MAMMALIAN OSMOLYTES and S-NITROSOGLUTATHIONE PROMOTE ΔF508 CFTR PROTEIN MATURATION AND FUNCTION

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Running Title: Small molecules and ΔF508 CFTR maturation/function

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ABSTRACT

CFTR is expressed in epithelial cells and functions as a cAMP-regulated chloride channel. Its importance in fluid secretion and exocrine function is underlined by the disease cystic fibrosis (CF). In CF, the absence of functional CFTR results in thick mucous secretions in the lung and intestines, as well as pancreatic deficiency. Although CFTR is expressed at high levels in the kidney, mutations in CFTR result in little or no apparent kidney dysfunction. In an effort to understand this phenomenon, we analyzed ΔF508 CFTR maturation and function in kidney cells under conditions that are common to the kidney, namely osmotic stress. Kidney cells (mIMCD3) were grown in culture and adapted to 250 mM NaCl and 250 mM urea. HPLC analysis of lysates from kidney cells adapted to these conditions identified an increase in the cellular osmolytes glycerophosphoryl-choline (GPC), myo-inositol, sorbitol and taurine. In contrast to iso-osmotic conditions, hyperosmotic stress led to the proper folding and processing of ΔF508 CFTR to its mature form (band C). Furthermore, three of the cellular osmolytes (myo-inositol, sorbitol and taurine) themselves, when added individually to cells, proved effective in promoting the proper folding and processing of the ΔF508 CFTR protein in both epithelial and fibroblast cells. Whole-cell patch clamping of osmolyte-treated cells showed that ΔF508 CFTR had trafficked to the plasma membrane and was activated by forskolin. Encouraged by these findings, we looked at other features common to the kidney that may impact ΔF508 maturation and function. Interestingly, a small molecule, S-nitrosoglutathione (GSNO) which is a substrate for gamma glutamyltranspeptidase, an abundant enzyme in the kidney, likewise promoted Δ508 CFTR maturation and function. GSNO-corrected ΔF508 CFTR exhibited a shorter half-life as compared to wt CFTR. These results demonstrate the feasibility of a small molecule approach as a therapeutic treatment in promoting ΔF508 CFTR maturation and function and suggests that an additional treatment maybe required to stabilize ΔF508 CFTR protein once present at the plasma membrane. Finally, our observations may help to explain why ΔF508 homozygous patients do not present with kidney dysfunction.
INTRODUCTION

The cystic fibrosis transmembrane regulator protein (CFTR) is a cAMP-regulated chloride channel expressed in epithelial cells. Mutations within this protein cause cystic fibrosis, a disease of altered electrolyte transport and dehydration of secretions. The hallmark of CF is the accumulation of thick mucous secretions and frequent colonization of pseudomonas bacterium within the lungs. Lung function deteriorates with age and lung pathology is the leading cause of morbidity and mortality. Other organs affected by this disease are the pancreas, the intestines and the liver. Interestingly, whereas CFTR is expressed in the kidney, CF patients show little or no kidney dysfunction. Indeed, Dr. di Sant’Agnese, who first identified abnormally high sodium chloride in the sweat of CF patients, also noted “the disassociation between increased electrolytes in the sweat and the low sodium and chloride levels in urine during the periods of low salt diet or heat waves appears to be a unique characteristic of CF” (1). In other words, the ability of the kidney to regulate sodium chloride levels in the urine of CF patients appeared normal.

The most prevalent mutation associated with the disease is the deletion of a phenylalanine residue at position 508 ($\Delta$F508 CFTR) (2). Loss of this single amino acid results in a failure of the newly synthesized protein to move out of the endoplasmic reticulum (ER) to its finale locale at the plasma membrane (3). This failure is due to the synthesis of a protein that apparently cannot fold properly. Consequently, the protein is retained in the ER and degraded by the ubiquitin-proteosome pathway (4,5). Validation for the idea that the $\Delta$F508 CFTR mutation leads to improper folding is supported by the facts that; (1) misfolding of the protein can be corrected by lowering the temperature of cells expressing the mutant protein (6), (2) correct folding of the protein can occur upon addition of protein stabilizing agents, such as glycerol and TMAO (7,8) and (3) once corrected, the $\Delta$F508 protein can traffic to the plasma membrane and function as a cAMP-regulated chloride channel (6-9).

During osmotic stress, yeast and sharks increase the intracellular levels of glycerol and TMAO respectively, two cellular osmolytes known to protect proteins from denaturation under harsh environmental conditions and as mentioned above, effective in correcting $\Delta$F508 CFTR protein folding (10,11). Similarly, mammalian systems have their own set of osmolytes, including sorbitol, myo-inositol, betaine, taurine and
glycerophosphorylcholine (GPC) (12,13). These compounds typically accumulate to high concentrations in the kidney medulla as part of the normal physiological process of concentrating urine. In contrast to other organs of epithelial origin, the kidney is considered unaffected in ΔF508 CFTR homozygotes. Therefore, we hypothesized that osmolytes that are synthesized and accumulated within kidney medulla might promote the proper folding and function of the ΔF508 CFTR protein and therefore help explain why ΔF508 CF patients do not present with kidney dysfunction.

The iso-osmotic renal cortex also expresses CFTR, but unlike the renal medulla, the absence of disease phenotype cannot be explained by elevated osmolality. Recently, Zaman and colleagues reported that S-nitrosoglutathione (GSNO) promotes the maturation of endogenous wild type and ΔF508 CFTR maturation, though functional data were not presented (14). Interestingly, GSNO is a substrate for gamma-glutamyltranspeptidase, (15) a transmembrane protein expressed at high levels in the lumen of the renal cortical proximal kidney (16-18). Here we show that GSNO promotes ΔF508 maturation and restores function while having no effect on wild type CFTR maturation. Thus, our results point to two possible mechanisms, intracellular osmolyte accumulation and hydrolysis of GSNO, carried out by the kidney, as potential explanations why CF patients exhibit little or no kidney dysfunction.

