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Strategies for Correcting the ΔF508 CFTR Protein-Folding Defect

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Many human diseases arise as a result of mutations within genes encoding essential proteins. In many cases, the mutations are not so severe as to render the protein biologically inactive. Rather, the mutations oftentimes result in only subtle protein-folding abnormalities. In the case of the CFTR protein, a mutation leading to the loss of a single amino acid is responsible for the diseased state in the majority of individuals with cystic fibrosis. Here the newly synthesized mutant CFTR protein, missing a phenylalanine residue at position 508 (ΔF508 CFTR), is unable to transit from the endoplasmic reticulum to the plasma membrane, where it functions as a regulator of chloride transport. All of the available evidence indicate that the newly synthesized ΔF508 CFTR protein adopts a slightly altered conformation and therefore is retained at the level of the endoplasmic reticulum, ostensibly by the actions of the cellular quality control system. Because the mutant protein is capable of functioning as a chloride channel, developing ways to elicit its release out of the ER and to the plasma membrane has important clinical implications. Herein, we discuss our recent studies showing that the protein-folding defect associated with the ΔF508 CFTR mutation, as well as a number of other temperature-sensitive mutations, can be overcome by strategies designed to influence protein folding inside the cell. Specifically we show that a number of low-molecular-weight compounds, all of which are known to stabilize proteins in their native conformation, are effective in rescuing the folding and/or processing defects associated with different mutations that oftentimes lead to human disease.

KEY WORDS: Cystic fibrosis; chemical chaperones; protein folding; glycerol; osmolytes; temperature sensitive.

INTRODUCTION

Cystic fibrosis is the most common fatal genetic disease in Caucasians. The gene responsible for cystic fibrosis, the so-called CFTR gene, encodes a protein which appears to function as a chloride channel (Kerem *et al.*, 1989; Riordan *et al.*, 1989; Rommens *et al.*, 1989). Since its initial discovery, numerous mutations have been identified within the CFTR gene, many of which appear responsible for the diseased state. The various mutations exert their negative effects via different mechanisms. For example, certain mutations result in errors in transcription. In other cases, the mutations result in the production of mRNA's which are unstable and are degraded very rapidly (Hamosh *et al.*, 1991). Accordingly, the cells fail to synthesize sufficient quantities of the CFTR protein. Yet other types of genetic errors result in the production of a CFTR protein which apparently fails to fold properly and never reaches the plasma membrane. For example, in the case of the ΔF508 CFTR mutant, the newly synthesized protein is inserted into the membrane of the endoplasmic reticulum (ER), but fails to move further along the secretory pathway (Cheng *et al.*, 1990). It is believed that retention at the level of the ER is due to the actions of accessory proteins including

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members of the molecular chaperone family (Yang *et al.*, 1993; Pind *et al.*, 1994) Finally, other genetic defects lead to the production of a CFTR protein which, while present at its proper site in the plasma membrane, does not function properly. Although the mechanisms are different, all of these disease-causing mutations ultimately result in the lack of a functional CFTR chloride channel at the plasma membrane (for a review of CFTR mutations, see Welsh, 1994).

STRATEGIES TO CORRECT THE MUTANT CFTR GENE AND/OR PROTEIN

Owing to this vast number of different mutations, cystic fibrosis is a rather complex disease that would not appear amenable to any one particular type of intervention. Accordingly, over the past few years a number of different treatment protocols have been designed, and are continuing to be tested. Here we briefly summarize some of the potential therapeutic strategies being contemplated and/or pursued. We will devote particular attention to our recent efforts to correct the folding of the ΔF508 CFTR protein.

Gene Therapy

Many have embraced the idea of using gene therapy as the best approach for combating cystic fibrosis. In theory, this type of treatment should be effective in correcting all classes of CFTR mutations since it would provide a gene encoding the wild type CFTR protein. While this avenue remains promising, there are still a number of issues that need to be solved. One problem involves a suitable method to deliver the CFTR genetic material into the cells of interest. Once properly delivered, there is also the problem of sustained expression of the gene. Finally there are a number of potential side effects that are now being recognized relating to the methods by which the genes are delivered. For example, viral vectors are the most prevalent vehicle being used to deliver the genetic material into the cell. Accordingly, immune responses against some of the virus components are being observed and will likely represent a major obstacle for repeated presentations of the gene (Zabner *et al.*, 1996). Consequently, different nonviral delivery systems now are being examined, with the hope of achieving sustained high level expression of the CFTR protein along with no accompanying adverse immune response (Alton *et al.*, 1993; Caplan *et al.*, 1995; Lee *et al.*, 1996).

Aminoglycoside Antibiotics

As an alternative to gene therapy, a number of pharmacological approaches are being pursued. Although obviously not being capable of "curing" the disease, many of the agents being used have the potential to either overcome the adverse effects associated with a nonfunctional CFTR protein, or in some cases act to stabilize or repair the mutant protein so that it will function in a manner similar to that of the wild type protein. As an example, a number of non-sense mutations within the CFTR gene result in the synthesis of truncated, nonfunctional forms of the CFTR protein. Howard and his colleagues have shown that treatment of cells expressing some of these truncation mutants (e.g., G542X and R553X) with different aminoglycoside antibiotics results in "translational read-through" and the production of low levels of a full-length and functional CFTR protein (Howard *et al.*, 1996). While these results are encouraging, non-sense mutations account for only approximately 5% of the cases of cystic fibrosis.

