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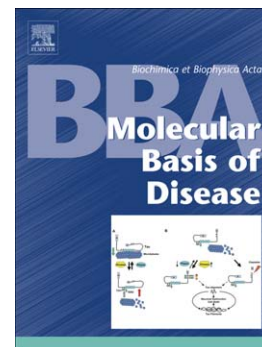
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Coupling cystic fibrosis to endoplasmic reticulum stress: differential role of Grp78 and ATF6.

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Abstract

Cystic fibrosis (CF) is the most common Caucasian autosomal recessive disease. It is due to mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene encoding the CFTR protein, which is a chloride (Cl⁻) channel. The most common mutation leads to a missing phenylalanine at position 508 (Δ F508). The Δ F508-CFTR protein is misfolded and retained in the endoplasmic reticulum and may trigger the unfolded protein response (UPR). Furthermore, CF is accompanied by inflammation and infection, which are also involved in the UPR. To date, the UPR transducer ATF6 and ER stress sensor Grp78 have been used as UPR markers. Therefore, our aim was to study the activation of ATF6 and Grp78 in transfected human epithelial cells expressing the Δ F508-CFTR protein, and we showed that they are activated in these cells. We investigated the effect of exogenous UPR inducers thapsigargin (Tg) and tunicamycin (Tu) on Grp78 and ATF6 expression. Whereas the cells reacted to the UPR induction, we show a difference in the electrophoretic pattern of ATF6. The Grp78/ATF6 complex was previously described, but its stability during UPR is controversial. Using co-immunoprecipitation we show that it is stable in Δ F508-CFTR-expressing cells and is maintained under UPR conditions. Finally, using siRNA, we show that decreased ATF6 expression induces increased cAMP-dependent halide flux through Δ F508-CFTR due to its increased membrane localization. Therefore, our results suggest that UPR may be triggered in CF and that ATF6 may be a therapeutic target.

Keywords: Cystic Fibrosis; Unfolded Protein Response; ATF6; Grp78; Endoplasmic Reticulum.

The abbreviations used are: CFTR, cystic fibrosis transmembrane conductance regulator; NBD, nucleotide-binding domain; ER, endoplasmic reticulum; ATF6, activating transporting factor 6; Grp78, glucose-regulated protein 78; Tg, thapsigargin; Tu, tunicamycin.

1. Introduction

Cystic fibrosis (CF) is the most common lethal autosomal recessive disease in the Caucasian population, and is due to mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene [1-3]. The normal CFTR protein, which is a member of the ATP-binding cassette transporters, functions as a chloride (Cl⁻) channel [2, 4-5]. It comprises two hydrophobic core regions, two nucleotide-binding domains (NBDs) with ATP-binding activity [5], and a regulatory domain. In CF, the gene encoding the CFTR protein is mutated, leading to an altered protein processing or expression. Therefore, altered chloride channel function through CFTR is the hallmark of CF. Nevertheless, the link between genotype and phenotype remains unclear in CF.

The most common mutation in CF is a missing phenylalanine at position 508 (Δ F508), which occurs in the first nucleotide-binding domain (NBD1) of the CFTR protein. The pathology associated with Δ F508-CFTR is believed to be a failure of the mutated protein to traffic correctly to the plasma membrane [6-9]. The Δ F508-CFTR protein is misfolded and is degraded by proteasomes after becoming a substrate for the Hsc70-CHIP E3 ubiquitin ligase [9-13]. Nevertheless, partial endoplasmic reticulum (ER) retention of Δ F508-CFTR was reported in some model systems [11, 14]. For example, in COS-7 cells the mutated CFTR protein is incompletely glycosylated and accumulates in the ER [6], and in pancreatic adenocarcinoma cells, which naturally express the Δ F508 mutated protein, and in the ER compartment [14]. Therefore, the misfolded CFTR is likely partially targeted to degradation. Interestingly, some studies indicate that the Δ F508-CFTR protein can function as a cAMP-dependent Cl⁻ channel, suggesting that if conditions could be created to allow the Δ F508-CFTR protein to exit the ER and reach the membrane, it might partially correct the CF defect.

Beside the accumulation of $\Delta F508$ -CFTR protein in the ER, inflammation and infection are the major features of CF [15]. Inflammation during CF produces local tissue damaging and cytokines are found to be increased [16]. This inflammation precedes the numerous infections due to specific micro-organisms such as *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Burkholderia cepacia* and *Haemophilus influenza* [17, 18, 19]. The consequence is an increased endobronchial inflammatory response [20]. Viral infections are also found during CF (13–52% of patients have a viral infection) with a worse prognosis than in normal patients [21-29].

Eukaryotic cells respond to the stress due to the accumulation of misfolded and unfolded proteins in the ER by activating a signalling pathway known as the unfolded protein response (UPR). Beside the accumulation of unfolded proteins in the ER, a link between inflammation, infection and the UPR pathway has been demonstrated in several models [30, 31, 32]. Because both inflammation and retention of misfolded protein are observed in CF, we hypothesized that UPR may be involved in CF.

The UPR induces the transcription of genes encoding the ER chaperones, the protein-folding enzymes, the membrane trafficking factors and components of the ER-associated degradation system, limiting new protein synthesis [33-37]. Whereas the UPR pathway is an adaptive process involved in restoration of ER homeostasis, it may also lead to apoptotic cell death [38]. To date, the UPR transducer ATF6 (activating transcription factor 6) and ER stress sensor Grp78 (glucose-regulated protein 78)/BiP have been used as UPR markers [39-44]. ATF6 is a bZIP transcription factor synthesized as an ER membrane precursor (90 kDa) with its C-terminus located in the ER lumen and its N-terminal DNA-binding domain facing the cytosol [41]. During the UPR process, ATF6 is cleaved in the Golgi by site-1 and site-2 proteases as an active form (50 kDa) which migrates to the nucleus of the stressed cells, where it acts as an activation factor, binding and activating the ER stress response element

[40, 41]. Grp78 (BiP, 78kDa), which is a peptide-dependent ATPase of the heat shock protein family, binds transiently to the newly synthesized proteins translocated into the ER. Grp78 is activated during the UPR process, which induces its redistribution from the ER lumen to the ER membrane [41]. Grp78 binds to the hydrophobic exposed patches on the surface of the unfolded proteins and is involved in the ATF6 activation due to their association and dissociation [43, 44]. IRE1 and PERK are also known as UPR transducers [39, 43]. They are both ER-resident transmembrane proteins which are bound to Grp78. Under UPR conditions they are released from Grp78 and activated. Through its interactions with ATF6, IRE1 and PERK, Grp78 is seen as the UPR sensor [43].

Our aim was first to study the activation of ATF6 and Grp78 in transfected human epithelial cells (A549 cells) expressing the mutated CFTR protein. We showed for the first time that the protein levels of ATF6 (active form) and Grp78 were increased in the Δ F508-CFTR protein-expressing cells. Because additional stress such as inflammation and infection, which are known to induce UPR, occur in CF, we investigated the effect of exogenous UPR inducers. The depletion of ER calcium (Ca^{2+}) stores using thapsigargin (Tg) and the effect of the blockage of N-linked protein glycosylation using tunicamycin (Tu) upon Grp78 and ATF6 expression were studied in Δ F508-CFTR protein-expressing cells. Whereas both cell lines react to UPR induction, we show a difference in the electrophoretic pattern of ATF6 in the Δ F508-CFTR-expressing cells under Tg or Tu treatment. The Grp78/ATF6 complex was previously described and, using co-immunoprecipitation, we show that it is stable in Δ F508-CFTR-expressing cells and is maintained under UPR conditions. Finally, using siRNA, we show that decreased ATF6 expression induces increased cAMP-dependent halide flux through Δ F508-CFTR, whereas Grp78 has no effect. Therefore, the present results suggest that ATF6 may be a therapeutic target in CF.

2. Materials and methods

2.1 Antibodies and drugs

Polyclonal anti-Grp78 (H-129): sc-13968 antibody, monoclonal anti-ATF6 antibody (clone 70B1413) and monoclonal anti-CFTR antibody (MM13-4) were from Santa Cruz Biotechnology, Stratagene and Interchim, respectively. Secondary antibodies and the ECL+ detection kit were from Amersham. The siRNA directed against Grp78 and ATF6 (human, sc 29338 and sc-37699, respectively; Santa Cruz Biotechnology), their negative control (scrambled siRNA) and the transfection buffer were all from Tebu-Bio. Tu (T7765), Tg (T9033), leupeptin, aprotinin, PMSF, iodoacetamide, pepstatin and DIFP were all from Sigma. The ER detection kit (SelectFX Alexa Fluor 488 ER labelling kit) and the 6-methoxy-N-(3-sulfopropyl) quinolinium (SPQ) were from Molecular Probes.

2.2 Cell culture conditions, plasmids and transfection

A549 cells, an alveolar type II epithelium-like cell line (ATCC, Rockville, USA), were cultured in Ham's F-12 medium, as modified by M.E. Kaighn, and supplemented with 10% foetal calf serum, 50 mg/ml streptomycin and 50 U/ml penicillin in a 5% CO₂- balanced air incubator at 37°C.

The wild type human CFTR (Wt-CFTR) cDNA (4.5 kb) inserted in the pTG5985 vector was tested by western blotting and provided by Transgene (Starsbourg, France). The cDNA (access Genbank no. M28668) was inserted in pcDNA3.1 plasmid (InVitrogen) between the KpnI and XhoI restriction sites. The cDNA encoding the Δ F508-CFTR protein was obtained by the use of QuickChange II XL Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's instructions. The primers were: 5'-CTGGCACCATTAAAGAAAATATCATTGGTGTTCCTATGATG-3' (sense) and 5'-CATCATAGGAAACACCAATGATATTTTCTTTAATGGTGCCAG-3' (antisense). In order to assess the mutated cDNA, it was sequenced using the ABI PRISM 310 sequencing

system (Applied Biosystems). A549 cells were transfected using lipofectamine 2000 (Life Technologies) according to the manufacturer's instructions, and stably transfected clones for normal and mutated construction were isolated by neomycin selection. Surviving individual colonies were inoculated, proliferated in the selective medium and used after 10 passages. To induce UPR stress, cells were cultured for 24 hours in the presence of Tu (10 $\mu\text{g/ml}$) or Tg (500 nM). At this time point, cell viability was assessed using the trypan blue exclusion test (not shown).

