

Correction of delF508-CFTR activity with benzo(c)quinolizinium compounds through facilitation of its processing in cystic fibrosis airway cells

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SUMMARY

A number of genetic diseases, including cystic fibrosis, have been identified as disorders of protein trafficking associated with retention of mutant protein within the endoplasmic reticulum. In the presence of the benzo(c)quinolizinium drugs, MPB-07 and its congener MPB-91, we show the activation of cystic fibrosis transmembrane conductance regulator (CFTR) delF508 channels in IB3-1 human cells, which express endogenous levels of delF508-CFTR. These drugs were without effect on the Ca²⁺-activated Cl⁻ transport, whereas the swelling-activated Cl⁻ transport was found altered in MPB-treated cells. Immunoprecipitation and *in vitro* phosphorylation shows a 20% increase of the band C form of delF508 after MPB treatment. We then investigated the effect of these drugs on the extent of mislocalisation of delF508-CFTR in native airway cells from cystic fibrosis patients. We first showed that delF508 CFTR was characteristically restricted to an endoplasmic reticulum location in approximately 80% of untreated cells from CF patients

homozygous for the delF508-CFTR mutation. By contrast, 60-70% of cells from non-CF patients showed wild-type CFTR in an apical location. MPB-07 treatment caused dramatic relocation of delF508-CFTR to the apical region such that the majority of delF508/delF508 CF cells showed a similar CFTR location to that of wild-type. MPB-07 had no apparent effect on the distribution of wild-type CFTR, the apical membrane protein CD59 or the ER membrane Ca²⁺,Mg-ATPase. We also showed a similar pharmacological effect in nasal cells freshly isolated from a delF508/G551D CF patient. The results demonstrate selective redirection of a mutant membrane protein using cell-permeant small molecules of the benzo(c)quinolizinium family and provide a major advance towards development of a targeted drug treatment for cystic fibrosis and other disorders of protein trafficking.

Key words: CFTR, delF508, Immunolocalisation, Human airway cells, Pharmacology, Trafficking

INTRODUCTION

Cystic fibrosis is a common fatal autosomal recessive disease, characterised by altered composition of epithelial secretions and caused by mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR) (Riordan et al., 1989). CFTR is normally located in the apical membrane of epithelial cells where it acts as a cyclic AMP-dependent chloride channel (Riordan, 1993) and also regulates protein secretion and plasma membrane recycling (McPherson et al., 1986; Pereira et al., 1998; Bradbury et al., 1992). The most common mutation in the cystic fibrosis gene is a three base pair deletion resulting in loss of a phenylalanine residue at position 508 in the protein, delF508-CFTR (Riordan et al., 1989). Cells transfected with delF508-CFTR show severely reduced Cl⁻ channel activity compared with wild-type (Anderson et al., 1991; Yang et al., 1993a) since the protein

is incorrectly processed and retained within the endoplasmic reticulum (Cheng et al., 1990; Denning et al., 1992a; Thomas et al., 1992; Yang et al., 1993b).

To gain insight into the pharmacology of CFTR chloride channel having disease-causing mutations at NBD1 such as delF508 it is necessary to develop compounds that can restore the defective processing of mutant CFTR. A limited number of molecules have been shown to restore some function in delF508 cells (Schultz et al., 1999), such as the xanthine derivatives IBMX (Becq et al., 1994) and CPX (Srivastava et al., 1999), the benzimidazolone NS004 (Gribkoff et al., 1994) and the isoflavone derivative genistein (Illek et al., 1995). CPX is believed to bind to delF508 protein and to affect the trafficking process of delF508 (Srivastava et al., 1999).

The degree of mislocalisation of delF508-CFTR in native epithelial cells, which also show reduced Cl⁻ channel activity (Kelley et al., 1996), is unclear because reports vary from

different tissues and type of preparation (Kartner et al., 1992; Engelhardt et al., 1992; Denning et al., 1992b; Puchelle et al., 1992; Kälin et al., 1999). We have developed a system to study the location of CFTR in freshly isolated native airway epithelial cells from cystic fibrosis patients, which allows quantification of the percentage of cells with a defined CFTR location. We have determined the degree of mislocalisation of delF508-CFTR and whether the new CFTR-activating benzo(c)quinolizinium compounds (Becq et al., 1999) affect delF508-CFTR activity and trafficking. Moving delF508-CFTR from within the cell to the apical membrane would be a major step forward in the development of a rational cystic fibrosis therapy and would be applicable to other disorders of protein trafficking (Aridor and Balch, 1999).

MATERIALS AND METHODS

Functional study of chloride channel activity in IB3-1 cells

Chloride channel activity was assayed in IB3-1 cells by measuring the rate of iodide (^{125}I) efflux (Becq et al., 1999). IB3-1 cells (delF508/W1282X) (a generous gift of P. Zeitlin) (Zeitlin et al., 1991) were routinely cultured in 5% CO_2 incubators in LHC-8 medium (Biofluids Inc., Rockville, MO) supplemented with 10% fetal bovine serum and 100 U/ml penicillin/streptomycin. Cells grown in 24-well plates were washed with efflux buffer containing 137 mM NaCl, 4.4 mM KCl, 0.3 mM KH_2PO_4 , 0.3 mM NaH_2PO_4 , 4.2 mM NaHCO_3 , 1.3 mM CaCl_2 , 0.5 mM MgCl_2 , 0.4 mM MgSO_4 , 5.6 mM glucose and 10 mM Hepes pH 7.5, and incubated in efflux medium containing 1 μM KI (1 μCi Na^{125}I /ml, NEN, Boston, MA) for 2 hours at 37°C. Cells were then washed with efflux medium. After 1 minute, the medium was removed and quickly replaced by 300 μl of the same medium. The first four aliquots were used to establish a stable baseline in efflux buffer alone. Efflux medium containing the appropriate drug was used for the remaining aliquots. At the end of the incubation, the medium was recovered and cells solubilized in 1 ml of 1 N NaOH. The radioactivity was determined using a gamma counter (Cobra II, Packard Bell). Curves were constructed by plotting rates of ^{125}I efflux versus time (Becq et al., 1999; Venglarik et al., 1990). All comparisons were based on maximal values for the time-dependent rates (peak rates) excluding the points used to establish the baseline. In Fig. 1, Fig. 2 and Fig. 3 the relative rates (r) correspond to: $r_{\text{peak}}/r_{\text{basal}}$. To stimulate CFTR, a cAMP-cocktail containing forskolin (10 μM), IBMX (500 μM) and cpt-cAMP (500 μM) was added after 4 minutes (t4). In some experiments, MPB-91 (250 μM) was added instead of cAMP cocktail. To stimulate the calcium-dependent chloride transport the calcium ionophore A23187 (1 μM , Sigma) was added to the efflux buffer. To stimulate the volume-sensitive chloride transport, the osmolarity of the efflux buffer was reduced from 300 mosm.l $^{-1}$ to 150 mosm.l $^{-1}$. Data are presented as the mean \pm s.e.m. of n separate experiments. Differences were considered statistically significant using the Student's t -test and the Anova test when the P value was <0.05 . All other chemicals were from Sigma.