Drugs That Affect Protein Phosphorylation

Owing to the apparent role protein phosphorylation plays in the function and regulation of CFTR chloride channel activity, drugs that affect CFTR protein phosphorylation have been examined and found to be effective for correcting some CFTR mutations. For the most part, these drugs are only effective in enhancing the activities of those CFTR mutants which are able to traffic to the plasma membrane (e.g., they are not very effective in inducing chloride conductance in the ΔF508 expressing cells). For example, phosphatase inhibitors can enhance the relative activities of the wild type protein, as well as certain CFTR mutants, but only if the mutant proteins are present at the plasma membrane (Becq *et al.*, 1994, 1996). Accordingly, when cells expressing ΔF508 are maintained at 23°C (which results in the movement of the CFTR protein to the plasma membrane), treatment with levamisole (a phosphatase inhibitor) is effective in inducing chloride efflux. Again, while the precise mode of action is not known, it is assumed that this compound exerts its effect via the stabilization of CFTR in its phosphoryl-

ated (and presumably active) state. In yet another study, the tyrosine kinase inhibitor genistein was found to induce cAMP independent activation of wild type CFTR chloride channels (Illek *et al.*, 1996). More recently, it has also been suggested that this compound may also be effective in activating the ΔF508 CFTR protein. Finally, the combination of the phosphodiesterase inhibitor milrinone and a beta agonist has been found to activate chloride efflux from cells expressing the ΔF508 CFTR protein, even when the cells are maintained at 37°C (Kelley *et al.*, 1996).

Compounds Targeting the ΔF508 CFTR Protein

The vast majority of patients with cystic fibrosis have a CFTR gene which contains a single mutation resulting in the lack of an apparently important phenylalanine residue at position 508. This mutation, ΔF508 CFTR, results in the production of a protein which appears unable to fold properly during the early stages of its biogenesis. As a consequence, newly synthesized ΔF508 CFTR is inserted into the membrane of the endoplasmic reticulum but fails to move further along the maturation pathway to the plasma membrane (Cheng *et al.*, 1990). Over the course of the last few years it has been shown that the ΔF508 CFTR protein can in fact function in chloride transport. First, when produced either in insect cells (Bear *et al.*, 1992; Li *et al.*, 1993) or in frog oocytes (Drumm *et al.*, 1991), ΔF508 CFTR appears functional, albeit not as active as the wild type protein. Capitalizing on the fact that insects and frogs live at lower temperatures, Welsh and colleagues subsequently showed that the ΔF508 mutation resulted in a protein product whose folding pathway was temperature sensitive (Denning *et al.*, 1992). Simply lowering the temperature of animal cells expressing ΔF508 CFTR (e.g., 30°C or less) now resulted in a small population of the protein which matured properly and was able to confer upon the cell cAMP-stimulated chloride transport.

The observation that the ΔF508 CFTR protein can function as a chloride channel has led many investigators to search for pharmacological ways to elicit movement of the protein out of the ER to the plasma membrane. Indeed, a number of compounds recently have been identified which appear effective for restoring chloride transport in cells expressing the ΔF508 CFTR mutant. In many cases, however, the precise mode of action of these compounds remains obscure. For example, the adenosine receptor antagonist CPX (8-cyclopentyl-1,3-dipropylxanthine) has been shown to activate the efflux of chloride from cells expressing the ΔF508 CFTR protein (Eidelman *et al.*, 1992; Guay-Broder *et al.*, 1995). The compound appears to exert its effect only on the mutated CFTR protein. For example, exposure of cells expressing wild type CFTR (e.g., T84 or HT-29) to CPX did not further increase chloride efflux. Although the molecular details by which this compound affects ΔF508 CFTR function are unknown, it does not appear to exert its effect on chloride efflux via binding to the adenosine receptor (Jacobson *et al.*, 1995).

Another compound receiving some attention is N-acetyl-L-cysteine. Because of its mucolytic properties, N-acetyl-L-cysteine is currently being used to treat patients with a variety of respiratory disorders, including cystic fibrosis. Interestingly, N-acetyl-L-cysteine has been found to activate chloride movement in cells expressing either the wild type or the ΔF508 form of CFTR, although again its mode of action remains unclear (Kottgen *et al.*, 1996). The compound presumably exerts its effect via the CFTR protein since no change in chloride conductance was observed for cells which did not express detectable levels of the CFTR protein.

Butyrate is yet another compound that appears to activate chloride conductance in cells expressing ΔF508 CFTR. Treatment of cells with sodium butyrate has been observed to increase the overall expression of the CFTR protein, be it the wild type or ΔF508 forms of the protein. Interestingly, low levels of the ΔF508 CFTR protein now appear to reach the plasma membrane in the butyrate treated cells (Cheng *et al.*, 1995). Recently, similar experiments have been tried using the butyrate derivative, phenylbutyrate (R. Rubenstein, personal communication). This compound, which already has been approved for use in the treatment of urea cycle disorders, also results in the correct processing of at least a portion of the ΔF508 CFTR protein and the cells ability to conduct chloride movement in a cAMP-dependent manner. Perhaps the increased expression of the ΔF508 CFTR protein in response to butyrate treatments somehow allows for a fraction of the mutant protein to escape from the ER and move to the plasma membrane. Alternatively, it is also possible that the different butyrate derivatives may be directly influencing the folding of the mutant protein, thereby allowing it to be properly processed and subsequently transported out of the ER to the plasma membrane. This latter suggestion is based on our observations (described below) that various low-

molecular-weight compounds can influence the folding of the ΔF508 CFTR protein and thereby restore cAMP-dependent chloride transport activities to the cell.

