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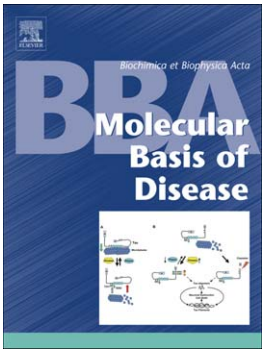

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Annexin A5 increases the cell surface expression and the chloride channel function of the ΔF508-cystic fibrosis transmembrane regulator.

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Short title: Annexin 5 increases the function of ΔF508-CFTR

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ABSTRACT

Cystic fibrosis (CF) is caused by a mutation in the cystic fibrosis transmembrane conductance regulator (CFTR) gene. In CF, the most common mutant ΔF508-CFTR is misfolded, is retained in the ER and is rapidly degraded. If conditions could allow ΔF508-CFTR to reach and to stabilize in the plasma membrane, it could partially correct the CF defect. We have previously shown that annexin V (anxA5) binds to both the normal CFTR and the ΔF508-CFTR in a Ca^{2+}-dependent manner and that it regulates the chloride channel function of Wt-CFTR through its membrane integration. Our aim was to extend this finding to the ΔF508-CFTR. Because some studies show that thapsigargin (Tg) increases the ΔF508-CFTR apical expression and induces an increased $[Ca^{2+}]_i$ and because anxA5 relocates and binds to the plasma membrane in the presence of Ca^{2+}, we hypothesized that the Tg effect upon ΔF508-CFTR function could involve anxA5. Our results show that raised anxA5 expression induces an augmented function of ΔF508-CFTR due to its increased membrane localization. Furthermore, we show that the Tg effect involves anxA5. Therefore, we suggest that anxA5 is a potential therapeutic target in CF.

Key words: Cystic fibrosis; annexin A5; CFTR; protein interaction; CFTR function

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INTRODUCTION

Cystic fibrosis (CF) is the most common severe autosomal recessive genetic disorder in Caucasians. It is due to a mutation in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR) [1-3]. The CFTR protein is a cAMP-regulated chloride (Cl^-) channel localized at the apical surface of epithelial cells. It is composed of two transmembrane segments, two nucleotide-binding domains (NBDs) and a regulatory domain (R domain) [1]. The R domain harbours multiple consensus phosphorylation sites by the protein kinase A (PKA) and the protein kinase C (PKC) [4]. The phosphorylation of the R domain together with the ATP binding and hydrolysis at the NBDs regulate the gating of the CFTR channel [4].

CF is caused by a defective Cl^- transport in the plasma membrane of epithelial cells in the lung, pancreas, liver, intestines, sweat duct and the epididymis [5]. Nevertheless, mutations in the CF gene lead to a variety of clinical defects, showing that the link between genotype and phenotype is complex [1, 6]. The most common mutation in CFTR leading to CF is a three-base-pair deletion which results in the absence of a phenylalanine residue at amino acid position 508 (ΔF508) in NBD1 [7]. The ΔF508-CFTR protein is misfolded [8], retained in the endoplasmic reticulum (ER) through interactions with the chaperones and targeted to degradation via the proteasome [7, 9]. Indeed, the misfolded ΔF508-CFTR interacts with the ER chaperones [10, 11] which exhibit an optimal activity when free calcium (Ca^{2+}) concentration is millimolar. Nevertheless, if some ΔF508-CFTR protein reaches the cell surface, it exhibits a normal Cl^- channel function despite a shorter half-life than the normal CFTR [11, 13]. Therefore, studies were performed to restore the membrane localization and function of ΔF508-CFTR [14] using CFTR overexpression [15], low temperature [16] and chemicals such as glycerol [17], phenylbutyrate [15], genistein [18] and thapsigargin (Tg) [19]. Tg is a natural tumour-promoting sesquiterpene lactone which increases the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in several cell lines by inhibiting the sarcoplasmic or ER Ca^{2+}-ATPase family SERCA [20]. Therefore, lowering the ER Ca^{2+} stores induces a decreased retention of ΔF508-CFTR by chaperones such as calnexin [19]. This decrease in the ER Ca^{2+} store, which is the largest Ca^{2+} store of cells, is accompanied by an increased $[Ca^{2+}]_i$.

We hypothesize that the ΔF508-CFTR function can also be restored by modulating its interacting partners. Nevertheless, the extent to which Wt-CFTR and ΔF508-CFTR channels

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are regulated by protein-protein interactions is largely unknown. The identification of the CFTR's interacting proteins should permit decoding the molecular mechanisms of the CFTR processing and function and should help to define new therapeutic targets to correct the CFTR defects in CF. Several CFTR-binding proteins are known to modulate the Cl^- channel function of CFTR. SNAP-23 and syntaxin 1A interact with the N-terminus of CFTR and inhibit its activity [21]. Na^+/H^+ exchanger regulatory factor (NHERF), also known as ezrin-binding protein of 50 kDa (EBP-50), and CFTR-associated protein of 70 kDa (CAP70) interact with the C-terminus CFTR via their PDZ domains and stimulate the Cl^- currents [22-24]. CFTR–associated ligand (CAL) also binds the C-terminus of CFTR via a PDZ domain and reduces its apical expression [25]. Recently, we identified annexin A5 (anxA5) as a new CFTR partner. We showed that anxA5 interacts with the NBD1 of the Wt-CFTR protein in a Ca^{2+}- and ATP-dependent manner, as well as with the ΔF508-CFTR [26]. Our previous study showed that the anxA5 expression is involved in the cAMP-dependent halide flux through CFTR as well as in its membrane localization. AnxA5 belongs to the ubiquitous annexin family of Ca^{2+}-binding proteins [27]. Whereas its role *in vivo* is still unclear, some of its properties have been depicted. It interacts with cytoskeletal proteins [28], exhibits anticoagulant activities [29], inhibits phospholipase A2 [30] and modulates some membrane proteins such as the Na^+/Ca^{2+} exchanger [31]. Furthermore, anxA5 binds to negatively charged phospholipids in a Ca^{2+}-dependent manner [32], may form a Ca^{2+} channel [33] and may be involved in exocytosis [34].