2.3 Immunofluorescence

Stably transfected A549 cells expressing human Wt-CFTR and ΔF508 -CFTR protein were grown on coverslips. The ER compartment was visualized using the SelectFX Alexa Fluor 488 Endoplasmic Reticulum Labelling Kit (S34200, Molecular Probes), according to the manufacturer's instructions. The kit contains the cell fixation and permeabilisation buffers. Cells were further incubated with the monoclonal anti-CFTR antibodies (1/10). After washing in PBS, the cells were incubated with secondary anti-mouse FITC-linked antibodies (1/100, Sigma) and the nuclei were stained with DAPI. First or secondary antibodies were omitted from controls.

2.4 Protein extraction and Western blotting

Cell lysates were prepared from transfected A549 cells by resuspending the cells on the plate in the lysis buffer (50 mM Tris HCl, 100 mM NaCl, 1% Triton X-100 [for co-immunoprecipitation] or 0.1% SDS [for Western blotting]), in the presence of 1.1 μM leupeptin, 0.7 μM aprotinin, 120 μM PMSF, 1 μM iodoacetamide, 0.7 μM pepstatin and 1 mM DIFP. For nuclear preparation, cells were washed with cold PBS and homogenized in NP-40 buffer. They were centrifuged (1500xg, 10 min.) and the supernatants were removed. The pellets were homogenized in CaCl_2 buffer and centrifuged (1500xg, 10 min.). The supernatants were discarded and the resuspended pellet was centrifuged (25,000xg, for 30

min). Finally, the pellet (nuclear envelope) was homogenized. Protein concentrations were determined by the method of Lowry et al., using bovine serum albumin as standard [45]. Equal amounts of total proteins for each sample were subjected to 10% or to 6% SDS/PAGE. After blotting, the membranes were incubated with either anti-GRP78 (1/200) or anti-ATF6 (1/200) antibodies and with the corresponding secondary antibodies. Visualization was performed using an ECL+ detection kit. Densitometric analysis of the signals was performed using a GelDoc 2000 apparatus (Bio-Rad, France) and each value was normalized to the total amount of loaded proteins per lane, which was estimated after Coomassie blue staining of the membranes and densitometric analysis.

2.5 Cell surface expression of CFTR by biotinylation

The presence of the CFTR protein in the plasma membranes of the non-transfected cells, in the Wt-CFTR-expressing cells and in the Δ F508-CFTR expressing cells, the proteins of the cell surface were biotinylated as previously described [46]. Four T75 cm² flasks of confluent cells were washed with ice-cold PBS (pH 8.0) and the cells were incubated in Sulfo-NHS-SS-Biotin solution (PinpointTM Cell Surface Protein Isolation Kit, Pierce, USA) for 30 min at 4°C. Cells were scraped and centrifuged for 3 min at 500 x g. The pellet was suspended in lysis buffer (50 mM Tris-HCl pH 6.8; 100 mM NaCl; 2% Triton X-100 and antiprotease cocktail) and incubated for 30 min on ice. The cell lysates were clarified (10,000 x g for 2 min at 4°C) and biotinylated proteins were isolated on Immobilized NeutrAvidinTM Gel (Pierce, USA). The CFTR protein was further detected by Western blotting as described above.

2.6 Co-immunoprecipitation

Cells expressing normal or mutated CFTR, with and without Tg or Tu incubation, were homogenized in Triton buffer and co-immunoprecipitations were carried out with Dynabeads (DynaL Biotech) on which anti-Grp78 or anti-ATF6 antibodies (2 µg per 100 µg of total protein) were linked, according to the manufacturer's instructions. The beads were

extensively washed and the immunoprecipitates were resolved by Western blot, and ATF6 or Grp78 was detected. The amount of protein in the immunoprecipitates was assessed by Coomassie blue staining of the membranes, in order to ensure that similar amounts were loaded in each lane. Controls with an irrelevant antibody linked to the beads and without primary or secondary antibody for detection were negative. Positive controls were performed by the use of beads to which anti-Grp78 or anti-ATF6 antibodies were linked when Grp78 or ATF6 was detected, respectively.

2.7 RNA interference

In cells expressing Wt-CFTR or Δ F508-CFTR protein, Grp78 and ATF6 inhibitions were performed using commercially available siRNA kits, in which the siRNAs are target-specific, designed to knockdown the gene expression. The minimal levels of Grp78 and ATF6 were assessed by Western blotting, as described above, after different post-transfection times (24, 48 and 72 hours). For each siRNA experiment, negative controls were performed using the irrelevant siRNA provided in the siRNA kits and using the siRNA buffers. The irrelevant siRNA was a scrambled sequence which did not lead to the specific degradation of any cellular mRNA (Santa Cruz Biotechnology).

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

Stably transfected A549 cells with either the cDNA encoding the Wt-CFTR or the cDNA encoding the Δ F508-CFTR protein were used in SPQ experiments as previously described, using forskolin as CFTR activator because it induces cAMP synthesis which regulates the CFTR chloride channel function [47].

Cells were loaded with intracellular SPQ dye by incubation in Ca^{2+} -free hypotonic (50% dilution) medium containing 10 mM SPQ, 15 min, at 37°C. Coverslips were mounted on the stage of an inverted microscope equipped for fluorescence and illuminated at 360 nm. The emitted light was collected at 456 ± 33 nm by a high-resolution image intensifier coupled

to a video camera connected to a digital image processing board controlled by FLUO software (Imstar, France). Cells were maintained at 37°C and continuously superfused with an extracellular solution containing in mM: 145 NaCl, 4 KCl, 1 MgCl₂, 1 CaCl₂, 5 HEPES and 5 glucose, pH 7.4. A microperfusion system allowed local application and rapid change of the different experimental mediums. Media containing iodide (I⁻) and nitrate (NO₃⁻) were identical to the extracellular solution except that I⁻ and NO₃⁻ replaced Cl⁻ as the dominant extracellular anions. All extracellular media also contained 10 μM bumetanide to inhibit the Cl⁻/Na⁺/K⁺ co-transporter. Single-cell fluorescence intensity was measured from digital image processing and displayed against time. Fluorescence intensity was standardized according to the equation $F = (F - F_0) / F_0 \times 100$, where F is the relative fluorescence and F₀, the fluorescence intensity measured in the presence of I⁻. Membrane permeability (p) to halides was determined as the rate of SPQ dequenching upon perfusion with nitrates. At least three successive data points were collected immediately after application of the NO₃⁻-containing medium, and then fitted using a linear regression analysis. The slope of the straight line reflects the membrane permeability to halides (p in min⁻¹) and was used as an index of CFTR activity [47].

2.9 Statistics

Results were obtained from at least 10 different experiments and were expressed as mean +/- standard error of the mean. Differences between experimental groups were evaluated by a two-tailed unpaired Student's *t* test. $p < 0.05$ was considered significant (*) and $p < 0.001$ was considered very significant (**).

3. Results

3.1 Localization and cAMP-dependent halide flux through Wt-CFTR and ΔF508-CFTR protein in the non-transfected and in the stably transfected A549 cells.

Two A549 cell lines were generated in order to express Wt-CFTR or Δ F508-CFTR protein. Because the present study is based on Δ F508-CFTR protein retention in the ER, we assessed the localization of the CFTR protein in both cell lines using immunofluorescence. Wt-CFTR and Δ F508-CFTR were labelled, as well as the ER compartment. The nuclei were DAPI-stained. We observed Wt-CFTR protein in the membranes (not shown). The Δ F508-CFTR protein was mostly present in the ER, around the nuclei. The controls performed without the first or second antibody were negative. In the biochemical assay, membrane proteins of the non-transfected and stably transfected A549 cells were purified and the CFTR protein was detected using Western blotting. As shown in Fig.1A, the CFTR protein was present in the membrane of Wt-CFTR-transfected cells and at a lower level in the Δ F508-CFTR-expressing cells. No CFTR protein was detected in the membrane of the native A549 cells.

The cAMP-dependent halide flux through CFTR was studied in both cell lines using SPQ experiments, and non-transfected cells were used as the control. As shown in Fig. 1B, the Wt-CFTR-expressing cells exhibited cAMP-dependent halide flux, whereas cells that were not transfected exhibited minimal fluorescence. The Δ F508-CFTR-expressing cells exhibited a cAMP-dependent halide flux which was reduced by nearly one-third compared with that of the Wt-CFTR-expressing cells. Although decreased, this functional activity in Δ F508-CFTR cells has been described before [46, 48].

3.2 Comparison of the protein expression of Grp78 and ATF6 between the CFTR- and Δ F508-CFTR protein-expressing A549 cells.

A large amount of work has established that specific induction of Grp78 is indicative of ER stress (for review see [43]). Therefore, we studied Grp78 protein expression in Δ F508-CFTR protein-expressing cells and compared the results with the levels observed in Wt-CFTR-expressing cells. Western blots were performed on whole cell lysates as described

elsewhere [43]. As shown in Fig. 2A, the level of Grp78 was increased in Δ F508-CFTR-expressing cells. Quantitative analysis of the signal (n=12) indicated that the observed over-expression of Grp78 protein in Δ F508-CFTR was significant (Fig. 2B).

