected with H_2O

so all experiments were done after this period. The resting membrane potential (Vm) of CFTR expressing oocytes was depolarized by ~13 mV with respect to the Vm of oocytes injected with H_2O (−49.0 ± 1.3 mV, $n = 10$), indicating that the expression of CFTR increased Cl^- permeability and depolarized the Vm. Figure 1a shows current traces in

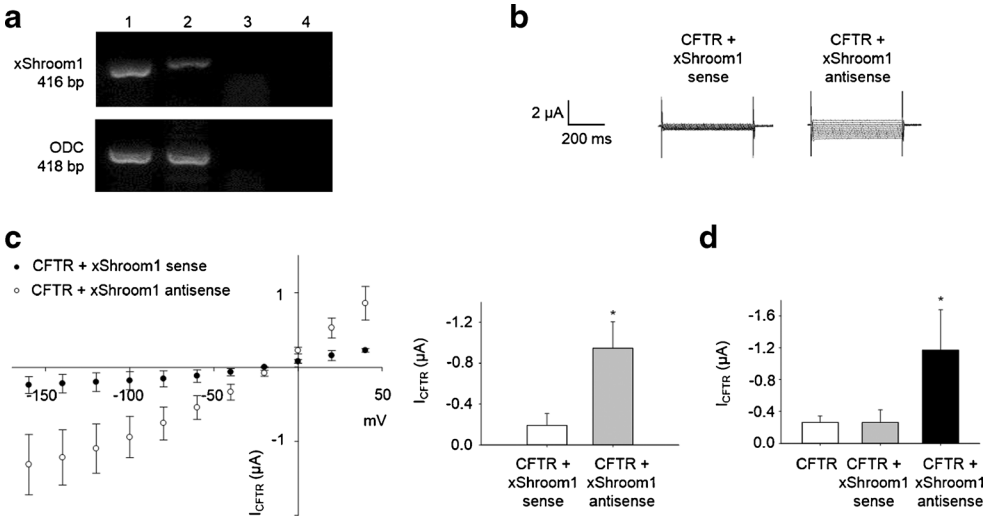

Fig. 2 Whole cell currents in oocytes coinjected with CFTR cRNA plus xShroom1 antisense or sense oligonucleotides. **a** RT-PCR in oocytes injected with xShroom1 sense or antisense oligonucleotides. Lanes *1* to *4*: sense-injected oocytes; antisense-injected oocytes; sense-injected oocyte samples without reverse transcriptase used as a negative control (absence of genomic DNA amplification); negative control with the reaction mix without a cDNA template. There was a marked reduction in the mRNA of xShroom1 (416 bp; *upper panel)* in antisense with respect to sense treatment. Ornithine decarboxylase (ODC) (418 bp; *bottom panel*) was used as internal control. The experiment was repeated three times. **b** Records from oocytes coinjected with CFTR cRNA and xShroom1 antisense (*open circles*) or sense (*filled circles*) oligonucleotides where it is evident that with the antisense oligonucleotides, the currents were much larger than in the oocytes injected with xShroom1 sense oligonucleotides. **c** I-V plots for oocytes injected with either xShroom1 sense ($n = 10$) or antisense oligonucleotides ($n = 8$) (*left*) and the mean inward currents measured at −100 mV (*right*) ($p < 0.05$). **d** Amplitude of the currents in oocytes coinjected with CFTR cRNA, CFTR + xShroom1 sense or CFTR + xShroom1 antisense. The *asterisk* indicates a significant difference in the I_{CFTR} in the xShroom1 antisense with respect to xShroom1 sense treatment

which the Vm was held at 0 mV and jumped to values between −160 and +40 mV for 500 ms. I_{CFTR} was not observed in oocytes injected with H_2O ($n = 10$). On the other hand, inward (downward deflections) and outward currents were observed in oocytes injected with CFTR cRNA. The reversal potential for the CFTR cRNA-injected oocytes was about −30 mV, a value similar to other published reports indicating the anion permeability [4, 16] (Fig. 1b). These currents were abolished by 1 mM of the Cl^- channel blocker diphenylamine-carboxylate (DPC) confirming the presence of CFTR currents (Fig. 1). The figure shows the I-V plot from these experiments in oocytes injected with CFTR cRNA (with and without DPC). The forskolin plus IBMX-activated current fraction was obtained by subtracting control currents to treated currents. The currents in oocytes injected with H_2O are also depicted for comparison. The mean values obtained at −100 mV from all sets of experiments are also shown.