Tg restores the ΔF508-CFTR in the membrane as well as its Cl^- channel function in a Ca^{2+}-dependent manner [35] and we previously showed that anxA5 interacts with Wt-CFTR and ΔF508-CFTR in a Ca^{2+}-dependent manner [26]. Therefore, we hypothesized that the effect of Tg may indirectly involve anxA5 by the increased $[Ca^{2+}]_i$. In the present paper, we show for the first time that anxA5 is involved in the cell surface localization of the ΔF508-CFTR and that the Cl^- channel function of the mutated CFTR is increased when anxA5 is overexpressed. Furthermore, we show that the Tg effect is decreased when the anxA5 expression is inhibited whereas it is increased when anxA5 is overexpressed. Therefore, we suggest that the positive effect of Tg involves anxA5 due to its CFTR-binding properties and to its relocalization to the membranes in the presence of Ca^{2+} [32]. Furthermore, we show here that the single effect of an anxA5 overexpression induces an increased ΔF508-CFTR function due to an increased localization in the plasma membrane of polarized and non-polarized cells, likely due to a reduced recycling. Therefore, we show for the first time that the modulation of anxA5 expression is a potential therapeutic target.

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EXPERIMENTAL PROCEDURES

Cloning of anxA5 in pcDNA3.1/His

The anxA5 cDNA was amplified by PCR using complete cDNA from HeLa cells as matrix. The primers were: sense 5'-ACCTGAGTAGTCGCCATGGCA-3'; anti-sense 5'-CCCCGTGACACGGTCATCTTC-3'. Reactions were carried out with the following parameters: denaturation at 94°C for 15 min, annealing at 62°C for 30 min and extension at 68°C for 1.5 min (30 cycles). The PCR products were purified (Qiaex II, Qiagen, Germany) and cloned into pcDNA3.1/His (TOPO TA Expression Kit, Invitrogen, USA). Double-strand sequencing (ABI 310, Applied Biosystems, USA) were performed to ascertain that the amplification did not introduce mistakes and that the coding sequence was in frame.

Site-directed mutagenesis

Mutagenesis was performed on the cDNA encoding CFTR (GenBank, M28668) using QuickChange® XL Site-directed Mutagenesis Kit (Stratagene, USA). The mutated oligonucleotides (Eurobio, France) corresponding to the ΔF508 mutation were: 5'-CTGGCACCATTAAAGAAAATATCGGTGTTTCCTATGATG- 3';
5'- CATCATAGGAAACACCGATATTTCTTTAATGGTGCCAG -3'. The mutation was controlled by double-strand sequencing (ABI 310, Applied Biosystems, USA).

Expression of short hairpin RNA (shRNA)

To decrease the anxA5 expression, a vector was constructed according to the Block-it™ RNAi Designer software (Invitrogen, USA). The single-stranded DNA oligos were: 5'-CACCGAACAAGATGCTCAGGCTTTACGAATAAAGCCTGAGCATCTTGTTC -3', 5'-AAAAGAACAAGATGCTCAGGCTTTATTCGTAAAGCCTGAGCATCTTGTTC-3'. The annealing to generate two double-strand oligos and their cloning in pENTR/U6 were performed with Block-it™ RNAi Entry vector kit, according to the manufacturer's instructions (Invitrogen, USA). The provided pENTR/U6/LacZ vector, encoding an irrelevant shRNA, was used as a negative control. Further controls were performed using an irrelevant scrambled shRNA and with buffer alone (not shown).

Cell culture and transfection

A549 cells, an immortalized human airway epithelial cell line (ATCC CCL-185, UK), were cultured in F-12K medium supplemented with 10% foetal bovine serum (FBS) and 100 U/ml penicillin-streptomycin in a humidified CO_2 incubator (37°C). They were transfected by Lipofectamine 2000 (Invitrogen, USA) with the pcDNA3.1 containing either the Wt-CFTR or the ΔF508-CFTR cDNA. Stably transfected cells were selected by neomycin. CFTR

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expression was assessed after 10 passages. Later, the cells were transfected with pcDNA3.1/anxA5 encoding anxA5 or with the shRNA against anxA5 (pENTR/U6/anxA5). AnxA5 expression was assessed by Western blotting as described below and compared with the negative controls. In some experiments, cells were treated with 500 nM Tg (16 hours, 37°C) [36].

Immortalized normal (16HBE14o-) and CF (CFBE41o-) polarized airway epithelial cell lines were from Pr D.C. Gruenert (University of California, San Francisco). The CFBE41o- cells are homozygous for the delF508 mutation whereas the 16HBE14o- cells have the characteristics of superficial epithelial cells and were originally derived by viral transformation of cells dissociated from human bronchial epithelium [37, 38]. The cells were cultured in Eagle's minimal essential medium (MEM) supplemented with 10% FBS and glutamine on an extracellular matrix (CellBind Corning, Bioblock).

Immunofluorescent labelling of CFTR after modulation of the anxA5 expression in cells with and without Tg treatment

Cells were grown on 22 x 22 mm glass coverslips and fixed in phosphate-buffered saline (PBS) containing 4% paraformaldehyde (w/v) for 30 min at room temperature (RT). The cells were permeabilized (0.25% saponin, 45 min, RT) and saturated (3% bovine serum albumin, (1 h, RT). Immunoflorescence was performed using successively anti-CFTR rabbit polyclonal antibodies (7.5 µg/ml; PA1-935, ABR, BD Bioscience Pharmingen, USA) for 1.5 h and goat anti-rabbit second antibodies coupled to Texas Red (1:250; GeneTex, Inc., USA) for 1 h. Nuclei were stained with DAPI. Actin cytoskeleton was labelled with phalloidin-FluoProbes®547 (1/40, Molecular Probes) according to the manufacturer's instructions.

Western blot analysis

Cells were lysed (50 mM Tris HCl, pH 6.8, 100 mM NaCl, 1% Triton X-100 and protease inhibitor cocktail [Sigma]) and protein concentrations were determined using the Lowry procedure [39]. The samples were resolved by SDS-PAGE (7.5%) and transferred on a PVDF membrane (Amersham Biosciences, UK). CFTR was detected by successive incubation of the membranes with a mouse monoclonal antibody anti-CFTR (1:500, Ab-2 (MM13-4), Interchim, France) and a peroxidase labelled secondary antibody (1:5000, Amersham Biosciences, UK). AnxA5 was detected by western blotting (12%) using a goat polyclonal IgG (sc-1929; Santa Cruz Biotechnology, USA) and a peroxidase anti-goat antibody (Sigma, France). Immunoreactive proteins were visualised by enhanced chemiluminescence (ECL+, Amersham Bioscience, UK). Densitometric analysis of the films was performed using a Biovision 1000 apparatus (Fischer Bioblock) and each value was normalized to the total

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amount of loaded proteins per lane as well as by G3PDH and actin quantifications (MAb to Glyceraldehyde-3-PDH (Biodesin International)). Actin expression was assessed by Western blotting (12%) using anti-nonmuscle-F-actin (1/200, Santa Cruz Biotechnology).