Beside Grp78, ATF6 is a widely used UPR marker (for review [49]). Therefore we studied its expression in both cell lines. ATF6 has an electrophoretic mobility of 90 kDa and is cleaved during UPR to an active fragment (50 kDa) [50]. As shown in Fig. 2A, the cleaved fragment was observed in the Δ F508-CFTR-expressing cells. Quantitative analysis of ATF6 (90 kDa form) clearly indicated its significantly increased expression in Δ F508-CFTR protein-expressing cells (Fig. 2B). Surprisingly, an additional band was observed in the 90 kDa form of ATF6 in both cell lines (Fig. 2A). This additional band was closer to the 90 kDa form of ATF6 in Wt-CFTR-expressing cells than in the Δ F508-CFTR protein-expressing cells. Such an additional band was previously reported and was referred to as the underglycosylated form of ATF6 involved in the sensing of the UPR pathway [51]. The ATF6 doublet, which appeared to migrate slower in Wt-CFTR-expressing cells, was previously described, and the nonglycosylated form of ATF6 was shown to be generated by Tu treatment of the cells [52]. This doublet was observed in our experiment (Fig. 2A).

Immunofluorescence was performed in order to investigate the translocation of ATF6 in the nuclei when Δ F508-CFTR was expressed. The cleaved form of ATF6 was only detected in Δ F508-CFTR-expressing cells (not shown). This result was further confirmed by a biochemical assay. Nuclear preparations were subjected to SDS/PAGE and ATF6 detection was performed by Western blotting. As shown in Fig. 2C, the cleaved form of ATF6 is only observed in the nuclei of the Δ F508-CFTR protein-expressing cells.

3.3 Comparison of the protein expression of Grp78 and ATF6 between the Wt-CFTR and Δ F508-CFTR protein-expressing A549 cells when an exogenous UPR stress is applied.

Because CF involves inflammation and infections, which both induce UPR, we studied the expression of Grp78 and ATF6 in both cell lines in the presence of the UPR inducers Tg and Tu. Tg is an ER calcium pump inhibitor which reduces the ER luminal calcium concentration, and Tu is an N-linked glycosylation inhibitor. Several experiments were performed in order to determine the best UPR induction conditions to be used in our cell type (not shown). Finally, non-transfected cells, Wt-CFTR- and Δ F508-CFTR-expressing cells were incubated for 24 hours in the presence of 500 nM Tg or 10 μ g/ml Tu. As shown in Fig. 3A, these conditions induced UPR since non-transfected cells exhibited an increased expression of Grp78 and the cleaved form of ATF6 under both Tg and Tu treatments. Wt-CFTR- and Δ F508-CFTR-expressing cells exhibited an increased expression of Grp78 in the presence of Tg or Tu (Fig. 3A). The quantitative analysis of Grp78 expression indicated that it was significantly increased under Tg and Tu treatment in both cell lines (Fig. 3B). ATF6 expression and cleaved ATF6 were observed when Tg or Tu was used (Fig. 3A). Quantitative analysis of ATF6 (50 kDa) expression indicated that it was significantly increased under Tg and Tu treatment in both the Wt-CFTR- and the Δ F508-CFTR-expressing cells (Fig. 3B). Therefore we concluded that both cell lines reacted under Tg or Tu treatment in a UPR-related way. Once more we noticed that ATF6 was cleaved in Δ F508-CFTR-expressing cells without any treatment (Fig. 3A).

Tg and Tu trigger UPR differently, so we performed a quantitative analysis in order to investigate a possible differential response between Wt-CFTR- and Δ F508-CFTR-expressing cells under Tg or Tu treatment. When the UPR stress was induced by Tu, no significant difference was observed in GRP78 or ATF6 expression between Wt-CFTR- and Δ F508-CFTR-expressing cells (Fig. 3C, right panel). The same result was observed when Tg was used (Fig. 3C, left panel). We concluded that the responses with Tg or Tu were not different between Wt-CFTR- and Δ F508-CFTR-expressing cells.

3.4 Comparison of the protein expression of Grp78 and ATF6 between non-transfected cells and cells transfected with the empty vector with and without Tg and Tu.

The transfection with the cDNA encoding either Wt-CFTR or Δ F508-CFTR could induce a cellular stress by itself. Therefore, we compared Grp78 and ATF6 expression in cells which were transfected with the empty vector with their expressions in non-transfected cells. As shown in Fig. 4A, no difference was observed. When the cells were treated with either Tg or Tu, the Grp78 and ATF6 expressions were identical in both cell types. The results were confirmed by the quantitative analysis of the blots (Fig. 4B).

3.5 Specific study of ATF6 expression in the absence and presence of Tu and Tg.

Using Western blotting, we studied the pattern of expression of ATF6 in Wt-CFTR- and Δ F508-CFTR-expressing cells and found that besides the fact that ATF6 expression was increased in Δ F508-CFTR cells, several bands were observed on the films (Fig. 5, upper panel). In order to study these bands after UPR induction, less protein was loaded on a 6% gel, as previously described [52], because with high protein loads the doublet pattern could merge into a single band. After Tg treatment, the nontransfected cells and the Wt-CFTR-expressing cells exhibited a doublet which migrated slower than the nonglycosylated form (Fig. 5, middle panel). After Tu treatment, nontransfected cells, Wt-CFTR- and Δ F508-CFTR-expressing cells all exhibited an additional band, close to the 90 kDa form of ATF6 (Fig. 5, lower panel). This band was previously described and referred to as a nonglycosylated form of ATF6 [41, 52]. The ATF6 doublet was not seen in Δ F508-CFTR cells and instead the nonglycosylated form of ATF6 was seen in Tg-treated cells, as previously described [52]. The level of the 90 kDa band was increased when the UPR induction was performed over a long period of time [52].

3.6 Study of the interaction between Grp78 and ATF6.

In unstressed cells, Grp78 binds to the luminal domain of ATF6, sequestering ATF6 in the ER and preventing its activation [53]. When UPR is triggered, ATF6 is released from Grp78. Grp78 also binds to newly synthesized proteins and more permanently to underglycosylated and misfolded proteins [54]. The formation and dissociation of the Grp78-ATF6 complex is a key event in UPR triggering. Nevertheless, the complex dissociation is unclear and two models have been proposed [44, 53]. The first assumes that unfolded proteins compete with Grp78 for ATF6 binding. The second assumes that the Grp78-ATF6 binding is stable and is actively dissociated by a signal from the UPR. Therefore, we compared the presence of Grp78 and ATF6 in immunoprecipitates from Wt-CFTR- and Δ F508-CFTR-expressing cells. Magnetic beads were covered with an anti-Grp78 antibody and further incubated with proteins from Wt-CFTR- or Δ F508-CFTR-expressing cells. The presence of ATF6 in the immunoprecipitated complex was investigated. As shown in Fig. 6 (upper panel), ATF6 is bound to Grp78 in Wt-CFTR and Δ F508-CFTR cells. This result was also observed when cells were treated with Tg or Tu. More ATF6 was bound when Δ F508-CFTR was expressed. The opposite experiment was performed. The anti-ATF6 was linked to the beads and Grp78 was detected (Fig. 6, lower panel). Once more we found that the interaction was stable when UPR was triggered, showing that the complex is stable as previously described [53]. More Grp78 was bound in Δ F508-CFTR cells. Because the dissociation of the complex is not due to the unfolded Δ F508-CFTR, since it was previously shown that CFTR does not interact with Grp78, the maintained ATF6 association could be due to the underglycosylated form of ATF6 [55, 56]. From these experiments we concluded that the Grp78-ATF6 complex in Δ F508-CFTR cells belongs to the previously described stable model [53].

3.7 Study of the cAMP-dependent halide flux through Δ F508-CFTR when Grp78 expression is decreased.

Under unstressed conditions, Grp78 binds to the luminal domain of IRE1 and PERK to maintain them within the ER and prevent their activation [39, 43]. Grp78 is described as the main UPR sensor which activates UPR transducers ATF6, IRE1 and PERK by releasing them [54].

Due to the key involvement of Grp78 in UPR triggering, we investigated its possible involvement in the cAMP-dependent halide flux through Wt-CFTR in Δ F508-CFTR-expressing cells. For this purpose we used the siRNA technology in order to decrease its expression and disrupt UPR triggering in the cells and investigate whether this decreased expression could release the Δ F508-CFTR from the ER and restore some cAMP-dependent halide flux in Δ F508-CFTR cells. As shown in Fig. 7A, the lowest expression of Grp78 was obtained 48 hours after the transfection of the specific siRNA (-80%), whereas no modification was observed at the corresponding time point when an irrelevant siRNA was used. Actin expression was not modified by the siRNA (Fig. 7B). As shown in Fig. 7C, the decreased Grp78 expression did not modify the level of the cAMP-dependent halide flux in the cells. The irrelevant siRNA had no effect upon this function.

3.8 Study of the cAMP-dependent halide flux through Δ F508-CFTR protein when ATF6 expression is decreased.

Because ATF6 is the main signalling regulator in UPR, we investigated its role in the cAMP-dependent halide flux via Δ F508-CFTR [39-44]. As shown in Fig. 8A, ATF6 expression was decreased 48 hours after siRNA transfection. At this time point, actin expression was not altered (Fig. 8B). The cAMP-dependent halide flux through Δ F508-CFTR was studied when ATF6 expression was at a minimum. As shown in Fig. 8C, the cAMP-dependent halide flux through Δ F508-CFTR was significantly increased when ATF6 expression was inhibited.

UPR may be triggered by inflammation and infection. Therefore, we studied the cAMP-dependent halide flux through $\Delta F508$ -CFTR in the presence of Tu or Tg. When Tu or Tg was applied to the Wt-CFTR cells, halide flux was decreased, with a greater effect for Tg (Fig. 9A). Surprisingly, neither Tu nor Tg decreased the residual cAMP-dependent halide flux in the $\Delta F508$ -CFTR-expressing cells. When $\Delta F508$ -CFTR cells, in which ATF6 expression was decreased, were treated with Tg or Tu, cAMP-dependent halide flux was increased (Fig. 9B and 9C).

