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Chemical chaperones correct the mutant phenotype of the ΔF508 cystic fibrosis transmembrane conductance regulator protein

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Abstract Mutations in the cystic fibrosis transmembrane conductance regulator protein (CFTR) often result in a failure of the protein to be properly processed at the level of the endoplasmic reticulum (ER) and subsequently transported to the plasma membrane. The folding defect associated with the most common CFTR mutation (ΔF508) has been shown to be temperature sensitive. Incubation of cells expressing ΔF508 CFTR at lower growth temperatures results in the proper processing of a portion of the mutant CFTR protein. Under these conditions, the mutant protein can move to the plasma membrane where it functions, similar to the wild-type protein, in mediating chloride transport. We set out to identify other methods, which like temperature treatment, would rescue the folding defect associated with the ΔF508 CFTR mutation. Here we show that treatment of cells expressing the ΔF508 mutant with a number of low molecular weight compounds, all known to stabilize proteins in their native conformation, results in the correct processing of the mutant CFTR protein and its deposition at the plasma membrane. Such compounds included the cellular osmolytes glycerol and trimethylamine N-oxide, as well as deuterated water. Treatment of the ΔF508 CFTR-expressing cells with any one of these compounds, which we now refer to as 'chemical chaperones', restored the ability of the mutant cells to exhibit forskolin-dependent chloride transport, similar to that observed for the cells expressing the wild-type CFTR protein. We suggest that the use of 'chemical chaperones' may prove to be effective for the treatment of cystic fibrosis, as well as other genetic diseases whose underlying basis involves defective protein folding and/or a failure in normal protein trafficking events.

INTRODUCTION

Defects in the processing and/or intracellular transport of the cystic fibrosis transmembrane conductance regulator protein (CFTR) appears to constitute the primary lesion associated with cystic fibrosis (Cheng et al 1990; Gregory et al 1991; Kartner et al 1992). A large number of mutations within the coding region of the gene encoding the CFTR protein have been described (Kerem et al 1989; Riordan et al 1989; Rommens et al 1989). Many of these mutations result in a failure of the newly synthesized protein to move out of the endoplasmic reticulum (ER) to the plasma membrane where it functions as a chloride channel. As a consequence, cells expressing these CFTR mutants are unable to transport chloride in response to increases in intracellular cAMP levels. Even in the case of wild-type CFTR, only 20–25% of the newly synthesized protein appears to exit from the ER and move to the plasma membrane (Cheng et al 1990). The remainder of the protein is retained at the ER and eventually is targeted for degradation, likely via the ubiquitin/proteosome-dependent pathway (Jensen et al 1995; Ward and Kopito 1995). Why only a fraction of the newly synthesized wild-type CFTR exits the ER and moves to the plasma membrane is still not clear.

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Deletion of a phenylalanine residue at position 508 of the CFTR protein (referred to as ΔF508 CFTR) is the most common mutation observed in patients with cystic fibrosis. This deletion is thought to result in the abnormal folding of the CFTR protein and its retention at the ER via the action of molecular chaperones. For example, both the ER-localized molecular chaperone, calnexin, and the cytosolic molecular chaperone, Hsp73, have been shown to interact transiently with the newly synthesized form of wild-type CFTR (Yang et al 1993; Pind et al 1994). The portion of wild-type CFTR that subsequently moves through the Golgi complex, on its way to the plasma membrane, is no longer observed to interact with Hsp73 and calnexin. In the case of the ΔF508 CFTR mutant, the newly synthesized protein is also observed to interact with the Hsp73/calnexin chaperones. In contrast to the situation with the wild-type protein, however, the interaction of mutant CFTR with these two chaperones appears relatively stable. As a consequence, the ΔF508 CFTR mutant is retained at the level of the ER, and over time is then targeted for degradation. Based on these observations, one suspects that at least one rate-limiting step in CFTR maturation is its interaction with, and subsequent release from, one or more members of the molecular chaperone family.

The folding defect associated with ΔF508 CFTR mutant is temperature sensitive (Denning et al 1992). When cells expressing the ΔF508 CFTR mutant are incubated at lower growth temperatures (e.g. 26°C), normal maturation of a portion of the mutant CFTR protein is observed. These cells now exhibit cAMP-dependent chloride transport, similar to that observed for cells expressing the wild-type CFTR protein. Based on this observation, we suspected that folding defect associated with the deletion of phenylalanine at position 508 was a relatively subtle one and, therefore, might be correctable by other methods. Consequently, we examined whether the incubation of cells expressing the ΔF508 CFTR mutant with a variety of low molecular weight compounds, all of which are known to stabilize proteins in their native conformation, might be effective in correcting the folding defect of the mutant CFTR protein. For example, previous studies have shown that agents such as glycerol, dimethylsulfoxide (DMSO) and deuterated water (D_2O), can effectively stabilize proteins against thermal denaturation, both in vitro (Gerlsma and Stuur 1972; Back et al 1979; Gekko and Koga 1983) as well as in vivo (Lin et al 1981; Henle et al 1983; Edington et al 1989). Likewise, methylamines such as trimethylamine N-oxide (TMAO) are known to protect proteins from denaturation by urea and high salt (Somero 1986). We show here that these various compounds, which we now refer to as 'chemical chaperones', when added to cells expressing the ΔF508 CFTR mutant, result in a portion of the mutant protein to be correctly processed at the level of the ER. These cells now appear competent for chloride transport in response to added forskolin.