Immunoprecipitation

Wt-CFTR-anxA5 and ΔF508-CFTR- anxA5 interactions were previously demonstrated [44]. The effect of the ΔF508 mutation upon the interaction was studied here by co-immunoprecipitation. Total protein was extracted from Wt-CFTR- and ΔF508-CFTR-expressing cells (50 mM Tris HCl, pH 6.8, 100 mM NaCl, 1% Triton X-100 and protease inhibitor cocktail). The anti-CFTR antibody (Ab-2 (MM13-4), Interchim, France) was coupled to Dynabeads Protein G (Dynal Biotech, Invitrogen, USA) overnight at 4°C with rotation. After centrifugation (6000 g, 15 min), clarified cell lysates were incubated with the beads. After incubation (16 hours, 4°C), the beads were washed four times in PBS and samples were resolved by SDS-PAGE (12%). AnxA5 was detected with the anti- anxA5 antibody. The commercially available purified anxA5 was used as molecular weight control (35 kDa). The negative controls were obtained with cells which did not express CFTR and using an irrelevant antibody (anti-V5 antibody, Invitrogen, USA) coupled to the beads.

Measurement Fura 2-AM

To ensure that the Tg treatment induced an increased $[Ca^{2+}]_i$, Wt-CFTR- and ΔF508-CFTR-expressing cells were loaded for 45 min with 2 µM Ca^{2+}-sensitive fluorescent probe fura-2 acetoxy-methyl ester (Fura-2 AM, Invitrogen Molecular Probes, USA) at RT in the dark. Cells were rinsed twice and incubated in Tyrode solution (20 min, 37°C). Fluorescence was recorded at 340 nm (saturated Ca^{2+}) and 380 nm (free Ca^{2+}) and was analysed using Fluostar software (IMSTAR, France). For each experiment, 20-30 cells were observed and the responses were compared with the controls obtained with untransfected cells or with cells that were not treated with Tg.

Methoxy-N-(3-sulfopropyl)quinolinium (SPQ) fluorescence assay

The cAMP-dependent halide flux through Wt-CFTR and ΔF508-CFTR was assessed by SPQ assays using forskolin as a CFTR activator. Cells were loaded with 10 mM SPQ (Molecular Probes) and placed in a quenching NaI buffer. The baseline fluorescence was measured in isotonic NaI buffer and cells were then perfused with isotonic dequench buffer ($NaNO_3$ replaced NaI). The perfusate was then switched to dequench buffer plus agonist and requenched at the end of the experiments. An inverted microscope equipped for fluorescence (IX-71, Olympus) was used and illumination was performed at 360 nm. The emitted light was collected at 456 ± 33 nm by a high-resolution image intensifier coupled to a video camera

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connected to a digital image processing board (FLUO software, Imstar, France). During the experiments (n=5) the cell morphology and number were checked to ensure that there was no difference due to the siRNA. SPQ experiments were also performed when anxA5 was overexpressed in cells transfected or not with the cDNA encoding CFTR. The controls were performed using cells transfected with the empty pcDNA3.1 vector. For the statistical analysis [40, 41], fluorescence intensity was standardized according to the equation F = (F - Fo) / Fo x 100, where F is the relative fluorescence and Fo, the fluorescence intensity measured in presence of I^-. The halides membrane permeability (p) was determined as the rate of SPQ dequenching upon perfusion with nitrates. At least three successive data points were collected immediately after the $NO3^-$ containing medium application, and then fitted using a linear regression analysis. The slope of the straight line reflecting the membrane permeability to halides (noted p in min-1) was used as an index of CFTR activity.

Patch clamp

A patch clamp technique was used to record the membrane currents under voltage clamped conditions [42]. The measurements of ionic currents were made by using a patch-clamp amplifier (Geneclamp 500B, Axon, USA) equipped with a current to voltage converter headstage (CV5 series). The signals were recorded with a micro-computer *via* an analogue to digital converter (CED 1401 plus, UK) running at 35 kHz. The WCP v. 3.5.9 program (Strathclyde University, UK) was used to record the currents and to deliver programmed voltage pulses. The stimulating pulses (-50 to 200 mV by 10 mV steps, duration 200 msec) were applied to the patch from the holding potential. The currents were analysed off-line with WCP and conductances were calculated from the slope of the current-potential relationship.

This procedure was performed with 75% confluent cells incubated with 500 nM Tg (16 hours, 37°C). In all experiments, cells were pre-treated with bumetamide as described above. In order to assess the CFTR activity in electrophysiological recordings, glibenclamide was used to block CFTR activity (data not shown).

Cell surface expression of CFTR by biotinylation

To estimate the CFTR (Wt and ΔF508) presence in the plasma membranes of the cells after modulation of anxA5 expression, coupled or not to a Tg treatment, the membrane proteins were biotinylated as previously described [43, 44]. Briefly, confluent cells were washed with ice-cold PBS (pH 8.0), incubated in Sulfo-NHS-SS-Biotin solution (Pinpoint™ Cell Surface Protein Isolation Kit, Pierce, USA) for 30 min at 4°C and quenching solution was added. Cells were scraped and centrifuged (3 min, 500g). The pellets were suspended in lysis buffer

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(Tris-HCl pH 6.8, 50 mM; NaCl 100 mM; Triton X-100 2% and antiprotease cocktail) and incubated for 30 min on ice. Cell lysates were clarified (2 min, 10,000 g, 4°C) and biotinylated proteins were isolated on Immobilized NeutrAvidin™ gel according to the manufacturer's instructions (Pierce, USA). The CFTR protein was further detected by Western blotting as described above.

Statistical analysis

Student's t-test was used and differences were considered significant when $p<0.05$ (*) and very significant when $p<0.001$ (**).

RESULTS

AnxA5 expression in non-polarized (A549) and polarized ΔF508-CFTR-expressing cells (CFBE41o-)

The basal anxA5 expression was assessed in A549 and in CFBE41o- cells by western blots. As shown in Fig. 1, anxA5 expression was higher in polarized cells than in non polarized cells. Although it was not significant, this expression tended to be lower in ΔF508-CFTR-expressing cells.