Affecting the Protein-Folding Environment inside the Cell: A New Strategy for Correcting the ΔF508 CFTR Protein-Folding Defect

The ΔF508 CFTR mutation results in a failure of the presumably abnormally folded protein to ever leave the ER. Although this trafficking defect can have severe consequences, the protein-folding abnormality itself appears to be relatively mild. For example, as mentioned above, simply lowering the growth temperature of cells expressing ΔF508 CFTR to 30°C or less results in a portion of the protein moving out of the ER to the plasma membrane. Moreover, these cells now exhibit cAMP-dependent chloride transport, even when they are shifted back to 37°C. Restoration of channel activity in the cells shifted back to 37°C, however, is only transient. Over time that population of the CFTR protein which is present at the plasma membrane is degraded, similar in kinetics to that of the wild type protein. Now at 37°C, the cells again are unable to produce any more functional ΔF508 CFTR protein.

Based on those observations showing that the ΔF508 CFTR mutation results in a temperature-sensitive protein-folding defect, we explored new ways to influence the folding of the protein in tissue culture cells (Brown *et al.,* 1996). Based on our experience examining the so-called heat-shock response we were aware of novel methods by which to stabilize proteins against heat denaturation. For example, a large body of work has shown that various low-molecular-weight compounds can stabilize proteins against thermal denaturation and aggregation (for a review, see Schein, 1990). Representative compounds include: polyols such as glycerol, solvents such as DMSO, and finally deuterated water (D_2O). In addition to their effects on protein stability *in vitro* (Germsla and Sturr, 1972; Back *et al.,* 1970; Gekko and Koga, 1983), some of these compounds have been shown to influence protein folding and/or stability *in vivo* (Lin *et al.,* 1981; Henle *et al.,* 1983; Edington *et al.,* 1989). For example, when incubated in the presence of either deuterated water or glycerol, cells can withstand temperatures (heat-shock treatments) that would otherwise be lethal. Presumably, following their uptake into cells, these compounds help to stabilize cellular proteins against the denaturing effect of the rather severe heat-shock treatment.

Owing to their ability to stabilize proteins against thermal denaturation, we wondered whether treatment of cells with any of these different agents might have an effect on the folding, processing, and/or function of the ΔF508 CFTR protein in animal cells maintained at 37°C. NIH 3T3 cells expressing ΔF508 CFTR (via transfection) were incubated in the presence of different concentrations of glycerol. After three days, the cells were harvested and examined for the presence of the mature CFTR protein. As shown by Western blotting (Fig. 1A), the ΔF508 CFTR-expressing cells now contained modest levels of the mature CFTR protein. Note that the optimum response was obtained using a relatively high concentration of glycerol (e.g., 1.25 M). Using 1.25 M glycerol, a time-course experiment revealed the highest levels of mature CFTR after three days of incubation (Fig. 1B). Importantly, following their treatment with glycerol, the ΔF508 CFTR cells now exhibited forskolin-dependent chloride transport, albeit less than that observed for those cells expressing the wild type CFTR protein (Fig. 1C).

Prompted by our success using glycerol, we examined a number of other low-molecular-weight compounds which also have been reported to act as protein stabilizers. Two of these compounds, deuterated water (D_2O) and trimethylamine N-oxide, also were found to be effective for partially correcting the processing defect associated with the ΔF508 CFTR protein. Moreover, both compounds resulted in the ΔF508 CFTR-transfected cells now exhibiting forskolin-dependent chloride transport (Brown *et al.,* 1996).

Thus our data had demonstrated that three totally unrelated compounds, when added to the ΔF508 CFTR-expressing cells, now resulted in the proper maturation of a small percentage of the newly synthesized ΔF508 CFTR protein and a restoration of chloride transport. Recent studies by Sato *et al.* (1996) similarly have demonstrated that glycerol treatment is effective for correcting the folding and processing defects associated with the ΔF508 mutation. Finally, Sorscher and colleagues have reported that the solvent dimethylsulfoxide (DMSO), at a final 2% concentration, can restore cAMP-dependent chloride transport to the cells, presumably via its positive effects on the maturation of ΔF508 CFTR (personal communication). Exactly how are these compounds influencing the folding of the mutant cystic fibrosis protein? As