3.9 Study of the cell surface expression of the $\Delta F508$ -CFTR protein when ATF6 is decreased.

To explain the increased the cAMP-dependent halide flux through $\Delta F508$ -CFTR when ATF6 expression is decreased, we studied the membrane expression of $\Delta F508$ -CFTR. Membrane proteins were biotinylated, purified and resolved by SDS/PAGE. The presence of the CFTR protein was assessed by Western blotting. As shown in Fig. 10, the presence of the $\Delta F508$ -CFTR protein in the membrane of the cells was increased when ATF6 expression was decreased. Nevertheless, its level was lower than in the Wt-CFTR-expressing cells. The control siRNA (irrelevant) was ineffective.

4. Discussion

In the most common genetic form of CF ($\Delta F508/\Delta F508$), incorrectly folded CFTR is retained in the ER and partially degraded. Because accumulation of unfolded or misfolded protein in the ER induces an adaptive response called UPR, we investigated a possible involvement of this response in CF. To date, the UPR triggering in CF has been little studied [57, 58].

One master regulator (Grp78/Bip) and three classes of UPR transducers (IRE1, PERK and ATF6) have been shown to be involved in UPR [54]. In addition, XBP1 was shown to participate in UPR. The present study focused on the UPR regulator Grp78 and the UPR

transducer ATF6 in a Δ F508-CFTR-expressing cell model. Grp78 is an ER resident protein whose synthesis is enhanced in cells that are stimulated by various environmental and physiological stress conditions that perturb ER function and homeostasis [43]. In unstressed cells, Grp78 binds to the UPR transducer ATF6, which is maintained in an inactive form. Upon ER stress, Grp78 releases ATF6 which is translocated from the ER to the Golgi where it is cleaved by the proteases S1P and S2P [50]. The cleaved part of ATF6 enters the nucleus where it functions as a transcription factor for the UPR target genes, including Grp78 [40, 41]. Although the use of transfected cells is widespread, we first showed that our cellular model is usable in studying the relationship between Δ F508-CFTR protein expression and UPR. Most of the Δ F508 protein was retained in the ER whereas the Wt-CFTR was present in the cell membranes. This localization was correlated with biochemical assays and the decreased cAMP-dependent halide flux through CFTR, as previously reported in the A549 transfected cells [46]. Grp78 protein expression was increased in the Δ F508-CFTR-expressing cells, which is a hallmark of UPR [39, 42, 43, 49, 54]. Beside this increased expression of Grp78, the cleaved form of ATF6 was observed. This represented further evidence of UPR triggering when Δ F508-CFTR is expressed [40, 41, 50, 51, 52]. Conversely, a recent paper by Nanua and co-workers indicates that in cultured cells of airway segments from three CF patients homozygous for the Δ F508 mutation, Grp78 expression was not increased at the mRNA or protein level [59]. In the same paper, the electrophoretic mobility of cleaved and uncleaved ATF6 was studied in the cells from these three patients. The immunoblots showed a decreased abundance of the full-length ATF6. This could be explained by the appearance of the cleaved form of ATF6. The observed discrepancy with the present results regarding Grp78 and ATF6 expression may be due to the cell type used. Furthermore, the results obtained for Grp78 and ATF6 mRNA expression in the cells from the three patients were normalized by glyceraldehyde-3-phosphate dehydrogenase (GAPDH). We normalized our protein expression

by the total amount of protein loaded on the gels, since the UPR pathway is not completely known. In the study using cells from patients, protein expression was normalized using actin. Nevertheless, GAPDH expression is modulated under various stress cell conditions [60] such as hypoxia [61, 62] and dysregulation of growth factors [63]. Additionally, GAPDH has been shown to be involved in other functions apart from basal cell metabolism, which may modify its level of expression in stressed tissues [64]. Furthermore, GAPDH has been related to a disease involving UPR [65] and some studies point to glucose metabolism alterations as a cause of clinical deterioration in patients with CF [66, 67]. Therefore, we believe that the use of the GAPDH to normalize the mRNA levels of proteins involved in the UPR pathway may lead to the observed discrepancy. Concerning the actin normalization, it was previously shown in cells treated with Tg that the increased Grp78 mRNA level was associated with decreased expression of actin mRNA [67]. Furthermore, renal proximal tubule epithelial cells (LLC-PK1) treated with a prostaglandin analogue exhibited increased Grp78 and actin expression, showing a direct link in the expression of both proteins [68]. Therefore, actin normalization of protein expression could also explain the discrepancy. More importantly, Grp78 regulation may be involved in the apparent discrepancy between our work and the data of Nanua et al. It was previously shown that the transcriptional regulation and translational regulation of Grp78 are independent events and that a rapid translational response permits the cells to adapt to small perturbations without a transcriptional response [69]. Due to the sensor properties of Grp78, we hypothesize that the stress level in the cells of Nanua et al. was not reached, whereas in our transfected cells the level of $\Delta F508$ -CFTR was sufficient to induce Grp78 activation. This latter point is more likely the explanation for the observed discrepancy, which should be correlated with another discrepancy: whereas proteasome inhibition, which is known to induce UPR, had no effect on CFTR protein abundance, a recent study shows that ER stress caused by brefeldin A or Tu results in an increase in spliced XBP1 levels and a

decrease in endogenous CFTR transcript levels very similar to that observed with proteasome inhibition [59]. The use of different cell types expressing endogenous or transfected CFTR could induce various UPR pathways. Indeed, Nanua experiments were performed in primary cell culture from three patients. Therefore, these cells may have been exposed to inflammation and infections that are present in CF. Furthermore, patients may have been under drug treatments which can modify the UPR pathway (for review, [70]). Furthermore, we used non-polarized epithelial cells in which the involved pathways may be different. The A549 cells were used because they are from human airways and do not express the CFTR protein [71] whereas the Wt-CFTR and $\Delta F508$ -CFTR expression, localization and function were previously described in these cells after transfection [72, 46]. A549 cells were also used because they were previously characterized regarding GRP78 [73]. Furthermore, they have recently been shown to elicit a classical UPR pathway in which translation attenuation, phosphorylation of eIF2 α , splicing of XBP-1 and nuclear translocation of ATF6 were observed [74]. Nevertheless, A549 cells are not polarized and may present a different trafficking process when compared to polarized cells. Polarized monolayers of human airway epithelial cells are the desirable model to study CFTR traffic but primary cell systems causes major limitations to the feasibility of the experiments since they are difficult to maintain in culture whereas we needed to perform culture for a long period of time in order to perform drug treatments. Moreover, their CFTR expression levels have been observed to decrease with increasing number of passages (for review, [75]). Finally, the present results are focused upon UPR and to a less extend upon CFTR trafficking since $\Delta F508$ -CFTR is retained in the ER.

Whereas Nanua and co-workers found that only a subset of elements of UPR is activated in $\Delta F508/\Delta F508$ CF cells, we concluded that in our cell model the Grp78 sensor and the AFT6 transducer were activated. These activations can not be due to overexpression of recombinant Wt-CFTR since this does not activate UPR [58]. Furthermore, we show here that

the transfection by itself does not induce the UPR pathway. Nevertheless, our results do not rule out the previously described atypical UPR in CF cells [57].

In the present paper, we show a differential involvement of Grp78 and ATF6 when the mutated CFTR protein is expressed. This is likely due to a different electrophoretic form of ATF6 in the $\Delta F508$ -CFTR cells, to the absence of Grp78-CFTR association, and to the stable Grp78/ATF6 complex in UPR, which is confirmed in the present study [53]. The unglycosylated form of ATF6 was previously studied. It was shown to trigger the UPR pathway by itself and to exhibit a reduced interaction with ER chaperones. It titrates away Grp78, releasing UPR signalling molecules and the full-length ATF6, which can then be cleaved. Furthermore, this form of ATF6 is transported faster to the Golgi, leading to a higher rate of ATF6 in the nucleus and in turn to triggering of the UPR pathway [51]. Therefore, the presence of this form of ATF6 in the $\Delta F508$ -CFTR-expressing cells could explain why the upregulation of Grp78 does not inhibit UPR. Our results indicate that UPR triggering in the $\Delta F508$ -CFTR cells also depends on the inducer even though both Wt-CFTR and $\Delta F508$ -CFTR cells react to the stress. This point is of interest due to the various potential inducers encountered in CF and should be correlated with the described positive effect of Tg on $\Delta F508$ -CFTR function [76]. Short treatment of the cells with Tg restores some $\Delta F508$ -CFTR function [76], whereas longer treatment induces UPR. This indicates that a threshold has to be reached to trigger UPR. Finally, we show that the downregulation of ATF6 leads to increased cAMP-dependent halide flux through CFTR due to its increased expression in the cell membrane. This can be explained by the decreased protein expression observed when UPR is triggered. By altering ATF6 expression, we decreased UPR triggering leading to arrest of the negative effect upon $\Delta F508$ -CFTR expression. Furthermore, ATF6 activates the ER stress response element, which induces GRP gene transcription [77]. Among GRP, calreticulin has recently been shown to downregulate the expression and membrane localization of CFTR [78,

79]. Therefore, the increased cAMP-dependent halide flux through $\Delta F508$ -CFTR when ATF6 expression is decreased can easily be explained by a decreased retention of the protein in the ER via decreased calreticulin expression.

The present results are of major importance because UPR is followed by significant changes in genomic CFTR mRNA and because endogenous CFTR biogenesis is regulated by UPR [58].

In conclusion, we show for the first time a specific involvement of ATF6 in $\Delta F508$ -CFTR cells and demonstrate that its decreased expression leads to restored cAMP-dependent halide flux in cells. We suggest that in CF, whereas the involvement of Grp78 is minor, because it does not interact with CFTR [53], ATF6 may be of a great importance because its decreased expression induces an increased membrane localization of CFTR. Furthermore, the unglycosylated form of ATF6 seems to be the main UPR effector in CF. Therefore, we provide new information regarding CF pathophysiology and open up a new field in the search for therapeutics.