The ΔF508 mutation does not disrupt the CFTR-anxA5 interaction

We previously showed direct anxA5 - CFTR and anxA5 - ΔF508-CFTR interactions [26]. Nevertheless, the presence of anxA5 in Wt-CFTR and in the ΔF508-CFTR protein complex was assessed here by co-immunoprecipitation. As shown in Fig. 2, anxA5 was present in the immunoprecipitated complexes from Wt-CFTR- and ΔF508-CFTR-expressing A549 cells, whereas it was not detected in the A549 cells which were not transfected with the cDNA encoding for the CFTR protein (NT) or when an irrelevant antibody was linked to the beads. This result indicated that the ΔF508 mutation did not disrupt the interaction. Pure anxA5 protein was used as molecular weight control to ensure the molecular weight of the observed bands.

Modulation of the anxA5 expression in Wt-CFTR- and ΔF508-CFTR-expressing A549 cells

To decrease the anxA5 expression, Wt-CFTR- and ΔF508-CFTR-expressing A549 cells were transfected with either the shRNA directed against anxA5 or the control shRNA. After the transfection (48 hours), anxA5 expression was assessed by Western blotting (Fig. 3A, upper panel). AnxA5 overexpression was obtained after transfection with the cDNA encoding anxA5 (pcDNA3.1/anxA5, Fig. 3A, lower panel). The pcDNA3.1 empty vector was used as a negative control. AnxA5 expression was assessed at increasing time points after the

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transfection (not shown), and peaked 48 hours after transfection. In all type of experiments, actin was used as a reference gene (Fig.3A). The statistical analysis was performed (Fig. 3B). AnxA5 expression was decreased in the presence of the shRNA by 73.5 ± 1.1% (n=3) and 75.6 ± 1.8% (n=3) in Wt-CFTR- and ΔF508-CFTR-expressing cells, respectively, when compared to the controls. Its expression was increased in the presence of the cDNA encoding anxA5 by 69.8 ± 0.8% and 71.4 ± 1.2% in Wt-CFTR- and ΔF508-CFTR- A549 expressing cells, respectively, when compared to the controls.

Because anxA5 is an actin-binding protein and because actin is involved in CFTR function, actin expression was studied. Actin expression was not modified in Wt-CFTR- or ΔF508-CFTR-expressing cells when anxA5 expression was modulated (not shown).

The cAMP-dependent halide flux through ΔF508-CFTR depends on anxA5 expression in A549 cells.

We previously showed that the cAMP-dependent halide flux through Wt-CFTR was correlated with anxA5 expression in A549 cells [26]. Therefore, we investigated the cAMP-dependent halide flux through ΔF508-CFTR when anxA5 expression was decreased and increased. Halide permeability was measured using the SPQ dye 48 hours after the transfection with pENTR/U6/anxA5 or with the irrelevant shRNA. The curves obtained after cAMP-stimulation of the CFTR channel and the blockage of other Cl^- channels showed a residual function of ΔF508-CFTR, as previously described [12]. Statistical analysis of the experiments (Fig. 4) indicated that the shRNA induced a significant decreased halide flux through ΔF508-CFTR in A549 cells when compared to the negative controls performed with the irrelevant shRNA which had no effect by itself.

The overexpression of anxA5 induced a very significant increased cAMP-dependent halide flux through ΔF508-CFTR in A549 cells (Fig. 4). In the controls performed with cells which did not express CFTR (not shown) and with the empty vector, no modification of the halide flux was observed. Therefore, our results indicate for the first time that the cAMP-dependent halide flux through ΔF508 CFTR is correlated with anxA5 expression

Tg treatment increases Wt and ΔF508-CFTR channel activity

It was previously shown that Tg increases the cell surface expression of ΔF508-CFTR due to the decreased ER Ca^{2+} store affecting the calnexin-CFTR interaction [35]. In a previous study, we demonstrated that the anxA5-CFTR interaction is Ca^{2+}-dependent [~~44,~~ 26]. These data, together with the known anxA5 relocalization in the membrane in the presence of Ca^{2+}, led us to postulate that Tg-induced increase in $[Ca^{2+}]_i$ could increase the Cl^- channel activities of CFTR. To test this hypothesis, we studied the CFTR channel activity in A549 cells when

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anxA5 expression was modulated in the presence or absence of Tg. We first checked that $[Ca^{2+}]_i$ was increased in our cell model after Tg treatment (16 hours) using Fura 2-AM. As shown in Fig 5A, a significant increase in $[Ca^{2+}]_i$ caused by Tg was observed in the A549 cells expressing the Wt-CFTR (+29.3 ± 1.8%) or the ΔF508-CFTR (+32 ± 1.2%) protein, when compared with untreated cells.

The Cl^- channel activity of CFTR was measured by patch-clamp in Wt-CFTR- and ΔF508-CFTR-expressing A549 cells after Tg treatment. Untransfected and untreated A549 cells were used as negative controls (not shown). As shown in Fig 5B, the I/V relationship obtained indicates that the Cl^- channel function of both the Wt-CFTR and the ΔF508-CFTR was increased in the presence of Tg in A549 cells. This result was in accordance with previous results [19, 35]. As shown in Fig. 5C, Tg had no effect upon anxA5 expression, which could have explained the increased Cl^- channel activity of both Wt-CFTR and ΔF508-CFTR in this cell types.

AnxA5 overexpression enhances the Tg effect in ΔF508-CFTR- expressing A549 cells

The combined effect of Tg and modulation of anxA5 expression was studied in the ΔF508-CFTR-expressing A549 cells. In a first set of experiments, we found that Tg treatment, shRNA and anxA5 overexpression had no effect upon Cl^- flux in the cells that did not express the CFTR protein (not shown).

The Cl^- channel activity of CFTR was assessed by the cell-attached patch-clamp technique. Fig. 6A shows the experimental conditions and an example of the corresponding waveform trace currents. The recordings showed that the forskolin treatment stimulated the ΔF508-CFTR Cl^- channel activity. The response of ΔF508-CFTR channels to the stimulation was lower than that of Wt-CFTR (not shown). These results can be correlated with those obtained in SPQ experiments. The patch-clamp recordings were converted to current-potential (I/V) relationship representations (Fig. 6B). When anxA5 expression was reduced in A549 cells the slope of the curves was decreased, indicating a reduced channel activity of ΔF508-CFTR. Conversely, the slopes of the curves were increased when anxA5 was overexpressed or when cells were treated with Tg. Finally, the slopes of the curves were increased when anxA5 was overexpressed in Tg-treated cells when compared with those of cells only submitted to Tg treatment or to anxA5 overexpression alone. The lower panel in Fig. 6B represents the current amplitudes (at +60 mV) and highlights statistical differences. The results show that the anxA5 expression reduced by shRNA significantly decreased the channel activities of the ΔF508-CFTR which function was significantly enhanced when anxA5 was overexpressed. It was further increased when anxA5 overexpression was coupled to the Tg treatment, when

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compared with the effect of each treatment singly applied. For each experiment, controls were performed with the irrelevant shRNA, with the empty vector and with non-transfected cells (not shown).