METHODS

Antibody preparation and characterization

The R domain of the CFTR protein (amino acids 590–830) was expressed in bacteria as a GST fusion protein. The GST-R fusion protein was purified using a GST affinity column, and the purified protein used as an antigen for the preparation of rabbit polyclonal antibodies. The polyclonal antibody obtained was characterized by its ability to immunoblot the GST-R antigen, as well as immunoprecipitate the full-length CFTR protein synthesized in vitro. For comparative purposes, a mouse monoclonal antibody specific for the C terminus of the CFTR protein was purchased from Genzyme.

Cell culture and Western blotting

NIH 3T3 cells stably transfected with either the wild-type or ΔF508 forms of CFTR (Anderson et al 1991) were used to examine the expression and maturation of the CFTR protein. Cells were maintained at 37°C in DMEM supplemented with 10% fetal bovine serum. For glycerol and TMAO treatments, concentrated stock solutions were prepared in DMEM, and the compounds added directly to growth medium of the cells. For D_2O experiments, deuterated water (Sigma) was used to reconstitute powdered DMEM media (Gibco) and the media then added to the cells. Following their treatment with the various chemical chaperones (times and concentrations presented in the Fig. legends), the cells were lysed in Laemmli sample buffer, the lysates heated to 50–60°C for 3–5 min, and the cell lysates applied to a 7.5% polyacrylamide gel. Following separation of the proteins by SDS-PAGE, the proteins were transferred to nitrocellulose and the blots probed with antibodies to the CFTR protein. Within a particular experiment an approximate equal amount of total protein was applied to the gels.

Chloride conductance

Cells were grown on glass coverslips in the presence or absence of glycerol or other chemical chaperones. Approximately 18 h prior to measurements, cells were loaded with the chloride-sensitive fluorophore SPQ (Verkman 1990; Verkman and Biwersi 1995) by addition of 5 mM SPQ to the cell culture medium. The SPQ-loaded cells were then washed in phosphate buffered saline (PBS) and mounted in a 200 ul perfusion chamber in which the cell-free glass surface made contact with the

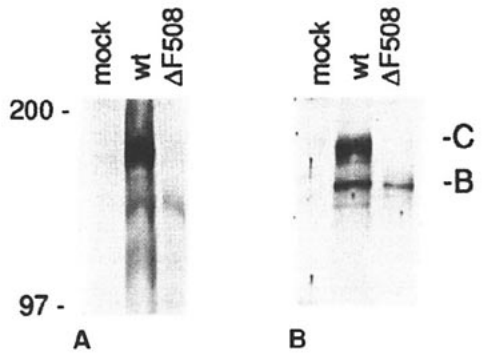

Fig. 1 Characterization of a CFTR-specific polyclonal antibody. A GST fusion protein containing the R domain (amino acids 590–830) of CFTR was expressed in bacteria, the protein purified, and used as an antigen for the production of rabbit polyclonal antibodies. Western blotting using a commercially available CFTR monoclonal antibody (panel A) or our polyclonal antibody (panel B), was performed using cell lysates from NIH 3T3 cells transfected with either the wild-type or ΔF508 forms of CFTR. Both the polyclonal and monoclonal antibodies recognized the 140 kDa (core glycosylated, band B) and 160 kDa (fully glycosylated, band C) forms of CFTR in the lysates from the wild-type CFTR-expressing cells. In contrast, only the 140 kDa core glycosylated ΔF508 CFTR protein was recognized by the two antibodies in the lysates from the cells transfected with the ΔF508 CFTR mutant.