**EXPERIMENTAL PROCEDURES**

**Cell culture and adenovirus infections**

All cells were maintained at 37° C and 5% CO2. Mouse inner medullary collecting duct cells (mIMCD3) isolated from the terminal one-third of the IMCD (19) were cultured in DMEM/F12 supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. 3T3 cells stably transfected with either the wild type or ΔF508 CFTR plasmid (20) were cultured in DMEM supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin. For osmotic adaptation experiments, cells were gradually adapted to increasing concentrations of equimolar NaCl and urea over a 3-week period to a final concentration of 250 mM NaCl and 250 mM urea. Cells were maintained at this concentration for an additional 2-3 weeks before the initiation of experiments. For cellular osmolyte treatments, the compounds were added directly to the
growth medium of the cells at the concentrations indicated in the text. Expression of either wild type or ΔF508 CFTR using recombinant adenovirus ((21), ΔF508 adenovirus, purchased from Dr. J. Wilson, University of Pennsylvania) was carried out as previously described (21). Twenty-four hours post-infection, 2 mM sodium butyrate was added to the cells to increase transcription of the CFTR cDNA. Eighteen hours later, cells were either harvested for biochemical analysis or used for whole-cell patch clamping.

**Immunoblotting**

Cells were washed twice in calcium free PBS and lysed in 50 mM Tris-HCl, pH 7.4; 1% NP-40; 0.25% sodium deoxycholate; 150 mM NaCl; 1 mM EGTA supplemented with protease inhibitors (Calbiochem). The lysates were clarified at 14,000 X g at 4°C. An aliquot of the supernatant was sampled for protein determination (BCA, Pierce) while the remaining sample was adjusted to 1X Laemmli sample buffer (40 mM Tris/HCl pH 6.8, 1% SDS, 50 mM DTT, 7.5% glycerol) and heated at 37°C for 20 minutes. Equal amounts of total protein were loaded on 7.5% polyacrylamide gels. Following SDS-PAGE, the proteins were transferred to nitrocellulose and probed with a monoclonal antibody specific for the CFTR protein (0.3 µg/ml M3A7, Upstate Biotechnology) and an HRP-conjugated goat anti-mouse secondary antibody at 1:5,000. CFTR protein was visualized by chemiluminescence.

**Radiolabeling and Immunoprecipitation**

Cells were incubated in methionine-free medium for 15 minutes prior to the addition of 50 µCi/ml of [35S] methionine for 4 hours in either the absence or presence of 100 µM GSNO. Increasing the concentration of GSNO resulted in an increase in ΔF508 CFTR protein maturation and thus increased levels of [35S] methionine incorporated into the nascent protein. This enabled better detection of the newly synthesized ΔF508 CFTR protein during the pulse-chase and immunoprecipitation experiments. After 4 hours of labeling, the cells were washed and one set of plates was harvested immediately for immunoprecipitation. To the remaining plates, the medium containing the radiolabel was removed, the cells washed with fresh medium and then incubated in medium containing 1 mM cold methionine for 4 hours in either the absence or presence of GSNO and then
harvested as described above. CFTR was immunoprecipitated from each sample (after being adjusted for equal protein concentration) at 4°C overnight with 2 µg each of M3A7 and L12B4 (Upstate Biotechnology). Protein G sepharose was added to the lysate for 1 hour at 4°C, pelleted at 5,000 X g for 10 seconds and washed twice with RIPA (1% Triton X-100, 1%sodium deoxycholate in PBS) buffer. The immune complexes were incubated with 2x LSB for 30 minutes at 37°C, the beads pelleted and the supernatant then loaded onto a 7.5% gel. Proteins were visualized by fluorography.

**Patch clamping**

Cells were whole cell patch-clamped at 37 °C as previously described (22). On the stage of an inverted microscope cells were bathed in (in mM): 136 N-methyl-D-glucamine (NMDG), 127 HCl, 1.7 CaCl₂, 1 MgCl₂, 10 Hapes, 10 glucose, 500 mannitol, pH 7.3. Patch pipettes were filled with (in mM): 130 NMDG, 130 HCl, 10 EGTA, 1 MgCl₂, 2 Hapes, 1 glucose, 500 mannitol, 5 Mg-ATP, 0.1 Li-GTP, pH 7.3. Single isolated cells were randomly selected for patch clamping. Only seals >10 GΩ were used. After establishing the whole cell configuration the membrane potential (Vm) was continuously clamped to −40 mV. The access resistance (Ra) and the cell membrane capacitance (Cm) were measured using the current transients caused by a 10-mV voltage pulse. Ra = 15 ± 1.2 MΩ (n = 104) and was not affected by the different treatments. Current-voltage (I-V) step protocols before and after addition of 20 µM forskolin were applied, from a resting potential of -40 mV, from -100 mV to +40 mV and the resulting currents were recorded. Larger voltage jumps reduced the success rate of experiments due to seal breakage and were generally not performed. Whole cell conductance (Gc) was calculated as the slope between −40 mV and −20 mV. For the calculation of the specific membrane conductance (Gm in pS/pF) Gc was corrected for Ra and normalized to Cm. Quantitative effects of different treatments were compared with factorial ANOVAs followed by Bonferroni-corrected t tests. P < 0.05 was considered significant. Calculations were done with StatView version 4.5 (Abacus Concepts, Berkeley, CA).
Determination of Cellular Osmolyte Concentration

Control cells (mIMCD3) and hyperosmolar-adapted cells (HT500) were grown on 10 cm plates. Intracellular osmolyte concentrations were determined via HPLC analysis using a modified protocol as described by Rauchman et al. (19). Briefly, cells were lysed in 7% perchloric acid and the lysates centrifuged at 14,000 g for 20 min. The acid insoluble pellet was resuspended in a 0.5 N NaOH solution and used for subsequent protein analysis using the BCA assay. The acid soluble material was adjusted to neutral pH and centrifuged at 10,000 g for 30 seconds. The supernatant was serially filtered through a C8 Sep-Pak column and a 0.45µm HV filter, lyophilized and resuspended in HPLC mobile-phase solution and loaded onto an HPLC column. Osmolytes were measured by refractive index.