Immunoprecipitation and phosphorylation of CFTR

IB3-1 cells were lysed in ice-cold RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 1% Triton X-100, 0.1% SDS and 1% Na deoxycholate) supplemented just before lysis with a protease inhibitor cocktail (0.2 mg/ml benzamide, 1 $\mu\text{g}/\text{ml}$ pepstatin A, 0.1 mM phenylmethylsulfonyl fluoride, 20 $\mu\text{g}/\text{ml}$ aprotinin, 20 $\mu\text{g}/\text{ml}$ leupeptin) for 30 minutes on ice. Non-dissolved material was pelleted by centrifugation at 10,000 g for 15 minutes. Cleared lysates, adjusted to 1 mg/ml of total protein, were incubated with 40 μl of protein A Sepharose beads (10% w/v, Amersham Pharmacia Biotech) for 30 minutes under gentle shaking and

centrifuged at 15,000 g . Then, the supernatants were reacted with CFTR C-terminal monoclonal antibody (Genzyme) for 90 minutes at 4°C, followed by addition of protein A Sepharose beads for 60 minutes. Immunoprecipitated proteins were washed and resuspended in phosphorylation buffer (50 mM Tris-HCl, 0.1 mg/ml BSA, 10 mM MgCl_2 , pH 7.5). CFTR attached to the beads was phosphorylated in vitro (60 minutes at 32°C) using 2 units of the catalytic subunit of PKA (Sigma) and 10 μCi [γ - ^{32}P] ATP (3000 Ci/mmol, Amersham Pharmacia Biotech). After several washes in ice-cold RIPA buffer, proteins were solubilized in 2 \times concentrated Laemmli sample buffer for 30 minutes at 25°C with frequent vortexing. Samples were then subjected to SDS-PAGE on a 7% separating gel and, after drying, proteins were visualized by autoradiography. The incorporation of ^{32}P into CFTR was quantified using a phosphor imager (Cyclone, Packard).

CFTR antibody

An antibody against a peptide consisting of the 23 C-terminal amino acids of CFTR (MPCT-1) was raised as previously described (Lloyd Mills et al., 1992; Pereira et al., 1998) and affinity-purified using peptide coupled to CH-Sepharose 4B (Pharmacia), with elution of antibody fractions in 0.1 M glycine-HCl and immediate neutralisation. The antibody has been previously shown to crossreact with CFTR in transfected but not mock transfected CHO cells (Pereira et al., 1998) and to recognise CFTR in native submandibular and pancreatic tissues (Lloyd Mills et al., 1992).

Incubation and fixation of nasal epithelial cells

Airway epithelial cells obtained by nasal brushing were from five non-CF individuals, four delF508/delF508 CF individuals and one delF508/G551D CF individual undergoing flexible bronchoscopy under sedation. Two brushing samples were obtained from the non-anaesthetised nostril by gently brushing a standard 0.5 cm cytology brush along the inferior turbinate. The study was approved by the local ethics committee of Bro Taf Health Authority. The brushes were placed immediately into DMEM/F12 medium and incubated in the presence or absence of MPB-07 or MPB-91 at 37°C, as described in the text. After incubation, the brushes were removed from the medium and cells smeared gently onto 'Snowcoat x-tra' microslides (Surgipath). The samples were left to air-dry, fixed for 5 minutes at -20°C in 5% acetic acid in ethanol, washed in PBS and stored at -20°C. The samples were treated with appropriate block (normal goat or rabbit serum; 1:20) for 30 minutes at room temperature.

Culture and fixation of nasal epithelial cells

Nasal polyps were obtained immediately following polypectomy from non-CF or delF508/delF508 CF individuals. Epithelial cells were digested from the surface with protease XIV (Sigma) for 1-2 hours at 37°C and cultured on glass coverslips in DMEM/F12 medium (GIBCOBRL) for 6-8 days before incubation in the presence or absence of MPB-07 at 37°C, as described in the text. Following incubation cells were washed twice with phosphate buffered saline (PBS) and fixed for 30 minutes in 4% paraformaldehyde in PBS. Cells were permeabilised with 0.2% Triton X-100 for 20 minutes and blocked by incubation first with 50 mM glycine for 30 minutes and then with 10% normal goat serum for 1 hour.

Immunofluorescence localisation

Anti-CFTR antibody (MPCT-1; 1:100), anti-cytokeratin (clone AE1/AE3; 1:50, from Sigma), and anti-CD59 (BRIC229; 1:100, from National Blood Service, Bristol, UK) were used as primary antibodies. The fixed cells were incubated with primary antibody overnight at 4°C, followed by three washes and then incubation with the appropriate secondary antibody (FITC- or Cy3-conjugated; 1:100) for 30 minutes at room temperature. For ER localisation, fixed cells were incubated for 2 hours at room temperature with 2 μM BODIPY-thapsigargin (Molecular Probes). Some cells from nasal brushings

were stained with the red nuclear stain propidium iodide (Sigma) for 5 minutes at room temperature. Slides were then mounted in Fluorosave Reagent (Calbiochem, La Jolla, CA). Fluorescence was detected using confocal laser scanning microscopy on a Leitz DMRBE microscope (Leica, Germany) fitted with a TCS4D scanner (Leica, Germany). Confocal images were collected at a magnification of $\times 100$ under oil immersion and displayed as Kalman averages on a screen 512 \times 512 pixels; 72 dpi. Images were processed using Scanware software (Leica, Germany) and image manipulation was performed using Adobe PhotoShop.