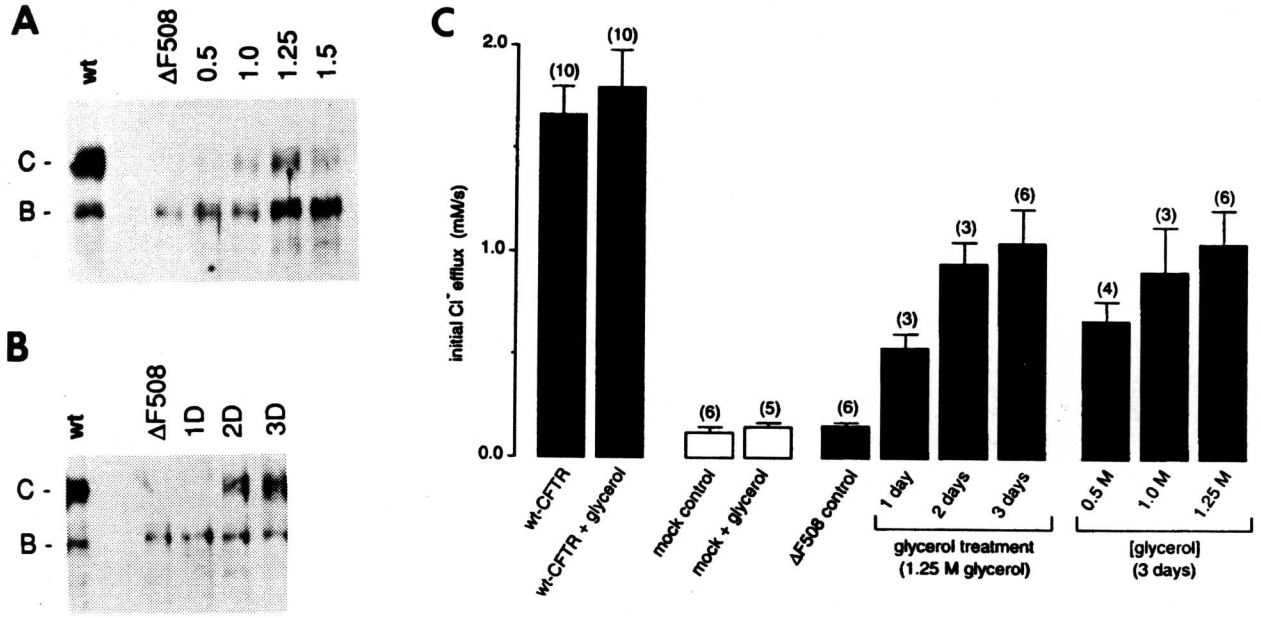


Fig. 1. The positive effects of glycerol on the processing and functioning of the ΔF508 CFTR protein, (**A**) Cells expressing ΔF508 CFTR were incubated in the absence (ΔF508) or presence of varying concentrations (0.5, 1.0, 1.25, or 1.5 M) of glycerol. After 3 days of growth at 37°C the cells were harvested and analyzed for their content of immature (band B) and mature (band C) forms of the CFTR protein by Western blotting using a rabbit polyclonal anti-CFTR antibody. Cells expressing wild type CFTR (wt) were included as a positive control. (**B**) Cells expressing ΔF508 CFTR were incubated at 37°C 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 both panels. (**C**) Cells expressing wild type CFTR, ΔF508 CFTR, or mock transfected cells were grown on glass coverslips at 37°C in the absence or presence glycerol. These cells then were examined for forskolin-stimulated chloride transport using SPQ fluorescence assays (performed by J. Biwersi and A.S. Verkman, UCSF). The results from several experiments are summarized in bar graph form. Indicated above the individual bars are the number of experiments performed, along with error bars (SEM). [Adapted from Brown *et al., Cell Stress Chap.* **1**, 117–125 (1996).]

was mentioned above, all of these different compounds are linked together via their ability to stabilize proteins, at least *in vitro,* against proteotoxic conditions, be it chemical denaturants or heat. Moreover, in the case of glycerol, the compound has been shown to effectively reduce the extent of protein denaturation following heat-shock treatments of animal cells (Lin *et al.,* 1981; Edington *et al.,* 1989). Hence, we suspect that these compounds somehow influence the "folding environment" within cells and thereby result in a population of the ΔF508 CFTR protein adopting its properly folded state. Based on a wealth of *in vitro* studies examining the effects of glycerol on protein folding and stability, we suspect that the compound must be altering protein–solvent interactions. For example, glycerol has been shown to be preferentially excluded from the immediate vicinity of protein domains (Gekko and Timasheff, 1981a,b). Thus, at high concentrations of glycerol the relative extent of solvent interaction with the mutant CFTR protein (or any protein for that matter) would be expected to increase. To offset this increase in its relative hydration, the CFTR protein at the ER membrane would likely try and decrease its degree of solvation via a tighter packing of its protein domains. This increased "self-association," due to a type of hydrophobic effect, somehow negates the adverse consequences of the phenylalanine deletion and thereby leads to a population of nascent ΔF508 CFTR which folds properly.

Based on our results with the ΔF508 CFTR protein, as well as with some other temperature-sensitive protein folding mutants described below, we now often refer to these different low-molecular-weight compounds which influence protein folding as "chemical chaperones." Similar to the proteinaceous molecular chaperones, the various chemical chaperones do not convey any direct information for the folding process. Instead, they seem to influence the overall fidelity of

folding reaction, likely by reducing the propensity of the nascent ΔF508 CFTR protein to go "off pathway" and adopt a conformation that eventually may lead to its aggregation and eventual degradation (Fig. 2A). We suspect that the different chemical chaperones help to lower a critical energy barrier that is rate limiting for the correct folding of the mutant protein (Fig. 2B). Once the protein has successfully overcome this energy barrier in the early stages of its folding pathway, the phenylalanine deletion may no longer have any deleterious consequences for the subsequent folding of the polypeptide. We refer the reader to the article by Thomas and colleagues in this volume which discusses further the thermodynamic parameters associated with CFTR folding, and the possible mechanisms by which the various chemical chaperones impact on the folding of the mutant CFTR protein.