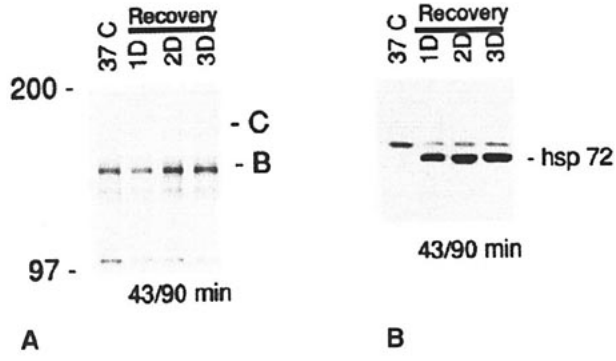

Fig. 2 Failure to correct the processing defect of ΔF508 CFTR by heat shock treatment. 3T3 cells expressing the ΔF508 CFTR protein (via stable transfection) were subjected to a 43°C/90-min heat shock treatment and then returned to 37°C for 12 h. One plate of cells was harvested, while to the remaining dishes the heat shock treatment was repeated on day 2 and day 3. Cells harvested on days 1 (1D), 2 (2D), and 3 (3D) were examined for their content of both the ΔF508 CFTR protein (panel A) and the heat-inducible Hsp72 stress protein (panel B) via Western blotting. In the first lane of each panel are cells which were maintained at 37°C (note that the slower migrating band detected by the Hsp72 antibody in panel B represents the related Hsp73 protein). Molecular mass markers are indicated on the left of panel A, while the positions of the immature (B) and mature forms (C) of the CFTR protein are indicated on the right of panel A.

immersion objective (Leitz, 40X magnification, glycerol emersion, quartz, numerical aperture 0.65). The cAMP-dependent chloride conductance was stimulated in cells by the addition of 10 μM forskolin in a chloride-free buffer containing nitrate. Nitrate, which does not quench SPQ fluorescence, can also be transported via the CFTR channel and exchanges with cellular Cl^-, resulting in an increased fluorescence intensity. SPQ fluorescence was excited at 365 nm and detected by a photomultiplier using a 410-nm dichroic mirror and 420-nm barrier filter in a Nikon inverted epifluorescence microscope. SPQ fluorescence was quenched by the addition of 150 mM KSCN.

RESULTS

A rabbit polyclonal antibody specific for the R domain of the CFTR protein was prepared and used to examine CFTR maturation. Previous work has identified three major forms of the CFTR protein:

- unglycosylated CFTR (band A)
- core glycosylated CFTR which is sensitive to endoglycosidase H and, therefore, most likely represents that form of CFTR present at the ER (band B)
- fully glycosylated CFTR which is endoglycosidase H-resistant and, therefore, represents the mature form of CFTR (band C).

As shown in Figure 1, both a commercially available CFTR monoclonal antibody (panel A) and our rabbit polyclonal CFTR antibody (panel B), recognized both the core glycosylated (band B) and fully glycosylated forms (band C) of wild-type CFTR expressed in NIH 3T3 cells. In the NIH 3T3 cells transfected with the ΔF508 CFTR mutant, only the core glycosylated, immature form of the CFTR protein was observed. The relatively low levels of the immature form of the ΔF508 CFTR protein (i.e. band B) are due to the protein being targeted for degradation very soon after its synthesis.

Previous studies have shown that newly synthesized wild-type CFTR is inserted into the membrane of the endoplasmic reticulum, with a small portion of the protein facing the lumen of the ER, and the vast remainder of the molecule present within the cytoplasm. As was mentioned earlier, at least one rate-limiting step in the maturation of the newly synthesized wild-type CFTR protein is its release from two molecular chaperones, calnexin within the ER and Hsp73 within the cytoplasm (Yang et al 1993; Pind et al 1994). In the case of the ΔF508 CFTR mutant, release of the newly synthesized protein from its Hsp73 chaperone is not observed. Thus, we reasoned that if we could somehow elicit the release of the Hsp73 chaperone from the newly synthesized CFTR mutant, we might induce a portion of the protein to undergo further maturation. Owing to previous studies demonstrating the rapid redistribution of the Hsp73