RESULTS

Effect of MPB compounds on Cl⁻ channel activity in IB3-1 cells

We investigated the effect of the novel CFTR channel activators benzoquinolizinium compounds MPB-07 (Becq et al., 1999) and its congener MPB-91 on Cl⁻ transport activity in the delF508-CFTR expressing IB3-1 cell line (Zeitlin et al., 1991). The cells express endogenous levels of delF508-CFTR and have been shown previously to be a good model for demonstrating the effects of chemical chaperones on delF508-CFTR trafficking (Rubenstein et al., 1997). As previously described (Rubenstein et al., 1997), untreated IB3-1 cells showed no stimulation of chloride transport following administration of a cyclic AMP stimulating cocktail (Fig. 1). However, when treated at 37°C for 2 hours with MPB compounds (250 μ M) during radiotracer loading, the IB3-1 cells showed a significant increase in chloride transport in response to a cyclic AMP-stimulating cocktail (Fig. 1A,B). Thus cells treated with either MPB-07 or MPB-91 gave significant increases (1.81-fold and 1.91-fold, respectively, $n=8$) in the rate of cAMP-stimulated iodide efflux (Fig. 1C). In addition, MPB-91 itself acutely stimulated iodide efflux in MPB-91-treated but not untreated cells IB3-1 cells (Fig. 2). Similar effects were observed using MPB-07 instead of MPB-91 (not shown).

To investigate the specificity of MPB compounds on chloride transport activity, we studied their actions on calcium-dependent and volume-sensitive chloride transport in IB3-1 cells (Fig. 3A,B). MPB-91 treatment (250 μ M for 2 hours at 37°C) did not alter the calcium-dependent chloride transport stimulated with 1 μ M of the calcium ionophore A23187 (relative rates to control: A23187 no MPB: 2.10 ± 0.16 , $n=4$ vs after treatment: 2.07 ± 0.15 , $n=4$) (Fig. 3A,C). By contrast, a volume-sensitive chloride transport that was stimulated following a hypo-osmotic challenge (relative rates to control 8.56 ± 0.89 , $n=4$) was significantly inhibited in treated cells (relative rates to control 5.83 ± 0.36 , $n=4$) (Fig. 3B,C). These data support an interaction between CFTR and the volume-sensitive chloride transport as suggested by Vennekens et al. (Vennekens et al., 1999).

Immunoprecipitation and in vitro phosphorylation of CFTR after two hours treatment with MPB-91 is shown Fig. 4. The low molecular weight (band B) is detected (Fig. 4, lanes 4,5). When IB3-1 cells were pretreated with 250 μ M MPB-91 a mature, fully processed form of CFTR was detected (Fig. 4, lane 5) at a molecular weight similar to that seen in Calu-3 cells expressing wt-CFTR (Fig. 4, lane 2). Densitometric analysis of CFTR after in vitro phosphorylation is shown Fig. 4B. The percentages of band C and B were compared in the presence

or absence of MPB-91. The band C form increased from 2.9% to 22% after MPB-91 treatment, whereas the band B form decreased from 97% in non-treated cells to 78% in MPB-91 treated cells.

To further investigate the mechanism by which MPB compounds restored CFTR Cl⁻ channel activity in delF508-CFTR expressing cells we examined whether the cellular location of delF508-CFTR in native cells from CF patients was affected by MPB compounds.

CFTR immunolocalisation in cells obtained by nasal brushing of non-cystic fibrosis and cystic fibrosis individuals

We investigated the location of wild-type and delF508-CFTR in freshly isolated nasal cells obtained from non-CF and

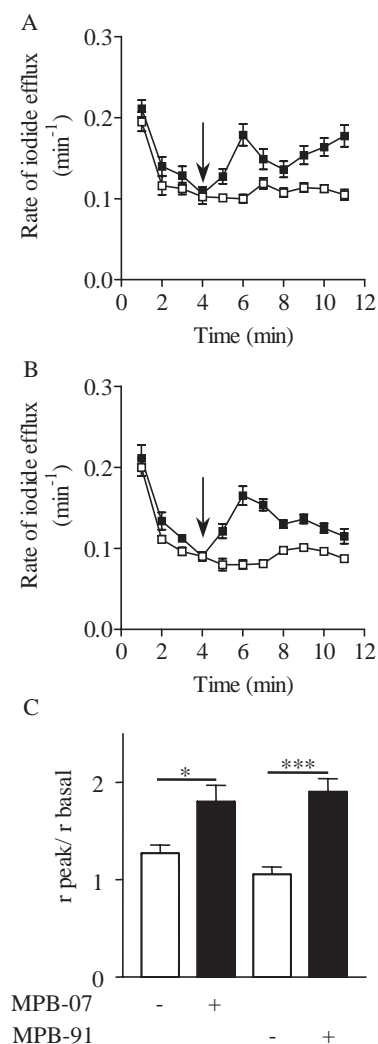


Fig. 1. Activation of CFTR-mediated iodide efflux by cAMP agonists in IB3-1 cells. Iodide efflux experiments were carried out following a 2 hour incubation at 37°C with 250 μ M MPB compound (closed symbols) or not (open symbols). (A,B) A cAMP elevating cocktail (Cock: 10 μ M forskolin, 500 μ M cpt-cAMP, 500 μ M IBMX) stimulated iodide efflux after MPB-07 (A) or MPB-91 (B) treatment ($n=8$ for each). (C) Bar graphs of the cAMP-dependent iodide efflux in IB3-1 cells after MPB treatment (filled bars). * $P < 0.05$; *** $P < 0.001$.