Chemical Chaperones Are Effective for Correcting Other Temperature-Sensitive Protein-Folding Defects

Abnormalities in protein folding constitute the molecular basis for a number of diseases (for reviews see Amara *et al.*, 1992; Bychkova and Ptitsyn, 1995; Thomas *et al.*, 1995, Welch and Brown, 1996). In addition to cystic fibrosis, some of these other diseases include cancer, chronic liver disease and emphysema, Tay-Sachs, and familial hypercholesterolemia, to name but a few. In addition, a number of neurological disorders (Kuru, scrapie, mad cow, and Alzheimer's disease) are thought to arise due to specific proteins which misfold and, over time, accumulate to levels that become pathogenic. Like the situation with the ΔF508 CFTR mutant, in many cases the mutations are not so severe as to render the protein biologically inactive. Rather, the mutant protein is sequestered in the wrong cellular compartment, oftentimes due to its being recognized as being abnormally folded. Retention of the abnormally folded protein likely occurs via the actions of one or more molecular chaperones acting as "quality control" monitors inside the cell.

Based on our success with the ΔF508 CFTR protein we turned our attention to some of these other proteins which also exhibit protein-folding abnormalities (Brown *et al.*, 1997). In particular, we were interested in proteins whose incorrect folding is thought to be responsible for the diseased state. Initially, we began by screening a number of other proteins which, like the ΔF508 CFTR protein, exhibited temperature-sensitive (ts) protein folding defects. The mutants chosen included a cell line (A1-5) expressing a temperature-sensitive form of the tumor suppressor protein p53 (Martinez *et al.*, 1991) and a cell line (ts20) expressing a temperature-sensitive form of the E1 enzyme (Chowdary *et al.*, 1994), an essential component of the ubiquitin-dependent pathway of protein degradation. In the A1-5 cells maintained at the permissive temperature (e.g., 32°C) p53 adopted a wild type conformation and was localized within the nucleus. In contrast, at the nonpermissive temperature (39°C) where the protein folds incorrectly, p53 accumulated within the cytoplasm (Fig. 3, panel A). Incubation of cells growing at 39°C with the different chemical chaperones now resulted in the protein apparently adopting a wild type conformation. For example, cells maintained at 39°C and incubated in media prepared with 100% deuterated water (D_2O) now exhibited a nuclear locale of p53 (Fig. 3, panel B). Similarly, inclusion of either 75 mM trimethylamine N-oxide (Fig. 3C) or 0.6 M glycerol (Fig. 3D) into the culture medium of the cells at 39°C also resulted in p53 assuming a wild type conformation and a corresponding nuclear locale.

Previous work has shown that the A1-5 cells proliferate normally at 39°C (Martinez *et al.*, 1991). Although expressing high levels of the p53 tumor suppressor protein (via transfection) the cells continue to grow, presumably because p53 is present in its abnormally folded and therefore biologically inactive conformation (Fig. 3E, con 39). Upon temperature downshift to 32°C the cells now accumulate the protein in its wild type conformation and as a result the cells exhibit growth arrest (Fig. 3E, con 32). Incubation of the 39°C cells in the presence of any one of the three different chemical chaperones (glycerol, TMAO, or D_2O) now resulted in the cells exhibiting a growth-arrested state, indicative that the p53 folding defect now had been corrected (Fig. 3E).

Positive results also were obtained when the analysis was performed with the ts20 cells that harbor a temperature-sensitive E1 enzyme, one of the first components involved in targeting proteins for degradation by the ubiquitin/proteasome-dependent pathway. At the permissive temperature of 32°C, the levels of p53 are relatively low in the ts 20 cells, ostensibly due to the normal activities of the ubiquitin/proteasome-dependent pathway, believed to be responsible for the degradation of this tumor suppresser protein (Fig. 3F). Upon raising the temperature of the cells to 39°C, the ubiquitin-dependent pathway of protein degradation is shut down. At this temperature p53 was observed to