We next examined the expression of CFTR on cell-surface using biotinylation of the oocyte membrane proteins with a mouse monoclonal antibody against the R domain of human CFTR (R&D Systems). As it was expected, the biotinylated CFTR protein was detected only in the oocytes injected with the CFTR cRNA (Fig. 1c) whereas it was absent in the H_2O-injected oocytes. A band of about 160–180 kDa was detected that corresponds to the glycosylated form of CFTR [3, 32].

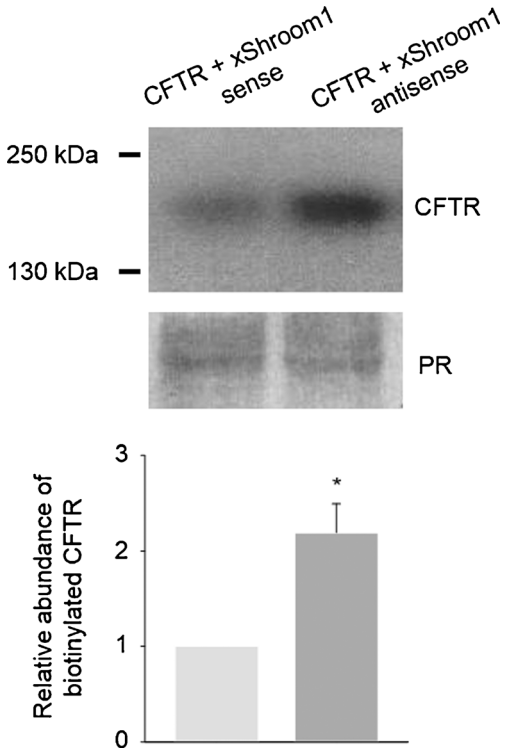

Fig. 3 Cell-surface expression of CFTR in the presence of xShroom1 sense or antisense oligonucleotides. Oocytes were subjected to cell-surface biotinylation prior to lysis. Representative immunoblots demonstrate an increase in surface expression of CFTR in oocytes coinjected with CFTR and the xShroom1 antisense oligonucleotides in comparison with the oocytes coinjected with CFTR and the xShroom1 sense oligonucleotides (CFTR) (*upper panel*). The CFTR expression was quantified by densitometric analysis (*bottom panel*). The intensities of the biotinylated signals were normalized to the staining with ponceau red (PR). The calculated values for CFTR in the sense treatment were set as 1. The *scale bars* represent the mean change in the expression of biotinylated CFTR at the antisense treatment compared with the sense treatment. Densitometric analysis shows that CFTR protein expression increased by a factor of 2 in oocytes coinjected with CFTR and the xShroom1 antisense oligonucleotides ($p < 0.05$, $n = 5$). The *asterisk* indicates a significant difference in the expression of CFTR in the xShroom1 antisense with respect to xShroom1 sense treatment

Inhibition of xShroom1 expression enhances CFTR currents

To corroborate if antisense oligonucleotides reduce the expression of xShroom1 in *Xenopus* oocytes coinjected with CFTR and xShroom1 antisense oligonucleotides, total RNA was extracted and reverse transcription polymerase chain reaction (RT-PCR) analysis was performed using xShroom1 primers (see "Material and methods"). As shown in Fig. 2a, an expected band of 416 bp corresponding to xShroom1 product was obtained in both treatments (upper panel). There was a marked reduction in xShroom1 expression in oocytes coinjected with CFTR and xShroom1 antisense oligonucleotides when compared with oocytes coinjected with CFTR and xShroom1 sense oligonucleotides. A band of 418 bp was detected, corresponding to ODC showing positive cDNA integrity (bottom panel), which did not change in either treatment. No product was detected in the absence of reverse transcriptase or cDNA template (negative controls).