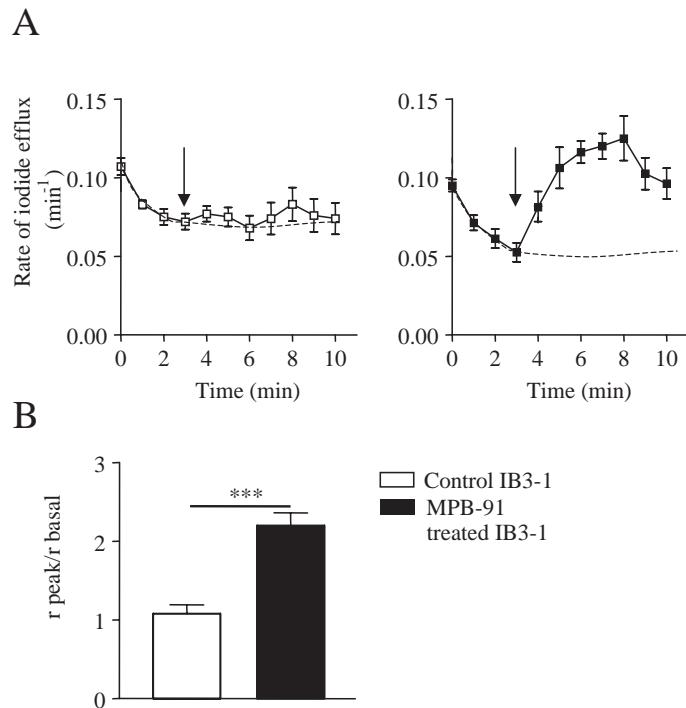


Fig. 2. Activation of CFTR-mediated iodide efflux by MPB-91 in IB3-1 cells. Iodide efflux experiments were carried out following a 2 hour incubation in the loading solution at 37°C with 250 μ M MPB-91 compound (closed symbols) or not (open symbols). (A) MPB-91 (250 μ M) stimulated iodide efflux only in MPB-treated cells ($n=8$ for each). (B) Bar graphs presenting the iodide efflux in IB3-1 cells after MPB treatment (filled bars). *** $P<0.001$.

delF508/delF508 CF individuals. Cells were predominantly ciliated and elongated, having a polarised morphology. Cilia, visible at the apical end of the cell (Fig. 5A), continued to beat for the 2 hour incubation prior to fixation. The nasal cells expressed cytokeratins (Fig. 5B, green), typical of epithelial cells, throughout the cell. Fig. 4B also shows that, in cells from non-cystic fibrosis individuals, wild-type CFTR (red) was located at the apical end of the cell. Wild-type CFTR (Fig. 5C, red) was shown to colocalise with the GPI-anchored apical membrane protein, CD59 (Davies et al., 1989) (Fig. 5D, green), also in dual-labelled cells. Fig. 6 shows a direct comparison between the cellular location of wild-type and delF508-CFTR. In non-cystic fibrosis cells (Fig. 6A), the apical location of wild-type CFTR (green) was readily observed in cells counterstained for nuclei with propidium iodide (red). By contrast, in cystic fibrosis nasal cells, delF508-CFTR was restricted to a region surrounding the nucleus (perinuclear) (Fig. 6C,E,G), or to a discrete region adjacent to the nucleus (juxtannuclear). In a small number (less than 10%) of untreated cystic fibrosis cells, delF508-CFTR was located throughout the cell (Fig. 6I).

Effect of MPB-07 on location of delF508-CFTR

MPB-07 treatment (250 μ M for 2 hours at 37°C) of nasal cells from delF508/delF508 CF patients, resulted in a marked increase in focussing of delF508-CFTR towards the apical region of the cell such that cystic fibrosis cells were indistinguishable from wild-type (compare Fig. 6D,F,H,J

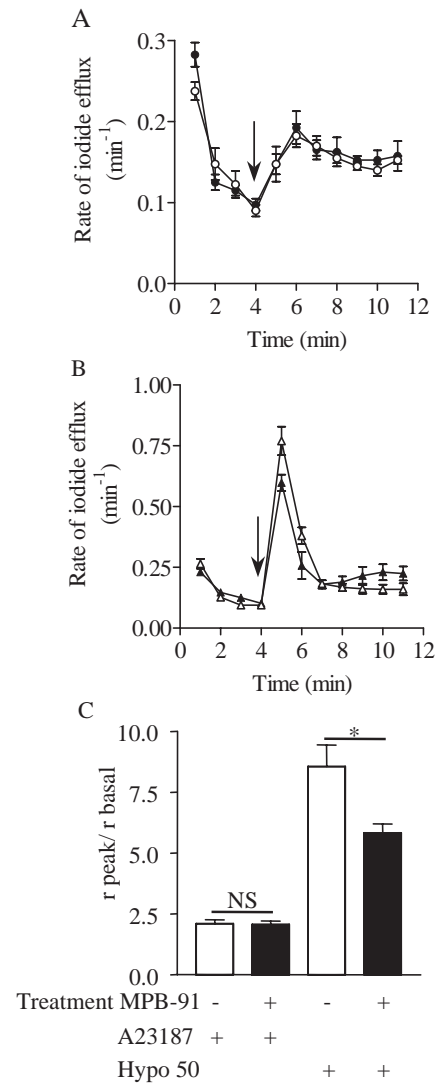


Fig. 3. Effect of MPB-91 on the calcium and swelling activated iodide efflux in IB3-1 cells. (A) Averaged iodide efflux curves showing the lack of effect of MPB-91 treatment on calcium-induced iodide efflux (1 μ M A23187 was used, $n=4$). (B) Demonstration of a small decrease of the volume-sensitive chloride transport observed after MPB-91 treatment (a 50% hypo-osmotic solution was present from the time indicated by the arrow to the end of the experiment, $n=4$). (C) Bar graphs presenting the effects of MPB-91 treatment on calcium and volume-sensitive chloride transport in IB3-1 cells. Arrows indicate the time of drug additions. NS, non-significant difference; * $P<0.05$.

with A). MPB-07 treatment (250 μ M for 2 hours at 37°C) had no effect on the apical location of wild-type CFTR (Fig. 6A,B). Immunofluorescence controls using pre-immune serum or preabsorption of CFTR antibody with C-terminal peptide showed no signal (data not shown). Location of CD59 or of BODIPY-thapsigargin, which binds to the endoplasmic reticulum Ca^{2+} , Mg-ATPase was not altered following treatment with MPB-07 (data not shown). Thus MPB-07 did not change the normal location of wild-type CFTR or of other apical membrane and endoplasmic reticulum proteins.