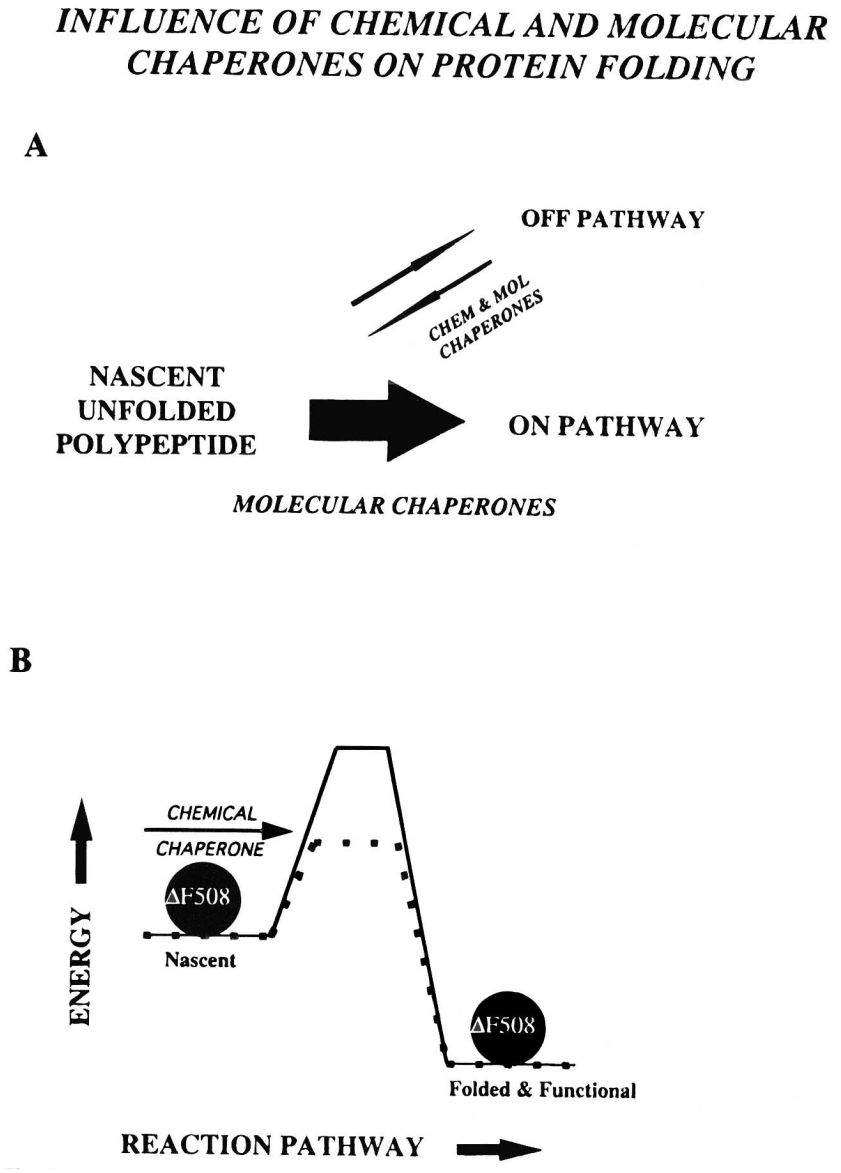
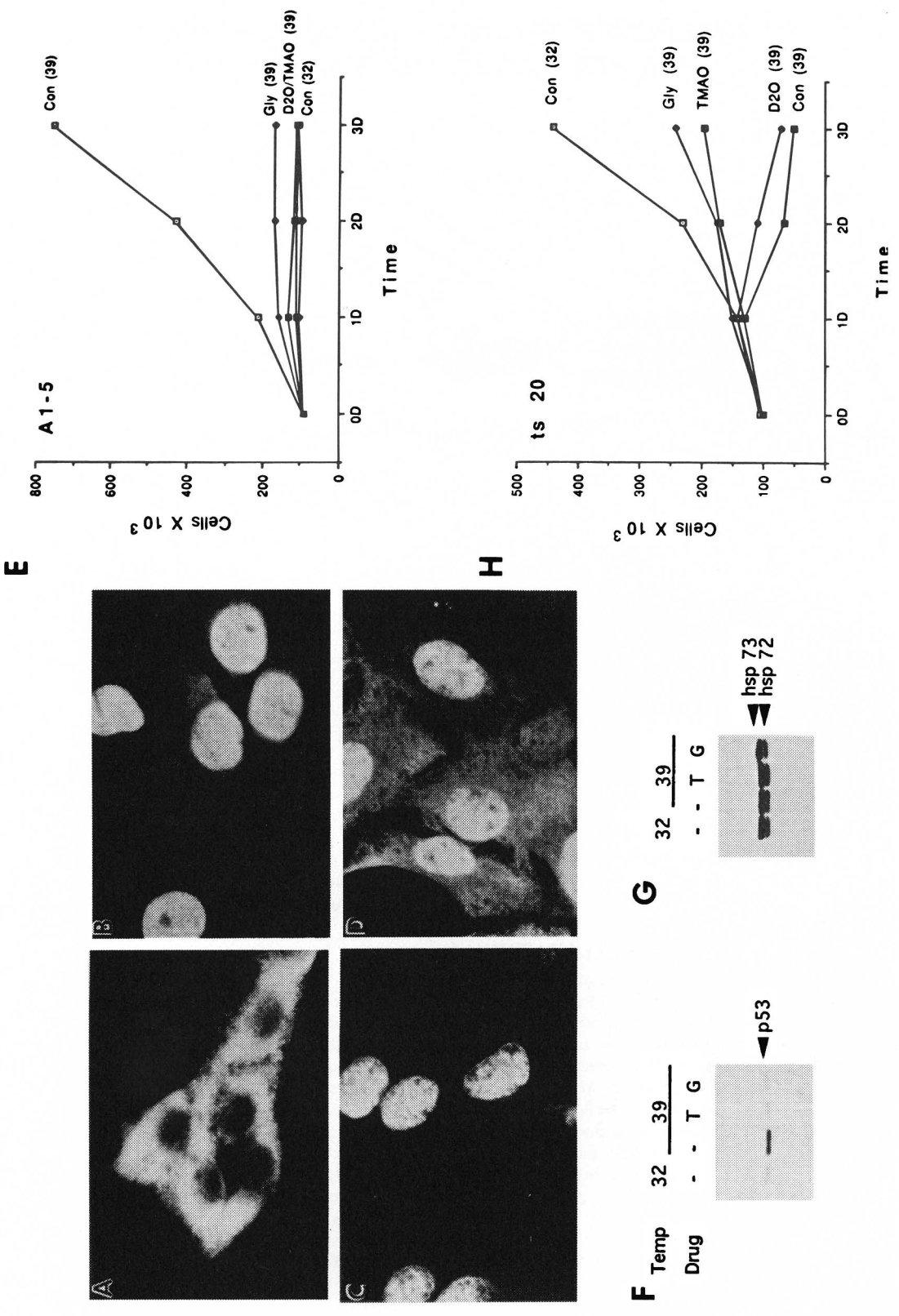