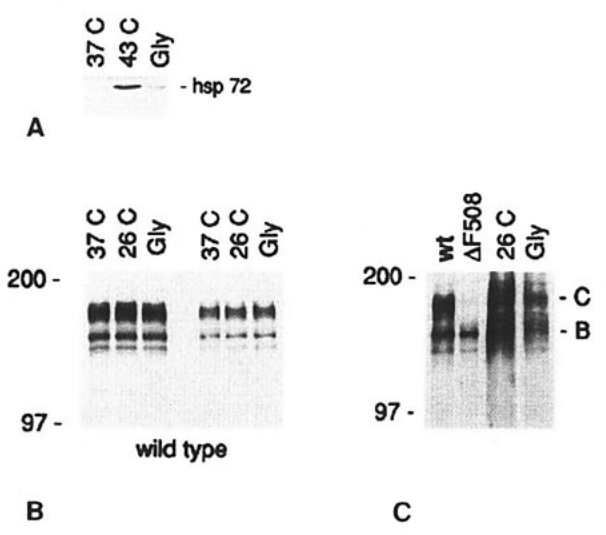

Fig. 3 Inclusion of glycerol into the culture medium influences the processing and maturation of the ΔF508 CFTR protein. **Panel A** – To determine an optimal concentration of glycerol which protects cells against heat shock treatment, NIH 3T3 cells were incubated in the absence or presence of 1 M glycerol. Cells in either the absence or presence of glycerol were subjected to a 43°C/90-min heat shock treatment, and then returned to 37°C for 18 h to allow for the accumulation of Hsp72. Following harvesting of the cells, the relative amounts of Hsp72 were determined by Western blotting using a monoclonal antibody specific for Hsp72. Shown are the results from the 37°C cells (37°C), or from the cells heat shock treated in the absence (43°C) or presence (Gly) of 1M glycerol. **Panel B** – NIH 3T3 cells expressing wild-type CFTR were grown at 37°C, 26°C, or 37°C in the presence of 1 M glycerol (Gly) for 3 days. The cells were harvested and the amounts of immature (band B) and mature (band C) CFTR examined by Western blotting using the rabbit polyclonal anti-CFTR antibody. Two different amounts of the cell lysates were examined to insure that any differences in the amount of the CFTR protein were detected. **Panel C** – Cells expressing the ΔF508 CFTR mutant were incubated at 37°C (ΔF508), 26°C (26°C), or 37°C in the presence of 1 M glycerol (Gly). Three days later the cells were harvested and the amounts of the immature (band B) and mature (band C) forms of the ΔF508 CFTR protein examined by Western blotting. Approximately equal amounts of total protein were applied to the gels. Cells expressing wild-type CFTR (wt) were included in the analysis as a positive control.

chaperone from the cytoplasm into the nucleus in cells subjected to heat shock treatment, cells expressing the ΔF508 CFTR were subjected to a heat shock treatment and then analyzed for their distribution of the ΔF508 CFTR protein by Western blotting (Fig. 2, panel A). Neither a single heat shock treatment, nor multiple heat shock treatments, administered to the cells over a 3-day period were effective in rescuing the maturation of the ΔF508 CFTR protein.

As an alternative approach to effect the proper maturation of the mutant CFTR protein, we turned our attention to the use of chemicals previously shown to stabilize proteins in their native conformation. For example, glycerol as well as a number of other low molecular weight polyols have been shown to protect proteins from thermal denaturation in vitro (Gekko and Timasheff 1981a, 1981b). Similarly, prior addition of glycerol or D_2O to cells in culture has been shown to be effective in reducing the extent of thermal injury, likely via the stabilization of intracellular proteins from thermally induced aggregation (Mclver et al 1980; Lin et al 1981; Henle et al 1983). Consistent with this latter suggestion is the fact that the amount of heat shock protein expression, an indicator of the extent of thermal injury, is reduced significantly in those cells first treated with glycerol and then subjected to heat shock treatments (Edington et al 1989). This is illustrated in Figure 3A where NIH 3T3 cells were incubated in either the absence or presence of 1 M glycerol, and the cells then subjected to a 43°C heat shock treatment for 90 min. The cells then were returned to 37°C to allow for the expression and accumulation of the heat shock proteins. The cells were harvested and analyzed via Western blotting using a mouse monoclonal antibody (C92) specific for the heat-inducible form of Hsp70, Hsp72 (Fig. 3A). In line with our previous work, NIH 3T3 cells, like most cells of rodent origin, do not express any appreciable amounts of Hsp72 when maintained at 37°C. In contrast, cells subjected to heat shock treatment accumulated significant amounts of Hsp72. Note, however, that the relative accumulation of Hsp72 was markedly reduced in those cells first treated with 1 M glycerol and then provided the heat shock treatment.

The effects of 1 M glycerol treatment were analyzed in the NIH 3T3 cells expressing either the wild-type or ΔF508 forms of CFTR. As a positive control in these experiments, cells expressing the ΔF508 CFTR mutant protein were incubated at 26°C in order to allow for the correct processing of the mutant protein. Incubation of the cells expressing wild-type CFTR at 26°C, or in the presence of 1 M glycerol for a period of 3 days, had no effect on the absolute amounts of mature CFTR protein accumulation (Fig. 3B). As was reported previously, incubation of the cells transfected with the ΔF508 CFTR mutant at 26°C for 3 days resulted in the appearance of the fully glycosylated, mature form of CFTR (Fig. 3C). Similar to the effects of low temperature treatment, incubation of the ΔF508 CFTR-expressing cells in the presence of glycerol appeared to rescue the processing defect associated with the ΔF508 CFTR mutant. For example, when the ΔF508-expressing cells were incubated at 37°C in the presence of 1 M glycerol for a period of 3 days, a significant amount of the mature ΔF508 CFTR protein was observed (Fig. 3C).