By counting cells with a defined pattern of delF508-CFTR

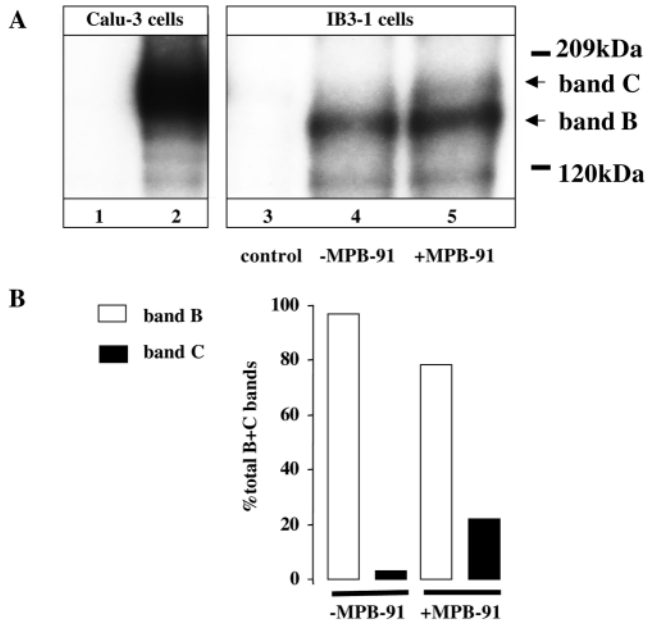


Fig. 4. Immunoprecipitation and in vitro phosphorylation of CFTR in IB3-1 cells. (A) Location of band-B and -C forms are shown together with the positions of molecular weight markers. Immunoprecipitation and in vitro phosphorylation of non-mutated CFTR from Calu-3 cells (lane 2) and delF508 from IB3-1 cells treated for 2 hours with 250 μ M MPB-91 (+MPB-91, lane 5) or without treatment (-MPB-91, lane 4). Lanes 1 and 3 are control experiments without CFTR antibody. (B) Densitometric measurement of CFTR after in vitro phosphorylation. Percentage of fully processed band C and B are shown after a 2 hour treatment of IB3-1 cells with 250 μ M MPB-91 (+MPB-91) or without treatment (-MPB-91).

location, we quantified the effect of MPB-07 in changing delF508-CFTR from a perinuclear to an apical location. Fig. 7A shows that in most wild-type cells CFTR had a distinct apical location (Fig. 5, Fig. 6A,B) with no cells showing a perinuclear CFTR location. In the rest of the wild-type cells CFTR (and also CD59) was present within the cell as well as apically located. The latter category of cells are not included in the histogram (Fig. 7) for clarity. The reason for the widespread distribution of both CFTR and CD59 in a minority of cells is not clear: this may reflect methodology in collecting and analysing native human cells but may equally reflect variation in CFTR distribution in the epithelial cell population. Nevertheless, MPB-07 did not change the pattern of wild-type CFTR location (Fig. 7B), which was very different from that of delF508-CFTR. Thus, in contrast to wild-type cells, the majority of untreated cystic fibrosis cells had a characteristically perinuclear location of delF508-CFTR (Fig. 6C,E,G) with less than 10% showing an apical location (Fig. 7C) and the remainder showing more widespread distribution throughout the cell. In a minority of delF508/delF508 cells, some delF508-CFTR may therefore escape to the apical region; however, the lack of complete uniformity of distribution in all delF508/delF508 cells may also reflect methodology in examining native human cells. Nevertheless, MPB-07 treatment resulted in a significant increase in the number of cells showing a marked focussing of delF508-CFTR towards the apical membrane (Fig. 7D), with a

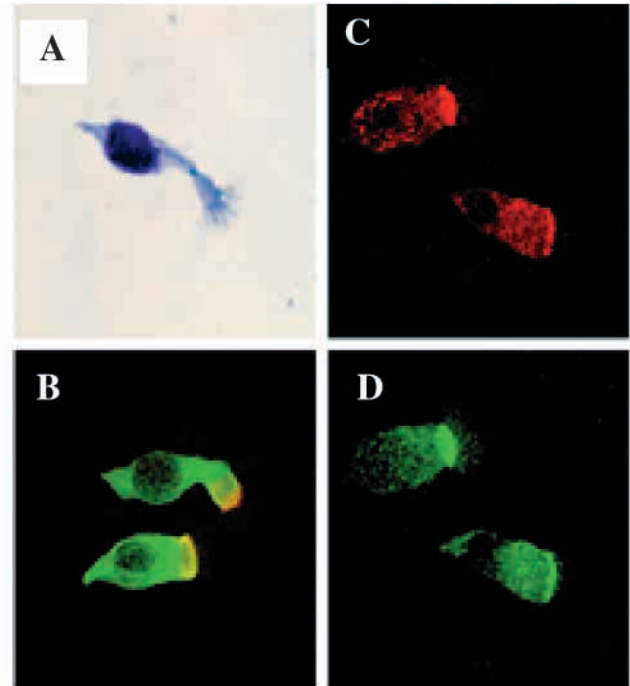


Fig. 5. Characteristics of cells from nasal brushings. (A) Single cell stained with Giemsa/May-Grünwald, showing cilia at apical end. (B) Immunofluorescent dual labelling of cytokeratin (green) and CFTR (red) in cells from a non-cystic fibrosis individual. (C,D) Immunofluorescent dual labelling of CFTR (C) and CD59 (D) showing the same cells, viewed with appropriate filter, from a non-cystic fibrosis individual. Results are representative data from five non-cystic fibrosis individuals. Magnification $\times 1000$.

corresponding decrease in the number of cells showing the perinuclear location characteristic of delF508/delF508 cystic fibrosis cells (Fig. 7D). MPB-07 did not change the percentage of cells showing juxtannuclear location of delF508-CFTR (20-25% with or without MPB-07 treatment) but did increase the number of cells in which delF508-CFTR was distributed throughout the cell (Fig. 6I) from $9.5 \pm 8.8\%$ to $28.1 \pm 5.4\%$ ($P < 0.05$ for $n = 4$ individuals). Thus, MPB-07 changed delF508-CFTR location such that, before treatment, 80% of cells showed a restricted intracellular location that could be distinguished from wild-type, whereas, after treatment, the majority of cystic fibrosis cells (60-70%) showed a more apical CFTR location that could not be distinguished from wild-type.