Chemicals and reagents

Forskolin (Calbiochem) was made as a 20 mM stock in DMSO and used at 20 µM. GSNO (Sigma) prepared as a fresh stock each time, was dissolved in water at 10 mM and used at 10 µM. Osmolytes (Sigma) were dissolved in media at 1M and used at 300 mM.

RESULTS

Hyperosmotic-adapted kidney cells accumulate osmolytes.

Mouse inner medullary collecting duct (mIMCD3) cells were cultured under iso-osmotic or hyperosmotic conditions. Cells were stepwise adapted to hyperosmotic conditions by the addition of a 50 mM solution of sodium chloride and urea every 4 days. The final concentration of sodium chloride and urea added was 250 mM each, and therefore the cells are referred to as HT500 (hypertonic 500 mM, 750 mosmoles). The cells retained a number of phenotypic characteristics typical of the kidney, such as tolerance to hyperosmotic conditions for long periods of time with minimal adverse effects on DNA, RNA and protein synthesis (19,23). Adaptation to the hyperosmotic conditions resulted in a significant increase in the intracellular levels of several osmolytes in the kidney cells, in particular, sorbitol, glycerophosphorylcholine, taurine and myo-inositol (figure 1A, filled bars). These changes in the levels of the osmolytes are consistent with levels seen in the kidney medulla, a segment of the kidney routinely
exposed to hyperosmolarity (13). Interestingly, no increase in betaine was observed in mIMCD cells adapted to 750 mosmol. On the other hand, Rauchman et al. (19) observed an increase in betaine in mIMCD cells adapted to 910 mosmol while Peterson et al. (24) reported that betaine does not increase in the inner medulla of rats subjected to a high protein diet e.g., a diet that increases urea levels in the kidney.

Hyperosmotic-adapted kidney cells exhibit proper maturation and functional ΔF508 CFTR. Having established conditions that increase osmolyte levels in the mIMCD3 cells, we tested whether the accumulation of these osmolytes would have any effect on the maturation of the ΔF508 CFTR protein. The rationale based on previous studies showing that osmolytes such as glycerol and TMAO, when added to cells, correct the folding and trafficking of ΔF508 CFTR (7,8). Control mIMCD3 cells maintained in iso-osmotic growth medium, and HT500 cells maintained in hyperosmotic growth medium were infected with a recombinant adenovirus expressing either wild type or ΔF508 CFTR. Neither mock-infected mIMCD3 cells nor mock-infected HT500 cells synthesized any detectable CFTR protein, as determined by Western blotting analysis (figure 1B, lanes 1 & 4 respectively). Infection of the mIMCD3 and HT500 cells with a recombinant adenovirus expressing wild type CFTR resulted in the synthesis and detection of both the immature core-glycosylated B band, and the mature fully glycosylated C band (figure 1B, lanes 2 & 5). Expression of the ΔF508 CFTR protein in mIMCD3 cells resulted in the appearance of only the core-glycosylated protein (band B), as is normally seen in cells expressing the ΔF508 mutant (figure 1B, lane 3). In the ΔF508 CFTR-infected HT500 cells, however, an increase in the amount of the core-glycosylated B band, as well as the processing of a portion of the protein to the mature form (C band) was observed (figure 1B, lane 6). Interestingly, ΔF508 CFTR expressed in the HT500 cells exhibited multiple electrophoretic species as evidenced by a smear of bands with slower mobility as compared to the immature B band. At the present time, the exact basis for this heterogeneous collection of CFTR species is not clear. Others, however, have similarly shown the mature CFTR protein to exist as a “broad smear” (5,25,26). Thus, it appears that hyperosmotic conditions promote the proper processing of at least a portion of the...
mutant ΔF508 CFTR protein, but have no apparent effect on the maturation of wild type CFTR.

To test whether the hyperosmotic stress resulted in the synthesis and folding of a functional ΔF508 CFTR protein, the HT500 cells were whole cell patch-clamped in order to quantify forskolin-stimulated chloride transport. Whole cell currents were measured under conditions selective for chloride currents in the presence of 20 μM forskolin (see Methods). Figure 1C shows typical examples of current step responses recorded from mock infected and ΔF508 CFTR-infected HT500 cells. HT500 cells expressing ΔF508 CFTR showed large forskolin-activated chloride currents. Measured currents were time- and voltage-independent (Fig. 1C) and showed linear current-voltage relationships (Fig. 1D). Figure 1E summarizes the calculated specific membrane conductances (Gm). Thus, whole cell patch-clamping demonstrated that ΔF508 CFTR expressed in HT500 cells showed significant function, exhibiting typical CFTR-specific characteristics, i.e. chloride selectivity, activation by forskolin, and linear current-voltage relationships.