Both the optimal concentration of glycerol, as well as the duration of glycerol treatment needed to elicit the highest levels of mature ΔF508 CFTR were examined

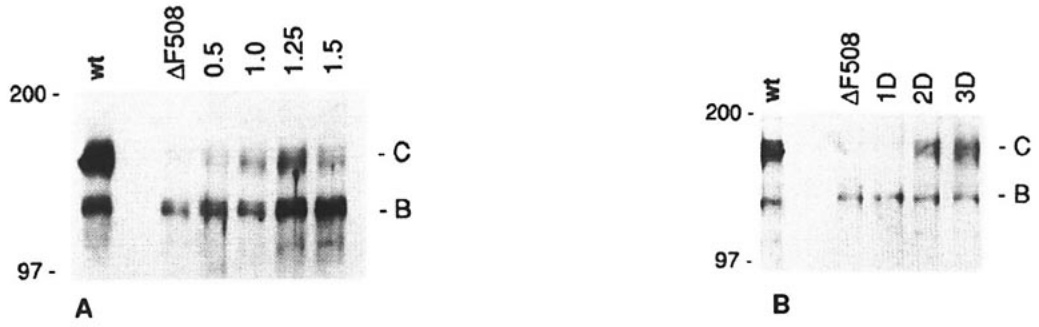

Fig. 4 The positive effect of glycerol on the processing of the ΔF508 CFTR protein is concentration- and time-dependent. **Panel A** – Cells expressing ΔF508 CFTR were incubated in the absence (ΔF508) or presence of varying concentrations (0.5, 1.0, 1.25 or 1.5 M) of glycerol. After 3 days of growth at 37ºC the cells were harvested and analyzed for their content of immature (band B) and mature (band C) forms of the CFTR protein by Western blotting using the rabbit polyclonal anti-CFTR antibody. Cells expressing wild-type CFTR (wt) were included as a positive control. **Panel B** – Cells expressing ΔF508 CFTR were incubated at 37ºC in the presence of 1.25 M glycerol for 1 (1D), 2 (2D) or 3 (3D) days. As a control, the same cells were incubated for 3 days in normal growth media (ΔF508). The cells were harvested and the amounts of the immature (band B) and mature (band C) forms of ΔF508 CFTR determined by Western blotting. Approximately equal amounts of total protein were applied to the gels in either panel A or panel B.

(Fig. 4). Glycerol concentrations of 1–1.25 M resulted in the maximal amount of the mutant CFTR protein which was properly processed (Fig. 4A). While lower doses resulted in less of the fully glycosylated CFTR protein, higher doses appeared to be somewhat inhibitory, perhaps due to adverse effects on the growth of the cells. Using 1.25 M glycerol, a time-course study was performed. Only after approximately 2 days of treatment could we observe appreciable levels of the mature ΔF508 CFTR protein by Western blotting. After 3 days of glycerol treatment, the cells appeared to have reached a steady state level of the mature ΔF508 CFTR protein (Fig. 4B).

Having shown that we could rescue the defective processing of the mutant CFTR protein, we examined whether the glycerol-treated cells expressing ΔF508 CFTR were competent for chloride transport. For this analysis we utilized the SPQ fluorescence assay, which previously has been shown to be a very sensitive indicator of chloride transport across the plasma membrane (Verkman 1990; Verkman and Biwersi 1995). This assay takes advantage of the fact that chloride quenches SPQ fluorescence, while nitrate, which is also transported by the CFTR Cl^- channels, does not. Therefore, the replacement of cellular Cl^- with nitrate results in an increase in fluorescence intensity. Cells were incubated in the presence of the chloride-sensitive fluorescent indicator, SPQ, for 12–18 h. The culture medium was then removed and the cells washed extensively with, and further incubated in Cl-free medium containing added NO_3^-. Subsequent addition of forskolin to the cells results in an increase in intracellular levels of cAMP and the activation of the CFTR protein Cl^- transporter. At the end of each experiment, the reaction was quenched via the addition of KSCN. Cells expressing the ΔF508 CFTR mutant were incubated in either the absence or presence of 1.25 M glycerol for 1 day, and then were examined for their ability to mediate chloride transport in response to added forskolin. Cells expressing wild-type CFTR, and grown in the absence of glycerol, served as the positive control in these studies. As is shown in Figure 5A, the wild-type cells maintained at 37°C exhibited a rapid increase in SPQ fluorescence in response to added forskolin. In contrast, the ΔF508 CFTR cells grown in the absence of glycerol at 37°C showed no forskolin-dependent changes in SPQ fluorescence. When cells expressing the ΔF508 CFTR protein were treated with 1.25 M glycerol for only 24 h, forskolin-stimulated chloride transport was observed. Note that, via Western blotting, we did not observe any mature CFTR in the 1-day glycerol-treated ΔF508-expressing cells (Fig. 4). Here, however, the more sensitive SPQ fluorescence assay indicated a partial restoration of chloride conductance in the ΔF508 CFTR-expressing cells after only 1 day of glycerol treatment.