In conclusion, these experiments show for the first time that anxA5 protein is intimately involved in the Cl^- channel function of ΔF508-CFTR. Finally, we demonstrate here that increasing anxA5 expression improved the effect of Tg upon the ΔF508-CFTR channel activity.

AnxA5 overexpression enhances the cell surface expression of both Wt-CFTR and ΔF508-CFTR

To determine whether the increased Cl^- currents were due to an increased channel activity or to an increased cell surface expression of the CFTR protein, the cell surface localization of the ΔF508-CFTR proteins was analysed by immunofluorescence studies (not shown) and by cell surface biotinylation experiments [43, 44].

In immunofluorescence experiments we observed that the irrelevant shRNA had no effect upon the ΔF508-CFTR localization whereas the shRNA directed against anxA5 induced its decreased membrane localization (not shown). We confirmed that Tg increases the cell surface expression of the ΔF508-CFTR [19] and showed that this positive effect was conserved when anxA5 expression was reduced.

When anxA5 was overexpressed, with or without Tg, ΔF508-CFTR localization was increased in the cell membrane. AnxA5 modulation had no effect upon actin distribution.

We confirmed these results by biochemical assays. The total cell surface proteins were biotinylated, purified on a streptavidin column and the CFTR protein was detected in collected fractions by Western blotting. As shown in Fig. 7, CFTR was observed in the membrane in the Wt-CFTR-expressing A549 cells, whereas a faint band was observed in the membrane proteins from the ΔF508-CFTR-expressing A549 cells, consistent with the retention and degradation of most of the mutated protein in the ER. The effect of the decreased anxA5 expression on the cell surface expression of CFTR in Wt-CFTR- and ΔF508-CFTR-expressing cells was assessed. When anxA5 expression was decreased, membrane expression of both Wt-CFTR and ΔF508-CFTR proteins was inhibited (Fig. 8).

Wt-CFTR and ΔF508-CFTR membrane localization was further studied when anxA5 was overexpressed in A549 cells. As shown in Fig 9, a faint band was observed in Wt-CFTR- and ΔF508-CFTR-expressing cells after transfection with the empty vector used as control. These bands were stronger when anxA5 was overexpressed. Therefore, anxA5 overexpression increased the cell surface expression of both Wt-CFTR and ΔF508-CFTR proteins. This cell

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surface expression of both Wt-CFTR and ΔF508-CFTR was increased when anxA5-overexpressing cells were treated with Tg. In summary, the cell surface expression of Wt-CFTR and ΔF508-CFTR proteins was decreased in the absence of anxA5 expression and, conversely, was increased when anxA5 protein was overexpressed and Tg enhanced the ΔF508-CFTR expression in plasma membrane in anxA5-overexpressing A549 cells.

Modulation of anxA5 expression in polarized cells

Our results were extended in the polarized cell line CFBE41o- in which anxA5 expression was modulated. As shown in Fig. 10A, shRNA induced a significant anxA5 decreased expression whereas a significant anxA5 overexpression was observed in the presence of the cDNA encoding anxA5. SPQ experiments were performed in the CFBE41o- cells when anxA5 expression was modulated. As shown in Fig. 10B, the cAMP-dependent halide flux was significantly decreased when anxA5 expression was inhibited. The anxA5 overexpression induced significant increased cAMP-dependent halide flux in CFBE41o- cells. Therefore, the results were similar in non-polarized and in polarized cells. Biotinylation experiments performed in the CFBE41o- cells overexpressing anxA5 indicated that the increased cAMP-dependent halide flux was likely due to an increased ΔF508-CFTR localization in the apical membranes in these polarized cells (Fig. 10C, left panel). Immunolocalization of the CFTR protein was performed in the CFBE41o- cells when anxA5 expression was increased (Fig. 10C, right panel). A faint increased localization of the mutated CFTR in the plasma membrane was observed when compared with cells transfected with the empty vector. Therefore immunofluorescence was in accordance with the biotinylation experiments.

DISCUSSION

The proteins or drugs that might increase the cell surface expression of the ΔF508-CFTR are potential targets to correct the Cl^- currents in CF. Because anxA1 is down-regulated in CF patients [45], because annexin 2 belongs to the functional complex of CFTR [46], and because anxA5 is overexpressed in CF epithelial cells from foetal trachea [47], annexins are involved in CF. In a previous study we identified anxA5 as a Ca^{2+}-dependent partner of CFTR whose expression was necessary for the CFTR membrane localization and channel function [26]. This led us to investigate whether anxA5 has any effect upon ΔF508 function or membrane localization.

The present study was performed in A549 cells, an immortalized human airway epithelial cell line which is routinely used as a model of pulmonary epithelium despite they

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are not polarized [26, 48]. The cells were stably transfected with the cDNA encoding the Wt-CFTR or ΔF508-CFTR proteins. Functional studies, immunofluorescent labelling and biotinylation assays showed that the Wt-CFTR was functional at the cell surface, whereas a small fraction of functional ΔF508-CFTR channels reached the cell surface, according to the previously described cell model [26].