Figure 2b shows records from oocytes coinjected with CFTR and xShroom1 antisense or xShroom1 sense oligonucleotides. With the xShroom1 antisense oligonucleotides, the currents were much larger than in the oocytes injected with the xShroom1 sense oligonucleotides. I-V plots for oocytes coinjected with CFTR and either xShroom1 sense or antisense oligonucleotides are shown in Fig. 2c, and the mean inward currents measured at −100 mV are also shown. The depolarization observed in the CFTR-expressing oocytes stimulated with forskolin plus IBMX is enhanced in the oocytes coinjected with CFTR and xShroom1 antisense oligonucleotides (CFTR + xShroom1 sense-expressing oocytes were depolarized 3.2 ± 0.8 mV, while CFTR + xShroom1 antisense-expressing oocytes were depolarized 8.1 ± 1.5 mV). The amplitude of the currents and the reversal potential for the CFTR cRNA and xShroom1 sense oligonucleotide-coinjected oocytes were similar to that obtained in the oocytes injected only with the CFTR cRNA ($n = 15$, Fig. 2d); thus, in all subsequent experiments, the latter were considered the control oocytes. Also, the increment in oocytes coinjected with CFTR and xShroom1 antisense oligonucleotides is shown.

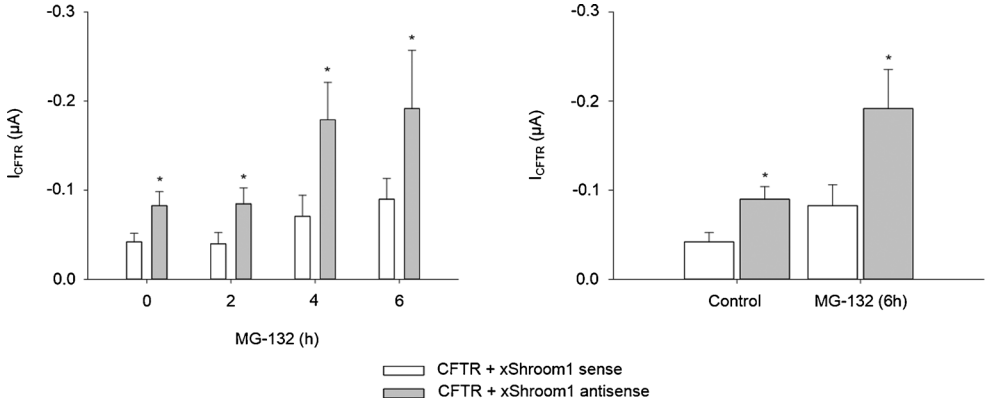

Fig. 4 Effect of MG-132, an inhibitor of the proteasome system, on CFTR currents. The oocytes were coinjected with CFTR and xShroom1 sense or antisense oligonucleotides together with CFTR cRNAs and incubated with 10 μM MG-132. As shown in the *right panel*, MG-132 (6 h) increased CFTR currents with both oligonucleotides although the currents were still much larger with the antisense than with the sense oligonucleotides ($p < 0.05$, $n = 5$). The time course (2–6 h) of the effects of MG-132 is depicted in the *left panel*. The *asterisk* indicates a significant difference in the I_{CFTR} in the xShroom1 antisense with respect to the xShroom1 sense treatment

CFTR cell-surface expression increases when xShroom1 expression is inhibited

To evaluate if the increase observed in the I_{CFTR} was associated with a change in cell-surface expression of the channel, we used biotinylation of plasma membrane proteins in oocytes coinjected with CFTR and xShroom1 antisense oligonucleotides. A single band of about 160–180 kDa was observed, which corresponds to mature glycosylated CFTR. Densitometric analysis of the signals indicates that the intensity of the biotinylated CFTR was higher in the oocytes coinjected with CFTR and the xShroom1 antisense (about twice) than in the oocytes with the xShroom1 sense oligonucleotides, normalized by relative amounts of proteins (Fig. 3). The mean value obtained from five experiments is shown at the bottom of the panel. This result supports the idea that an increment in the channel expression in the plasma membrane is responsible for the larger currents when xShroom1 is suppressed. The following experiments were conducted to further examine this possibility.