The positive effects of glycerol treatment on chloride conductance were concentration- and time-dependent (Fig. 5B). Increased chloride transport was observed for cells exposed to as little as 0.5 M glycerol for 3 days. Maximal rates of chloride efflux was observed in those cells incubated in 1.25 M glycerol for a period of 3 days, consistent with the results obtained using Western blotting. Note, however, that we were never able to restore the magnitude of chloride transport in the glycerol-treated ΔF508 cells to the levels observed for those cells expressing the wild-type protein. In line with our biochemical observations, glycerol treatment of the cells

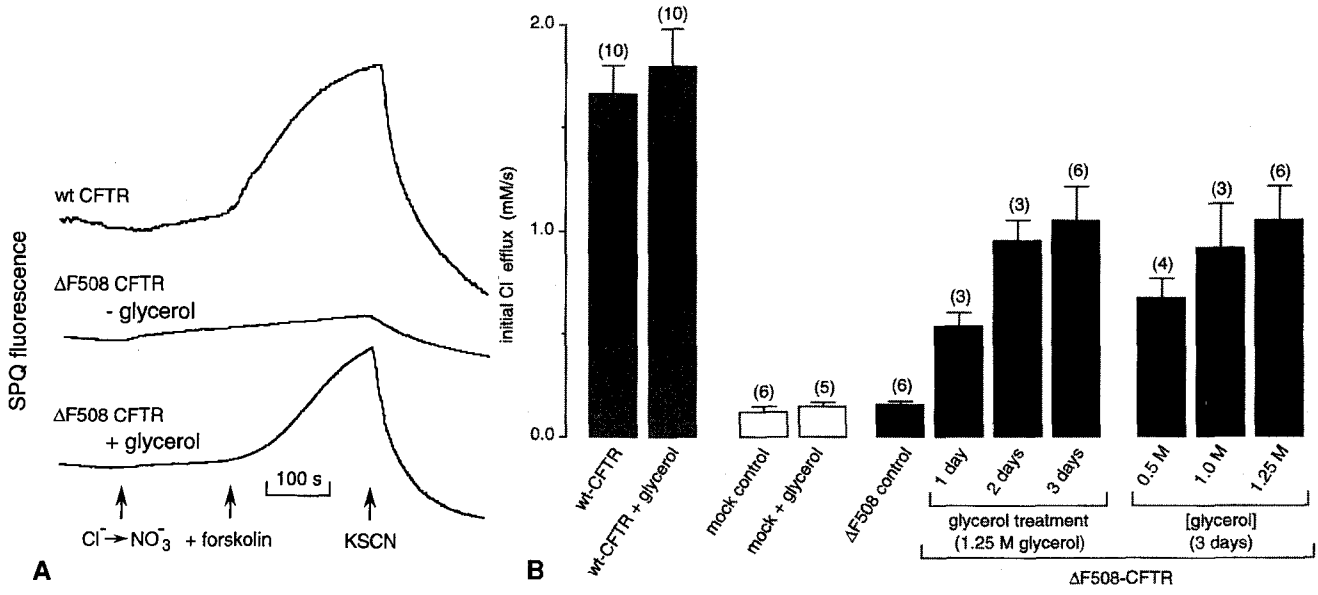

Fig. 5 ΔF508 CFTR-expressing cells incubated in the presence of glycerol now exhibit forskolin-dependent chloride transport. **Panel A** – ΔF508 CFTR-expressing cells were grown on glass coverslips at 37°C in the absence or presence of 1.25 M glycerol for 1 day. These cells, along with cells expressing wild-type CFTR, were then examined for forskolin-stimulated chloride transport using SPQ fluorescence assays as is described in Methods. Incubation of the ΔF508 CFTR-expressing cells in the presence of glycerol resulted in an increase in forskolin-dependent chloride transport. **Panel B** – NIH 3T3 cells transfected with: wild-type CFTR (wt-CFTR), ΔF508 CFTR, or mock-transfected cells (mock) were incubated in the absence or presence of glycerol as indicated at the bottom of the Figure. The wild-type and mock-transfected cells were incubated in the presence of 1.25 M glycerol for a period of 3 days. Chloride efflux measurements as a function of added forskolin were performed as described in panel A, and the results summarized in bar graph form. Indicated above the individual bars are the number of experiments performed, along with error bars (SEM).

expressing wild-type CFTR had little or no effect on the extent of chloride conductance. Finally, the mock-transfected 3T3 cells, either in the absence or presence of glycerol, did not display significant cAMP-stimulated chloride movement, indicating that glycerol was influencing chloride conductance via the CFTR protein and not through some other mechanism.