Because anxA5 expression is necessary for CFTR function and because anxA5 interacts with the both the normal and mutated CFTR, we hypothesized that anxA5 could also regulate the function and membrane localization of ΔF508-CFTR. To test this hypothesis, we modulated anxA5 expression in both Wt-CFTR- and ΔF508-CFTR-expressing cells. The results showed that the decreased anxA5 expression reduced the Cl^- channel activities of both Wt and ΔF508-CFTR channels whereas they were increased when anxA5 was overexpressed. Nevertheless, these experiments did not show whether anxA5 regulates the channel activity or the plasma membrane expression of CFTR. Therefore, its cell surface expression was analysed. Our results show that anxA5 underexpression decreases the cell surface localization of both normal and mutated channels and that its overexpression increases it, suggesting that the membrane expression of CFTR in polarized and non-polarized cells depends on anxA5 expression. These data led us to hypothesize that anxA5 might be involved in the regulation of the CFTR trafficking or in its stability in the plasma membrane. Indeed, annexins are involved in membrane organization, membrane trafficking and membrane-cytoskeleton-linkage [49]. Furthermore, the main property of anxA5 is to bind to negatively charged phospholipids of the plasma membrane and to the membranes of the biosynthetic or endocytic pathway, in a Ca^{2+}-dependent manner [32]. Therefore, because anxA5 binds to both Wt and ΔF508-CFTR and increases their cell surface expression and because annexins are involved in membrane trafficking, we hypothesize that anxA5 might regulate CFTR trafficking to the cell surface. Because anxA5 is a non-membrane-aggregating annexin [50], we speculate that it is involved in the stability of CFTR in the plasma membrane rather than in the trafficking process. Moreover, because anxA5 is involved in the stabilization of the membranes [51], because it binds to the membranes and to actin, because it regulates the function and the distribution of membrane glycoproteins [52] and because CFTR binds to actin [53], we suggest that anxA5 belongs to the three-dimensional network that might stabilize the mutated CFTR at the cell surface increasing its half-life in the plasma membrane. This could explain why the cell surface expression of both Wt-CFTR and ΔF508-CFTR depends on anxA5 expression. The findings that increased anxA5 expression leads to the transport of ΔF508-CFTR to the plasma membrane seem in contradiction with the results showing an increased

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anxA5 expression in fetal CF cells. Nevertheless, the expression of CFTR-specific mRNA is approximately 75-fold greater in the fetal lung than in the adult lung and anxA5 accumulates almost linearly from birth to adulthood. Because anxA5 expression tends to be higher in CF cells, our hypothesis that it may in act upon the constitutive internalization and recycling of CFTR.

Since the anxA5-CFTR interaction and the anxA5 relocalization to the plasma membrane both depend on $[Ca^{2+}]_i$, we hypothesized that increasing the $[Ca^{2+}]_i$ might help to maintain the cell surface expression of both Wt and ΔF508-CFTR. This was tested using Tg, the most widely used SERCA inhibitor, which increases the $[Ca^{2+}]_i$ by mobilizing the sequestered ER calcium stores. Furthermore, Tg induced the redistribution of the ΔF508-CFTR from the ER to the apical membrane [19, 35] by disrupting the interaction between the newly synthesized CFTR and calnexin, a Ca^{2+}-dependent chaperone protein involved in ΔF508-CFTR retention in the ER. We suggest that anxA5 could be involved in the positive effect of Tg hitherto only attributed to the disruption of the calnexin-ΔF508-CFTR interaction.

In the present study, we demonstrate that Tg increases the cell surface expression of both Wt and the mutated CFTR in A549 cells. However, this effect was decreased when anxA5 expression was reduced, but was not totally abolished. This indicates that the positive effect of Tg involves anxA5. Because Tg does not induce increased anxA5 expression, this effect is likely due to the previously described relocalization of anxA5 to the membrane when $[Ca^{2+}]_i$ is increased.

In conclusion, because anxA5-CFTR, anxA5-membrane and anxA5-actin interactions are regulated by $[Ca^{2+}]_i$, we suggest that the increased $[Ca^{2+}]_i$ induced by Tg might enhance the formation of the three-dimensional network. So, enhancement of anxA5 expression may increase the presence of the mutated CFTR protein in the plasma membrane where it exhibits some Cl^- channel functions.

This study shows for the first time that anxA5, which interacts with the NBD1 of the CFTR [26], regulates the cell surface expression of both the Wt and the mutated CFTR by increasing their presence in the plasma membrane in polarized and in non-polarized cells. We show that an increased $[Ca^{2+}]_i$ induces an increased anxA5 effect upon CFTR function. Therefore we propose a synergic and Ca^{2+}-dependent effect between Tg and anxA5. This positive effect of anxA5 has now to be taken into account when drugs such as Tg are used in order to restore the function of a mutated protein which is released from the ER. Furthermore,

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we propose that increased anxA5 expression *in vivo* might partially correct the altered Cl^- channel in CF.

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Legends to figures

Figure 1. anxA5 expression in non-polarized (A549) and polarized ΔF508-CFTR-expressing cells (CFBE41o-).

The left panel shows an example of western blot representing the expression of anxA5 (35 kDa) in wt-CFTR- and ΔF508-CFTR A549 cells (1 and 2, respectively), in 16HBE14o- (3) and CFBE41o- (4) cells. The right panel shows the corresponding quantification (percentage). The anxA5 levels tended to be lower in ΔF508-CFTR expressing cells.

Figure 2. Co-immunoprecipitation of anxA5 with Wt-CFTR and ΔF508-CFTR.

CFTR protein was immunoprecipitated from Wt-CFTR- and ΔF508-CFTR-expressing A549 cells. The complexes were resolved by SDS/PAGE (12%) and transferred onto a PVDF membrane. AnxA5 was then detected by immunoblotting and was observed in the complexes. It was not detected in the complex from A549 cells which did not express CFTR (NT) or when an irrelevant antibody was linked on the beads (negative control). Pure anxA5 was used as molecular weight control (35 kDa).

Figure 3. Modulation of anxA5 expression in A549 cells

Example of detection of anxA5 in Wt-CFTR- and ΔF508-CFTR-expressing A549 cells by Western blotting. **A.** Wt-CFTR and ΔF508-CFTR-expressing A549 cells were transfected with either the shRNA directed against anxA5 or the irrelevant shRNA. Decreased anxA5 expression was observed in both cell types in the presence of its specific shRNA, when compared to the control (upper panel). Overexpression of anxA5 was obtained in Wt-CFTR and ΔF508-CFTR-expressing- A549 cells by transfection with the cDNA encoding anxA5 (lower panel). 48 hours after the transfection, anxA5 expression was increased in both cell types when compared with the control (empty pcDNA3.1 vector). **B.** Representative

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histograms (percentage) showing the statistical decreased anxA5 expression due to the shRNA and its significant increase in transfected cells. (***: $p<0.001$).

Figure 4. Effect of the modulation of anxA5 expression upon the cAMP-dependent halide flux through ΔF508-CFTR in A549 cells. Bar graph representation of the effect of the decreased and increased anxA5 expression upon the cAMP-dependent halide flux through ΔF508-CFTR in A549 cells channel which was measured by fluorescence digital imaging microscopy using SPQ dye. The obtained curves were used to determine the halide permeability (p) as the rate of SPQ dequenching in NO_3^- medium under base-line conditions and under cAMP stimulation. When anxA5 expression was decreased the cAMP-dependent halide flux through ΔF508-CFTR in A549 cells was significantly decreased when compared to the control (light grey). When anxA5 was overexpressed, the halide flux was significantly increased (dark grey). Comparison with non-treated ΔF508-CFTR expressing A549 cells (white) indicate that the controls did not modify the halide flux. Bars represent SEM, *: $p<0.05$, **: $p< 0.001$.