*Individual osmolytes in fibroblast cells promote the maturation of functional of ΔF508 CFTR*

Having shown that hyperosmotic stress both increases the levels of different osmolytes and promotes the maturation of ΔF508 CFTR, we tested whether the individual osmolytes themselves were effective in correcting ΔF508 misfolding and thereby would restore chloride channel function. If so, this would indicate that the osmolytes themselves, rather than some other effect due to salt and urea adaptation of renal cells, can help promote ΔF508 CFTR protein maturation. For these experiments, we utilized 3T3 fibroblasts stably expressing ΔF508 CFTR under the control of the CMV promoter (20). Using these cells, we previously showed that two other osmolytes, glycerol and TMAO, were effective in correcting the misfolding of the ΔF508 CFTR mutant (7). Furthermore, Denning and colleagues used these cells to show that incubation at low temperatures (e.g., 27°C) also corrected the misfolding of ΔF508 CFTR protein (6). Cells were incubated in growth medium containing the individual osmolytes for 3 days at a concentration of 300 mM, after which the cells were lysed and CFTR
expression examined by western blot (figure 2A). Cells not incubated with osmolytes nor stimulated with the general transcriptional activator, sodium butyrate, and cells stimulated with sodium butyrate alone to increase CFTR transcription, showed little or no ΔF508 protein in the cells maintained at 37°C (figure 2A, lanes 1 & 2). Treatment with the osmolytes myo-inositol, sorbitol, or taurine resulted in a portion of the ΔF508 CFTR protein being correctly folded and processed to the mature form (figure 2A, lanes 4-6). Sorbitol treatment elicited the highest levels of the mature form of the protein (figure 2A, lane 5) while betaine had little or no effect on ΔF508 CFTR maturation (figure 2A, lane 3). Curiously, betaine itself proved to be somewhat toxic to the fibroblast cells with the cells appearing rounded after 3 days of incubation with 300 mM betaine (data not shown). This result is consistent with a previous study showing betaine is toxic to renal cells when added alone to the extracellular medium but is nontoxic when added in conjunction with urea (27).

Incubation of the cells at 27°C along with the osmolytes revealed a synergistic effect on ΔF508 maturation. When incubated at 27°C, low levels of the immature form of ΔF508 CFTR were now evident and increased upon addition of the general transcriptional activator, sodium butyrate (figure 2A lanes 1 & 2). Note as well, and consistent with previous reports showing ΔF508 CFTR folding to be temperature-sensitive, the appearance of the mature form of the protein (band C) in those cells maintained at the lower temperature. When the cells growing at 27°C now were treated with the various osmolytes, significant amounts of mature ΔF508 CFTR (band C) were observed (figure 2A; lanes 4-6, 27°C). Similar to the situation at 37°C, addition of betaine (300 mM) to the cells proved to be somewhat toxic after three days of incubation (figure 2A, lane 3). Thus, ΔF508 CFTR maturation in 3T3 cells can be rescued by either lowering the temperature of the cells, or by the addition of various cellular osmolytes. Moreover, osmolyte treatment along with a lower growth temperature resulted in a synergistic enhanced effect on the maturation of the mutant protein.

To determine whether the osmolyte-corrected ΔF508 CFTR protein was in fact functional, the 3T3 cells were whole cell patch-clamped. The cells were incubated in myo-inositol, taurine, TMAO, or sorbitol (300 mM each) for 3 days and whole cell currents were measured under conditions selective for Cl currents (see Methods). Figure
2B shows examples of current step responses recorded from the unstimulated cells (top panels) or from the forskolin-stimulated cells (bottom panels) incubated in the absence or presence of the osmolytes. Wild type CFTR-expressing 3T3 cells showed typical CFTR mediated chloride currents after forskolin stimulation (Fig. 2B, right panels). Untreated ΔF508 CFTR expressing cells, either without or with forskolin stimulation, showed no chloride currents (Fig. 2B, left panels). In contrast, three days of treatment with the different osmolytes resulted in the appearance of large forskolin-activated Cl currents. All measured currents were time- and voltage-independent (Fig. 2B) and showed linear current-voltage relations (Fig. 2C).

Figure 2D summarizes the specific conductance (Gm) from 47 patch-clamped cells. Treatment with myo-inositol, but not any of the other osmolytes resulted in a significant increase in Gm in the absence of forskolin stimulation. As a control for the effects of myo-inositol on basal currents we used its isomer scyllo-inositol. Cells incubated with scyllo-inositol showed no increase of basal Gm, but upon forskolin stimulation did exhibit an increase in Gm (data not shown). In cells treated with myo-inositol, TMAO, or sorbitol, stimulation with forskolin (20 µM) increased Gm to levels similar to that found for cells expressing wild type CFTR. Treatment with taurine resulted in the largest recovery of a forskolin-stimulated Gm, which was significantly greater than Gm measured in the wild type CFTR expressing cells (Fig. 2D).

Osmolytes promote ΔF508 maturation in epithelial cells.

Individual osmolytes proved to be effective in promoting ΔF508 CFTR maturation in fibroblasts after three days of treatment. However, because CFTR is expressed in epithelial cells we tested whether kidney cells that are normally exposed to osmolytes would be even more effective in correcting ΔF508 misfolding. mIMCD3 cells were treated with the individual osmolytes for either 24 or 72 hours. In the case of the 24-hour treatments, the cells were first infected with adenovirus expressing ΔF508 and then incubated in myo-inositol, sorbitol or taurine at a final concentration of 300 mM. For the 72-hour treatments, the mIMCD3 cells were first incubated in myo-inositol, sorbitol or taurine at a final concentration of 300 mM for 24 hours and then infected with the ΔF508 CFTR adenovirus. The infected cells then were further incubated with the
individual osmolytes for an additional 48 hours prior to cell harvest. Twenty-four hours of treatment resulted in the appearance of low levels of mature ΔF508 CFTR (figure 3A, lanes 2-4). A novel electrophoretic species of CFTR (indicated as B1) was observed in the epithelial cells treated with the osmolytes. Following 72-hours of treatment, large increases in the amount of the ΔF508 CFTR band C were now observed (figure 3B, lanes 2-4). Thus, individual osmolyte treatment of epithelial cells promotes ΔF508 CFTR protein maturation and reveals a putative folding intermediate (i.e., B1) not normally observed for wild type CFTR maturation.