A number of other compounds, also known to protect proteins from aggregation, were examined for their ability to correct the processing defect associated with the ΔF508 mutation (Fig. 6). Incubation of cells expressing mutant CFTR with cell culture medium prepared in D_2O for a period of 2 days resulted in significant forskolin-dependent chloride transport. Similar results were obtained when the cells were exposed to the solvent DMSO (data not shown). Finally, cells were incubated with TMAO, a naturally occurring cellular osmolyte (Somero 1986) that accumulates in the cells of cartilaginous fish (e.g. sharks) in response to high concentrations of the protein chaotrope urea. Again, a significant increase in forskolin-dependent chloride transport was observed for the ΔF508- expressing cells treated with 100 mM TMAO over a period of 2 days.

DISCUSSION

Our studies presented here demonstrate novel ways by which to influence the maturation of the ΔF508 cystic fibrosis transmembrane regulator protein. The impetus for these experiments was the previous observation of Denning et al (1992) showing that the ΔF508 CFTR protein was temperature sensitive. Cells at their normal growth temperature (e.g. 37°C) exhibited a block in the maturation of ΔF508 CFTR, but when incubated at 26°C a portion of the mutant protein was observed to undergo normal maturation, similar to that observed for the wild-type protein. Owing to its temperature sensitivity, we suspected that the deletion of phenylalanine at position 508 likely resulted in only a subtle alteration in the folding of the mutant ΔF508 CFTR protein. Therefore, we examined whether there might be other ways by which to influence the proper folding of the ΔF508 mutant. Our rationale for using glycerol was based on previous studies demonstrating its ability to protect proteins from thermal denaturation, either in vitro (Gerlsma and Stuur 1972; Back et al 1979; Gekko and Koga 1983) or in vivo (Lin et al 1981; Henle et al 1983; Edington et al 1989). As was

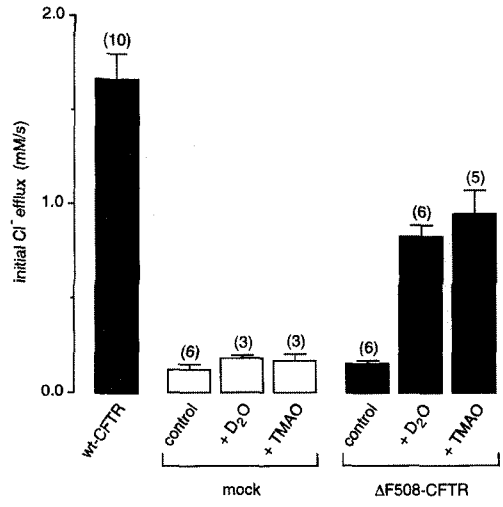

Fig. 6 Two other chemical chaperones, deuterated water and TMAO, restore forskolin-dependent chloride transport ability in the ΔF508 CFTR-expressing cells. Mock-transfected, or ΔF508 CFTR-transfected cells were incubated in: normal growth medium (control), medium supplemented with 100 mM of trimethylamine N-oxide (TMAO), or growth medium prepared in 100% deuterated water (D_2O). After 2 days of growth under the different conditions, forskolin-stimulated chloride efflux was examined using the SPQ fluorescence technique. Cells expressing wild-type CFTR (wt-CFTR) were included in the analysis as a positive control. Indicated above the individual bars are the number of experiments performed, along with error bars (SEM).

shown here, inclusion of glycerol, as well as a variety of other low molecular weight compounds, into the culture medium of cells stably transfected with ΔF508 CFTR cDNA and expressing the ΔF508 CFTR mutant protein resulted in the appearance of the fully glycosylated and mature form of the ΔF508 CFTR protein. Moreover, the ΔF508 CFTR-expressing cells treated with these different 'chemical chaperones' were competent to carry out chloride transport in response to added forskolin. Similar results have recently been reported by Sato et al (1996). Prompted by our suggestion, these investigators examined the effects of glycerol on the maturation of a variety of different CFTR mutants. Interestingly, those CFTR mutants which exhibit a temperature sensitive phenotype were all rescued, albeit to different extents, via the exposure of the cells to glycerol. Moreover, via patch clamp techniques, these investigators similarly reported the restoration of cAMP-dependent chloride transport in the ΔF508-expressing cells incubated in the presence of glycerol.

The mechanism by which these different chemical chaperones elicit proper maturation of at least a portion of the mutant CFTR protein remains unclear. We suspect that, like lower temperatures, these compounds result in a portion of the mutant protein adopting a wild-type-like conformation, thereby resulting in its release from the ER and its subsequent movement to the plasma membrane.