Fig. 2. Influence of chemical and molecular chaperones on protein folding. (**A**) Nascent polypeptides are believed to interact with different molecular chaperones, either during or shortly following their synthesis. All of the available evidence indicates that the different molecular chaperones are capable of recognizing proteins that appear "non-native" and thereby bind to such proteins in order to prevent their going "off pathway" where they may misfold and/or aggregate. Molecular chaperones likely feature in the synthesis and folding of most intracellular proteins, and therefore represent an essential part of the normal pathway of protein folding. Both molecular chaperones and chemical chaperones may help prevent proteins from going off-pathway. Limited data indicate that both classes of chaperones also may be effective in rescuing off-pathway proteins back on to the productive pathway of protein folding. (**B**) We suspect that the different chemical chaperones, via their effect on protein–solvent interactions, act to lower a critical energy barrier needed for the nascent ΔF508 CFTR protein to overcome in order to reach its properly folded state. Once overcome, the protein now folds properly, transits to the plasma membrane, and is functional as a chloride channel.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accumulate to rather high levels. Incubation of the 39°C ts 20 cells in the presence of either TMAO or glycerol, however, resulted in a decline of p53 levels, similar to that observed for the cells maintained at the permissive temperature (Fig. 3F). Note that neither of the two different temperatures used to culture the cells, nor the inclusion of the different chemical chaperones, had any obvious effect on the levels of two major cytosolic molecular chaperones, hsp73 and hsp72 (3G). Indeed, we have no evidence at all which would indicate that the different chemical chaperones are affecting either the levels or activities of the cell's own protein-folding machinery.

ts 20 cells, when incubated at the nonpermissive temperature, exhibited a growth-arrested phenotype (Fig. 3H, con 39). Such a phenotype likely is due, at least in part, to a failure of the cells to normally degrade important cell cycle regulatory proteins like p53. In contrast, when the cells were incubated at 32°C the cells now exhibited normal log phase growth (Fig. 3H, con 32). Inclusion of either TMAO or glycerol into the culture medium of those cells maintained at 39°C resulted in a partial restoration of proliferative activities. With p53 levels now approaching the same levels as that found for cells maintained at 32°C (see Fig. 3F) the cells apparently resumed their growth.

For each of these different temperature-sensitive protein folding mutants we believe the chemical chaperones are having an impact only on the folding of the newly synthesized protein. None of our data is consistent with the idea that those pre-existing proteins which already have adopted the mutant conformation are amenable to correction via the chemical chaperone approach. As was discussed above, we suspect that the different mutations interfere with a critical folding step at some point during the early stages of protein maturation. We do want to emphasize, however, that it is not just temperature-sensitive protein-folding abnormalities that can be corrected via the use of chemical chaperones. Recently we have shown that formation of the scrapie prion protein, thought to be responsible for severe neurological disorders in sheep, the related mad cow disease, and finally disease in humans, is blocked in those cells incubated in the presence of any one of three different chemical chaperones (Tatzelt *et al.*, 1996)

Cellular Osmolytes Are Nature's Own Chemical Chaperones

While our use of chemical chaperones to correct protein-folding abnormalities responsible for various pathological conditions is perhaps novel, it should be emphasized that this type of approach has been noted in the literature for some time. Indeed, the concept of using protein-stabilizing agents *in vivo* to correct protein-folding defects was first proposed more than 30 years ago. For example, in both yeast and bacteria it was recognized that different mutations (usually missence mutations) could be corrected by either lowering the growth temperature or by increasing the osmotic

Fig. 3. A1-5 cells and ts20 cells incubated at the nonpermissive temperature in the presence of different chemical chaperones now exhibit a wild type phenotype. A1-5 cells were grown on glass coverslips and incubated for 1 day at 39.5°C. Some of the coverslips were then transferred into medium supplemented with either D_2O (100%), TMAO (75 mM), or glycerol (0.6M) and the cells further incubated at 39.5°C. After 2 days of incubation at 39.5°C, the cells were analyzed for the intracellular distribution of p53 by indirect immunofluorescence. (**A**) control cells incubated in normal medium. (**B**) Cells incubated in medium prepared with 100% D_2O. (**C**) Cells incubated in the presence of 75 mM TMAO. (**D**) Cells treated with 0.6 M glycerol. (**E**) A1-5 cells treated with protein stabilizers at the nonpermissive temperature now exhibit wild type cell proliferation rates. Equal numbers of A1-5 cells, growing at 37°C, were plated on 60-mm dishes in DMEM containing 10% serum. After plating, the cells were moved to 39.5°C. The next day (day 0) the media were removed, and fresh medium supplemented with either: nothing (con); 0.6 M glycerol (Gly); 100% D_2O (D_2O); or 75 mM TMAO (TMAO), was added to the cells. The cells were further incubated at 39.5°C for 1, 2, or 3 days. After each day, a plate of the cells was removed and cell number determined. (**F, G**) ts20 cells incubated at 39.5°C in the presence of chemical chaperones now exhibit low levels of the p53 protein as determined by Western blotting. ts20 cells were plated on 35-mm dishes and incubated at 32.5°C for 1 day. One plate of cells was maintained at 32.5°C, while the other plates were incubated at 39.5°C in the absence or presence of either TMAO (T) or glycerol (G). One day later the cells were harvested and the relative levels of p53 (**F**) or the cytosolic chaperones hsp73/72 (**G**) determined by Western blotting. (**H**) ts20 cells incubated in the presence of chemical chaperones now proliferate at the nonpermissive temperature. ts20 cells were plated on 60-mm dishes at low confluence and incubated for 24 hr at 32.5°C. Control cells (no added chemicals) were then placed at either 32.5°C (permissive temperature) or 39.5°C (nonpermissive temperature). In parallel, some of the cells were incubated in the presence of the three different compounds and then incubated at 39.5°C. After 1, 2, or 3 days of incubation, the cells were collected and cell number determined. Cell number as a function of the days (D) of incubation at either 32.5°C or 39.5°C are presented. [Adapted from Brown *et al., J. Clin. Invest.*, **99**, 1432–1444 (1997).]

strength of the growth medium. Indeed, Hawthorne and Friis in a 1964 report demonstrated that a variety of mutations in *S. cerevisiae* could be corrected by the inclusion of compounds such as glucose, potassium chloride, glycerol, or diethylene glycol into the growth medium. Even then, the investigators suspected that the osmotic remediation was working via the correction of specific protein-folding abnormalities.