*S-nitrosoglutathione promotes the maturation resulting in functional ΔF508 CFTR.*

Osmolytes accumulate in the cells of the inner medullary collecting duct in response to hyperosmotic stress as the kidney concentrates the urine. As shown above, some of these osmolytes can help to promote the folding of the ΔF508 CFTR, resulting in functional CFTR at the plasma membrane. However, CFTR also is expressed in other segments of the kidney that are not subjected to high osmotic stress. As part of our effort to explain the lack of kidney phenotype in patients homozygous for ΔF508 CFTR, we searched for other aspects unique to the kidney that might affect CFTR maturation. In searching the literature, we found a study by Zaman and colleagues reporting that GNSO treatment promoted the maturation of both wild type and ΔF508 CFTR (14). Although data demonstrating that the corrected ΔF508 could now function as a chloride channel were not presented, we were intrigued since GSNO is a substrate for gamma glutamyltranspeptidase (GGT) an enzyme that is expressed at high levels in the kidney (16-18). GGT hydrolyses glutathione and S-substituted GSH derivatives within the kidney filtrate (28) for subsequent uptake into the cells by amino acid transporters. GSNO treatment has been shown to have modifying effects (e.g., nitrosylation) on other cellular proteins thereby impacting their function (29-32). Thus, we tested whether GSNO would have any effect on ΔF508 maturation and if so, whether the corrected ΔF508 CFTR would exhibit cAMP-regulated chloride channel function. Treatment of mIMCD3 cells expressing ΔF508 CFTR with 10 μM GSNO for either 1 or 2 hours
resulted in the proper maturation of the mutant protein (figure 4A lanes 2-4). Treatment of the cells expressing wild type CFTR with GSNO had no obvious effect on the amounts of mature CFTR produced (figure 4B lanes 2-4). Next, we examined whether the GSNO-corrected ΔF508 protein exhibited functional chloride channel activity. Whole cell currents were recorded from forskolin-stimulated (20 μM) ΔF508 CFTR-expressing mIMCD3 cells pre-treated with 10 μM GSNO and compared to untreated or mock-infected cells (figure 4C). The GSNO-treated cells, but not the untreated ΔF508 CFTR-infected mIMCD3 cells, showed linear time- and voltage-independent current characteristics (figure 4, panel D). In comparison, mock-infected or untreated ΔF508 CFTR infected cells showed very small Gm values that were not statistically different from one another (figure 4, panel E.). Thus, short term GSNO treatment results in a portion of ΔF508 CFTR to mature into the C band resulting in CFTR forskolin-stimulated chloride transport.

**GSNO-corrected ΔF508 CFTR is unstable compared to wild type CFTR.**

The relatively low concentration required and the fast kinetics of GSNO-induced ΔF508 CFTR protein maturation highlighted this naturally occurring compound as a potential therapeutic agent. To pursue this possibility further, we investigated the stability of GSNO-corrected ΔF508 CFTR protein. The impetus here being based on Sharma et al. who reported that ΔF508 CFTR, corrected either by low temperature or by glycerol, was unstable when the cells expressing the corrected ΔF508 CFTR were returned to 37°C (26). mIMCD3 cells expressing either wild type or ΔF508 CFTR were pulse-labeled with [35S] methionine for 4 hours in either the absence or presence of 100 μM GSNO. The medium containing the radiolabel was removed; fresh medium was added to the cells and further incubated for 4 hours (i.e., chase) either in the absence or in the presence of GSNO prior to quantitative immunoprecipitation (figure 5). Immediately following the pulse labeling, significant amounts of the mature form (C band) of the ΔF508 CFTR protein were observed. The ΔF508 CFTR protein corrected by GSNO treatment proved to be unstable and little of the radiolabeled protein was detected after the four-hour chase period, either in the absence or in the presence of GSNO. Thus,
while our western blot analysis showed the corrected ΔF508 CFTR, presumably due to continuous maturation of nascent ΔF508 CFTR protein in the presence of GSNO, our pulse-chase studies showed that once ΔF508 CFTR protein had folded and trafficked to the plasma membrane, the protein was unstable.

**DISCUSSION**

Cystic fibrosis is a disease affecting epithelial cell function in various organs such as lung, intestines, pancreas, and liver as well as the reproductive system. However, even though CFTR is expressed in the kidney (33,34), cystic fibrosis patients present with little or no kidney dysfunction. The absence of kidney disease suggests that either other chloride channels can complement the function of CFTR in the kidney (e.g., there is a redundancy in the kidney with regards to chloride channels), or that some other mechanisms are operative in the kidney that prevail over the particular CFTR mutation. In this regard, studies by Morales and colleagues reported a unique isoform of CFTR expressed in the renal medulla comprising only the first transmembrane domain, the nucleotide binding domain and the R domain (34). The authors suggested that the generation of this unique functional isoform of CFTR in the kidney may be one mechanism by which the kidney is protected from functional defects of CF. In their study, the authors demonstrated that this unique isoform functioned as a cAMP-regulated chloride channel, and was effective in correcting the regulation of the outwardly rectifying chloride channel (ORCC). Thus, for CF patients with disease-associated mutations in the carboxy-half of CFTR, expression of this alternate isoform expressing only the amino terminal half of CFTR would maintain chloride transport necessary for normal kidney function. Alternatively, the kidney by the very nature of its function (i.e., to concentrate urine) is routinely exposed to hyperosmotic conditions, specifically in the medulla. While similar hyperosmotic conditions likely would be deleterious to most cell types, certain cells within the kidney are able to adapt to and continue their normal physiologic functions even in the presence of high osmotic stress. Under these conditions, kidney homeostasis is preserved, at least in part, by the accumulation of cellular osmolytes that help prevent general protein denaturation (discussed further below).
In our studies here, we adapted mIMCD3 kidney cells to hyperosmotic conditions by the addition of 250 mM sodium chloride and 250 mM urea. By HPLC analysis, we found that the hyperosmotically-adapted mIMCD3 cells accumulated a number of intracellular osmolytes including GPC, myo-inositol, sorbitol and taurine. The accumulation of the osmolytes was accompanied by an ability of the cells to produce a functional form of the ΔF508 CFTR mutant. Furthermore, when these different osmolytes were added individually to the medium of ΔF508 CFTR expressing cells, a portion of ΔF508 CFTR was processed to its mature form and resulted in the cells exhibiting cAMP-regulated CFTR chloride channel function. Interesting, myo-inositol activated CFTR channels in the absence of agonist. While not within the scope of this study, signaling roles of inositol in both calcium release from the ER as well as activation of PKC may be involved. In this regard, others have shown that PKC has a modulating effect on CFTR channel activity (35,36).