Figure 5. Study of the effect of Tg in Wt-CFTR- and ΔF508-CFTR-expressing A549 cells.

A. The effect of Tg upon $[Ca^{2+}]_i$ was checked by Fura 2-AM measurement. Non-transfected A549 cells, Wt-CFTR- and ΔF508-CFTR-expressing A549 cells were loaded with Fura-2 AM after Tg treatment. For each cell type, fluorescence was recorded at 340 nm (saturated calcium) and 380 nm (free calcium) and compared with that of untreated cells. In all cell types, Tg induced a significant increase in $[Ca^{2+}]_i$. Bars represent SEM (n=5). **B.** The effect of Tg upon the Cl^- channel function of Wt-CFTR and ΔF508-CFTR was measured by patch-clamp. The I/V relationships show that in both Wt-CFTR- and ΔF508-CFTR-expressing cells, Tg increased CFTR channel function. Cells which did not express CFTR and untreated cells were used as negative controls. Bars represent SEM. **C.** Analysis of Tg effect on anxA5 expression in Wt-CFTR- and ΔF508-CFTR-expressing A549 cells by Western blotting. Tg treatment did not modify anxA5 expression in either cell type. The same amount of protein was loaded in each lane (50 µg).

Figure 6. Cell-attached patch-clamp experiments performed in ΔF508-CFTR-expressing A549 cells submitted to different levels of anxA5 expression in native conditions and after Tg treatment. A. The upper panel indicates the cell conditions used in the patch-clamp analysis. The upper waveforms trace currents recorded in each set of experimental conditions

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are representative of the currents recorded in ΔF508-CFTR before (basal currents) and after cAMP-stimulation underexpressing or overexpressing anxA5 with or without Tg in A549 cells. The lower trace represents the voltage steps applied to the membrane. Horizontal bar, 20.88·ms; vertical bars, 1·nA (top) and 1·mV (bottom). **B.** I/V relationship in all experimental conditions (mean ± SEM). Bar graphs represent the statistical analysis of the current amplitudes (mean ± SEM, n=5) at + 60 mV in all experimental conditions. The conditions where only forskolin and IBMX were applied to the cells were taken as reference for the analysis. *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$

Figure 7. Cell surface detection of Wt-CFTR and ΔF508-CFTR by biotinylation in A549 cells.

Cell surface proteins of the cells expressing Wt-CFTR or ΔF508-CFTR were labelled by biotinylation, purified and analysed by Western blotting (7% gel electrophoresis). The mature CFTR (170 kDa) band is observed and is stronger in Wt-CFTR-expressing A549 cells than in ΔF508-CFTR A549 cells. Non-transfected A549 cells which do not express CFTR were used as negative controls and the same amount of protein was loaded in each lane.

Figure 8: Cell surface detection of Wt-CFTR and ΔF508-CFTR by biotinylation, when anxA5 expression is reduced.

When both Wt-CFTR- and ΔF508-CFTR-expressing A549 cells were transfected by the irrelevant shRNA, cell surface expression of CFTR protein was detected in membrane proteins after biotinylation and Western blotting. CFTR was not detected in membrane proteins from non-transfected cells (negative control). The plasma membrane expression of Wt-CFTR and ΔF508-CFTR in A549 cells was observed when the irrelevant shRNA was transfected, whereas neither protein was detected when anxA5 expression was reduced. The same amount of protein was loaded in each lane.

Figure 9. Cell surface detection of Wt-CFTR and ΔF508-CFTR in A549 cells by biotinylation, when anxA5 is overexpressed, with and without Tg.

Biotinylation experiments and CFTR detection were performed. When anxA5 is overexpressed, both Wt-CFTR and ΔF508-CFTR are detected in membrane proteins at a higher level when compared with the empty vector transfected cells. The cell surface expression of Wt-CFTR and ΔF508-CFTR in A549 cells is further increased in the presence

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of Tg. A549 cells which did not express the CFTR proteins (NT) were used as a negative controls.

Figure 10. Modulation of anxA5 expression in polarized cells (CFBE41o-).

A. Example of western blot (left panel) performed to assess the expression of actin and anxA5 in 16HBE14o- (1) and in CFBE41o- cells (2-5). CFBE41o- cells were transfected whith either the control shRNA (2), the shRNA directed against anxA5 (3), the control plasmid (4) and the cDNA encoding anxA5 (5). The anxA5 expression was quantified (right panel) showing that the shRNA induced a significant anxA5 decreased expression whereas a significant anxA5 overexpression was observed in the presence of the cDNA encoding anxA5. **B.** SPQ experiments were performed in CFBE41o- cells and p (min^{-1}) was calculated as described above. The cAMP-dependent halide flux was significantly decreased when anxA5 expression was inhibited and it was increased when anxA5 was overexpressed. **C.** Biotinylation experiments were performed as described above and CFTR was detected by immunoblotting. In CFBE41o- cells transfected with the control cDNA, the CFTR protein was not distinguished (1) whereas it was observed in the cells overexpressing anxA5 (2). Immunofluorescence was performed in CFBE41o-. In the CFBE41o- cells which were transfected with the empty vector, CFTR was observed mainly around the nuclei (left image). In the CFBE41o- cells which were transfected with the cDNA encoding anxA5, a faint labelling in the membrane was observed (right image).