Effect of longer-term treatment with MPB-07 on cultured surface nasal epithelial cells from delF508/delF508 cystic fibrosis individuals

We showed correction of delF508-CFTR mislocalisation and maintenance of integrity of cells following 18 hour exposure to MPB-07 (500 μ M). As in the freshly isolated nasal brushing cells, some delF508-CFTR was detectable at the apical region in cultured nasal cells (Fig. 8A), but a large amount was present within the cell (Fig. 8C). By contrast, wild-type CFTR colocalised with CD59 at the apical membrane and the distribution was not altered by long term (500 μ M for 18 hours) MPB-07 treatment (data not shown). However, MPB-07

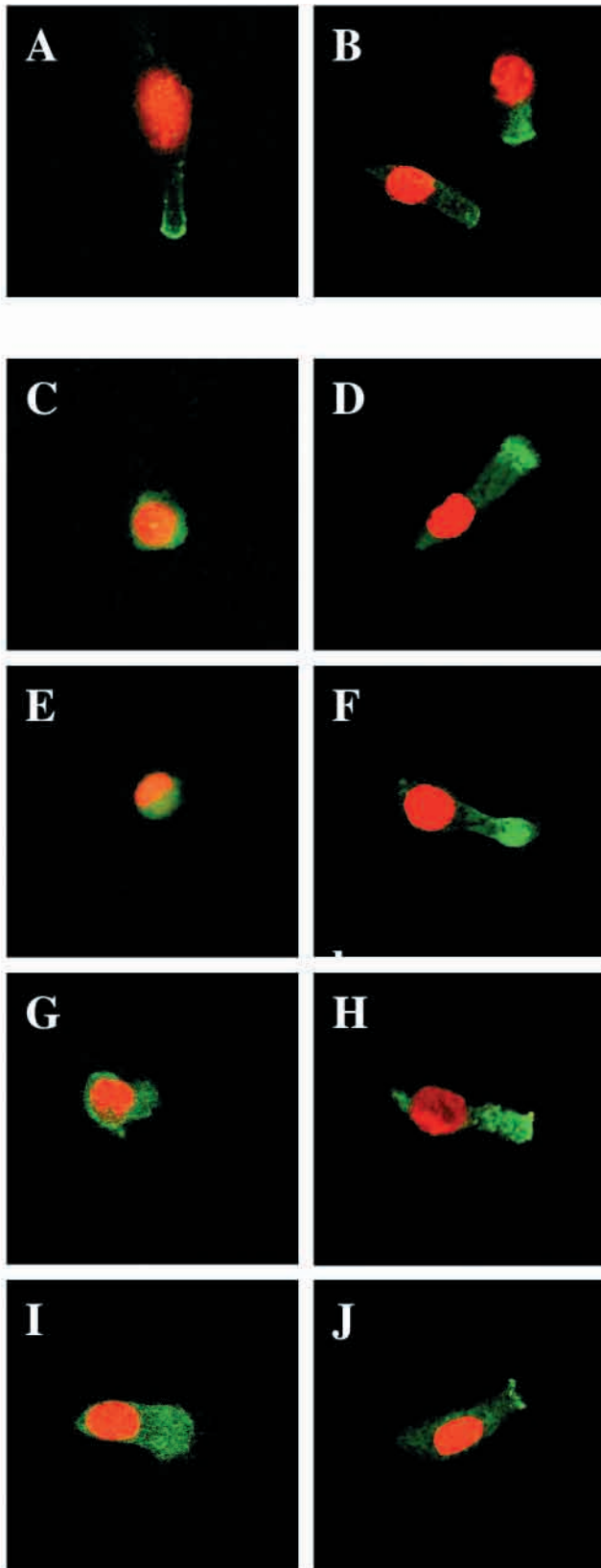


Fig. 6. Effect of MPB-07 on confocal immunofluorescent labelling of CFTR in non-cystic fibrosis and delF508/delF508 cystic fibrosis epithelial cells from nasal brushings. Images show CFTR immunofluorescence in green with the nucleus counterstained with propidium iodide (red). (A,B) Non-cystic fibrosis cells. (C-J) Cystic fibrosis cells. (A,C,E,G,I) Untreated cells. (B,D,F,H,J) Cells treated for 2 hours at 37°C with MPB-07 (250 μ M). Results are representative and show the predominant cell type from five non-cystic fibrosis individuals and from four delF508/delF508 cystic fibrosis individuals with or without MPB-07 treatment with the exception of I. This shows an example of delF508-CFTR present throughout the cell, a pattern of distribution present in less than 10% of untreated cells, although in this cystic fibrosis individual, the predominant cell type had a perinuclear delF508-CFTR location similar to that seen in C,E,G. Magnification \times 1000.

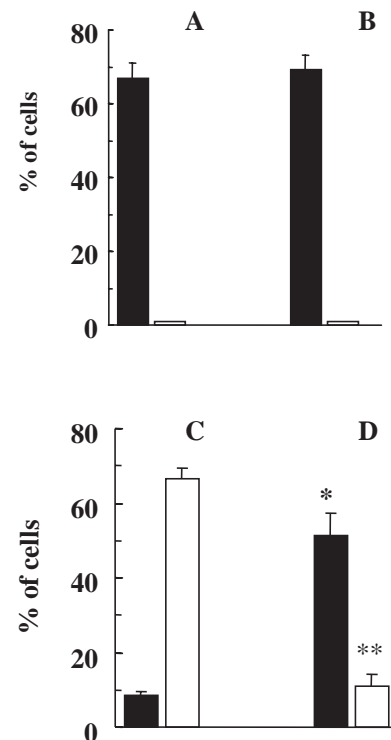


Fig. 7. Quantification of the effect of MPB-07 on location of wild-type and delF508-CFTR in epithelial cells from nasal brushings. Cells were examined (up to 140 cells from each brushing) and categorised as having a distinct CFTR location of either predominantly apical (filled bars) or perinuclear (open bars). A and B show wild-type cells, C and D show delF508/delF508 cells; A and C are untreated, and B and D are MPB-07 treated. Cells showing intermediate distributions that were either a discrete location adjacent to the nucleus, characteristic of delF508 cells (juxtannuclear), or more evenly distributed throughout the cell including the apical region were counted but were not included in the histogram for clarity (see text). The data are mean \pm s.e.m. for cells from five non-cystic fibrosis and four delF508/delF508 cystic fibrosis individuals (>100 cells counted). * P <0.001; ** P <0.0001 for difference from untreated delF508 cells as assessed by Student's t -test.

treatment (500 μ M for 18 hours) of cystic fibrosis cells resulted in a dramatic change in delF508-CFTR distribution (Fig. 8B,D) such that delF508-CFTR was almost undetectable in sections

through the interior of the cell (Fig. 8D) with markedly increased fluorescence intensity in the apical section (Fig. 8B). The XZ scans (Fig. 8E,F) also reflected the increased amount of delF508-CFTR present at the apical surface following MPB-07 treatment.