The mechanisms by which cellular osmolytes impact protein folding and/or stability has been an active area of research (see reviews (37,38)). Early work by Timasheff and colleagues (39) concluded that osmolytes help stabilize proteins in their native conformation due to their preferential exclusion from the immediate vicinity of the protein. Preferential exclusion of the osmolytes leads to an increase in the local concentration of water surrounding the protein. The resultant increase in surface tension would then favor a decrease in surface area of the protein, thereby promoting the folded state (i.e. one with the lowest energy conformation). Subsequent studies by Bolen and others (40-42) concluded that in the presence of osmolytes, unfavorable interactions of the peptide backbone (but not the R groups) with the osmolytes enhances proper protein folding, thereby helping to minimize these unfavorable interactions. In other words, the same general principles that govern protein folding events under iso-osmotic conditions, in particular the hydrophobic effect, are likely amplified in solutions containing high concentrations of the osmolytes. Thus, in the case of ΔF508 CFTR where a potentially critical hydrophobic amino acid phenylalanine is absent, we suspect that an osmolyte-enhancement of the hydrophobic effect may help drive (and/or stabilize) an early folding intermediate requiring hydrophobic interactions in and around position 508. As a consequence, at least a portion of the folding intermediates continues to fold to the native
state and thereby escape the quality control pathway operative in the endoplasmic reticulum. Subsequent maturation in the Golgi followed by localization at the plasma membrane results in a ΔF508 CFTR protein that appears functional as assayed by forskolin-dependent Cl transport.

Our results demonstrating that osmotic-stress induced osmolyte accumulation, or the addition of individual osmolytes to the medium of cells results in the rescue of ΔF508 CFTR maturation confirms and extends earlier work. Specifically, previous studies have shown that high concentrations of either glycerol or TMAO, when added to fibroblast cells in culture, restored wild type-like ΔF508 CFTR localization and chloride channel function (7,8). Moreover, both glycerol and TMAO were found to be effective in promoting the proper folding of a variety of other temperature sensitive protein folding mutants including the tumor suppressor p53, the viral oncogene protein pp60src, the ubiquitin activating enzyme E1 (43), the water channel protein aquaporin-2 (44), α1-antitrypsin (45), and α-ketoacid decarboxylase (46). In addition, cellular osmolytes appeared effective in slowing and even preventing the formation of the pathogenic form of the so-called scrapie prion protein (47). Based on these observations we have collectively referred to the cellular osmolytes as “chemical chaperones” to reflect their wide range of action in promoting the proper folding of proteins, which due to mutation, oftentimes fail to reach the native state.

Within the kidney, the inner medullary collecting ducts are routinely exposed to hyperosmotic stress and thus the accumulation of intracellular osmolytes could help to explain the lack of adverse phenotype in individuals homozygous for ΔF508 CFTR. The renal cortex, another segment of the kidney that expresses CFTR, is typically exposed to iso-osmotic conditions and therefore osmolyte accumulation would not likely explain any lack of adverse phenotype. During the course of our studies however, Zaman and colleagues reported that another small molecule, S-nitrosoglutathione (GSNO) acted to promote the proper maturation of both the wild type and ΔF508 forms of CFTR (14). Consistent with their report we found that the addition of GSNO to the medium of ΔF508 expressing IMCD3 cells resulted in the proper maturation of ΔF508 CFTR. In addition, we showed that the GSNO-treated cells expressing the ΔF508 CFTR mutant now were
capable of carrying out forskolin-mediated Cl transport. Recently, Andersson and colleagues reported similar effects of GSNO on ΔF508 CFTR expressing cells using a fluorescent-based assay (48). Our data showed that the maturation of ΔF508 CFTR occurred using rather low concentrations (e.g., 10 µM) of GSNO, and was very rapid (within 1 hour the mature form of the ΔF508 CFTR protein was readily apparent by western blot analysis).

The mechanism by which ΔF508 CFTR maturation and subsequent function are rescued by GSNO treatment is currently unknown. GSNO (a thiol-derivative of glutathione) is a reservoir of both nitric oxide (NO) and glutathione. NO at low concentrations can act as a signaling molecule, activating guanylate cyclase and the subsequent formation of the second messenger cGMP (see review (49)). cGMP in turn is known to activate various ion channels and kinases (49). Higher concentrations of NO can lead to the modification of cysteine thiols (nitrosylation) leading to either activation or inactivation of the protein target and thus affect various cellular functions including transcription, ion channel function, chaperone function and proteolysis (reviewed in (50)). Contradictory evidence of the effect of NO on CFTR channel function and biogenesis has been reported. For example, NO has been shown to activate CFTR in human T lymphocytes (51) while Ruckes-Nilges et al. reported that NO had no effect on CFTR or any other chloride channel activation in primary nasal epithelial cells (52). GSNO, on the other hand, was found to activate chloride channels in lung epithelial cells (53). Finally, other studies have shown that NO inhibits CFTR protein expression (54,55). In the case of glutathione, this tripeptide is an important antioxidant necessary for the maintenance of the cell’s redox state. While others have suggested that the redox state of the cell is important for the activation of CFTR channels (56), in our study, the low concentration of GSNO required to promote ΔF508 CFTR maturation likely would not impact glutathione levels. Thus, we suspect that the operative mechanism by which GSNO promotes ΔF508 CFTR maturation is by modification of either cysteine thiols of the nascent ΔF508 CFTR protein, or cysteine thiols of a cellular protein(s) involved in the monitoring of quality control mechanisms of protein folding.