The proteosomal pathway is not involved in the effect on CFTR by xShroom1

To test the hypothesis that xShroom1 enhances CFTR degradation through the proteasome pathway, we incubated with MG-132 (10 μM) for 6 h the oocytes coinjected with CFTR and xShroom1 sense or antisense oligonucleotides [41]. MG-132 in the concentration range used in this study is a potent disrupter of the proteolytic activity of the proteasome system increasing the accumulation of CFTR, although the detailed mechanism of this process is not clearly established. As shown in Fig. 4, MG-132 increased CFTR currents in both conditions, although the currents were still much larger when the oocytes were coinjected with the xShroom1 antisense than with the xShroom1 sense oligonucleotides. This suggests that xShroom1 does not enhance degradation of CFTR through the proteasome pathway.

The half-life of CFTR in the plasma membrane is higher when xShroom1 expression is inhibited

To analyze if xShroom1 antisense treatment changes the half-life of plasma membrane CFTR, we used brefeldin A (BFA), a fungal toxin that blocks the transport of proteins by disassembly of the Golgi complex into the endoplasmic reticulum, thereby inhibiting the delivery of newly synthesized proteins to the plasma membrane [10, 11]. Two days following the coinjection of oocytes with CFTR cRNA and either

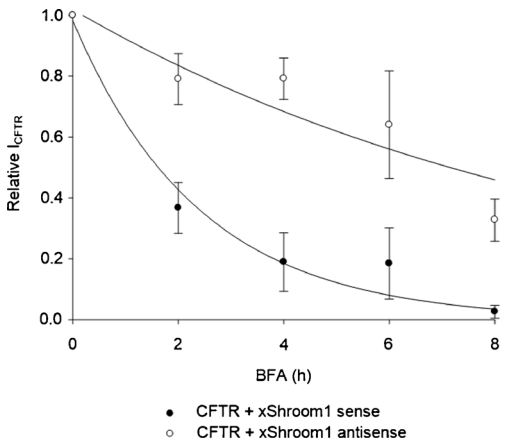

Fig. 5 Effect of BFA on CFTR currents. The experiment started following 48 h the coinjection of oocytes with CFTR and either sense (*filled circles*) or antisense xShroom1 oligonucleotides (*open circles*). For the current measurement, oocytes were continuously perfused with 5 μM BFA and relative currents normalized at time 0 h at a holding potential of 0 mV and measured every 2 h. The half-life of I_{CFTR} was calculated using SigmaPlot software (Systat Software). The data were best fit to a single exponential

xShroom1 sense or antisense oligonucleotides, the decay of currents was measured every 2 h in the presence of 5 μM BFA, a concentration similar to that used by Carattino et al. [10] in *Xenopus* oocytes. The currents were normalized to time 0 h. The curves in Fig. 5 gave us an estimate of the half-life of CFTR channels in the plasma membrane. Thus, the suppression of xShroom1 increased the half-life of the plasma membrane channels from 1.66 ± 0.24 to 6.97 ± 1.84 h ($n = 3$). These data suggest that xShroom1antisense oligonucleotides increase CFTR surface expression probably through an increased retention or recycling of the channel at the plasma membrane.