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Other

Figure 1

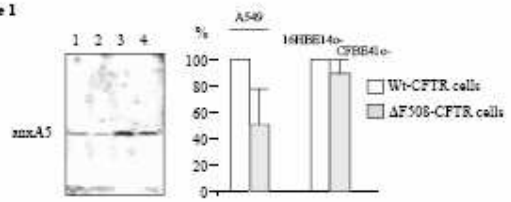
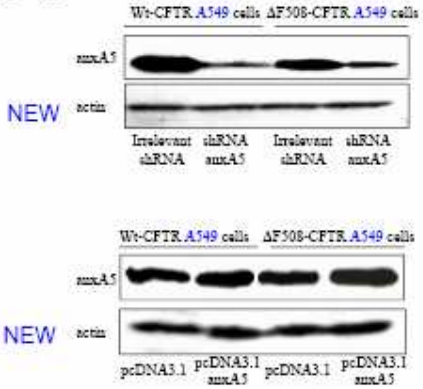


Figure 2

Figure 3 A



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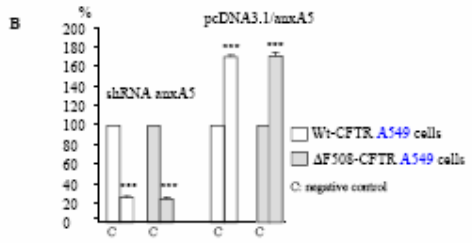

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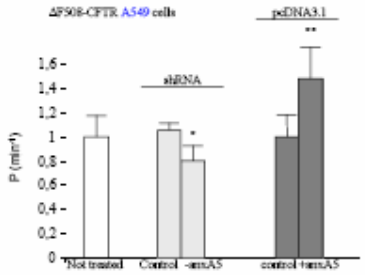

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ACCEPTED MANUSCRIPT

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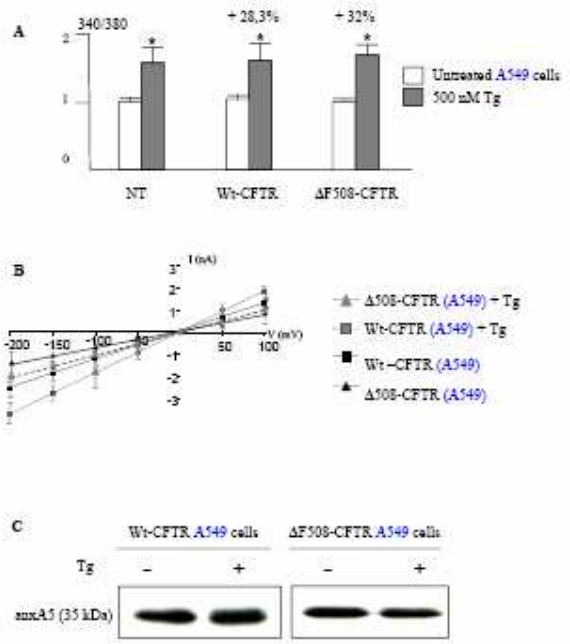

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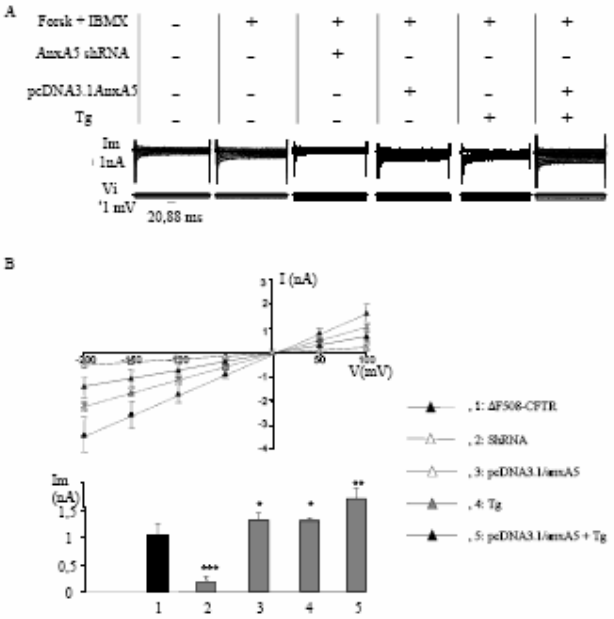

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ACCEPTED MANUSCRIPT

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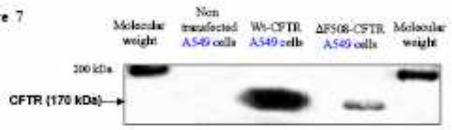

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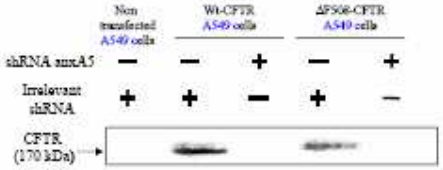

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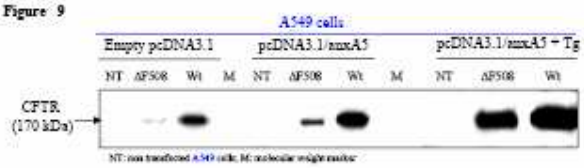

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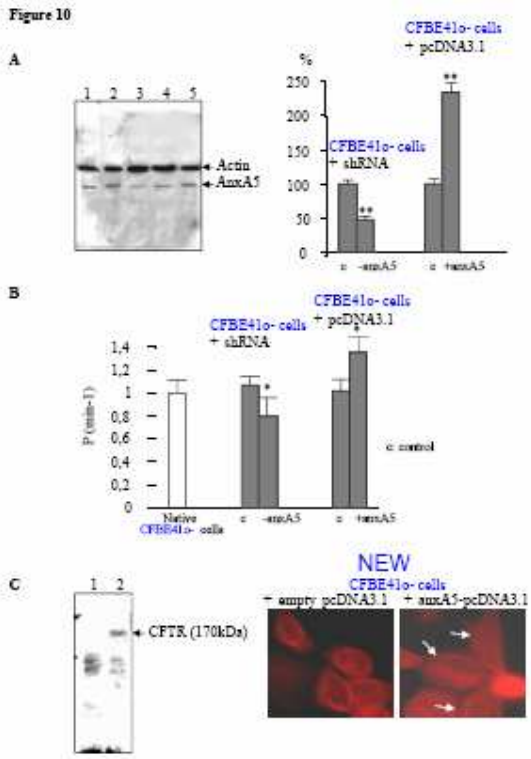

ACCEPTED MANUSCRIPT

Figure

Figure 1

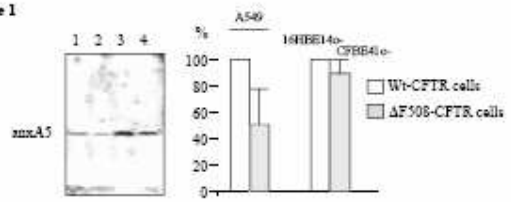

Figure 2

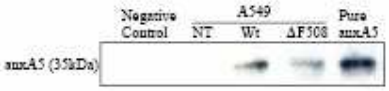
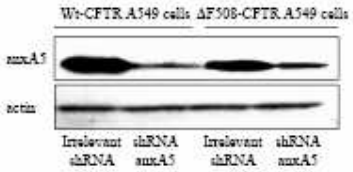
Figure 3 A