While GSNO levels in the blood of healthy individuals has been questioned, it may be possible that plasma GSNO is formed in vivo (57-59) due to inflammation as is
common in CF patients. In addition, intracellularly synthesized GSNO could be exported out of the cells by the MRP2 transporter, which is expressed in the proximal tubule luminal membrane of the kidney (60). Whatever the case, extracellular glutathione and S-substituted GSH derivatives are a substrate for gamma-glutamyltranspeptidase, an enzyme expressed at high levels in the luminal membrane of the kidney. Hydrolysis of GSNO would produce glutamate and S-substituted cysteinylglycine, in the kidney filtrate (15,28). Subsequent uptake of the S-substituted cysteinylglycine by amino acid transporters and further protein modifications by S-nitrosylation could be one mechanism by which ΔF508 CFTR protein maturation is rescued. Studies are underway to test this and other possibilities.

Our pulse-chase studies demonstrated that once ΔF508 CFTR was corrected by GSNO, the mature form of the protein was unstable. Whether ΔF508 CFTR is corrected by temperature, glycerol (26) or GSNO, the protein appears to be unstable relative to its wild type counterpart. Thus, the ΔF508 CFTR protein is not a typical temperature-sensitive folding mutant since most temperature-sensitive protein folding mutants, once properly folded, usually exhibit temperature-dependent stability profiles similar to the wild type protein (61). This then indicates that ΔF508, whether acquiring a functionally competent conformation via low temperature, osmolyte or GSNO treatments, presumably does not achieve a conformation identical to that of the wild type protein. Our results together with the results of Sharma and colleagues highlight the possibility that a second drug may be required to stabilize the ΔF508 CFTR protein once it has exited the ER and Golgi and trafficked to the plasma membrane.

In summary, our studies point to two mechanisms that may help to explain the absence of kidney dysfunction in CF patients; osmolyte accumulation and GSNO catabolism. In addition, our work further demonstrates the feasibility of a small molecule approach as a clinical therapy to correcting ΔF508 misfolding, while at the same time revealing a second issue that needs to be addressed in the search for therapeutic candidates i.e., the stability of the fully “mature” ΔF508 CFTR protein. While using high concentrations of osmolytes may not be clinically desirable, GSNO, a substrate for GGT, an enzyme also expressed in the lung (62), is one potential therapeutic candidate. In this regard, a study of 9 CF patients receiving one dose of aerosolized GSNO showed that the
compound was safely tolerated (63). Moreover, there are known beneficial effects of GSNO in the lung including improved airway ciliary motility, airway smooth muscle relaxation, inhibition of ENaC sodium transport (64), and antimicrobial activity (for review (65). Thus, investigating the possible mechanisms by which the kidney appears to prevail over disease-associated CFTR mutations has led to the identification of potential therapeutic candidates for the treatment of CF.

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References

Figure 1: Hyperosmolarity increases intracellular osmolyte levels and promotes the maturation of ΔF508 CFTR in kidney cells. Mouse inner medullary collecting duct cells were adapted to 250 mM sodium chloride and 250 mM urea over a three week period by increasing the osmolarity of sodium chloride and urea by 50 mM every four days. **Panel A.** Intracellular osmolyte levels of both the parental and the 3 week salt-adapted cells (HT500) were determined using HPLC as described in the methods. Bars represent the average osmolyte concentration from 4 different samples for both HT500 and parental lysates. Before treatment (parental, open bars), the betaine concentration was significantly larger than the other osmolytes (p<0.0001, ANOVA). Treatment with salt and urea (HT500, filled bars) significantly increased the concentrations of myo-inositol (p<0.0001), taurine (p=0.0014), GPC (p<0.0001) and sorbitol (p<0.0001), but not betaine (p=0.13, unpaired t tests). Asterisk indicates statistical significance from parental cells. **Panel B.** Parental mIMCD cells and the salt/urea adapted cells (HT500) were infected with adenovirus expressing either wild type or ΔF508 CFTR. Thirty-six hours post-infection the cells were treated with 2 mM sodium butyrate and then harvested 18 hours later. Equal amounts of total protein were separated by SDS-PAGE and CFTR protein expression was determined by western blot analysis. Lanes 1 & 4, mock infected cells; Lanes 2 & 5, wild type CFTR protein expression; Lanes 3 & 6, ΔF508 CFTR protein expression. B demarcates the immature core-glycosylated CFTR protein; C demarcates the fully-glycosylated mature form of CFTR protein. **Panel C.** Representative recordings of currents elicited by voltage steps (from −100 mV to +40 mV) in mock- and ΔF508 CFTR adenovirus infected HT500 cells after stimulation with 20 μM forskolin. **Panel D.** Current-voltage relationship of currents recorded in panel C.
Open circles, mock-infected cells; filled circles, ΔF508 CFTR-infected HT500 cells.

Panel E. Specific conductance (Gm) for mock- and ΔF508 CFTR adenovirus infected HT500 cells. ΔF508 CFTR protein expressing cells showed a significantly higher Gm (p<0.001, t test). Average capacitance of these cells was 53+/−14 pF (n=10) and was not different between mock and ΔF508 CFTR adenovirus infected cells.