The endocytic/lysosomal pathway is involved in the effect on CFTR channel by xShroom1

The effect of disrupting the endocytic/lysosomal pathway with chloroquine was also examined. Chloroquine, a weak base that blocks the late endosomes/lysosomes thereby inhibiting proteolysis in these compartments [37], is reported to prevent traffic of plasma membrane-derived vesicles to lysosomal and other degradation pathways [29], although its mechanism of action seems to be more complex [8]. In our hands, chloroquine (50 μM) led to a significant increment in CFTR currents in oocytes coinjected with CFTR and xShroom1 sense oligonucleotides but not in oocytes coinjected with CFTR and xShroom1 antisense oligonucleotides, compared to those untreated (Fig. 6, right panel). Thus, the expression of functional CFTR is not increased with chloroquine treatment when xShroom1 expression is inhibited by antisense oligonucleotides, suggesting the involvement of the endocytic/lysosomal pathway in the effect of xShroom1.

Discussion

In this work, we analyzed the regulation of CFTR by xShroom1 protein in oocytes coinjected with CFTR and either xShroom1 sense or antisense oligonucleotides and we observed a marked increment in CFTR currents and CFTR cell-surface expression in oocytes coinjected with xShroom1 antisense oligonucleotides. A number of diverse mechanisms including the regulation of channel synthesis, intracellular channel trafficking, membrane insertion, degradation and open probability are the main factors in controlling the expression or activity of CFTR [1, 28, 36]. Any of these factors could be responsible for the increment in current and expression observed in our experiments.

Shroom is a family of proteins involved in the regulation and maintenance of cytoskeleton architecture by binding to actin cytoskeleton with many functions in the development of the cell. The best characterized shrooms are xShroom3 and mShroom3, which are essential for neural tube morphogenesis in *Xenopus* and in mouse [17, 22, 25]. Another member of the Shroom family is xShroom1, which has been associated with membrane proteins. In this regard, it is important to note that results from Zuckerman et al. [42] and from our laboratory [2] demonstrated that xShroom1 was required for functional expression of ENaC in *X. laevis* cells probably due to a regulation in the number of channels inserted in the plasma membrane. In addition, it is evident from our previous studies that there is a dual action of xShroom1 on the expression and level of activity of ENaC and CFTR in oocytes from *X. laevis*. Suppression of xShroom1 resulted in a decrement in ENaC function [2, 42] whereas the opposite was found on CFTR (this paper). The details of this dual action are poorly understood but it is interesting to note that in some cell types, CFTR inhibits the ENaC conductance (see "Introduction").

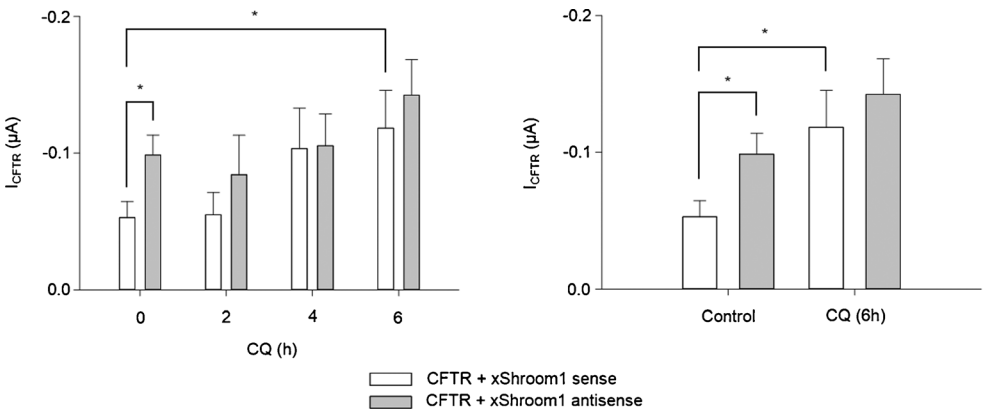

Fig. 6 Effect of chloroquine (50 μM), an inhibitor of the late endosome/lysosome, on whole cell currents. The oocytes were coinjected with CFTR and xShroom1 sense or antisense plus CFTR cRNA and incubated during 6 h with chloroquine. The *right panel* shows the effect of chloroquine on the mean value of currents elicited by a −100mV pulse where it is evident a significant increment in the currents in the oocytes coinjected with CFTR and xShroom1 sense oligonucleotides but not in the oocytes coinjected with CFTR and xShroom1 antisense oligonucleotides ($p = 0.1$, $n = 14$). The *left panel* shows the time course of the chloroquine effect. The *asterisk* indicates a significant difference between indicated datasets