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Figure legends

Figure 1. Membrane localization and cAMP-dependent halide flux in non-transfected and in transfected A549 cells. **A.** Membrane proteins were biotinylated and CFTR was detected by Western blotting (7.5% gel electrophoresis). Whereas the CFTR protein is observed in the cell membrane of CFTR-expressing cells, its expression was lower in the $\Delta F508$ -CFTR-expressing cells. No CFTR protein was detected in the non-transfected A549 cells. **B.** Study of the cyclic AMP-induced Γ efflux from CFTR stably expressing A549, $\Delta F508$ -CFTR-expressing and control cells. Data represent the mean (\pm S.E.M.) of the relative fluorescence (F/F_0) from 10 randomly selected cells, where F is the observed SPQ fluorescence and F_0 the fluorescence in the presence of Γ (maximal fluorescence) measured initially in buffer (consisting of 135 mM NaNO_3 , 1 mM Ca_2SO_4 , 2.4 mM K_2HPO_4 , 0.6 mM KH_2PO_4 , 10 mM HEPES and 10 mM glucose). Following 20-min incubation, basal fluorescence was measured after substitution of nitrate for Γ . Cells were stimulated with forskolin (20 μM)/IBMX (100 μM). Intracellular SPQ was completely quenched by addition of Γ -containing buffer (NaI, 150 mM) to obtain the minimum fluorescence. Each curve was obtained by the mean of at least 5 different experiments. The maximal fluorescence was observed in cells expressing CFTR protein (upper curve). In cells expressing $\Delta F508$ -CFTR, the fluorescence was decreased (middle curve). The fluorescence was residual in cells which did not express CFTR protein (not transfected, lower curve). The slopes of the lines (a, b) correspond to the rate of SPQ dequenching in NO_3^- medium under baseline conditions (p_{baseline}) and under cAMP stimulation (p_{cAMP}). They were used to calculate the halide permeability (p) as the rate of SPQ dequenching presented in figures 7, 8 and 9. The slopes of the two lines correspond to the rate of SPQ dequenching in NO_3^- medium under baseline conditions (p_{baseline} , a) and under cAMP stimulation (p_{cAMP} , b). Data are the means \pm S.E. of three mock injected cells and three CFTR injected cells.

Figure 2. Comparison of Grp78 and ATF6 expression in CFTR- and Δ F508-CFTR-expressing cells. **A.** Grp78 and ATF6 were detected by Western blotting in cell lysates. Increased expression of the Grp78 was observed in Δ F508-CFTR-expressing cells when compared with CFTR-expressing cells (upper panel). The right panel shows the result of a Western blot experiment performed to compare ATF6 expression. The 90 kDa form of ATF6 was increased in Δ F508-CFTR-expressing cells. In the CFTR-expressing cells, a previously described doublet was seen (*). In the Δ F508-CFTR cells, the under-glycosylated form (**) and the cleaved form of ATF6 were observed. An example of actin detection (lower panel) shows that the loading was identical for both the CFTR and the Δ F508-CFTR cells. **B.** Densitometric analysis of the signal was performed. It was normalized by the total amount of loaded protein in each lane and statistical analysis was performed (n=12). Grp78 and ATF6 expression was significantly increased in Δ F508-CFTR cells. **C.** Detection of the cleaved ATF6 in the nuclei by Western blotting. Nuclei extracts were obtained from transfected cells with the empty vector, the cDNA encoding CFTR and the cDNA encoding Δ F508-CFTR. These extracts together with the extract from non-transfected cells were subjected to Western blotting. Cleaved ATF6 was only detected in the extract from Δ F508-CFTR cells.

Figure 3. Comparison of Grp78 and ATF6 expression in the CFTR- and Δ F508-CFTR-expressing cells under normal conditions and under Tu and Tg conditions. **A.** Example of a Western blot indicating that the Grp78 expression is increased in all cell lines when they are cultured in the presence of Tg or Tu (upper panel). The middle panel represents Western blotting used to detect ATF6 expression in the same cell lines. Whereas the cleaved fragment of ATF6 is observed in Δ F508-CFTR-expressing cells in the absence and presence of Tg or Tu treatment, in CFTR-expressing cells and in non-CFTR-expressing cells, this cleaved fragment is observed with Tg and Tu. The lower panel shows an example of actin detection indicating that the loading was identical in all lanes. **B.** Quantitative analysis of Grp78 and ATF6 expression. They are significantly increased in CFTR-expressing cells under Tu or Tg conditions (left panel). Both Grp78 and ATF6 expression are significantly increased in Δ F508-CFTR cells under Tu or Tg conditions (right panel). **C.** Grp78 expression and ATF6 expression are not different between CFTR- and Δ F508-CFTR-expressing cells in Tu conditions (left panel) and Tg conditions (right panel).

Figure 4. Comparison of Grp78 and ATF6 expression in the non-transfected cells (NT) and in the cells transfected with the empty pcDNA3.1 vector under normal conditions and under Tu and Tg conditions. **A.** Example of a Western blot indicating that the Grp78 expression is increased in the NT cells and in the cells transfected with the empty vector when they are cultured in the presence of Tg or Tu (upper panel). The lower panel represents an example of Western blot used to detect ATF6 expression in the same cell lines. Whereas the cleaved fragment of ATF6 is clearly observed in the presence of Tg or Tu, it is faint when cells are not treated. **B.** The quantitative analysis of the Grp78 and the ATF6 expression indicates that there is no difference between non-transfected cells and cells transfected with the empty plasmid.

Figure 5. Analysis of the different forms of ATF6. 6% gel electrophoresis was performed in order to visualize the 90 kDa form, the doublet and the unglycosylated forms of ATF6. Without any treatment, the unglycosylated form of ATF6 is only observed in Δ F508-CFTR-expressing cells. Under Tg conditions, the ATF6 doublet is observed in the nontransfected and CFTR-expressing cells (middle panel). Under Tu conditions, the doublet is observed in the nontransfected cells, and in CFTR- and Δ F508-CFTR-expressing cells (lower panel). The

unglycosylated form of ATF6 is only observed in the Δ F508-CFTR-expressing cells, whereas the doublet is not seen.

Figure 6. Study of the Grp78-ATF6 complex by co-immunoprecipitation. The Grp78/ATF6 complex was studied by the use of beads covered with anti-Grp78 (upper panel) or anti-ATF6 (lower panel) antibodies. They were incubated with protein extracts from nontransfected, CFTR- and Δ F508-CFTR-expressing cells, with or without Tg or Tu. The complex was analysed by Western blotting using a first antibody directed against ATF6 (upper panel) or Grp78 (lower panel). Both images indicate that the Grp78/ATF6 complex is stable in all conditions. Negative controls were performed with an irrelevant antibody on the beads. Positive controls were performed with the beads covered with the antibody used in Western blotting.

Figure 7. Analysis of the effect of the specific siRNA directed against Grp78 upon the cAMP-dependent halide flux through Δ F508-CFTR. **A.** Example of Western blot performed to detect Grp78 in Δ F508-CFTR-expressing cells. The left panel represents Grp78 detection when cells were transfected with an irrelevant siRNA, and the right panel represents Grp78 detection in the presence of the specific siRNA. Histograms represent the corresponding quantification of Grp78. Whereas the irrelevant siRNA had no effect upon Grp78 expression, the specific siRNA significantly decreased expression, 48 hours after the transfection. **B.** Western blot performed to assess actin expression in the presence of siRNA, showing that the amount of protein was the same at the different transfection times and that actin was not altered by siRNA. **C.** SPQ experiments were performed using Δ F508-CFTR-expressing cells without siRNA, in the presence of the irrelevant siRNA and in the presence of the specific siRNA directed against Grp78. No significant difference was observed between the cell types. The membrane permeability to halides (p) was determined as the rate of SPQ dequenching upon perfusion with NO_3^- medium [47]. The data represent the mean \pm S.E.M. ($n = 5$).

Figure 8. Analysis of the effect of the specific siRNA directed against ATF6 upon the cAMP-dependent halide flux through Δ F508-CFTR. **A.** Example of Western blot performed to detect ATF6 in Δ F508-CFTR-expressing cells. The left panel represents ATF6 detection when cells were transfected with an irrelevant siRNA, and the right panel represents ATF6 expression in the presence of the specific siRNA. Histograms represent the corresponding quantification of ATF6. Whereas the irrelevant siRNA had no effect upon ATF6 expression, the specific siRNA significantly decreased expression 48 hours after the transfection. **B.** Western blot performed to assess actin expression in the presence of siRNA, showing that the amount of protein was the same at the different transfection times and that actin was not altered by siRNA. **C.** SPQ experiments were performed using Δ F508-CFTR-expressing cells without siRNA, in the presence of the irrelevant siRNA and in the presence of the specific siRNA directed against ATF6. The membrane permeability to halides (p) was determined as the rate of SPQ dequenching upon perfusion with NO_3^- medium [47]. A significant increase in cAMP-dependent halide flux through Δ F508-CFTR was observed (the data represent the mean \pm S.E.M., $n = 6$, **: $p < 0.01$).

Figure 9. Study of cAMP-dependent halide flux through Δ F508-CFTR by SPQ. **A.** Histograms represent the halide permeability through CFTR and Δ F508-CFTR in the absence or presence of Tg or Tu ($n = 5$). p was calculated as previously described [47]. Whereas Cl⁻ channel activity is decreased when UPR is triggered, it is not affected in Δ F508-CFTR cells.

B. Histograms represent the halide permeability through $\Delta F508$ -CFTR in the absence or presence of the siRNA directed against ATF6 under Tg treatment ($n = 6$). The results indicate that the inhibition of ATF6 increased halide permeability through $\Delta F508$ -CFTR. **C.** The same experiment was performed as in B, but Tg was replaced by Tu. The cAMP-dependent halide flux through $\Delta F508$ -CFTR was increased when ATF6 expression was decreased ($n = 6$).

Figure 10. Increased membrane localization of the CFTR protein in the $\Delta F508$ -CFTR-expressing cells in the presence of the specific siRNA directed against ATF6. Membrane proteins of the Wt-CFTR, the $\Delta F508$ -CFTR and the non-transfected cells were biotinylated. Biotinylated proteins were purified, submitted to SDS/PAGE (7.5% gel electrophoresis) and CFTR was detected by Western blotting. The CFTR protein (mature band, 170 kDa) is observed in the cell membrane of CFTR-expressing cells. Whereas its expression was lower in the $\Delta F508$ -CFTR expressing cells, it was increased in the presence of the siRNA directed against ATF6. No CFTR protein was detected in the non-transfected A549 cells although the cells were transfected with the specific siRNA. The control was performed with the irrelevant siRNA.