Figure 2: Sorbitol, myo-inositol and taurine promote the maturation of ΔF508 CFTR and results in cAMP-regulated chloride transport in mouse fibroblast cells.

Panel A. Osmolytes were added to a final concentration of 300 mM to the extracellular medium of 3T3 cells stably expressing ΔF508 CFTR over a 24-hour period as described in the methods. The cells were maintained in the medium containing the osmolytes and further incubated at either 27° C or 37° C for 24 hours. One plate of cells was left untreated while all the other cells were treated with 2 mM sodium butyrate. Eighteen hours later the cells were harvested, equal amounts of total protein were separated by SDS-PAGE and CFTR expression was determined by western blot. Lane 1, no sodium butyrate; Lane 2, sodium butyrate added; Lane 3, betaine; Lane 4, myo-inositol; Lane 5, sorbitol; Lane 6, taurine. The temperatures at the top of the panels indicate the incubation temperatures of the cells. Panel B. 3T3 cells were adapted to 300 mM osmolytes as described above and analyzed for cAMP-regulated chloride channel function by whole cell patch clamp. Current step responses to a voltage protocol from -100 mV to +40 mV in 20 mV increments from a -40 mV holding potential as depicted in inset. Top panels show unstimulated and the bottom panels show forskolin-stimulated recordings for the given treatments (left to right). Panel C. Current voltage relations of
forskolin-activated currents. **Panel D.** Averages of specific membrane conductance Gm of unstimulated (filled bars) or forskolin-stimulated (20 µM, open bars) cells treated with osmolytes. Mean +/- SEM are given, number of cells investigated for every condition is given above each bar. * indicates significant difference from untreated cells, # indicates significant difference from respective unstimulated value (p<0.05, factorial ANOVA).

Effect of taurine treatment resulted in significantly larger Gm than wild type CFTR.

**Figure 3: Osmolyte treatment of epithelial cells promotes processing of ΔF508**

**CFTR protein.** mIMCD3 epithelial cells expressing ΔF508 CFTR were incubated with osmolytes for 24 or 72 hours prior to cells lysis. In the case of the 24-hour treatments, the mIMCD3 cells were first infected with adenovirus expressing ΔF508 and then incubated in either myo-inositol, sorbitol or taurine at a final concentration of 300 mM before cell lysis (**Panel A**). For the 72-hour treatments, the mIMCD3 cells were first incubated in myo-inositol, sorbitol or taurine at a final concentration of 300 mM for 24 hours and then infected with the ΔF508 CFTR adenovirus. The infected cells were further incubated with the individual osmolytes for another 48 hours prior to cell lysis (**Panel B**). Cells were stimulated with 2 mM sodium butyrate and harvested 18 hours later. Equal amounts of total protein were separated by SDS-PAGE and CFTR expression was determined by western blot. Lane 1, no osmolytes; Lane 2, myo-inositol; Lane 3, sorbitol; Lane 4, taurine.

**Figure 4: GSNO promotes the maturation and function of ΔF508 CFTR but has no effect on wild type CFTR maturation.** mIMCD cells were infected with either ΔF508
or wild type CFTR recombinant adenovirus as described previously. Cells were left untreated or were treated with 10 μM GNSO for 1 or 2 hours prior to cell lysis. Equal amounts of total protein were separated by SDS-PAGE and CFTR expression was determined by western blot. **Panel A**, ΔF508 CFTR: Lane 1, mock-infected parental cells; lane 2, ΔF508 CFTR expressing cells, untreated; lane 3, 1 hour treatment of 10 μM GSNO; lane 4, 2 hour treatment with 10 μM GSNO. **Panel B**, wild type CFTR: Lane 1, mock-infected parental cells; lane 2, wt CFTR expressing cells, untreated; lane 3, 1 hour treatment of 10 μM GSNO; lane 4, 2 hour treatment with 10 μM GSNO. **Panel C**. Representative recordings of currents elicited by voltage steps (from −100 mV to +40 mV) in ΔF508 CFTR-infected mIMCD cells after stimulation with 20 μM forskolin. Very small currents were recorded in untreated cells. Treatments with GSNO resulted in forskolin-stimulated time- and voltage-independent currents. **Panel D**. Current-voltage relations of currents recorded in C. Open circles, untreated cells: filled circles, GSNO-treated cells. **Panel E**. Specific conductance (Gm) for GSNO-treated and untreated ΔF508 CFTR-or mock-infected mIMCD cells. GSNO-treated ΔF508 CFTR-infected cells showed a significantly higher Gm (P= 0.033, factorial ANOVA).

**Figure 5**: GSNO-corrected ΔF508 CFTR protein is less stable than wild type CFTR protein. mIMCD3 cells were infected with either wild type or ΔF508 CFTR recombinant adenovirus. Twenty-four hours post-infection, cells were treated with 2 mM sodium butyrate. Fourteen hours post-induction, 50 μCi/ml of [35S] methionine was added to the medium either in the absence or presence of 100μM GSNO. After 4 hours, cells were washed and either harvested immediately (pulse-labeled) or the medium was
removed, the cells washed and further incubated in medium supplemented with 1 mM cold methionine for 4 hours either in the absence or presence of GSNO before being harvested (pulse-labeled and chase). The amount of radiolabeled CFTR present in the lysates was determined by quantitative immunoprecipitation as described in the methods. Shown is a fluorogram of the gel. Treatment of the cells without (-) or with (+) GSNO during the pulse and chase is indicated at the top.
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- Pulse 4h
- Chase 4h

Figure 5
Mammalian osmolytes and S-nitrosoglutathione promote δF508 CFTR protein maturation and function
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