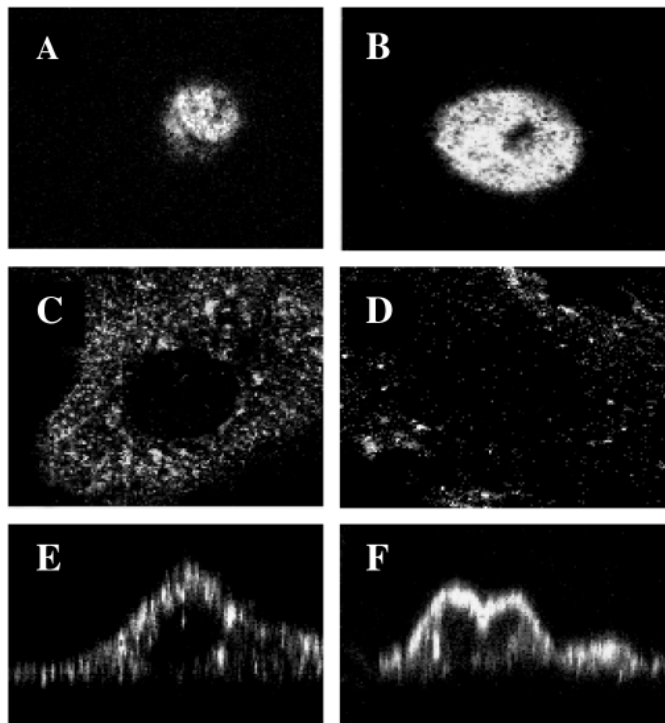


Fig. 8. Confocal immunofluorescent labelling of delF508-CFTR in cultured surface nasal epithelial cells from a delF508/delF508 cystic fibrosis individual. (A,B) XY confocal sections $<1\ \mu\text{m}$ from the apical surface. (C,D) XY confocal sections $7\ \mu\text{m}$ from the apical surface. (E,F) XZ scans. A, C and E are untreated cells. B, D and F are cells treated for 18 hours at 37°C with MPB-07 (500 μM). Results are representative of data from three delF508/delF508 cystic fibrosis individuals. Magnification $\times 2500$.

Effect of MPB compounds on the location of CFTR from delF508/G551D cystic fibrosis individuals

The technique allows us to investigate the effect of MPB compounds on other naturally occurring genotypes and we show CFTR distribution in cells obtained from nasal brushings from a CF individual with a delF508/G551D genotype. Fig. 9A-D shows examples of confocal images that illustrate the range of CFTR distribution. The bar graph shows quantification of the distribution by counting the number of cells with a particular CFTR location. As in delF508/delF508 cells, the majority (80%) of the cells showed CFTR located to a restricted region around the nucleus (perinuclear, Fig. 9A), with a minority of cells showing a juxtannuclear location (Fig. 9B) or more evenly distributed throughout the cell (Fig. 9C). A small percentage ($<10\%$) of cells showed delF508-CFTR at the apical membrane (Fig. 9D). MPB-91 treatment (250 μM , 2 hours at 37°C) resulted in a significant increase in the number of cells showing a focussed apical location (Fig. 9F), with a marked decrease in the number of cells with a perinuclear location. Thus MPB-91 dramatically changed the cellular distribution of mutant CFTR from a perinuclear towards an apical location in nasal cells from a delF508/G551D CF patient.

DISCUSSION

We have shown dramatic and acute effects of the new CFTR-activating benzo(c)quinolizinium compounds in causing the appearance of CFTR Cl^- transport activity in cells expressing delF508-CFTR, which our data suggest is caused by redirecting delF508-CFTR to the apical membrane in native airway cells. Immunoprecipitation and in vitro phosphorylation study shows a sevenfold increase of the band

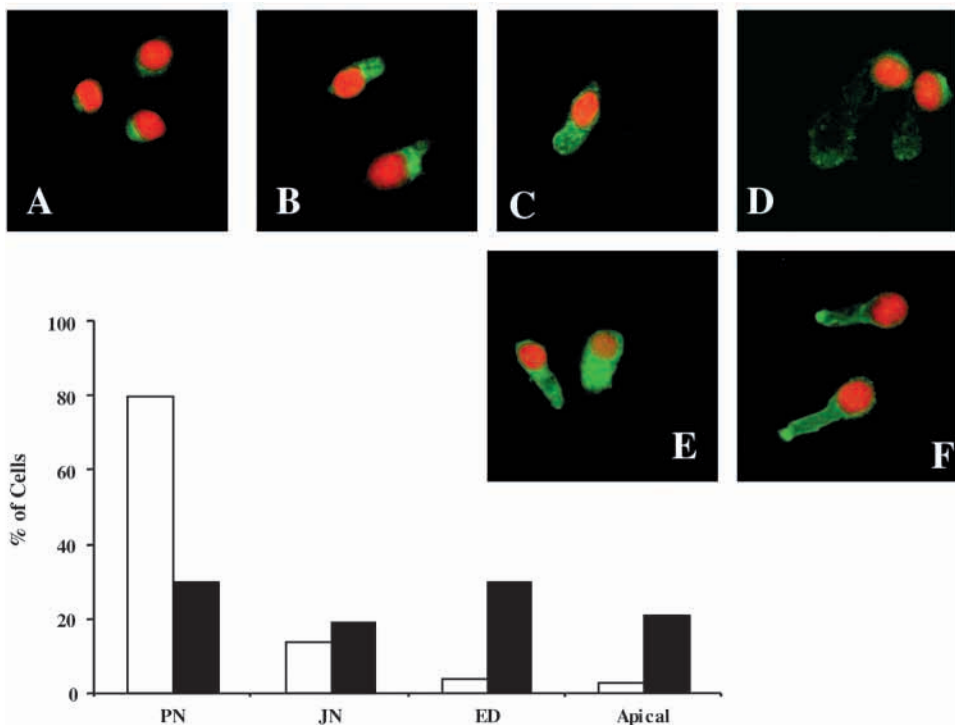


Fig. 9. Effect of MPB-91 on confocal immunofluorescent labelling of CFTR in delF508/G551D cystic fibrosis epithelial cells from nasal brushings. Images show CFTR immunofluorescence in green with the nucleus counterstained with propidium iodide (red). A-D are untreated cells; E and F are cells treated for 2 hours at 37°C with MPB-91 (250 μM). The bar graph shows quantification of the effect of MPB-91 on CFTR location. 80 cells were examined from each condition and categorized as having a distinct CFTR location of either perinuclear (PN), juxtannuclear (JN), more evenly distributed (ED) throughout the cell including the apical region, or predominantly apical (Apical) as described in the text. Open bars are untreated cells; filled bars are cells treated with MPB-91.