Figure 1

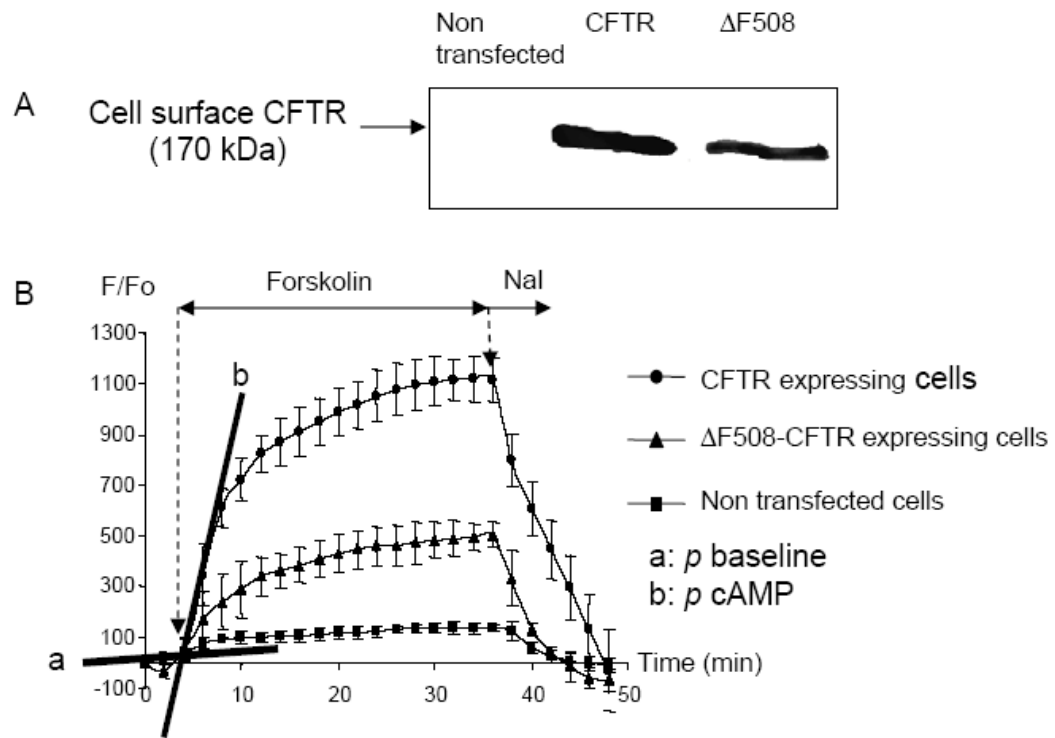


Figure 2

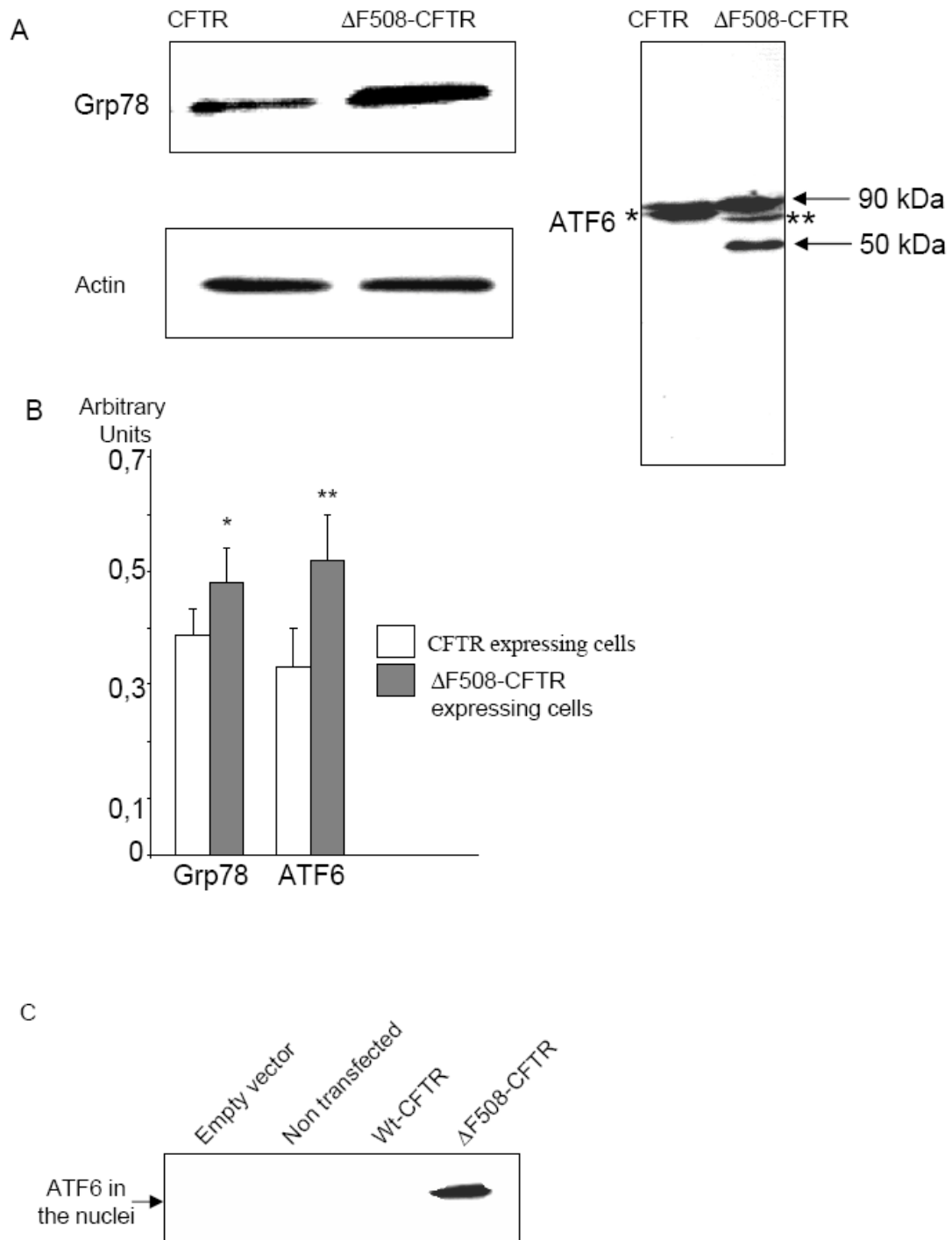


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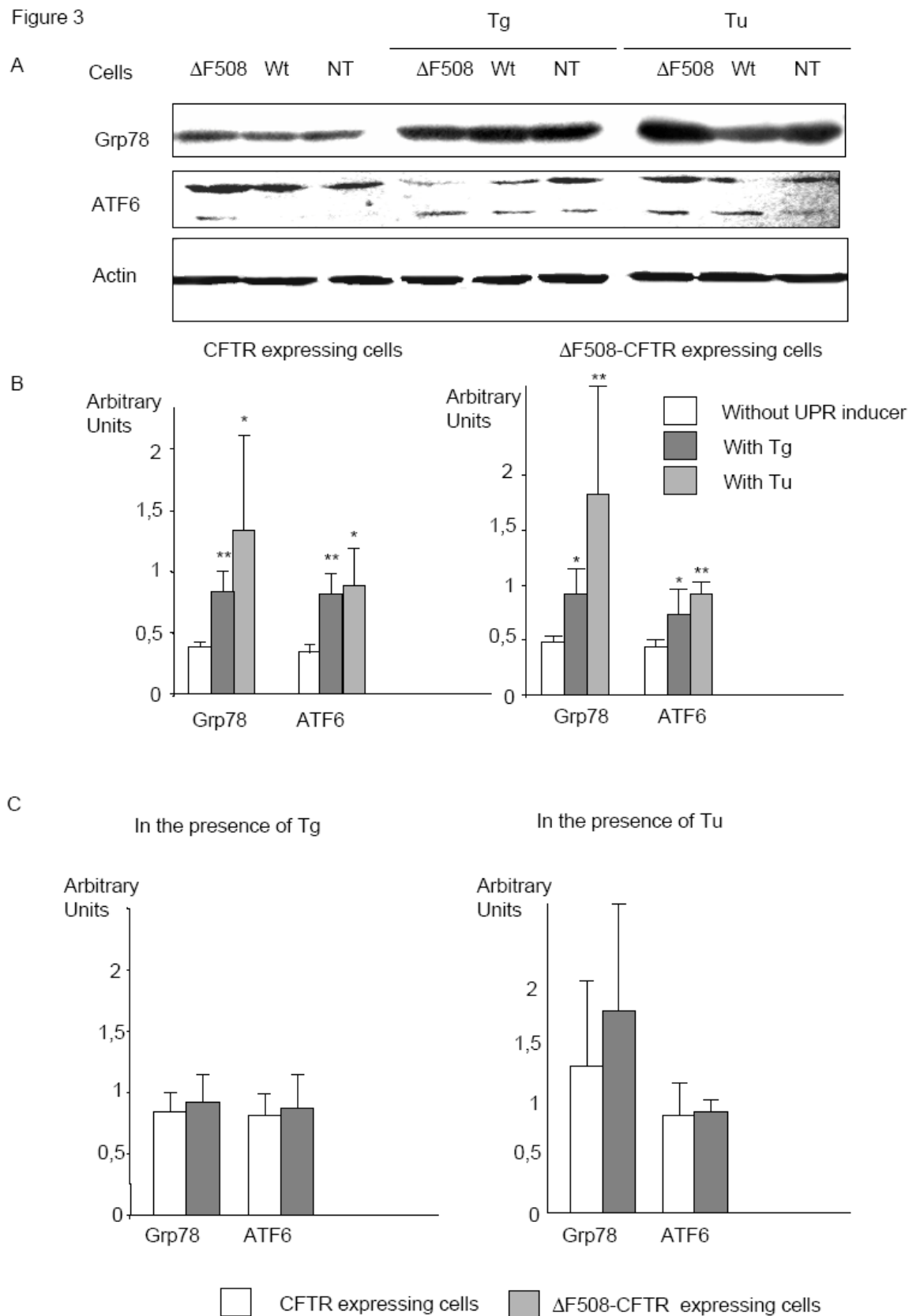