Previous studies have shown that at least two molecular chaperones may be involved in the maturation of the CFTR protein, and that their interaction with, and subsequent release from, the newly synthesized CFTR protein may be rate-limiting in CFTR protein maturation. For example, both calnexin, present within the lumen of the ER, and Hsp70 within the cytoplasm have been shown to interact transiently with the newly synthesized form of wild-type CFTR (Yang et al 1993; Pind et al 1994). Subsequent movement of the newly synthesized wild-type CFTR protein through the Golgi complex to the plasma membrane was accompanied by its release from the Hsp70 chaperone. In contrast, the newly synthesized ΔF508 form of CFTR was never observed to be released from its Hsp70 chaperone, and over time was degraded. Thus, one suspects that the newly synthesized ΔF508 CFTR protein does not fold properly while present at the ER and, as a consequence, is recognized as being an abnormally folded protein by the Hsp70 chaperone. Failure to ever reach its properly folded state likely results in a continued interaction with, and retention by, the Hsp70 chaperone, at least until being targeted to a degradative pathway. Indeed, it is this kind of scenario which has prompted the suggestion that members of the molecular chaperone family act as a type of 'quality control system' allowing properly folded proteins to continue along their maturation pathway, but retaining those which appear misfolded.

How might the presence of glycerol, or these other chemical chaperones, influence the correct folding of the mutant CFTR protein? As was mentioned earlier, many of these compounds have been shown to stabilize proteins in their native conformation and thereby reduce their potential for aggregation in response to thermal treatments (for a review see Schein 1990). In the case of glycerol, the compound has been shown to be preferentially excluded from the immediate domains of polypeptides (Gekko and Timasheff 1981a, 1981b). Therefore, at high concentrations of glycerol, the relative degree of solvent interaction with the protein would be expected to increase. To offset this increase in its relative hydration, the polypeptide would decrease its area of solvent-protein interaction via tighter packing, or increased self-association. In other words, high concentrations of glycerol likely result in an enhancement of the 'hydrophobic effect' thereby increasing the interactions between internal domains of the protein. This increase in the rigidity of the protein would act to stabilize the protein and thereby explain its increased resistance to denaturation by heat. In the case of the ΔF508 form of CFTR, one suspects that the deletion of phenylalanine at position 508 may perturb or interfere with the localized folding or internal packing of the polypeptide. In the presence of glycerol, the resultant increased hydration

surrounding this portion of the ΔF508 CFTR polypeptide may drive an important hydrophobic interaction that normally is mediated (or enhanced) by the presence of the phenylalanine residue.

Although we favor a model akin to the one described above to explain the effects of chemical chaperones on mutant CFTR folding, we should also point out another effect of these compounds when added to cells in culture. Specifically, owing to a previous study which reported an inhibition of cell proliferation by glycerol treatment (Wiebe and Dinsdale 1991), we examined the relative extent of protein synthesis in the NIH 3T3 cells exposed to the different chemical chaperones, or when cultured at lowered growth temperatures (e.g. 26°C where the ΔF508 CFTR protein is processed correctly). In each case, the overall extent of protein synthesis (as determined by the incorporation of [35]S-methionine) was reduced approximately 30–50% as compared to the control, untreated cells maintained at 37°C (data not shown). Whether this reduction in [35]S-methionine incorporation simply correlates with a slow down in cell growth, or alternatively results from a reduced rate of protein synthesis, is currently under study. It would be interesting to examine whether slowing down the actual rate of polypeptide synthesis (i.e. the rate of elongation) might have any positive effects on the proper folding of the mutant ΔF508 CFTR protein.

Abnormal protein folding, due to specific point mutations or deletions, represents the molecular basis of a number of diseased states (for reviews see Amara et al 1992; Bychkova and Ptitsyn 1995; Thomas et al 1995; Welch and Brown 1996). Relevant examples include familial hypercholesterolemia (LDL receptor), Tay-Sachs (B-hexosaminidase), emphysema and cirrhosis (α1-antitryspsin) and leprechaunism (insulin receptor). Similar to the situation with the ΔF508 CFTR protein, the mutations involved often are not so severe as to totally inhibit the activities of the particular protein product. Instead, the abnormal phenotype arises due to a failure of the newly synthesized mutant protein to be delivered to its correct locale, either within a specific subcellular compartment or secreted out of the cell. Owing to an inability to fold properly, the mutant proteins are recognized by, and retained at the level of the ER via the action of one or more members of the molecular chaperone family. Thus, it could be argued that by virtue of their normal role in recognizing and binding to abnormally folded proteins, the molecular chaperones may actually contribute to the diseased state in those cases where the folding abnormality is not so severe as to inhibit the biological activities of the particular protein. We suggest that, like the ΔF508 CFTR protein, those mutant proteins which exhibit only a subtle defect in their folding pathway might be 'rescued' when synthesized in the presence of chemical chaperones. We have begun to test this idea using cell lines expressing proteins which, like the ΔF508 CFTR mutant, exhibit a temperature sensitive phenotype. In many cases, the inclusion of chemical chaperones into the culture medium appears to rescue the biological activity of the mutant protein at the non-permissive temperature (Brown et al, MS in preparation). We hope that our studies presented here will prompt further efforts to identify other small molecules which at low concentrations and negligible toxicity, will prove effective in correcting the folding defects associated with genetically-based diseases.

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