C form of delF508 after MPB treatment in IB3-1 cells. This corresponds approximately to one-fifth to one-fourth of total immunoreactive material as fully glycosylated band C. Similar proportions of band C were observed by Rubenstein et al. (Rubenstein et al., 1997) in 4PBA-treated IB3-1 cells or IB3-1 cells incubated at 27°C. The observation that MPB compounds caused only a modest increase in the amount of mature delF508-CFTR (Fig. 4) compared with the marked effect on its relocation in nasal epithelial cells may be reconciled by suggesting: (1) that the magnitude of the effect on delF508 redistribution is more marked in native cells that have not been transformed or cultured; and (2) that a proportion of immature protein escapes to the apical membrane where it can be activated. MPB treatment of delF508-CFTR cells did not affect calcium-activated Cl⁻ channel activity but volume-sensitive anion transport was inhibited when compared with non-treated cells. This result is in good agreement with the observations made on endothelial cells (Vennekens et al., 1999), in which the volume-sensitive anion transport was inhibited following expression of delF508 CFTR. This effect did not require an active CFTR, but only a membrane resident protein (Vennekens et al., 1999). Our data further support a role for CFTR (and mutant forms of CFTR) in the regulation of other membrane transport proteins, including volume-sensitive. These results also show that a pharmacological approach may help not only to restore the defective transport activity of mutant CFTR but also to correct the regulatory function of CFTR.

Other non-selective treatments such as low temperature (25-27°C), glycerol, 4-phenylbutyrate or DMSO have required long-term exposure to show modest effects on delF508-CFTR trafficking in transfected cells or cultured cell lines (Cheng et al., 1990; Denning et al., 1992a; Rubenstein et al., 1997; Sato et al., 1996; Bebök et al., 1998). Here we have shown for the first time a dramatic effect of a pharmacological agent in native cells in which the naturally occurring delF508 CFTR protein is abnormally retained in the endoplasmic reticulum. The action of MPB compounds on delF508-CFTR trafficking and appearance of CFTR Cl⁻ channel activity was selective and readily observed after 2 hours, indicative of an acute effect on release from the endoplasmic reticulum by a direct action on the misfolded protein. Interestingly, correction of defective protein kinesis of human P-glycoprotein mutants in the presence of substrates and modulators such as capsaicin, cyclosporin, vinblastine or verapamil has been demonstrated (Loo and Clarke, 1996). These effects occurred within a few hours (2-4 hours) after the addition of drugs (Loo and Clarke, 1996), a time scale remarkably similar to our study. Both the Loo and Clarke study and the present report may indicate a common mechanism of action for compounds that can modulate the activity of P-glycoprotein and CFTR. Upregulation of CFTR expression was shown using butyrate and its analog sodium 4-phenylbutyrate (Rubenstein et al., 1997). Unexpectedly, a direct inhibitory action of both compounds on wild-type CFTR was recently demonstrated at a single-channel level (Lindsell, 2001) further indicating that some drugs that act on the biosynthesis of CFTR may also be able to directly interfere with the channel transport function. One hypothesis is that within the CFTR structure, occupation of a drug-binding site by MPB (and/or other compounds) may stabilize the conformation of delF508-CFTR protein such that

correction of misfolding occurs. This would allow the complex formed by MPB and the protein to escape the quality control system and reach the apical compartment of cells, where activation of chloride transport by cAMP agonists occurs.

The data indicate that, in the majority of untreated delF508/delF508 cystic fibrosis cells, delF508-CFTR is restricted to the endoplasmic reticulum. The juxtannuclear and more widespread cellular distribution, present in a small percentage of cells (Fig. 6I) and increased by MPB-07 treatment, are likely to represent more distal compartments on the pathway to the apical membrane. This data broadly agrees with other recent studies on nasal epithelial cells from polyps or brushings (Kälin et al., 1999; Penque et al., 2000), which also suggest that at least some delF508-CFTR escapes from the endoplasmic reticulum in native cystic fibrosis cells. However, the demonstration that the majority of delF508-CFTR is mislocalised emphasizes the importance of the present findings that MPB compounds correct delF508-CFTR location. Their application to the majority of CF patients is highlighted by our results in the present report that nasal cells obtained from a compound heterozygote individual (delF508/G551D) have an altered CFTR location similar to delF508/delF508 cells. The data suggest that delF508-CFTR is more strongly expressed than G551D or that its restriction to the endoplasmic reticulum also retains G551D. Incubation of delF508/G551D cells with MPB-91 markedly altered the location of mutant CFTR towards a wild-type distribution, although we cannot distinguish whether the apically located CFTR is delF508 or G551D. Thus, MPB-91 provides a new class of compounds that not only activate G551D-CFTR (R.D., L.B.-P. and F.B., unpublished) but also traffic mutant CFTR to the apical membrane in CF compound heterozygotes with a delF508/G551D mutation.

In conclusion, the present study demonstrates that benzoquinolizinium compounds, which directly activate wild-type (Becq et al., 1999) and G551D-CFTR Cl⁻ channels (R.D., L.B.-P. and F.B., unpublished), have a dramatic effect in restoring defective chloride transport in CF cells by increasing delF508-CFTR at the apical membrane. This is a major step forward in the goal of devising a rational drug treatment aimed at repair or rescue of the activity of the mutant cystic fibrosis gene protein and has implications for other disorders of protein trafficking (Aridor and Balch, 1999).

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