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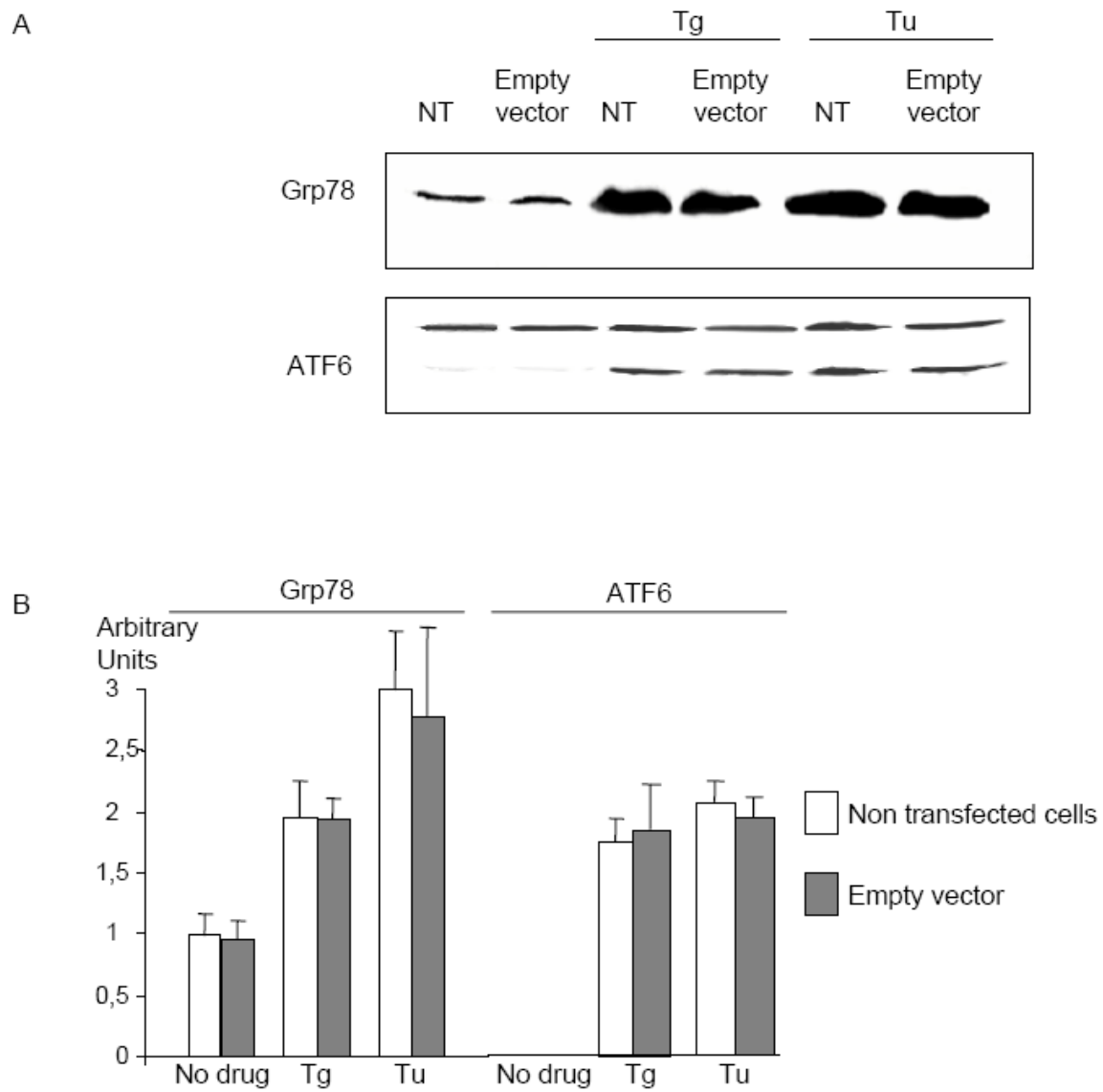


Figure 5

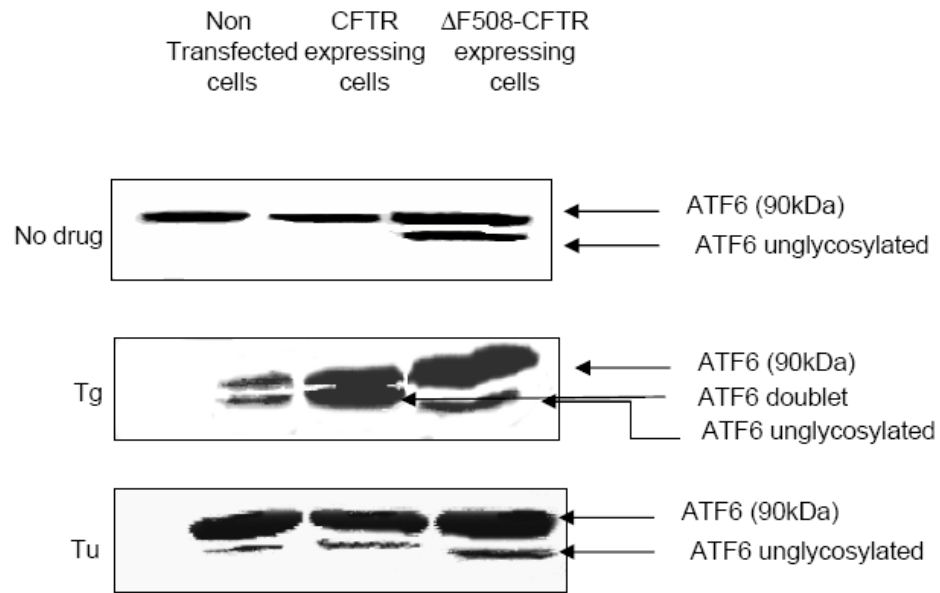


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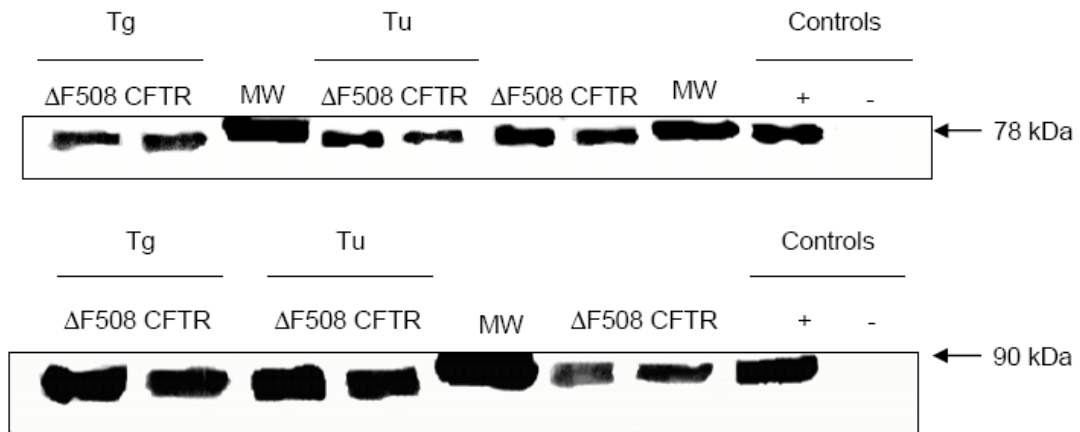