The present results suggest that inhibition of the xShroom1 protein resulted in larger CFTR currents by an increment in the CFTR channels on the surface membranes possibly by reducing its delivery to the endocytic/lysosomal pathway. This conclusion was drawn from the following observations: (1) MG-132, a proteasome inhibitor [41], did not inhibit the increment in CFTR currents when xShroom1 was suppressed with antisense oligonucleotides; (2) when BFA was used, a compound that inhibits the delivery of newly synthesized proteins to the plasma membrane [11], we obtained a prolonged half-life of plasma membrane CFTR in oocytes coinjected with CFTR and the xShroom1 antisense; and (3) with chloroquine, an inhibitor of the late endosomes/lysosomes [8, 29], CFTR current increment was not observed in oocytes coinjected with CFTR and xShroom1 antisense oligonucleotides when compared with control oocytes coinjected with CFTR and xShroom1 sense oligonucleotides.

The number of channels in the cell-surface is determined by the balance between insertion of new channels into the plasma membrane and the endocytosis and degradation of channels from the membrane. We found that BFA decreases CFTR currents in agreement with Weber et al. [40] who found that BFA reduced the activation of CFTR currents in a time-dependent manner and also reduced capacitance in oocytes indicating that it effectively blocks the transport of channels to the plasma membrane. The reported values for the half-life of CFTR in the plasma membrane vary widely up to over 48 h [24, 39], which may be due to the use of different cell lines, expression systems and experimental protocols performed at different temperatures. When BFA was used in our experiments, an increment in the half-life of plasma membrane CFTR was obtained in oocytes coinjected with CFTR and the xShroom1 antisense oligonucleotides. This half-life reflects the rate of endocytic retrieval and the recycling of CFTR, and the obtained results are consistent with the idea that the inhibition of xShroom1expression augmented the number of surface CFTR by an increased retention or recycling of the channel at the plasma membrane.

CFTR channels enter the degradation pathway via clathrin-mediated endocytosis [6] and are subsequently degraded via the lysosomal pathway [14, 36]. When we analyzed the endosomal/lysosomal pathway, chloroquine led to a significant increment in CFTR currents in sense oocytes in comparison with control oocytes (without chloroquine). This is in concordance with other results obtained in Kir2.1 channels where there is an increment in I_{K1} currents in the presence of chloroquine [27]. Probably, our treatment with chloroquine results in a saturation of the CFTR degradation pathway which likely affects the internalization capacity of the functional CFTR channels, culminating in increased I_{CFTR}. Also, chloroquine treatment did not significantly increase CFTR currents when xShroom1 expression was inhibited suggesting a participation of xShroom1 in the endosomal/lysosomal pathway, and not in CFTR recycling. Additionally, the measurements of cell-surface CFTR expression showed a band of about 160–180 kDa that corresponds to the glycosylated form of the channel [3, 32], with an expression and subsequent CFTR protein density increased by treatment with xShroom1 antisense oligonucleotides. This is in concordance with the functional studies presented above, suggesting the possibility that an increment in the number of channels inserted in the plasma membrane is responsible for the larger currents when xShroom1 is suppressed.

In conclusion, the suppression of xShroom1 resulted in an increment in CFTR currents by an increase in the CFTR channels on the cell-surface membrane. Possibly, this is due to a reduction in the delivery to the later stages of the endocytic trafficking that directs CFTR to the degradative pathway, but we cannot however rule out that the suppression of xShroom1 affects the open probability of CFTR. Our data suggest that xShroom1 plays an important role in regulating CFTR expression and thus its function. This could be relevant in the understanding of the channel malfunction in several pathologies.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Ethical approval All applicable international, national and/or institutional guidelines for the care and use of animals were followed.

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