Nature has developed her own chemical chaperones to help particular organs cope with environments hostile to protein folding and/or function. For example, exposure of tissues and organs to hyper-osmotic stress often results in the accumulation of a variety of different low-molecular-weight compounds commonly referred to as cellular osmolytes (reviewed in Yancey *et al.*, 1982; Somero, 1986; Garcia-Perez and Burg, 1991). These compounds typically fall into one of three classes: (a) carbohydrates such as glycerol, sorbitol, arabitol, myoinositol, and trehalose; (b) free amino acids and derivatives such as glycine, alanine, proline, and taurine; (c) methylamines such as betaine, trimethylamine N-oxide (TMAO), and glycerolphosphocholine. Although the mechanisms responsible for the accumulation of these osmolytes vary, the intracellular level of osmolytes is typically regulated either by uptake mediated by specific transporter proteins or, alternatively, by the direct synthesis of the compounds in the affected cells. Furthermore, the particular type of stress itself (e.g., salt stress, glucose stress, urea stress, etc.) can influence both the type of osmolyte which accumulates and its mode of accumulation.

The above-described osmolytes often are classified either as compatible osmolytes (e.g., carbohydrates and amino acids) or counteracting osmolytes (e.g., the methylamines). The former can accumulate within the cell to rather high concentrations without significant adverse effects on other macromolecules; in particular, they do not perturb the structure/function of already folded polypeptides. Counteracting osmolytes are produced to offset the protein-denaturing effects of naturally occurring protein denaturants like urea. In the tissues of some saltwater organisms (e.g., sharks), or in the mammalian renal medulla, urea concentrations can reach levels that are potentially dangerous as it relates to protein function. To offset this, methylamines like TMAO are produced, at an approximate 1:2 ratio (TMAO:urea). Under these conditions, the protein denaturing effects of urea are greatly minimized (Yancey *et al.*, 1982; Somero, 1986).

Manipulating Cellular Osmolytes/Chemical Chaperones for the Treatment of Disease

Taken altogether, the observations that small molecules can influence protein-folding pathways has significant implications for our developing novel strategies to correct diseases due to abnormal protein folding. The fact that nature, via the use of cellular osmolytes, already employs such an approach on a daily basis for organs experiencing metabolic stress is encouraging, and we believe yet a further impetus for pursuing this type of approach to combat diseases caused by abnormal protein folding. We envision two ways by which this type of technology can be pursued. The first is to simply search for small molecules that are able to effect protein folding, are diffusable into cells, and, of course, pose no significant toxicological problems. Toward these ends, we have described a number of biological assays, at both the biochemical and cellular levels, that can help in the identification of potential chemical chaperones. In addition to many of the well-known cellular osmolytes, we believe it also would be prudent to start examining some of the existing drug libraries that have been developed in the pharmaceutical and biotechnology arenas. Further refinement of this kind of approach may eventually require the creation of small peptides which can effectively "target" the particular polypeptide in need of correction (e.g., ΔF508 CFTR).

Yet another approach would be to learn more regarding the mechanisms by which cells respond to osmotic stress. Being able to manipulate the expression and accumulation of endogenous cellular osmolytes within different tissues and organs might be a better approach than an empirically driven search for effective compounds. While some progress has been made regarding the mechanism by which cells manipulate their content of cellular osmolytes, we still have much to learn. Within the last few years, a number of different transporters and enzymes involved in the uptake and biosynthesis of cellular osmolytes have been identified, in some cases their genes cloned, and the proteins themselves rather well characterized (see Burg, 1995 for a review). Eventually, this new information regarding these components may enable us to pharmacologically manipulate the levels of osmolytes within tissues and organs and thereby facilitate correction of the particular protein-folding defect responsible for the pathological condition.

While the concept of correcting protein-folding abnormalities by changing the protein-folding environ-

ment is not new, we suggest that this type of approach may represent a novel strategy to interfere with those protein-folding abnormalities which constitute the molecular basis of different diseases. The list of diseases known to result from protein-folding abnormalities continues to grow as investigators identify new genes and determine that specific mutations correlate with the diseased state. In addition, we wonder how many of these genetically inherited diseases result in the production of mutant proteins that exhibit temperature-sensitive folding defects? We remind the reader that in the case of the ΔF508 CFTR mutation, it took the field a few years before realizing that the mutation caused a temperature-sensitive folding abnormality, and that the mutation was not so severe as to render the protein biologically inactive. Considering the results presented here, we predict that most any temperature-sensitive protein-folding mutant will be amenable to correction *in vivo* by one or more of the chemical chaperones. We hope that our observations will encourage others to search for additional low-molecular-weight compounds which may prove to be effective for correcting those protein-folding abnormalities which constitute the molecular basis of the diseased state.

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