Figure 7

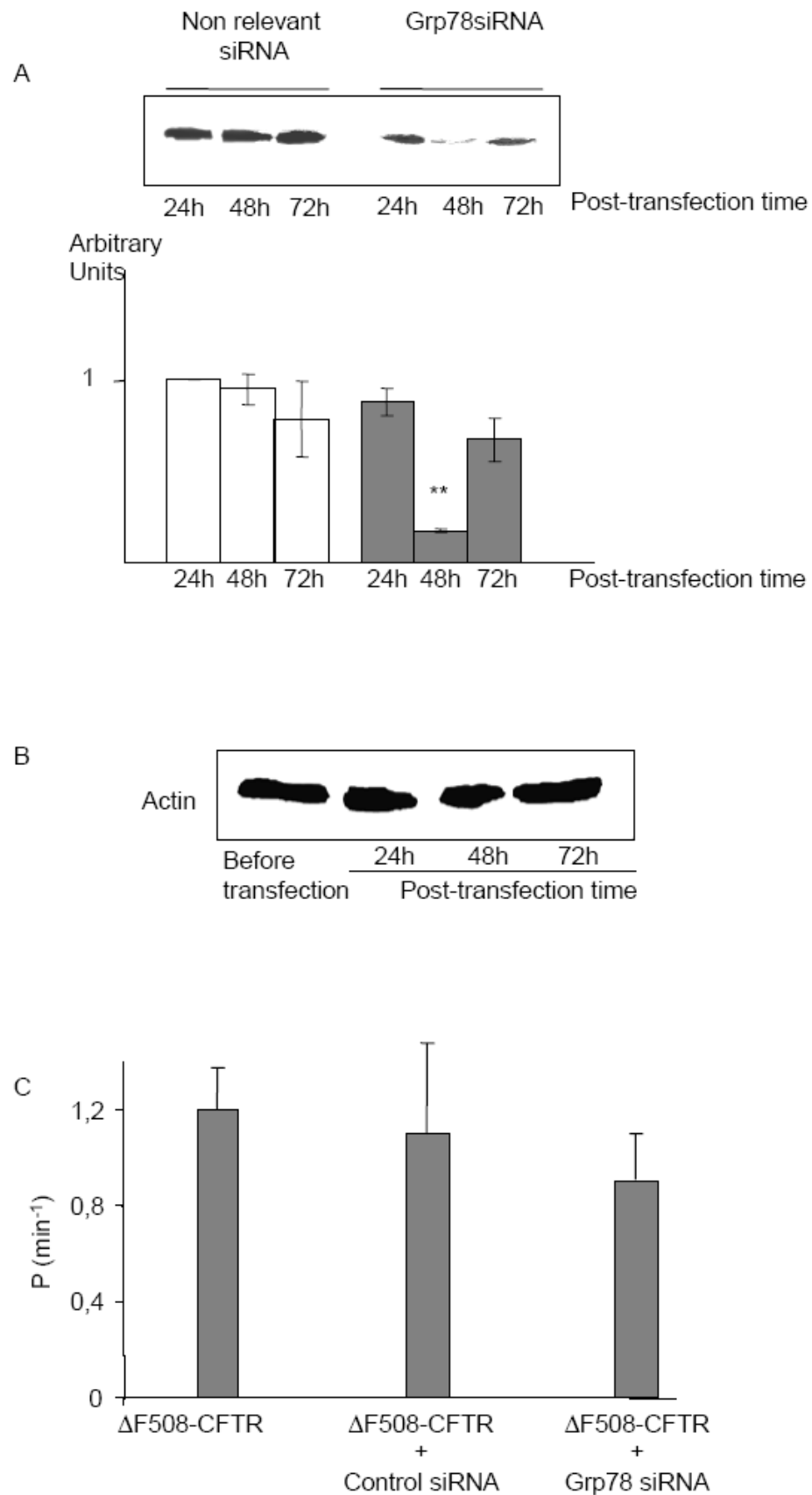
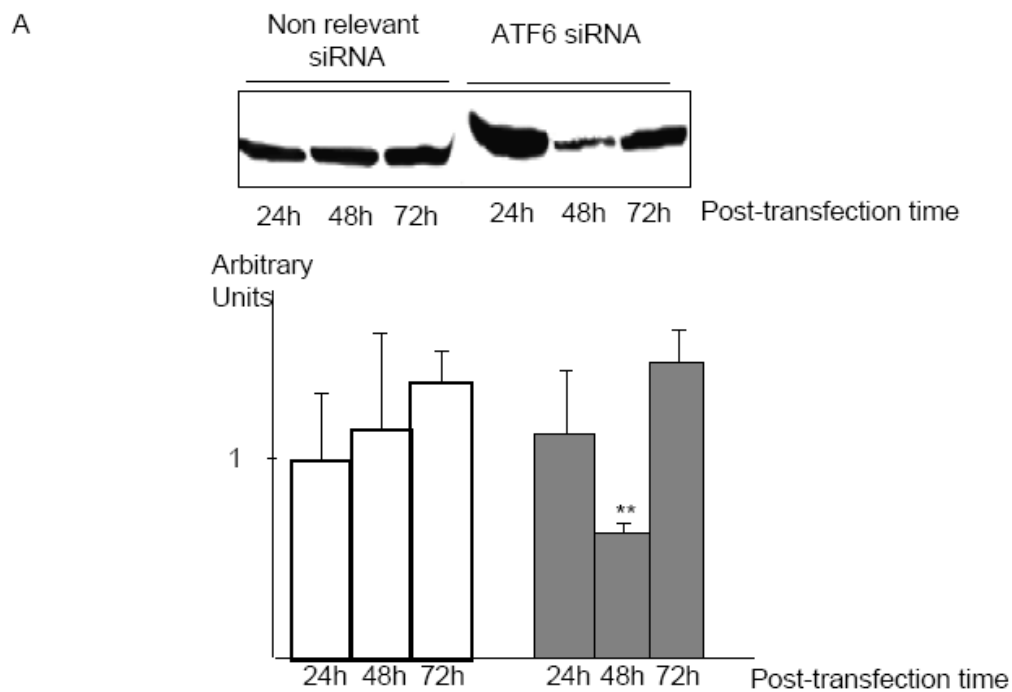
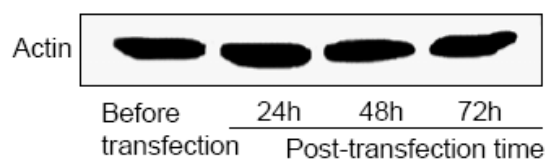


Figure 8 A



B



C

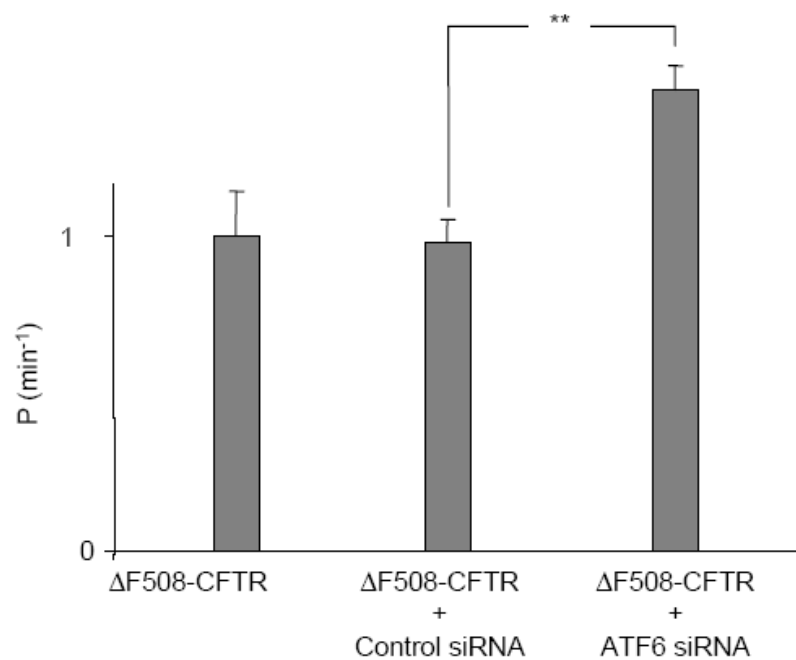


Figure 9

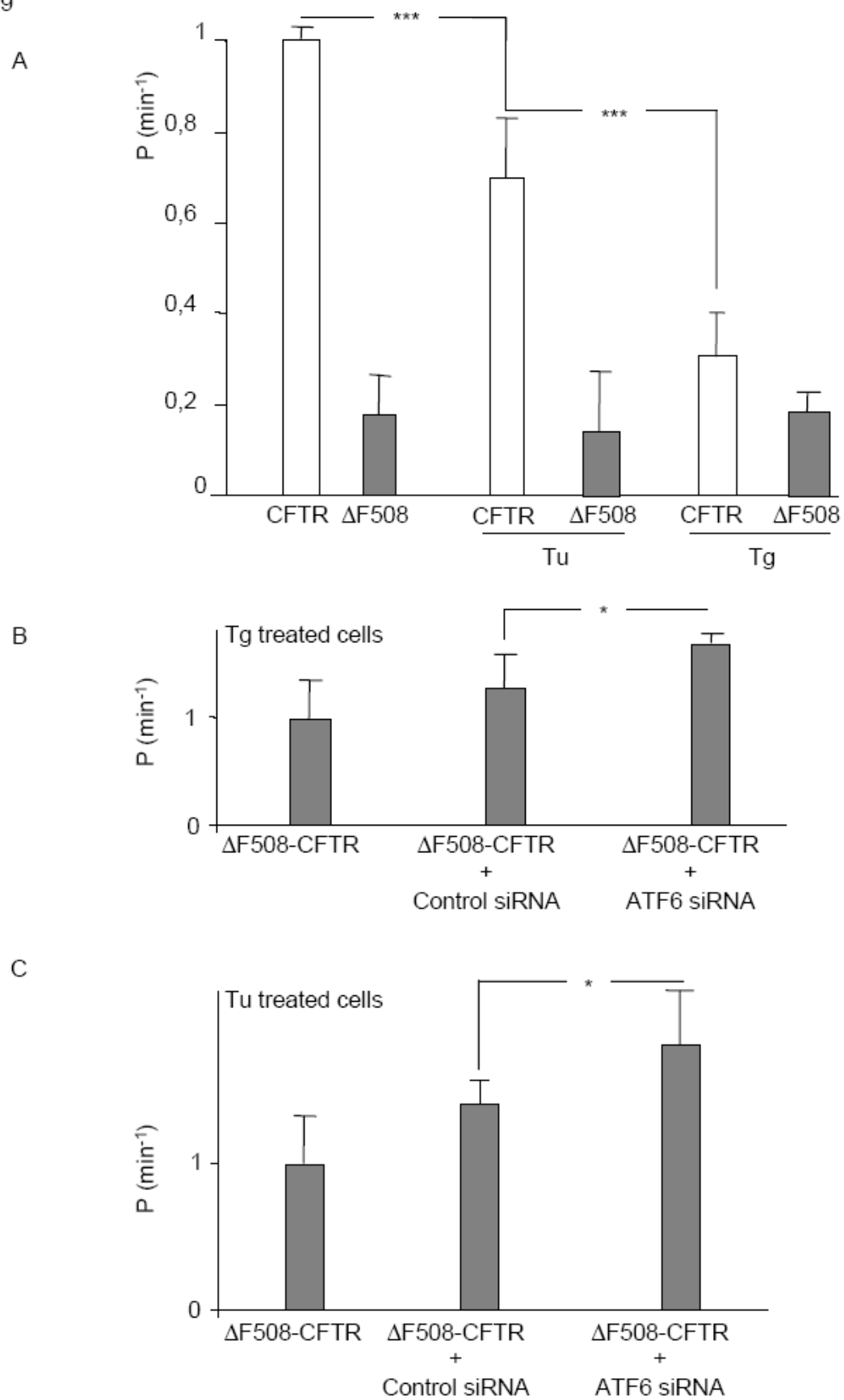


Figure 10

