Glycerol Reverses the Misfolding Phenotype of the Most Common Cystic Fibrosis Mutation*

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The common ΔF508 mutation in the cystic fibrosis transmembrane conductance regulator (CFTR) interferes with the biosynthetic folding of nascent CFTR polypeptides, leading to their retention and rapid degradation in an intracellular compartment proximal to the Golgi apparatus. Neither the pathway by which wild-type CFTR folds nor the mechanism by which the Phe508 deletion interferes with this process is well understood. We have investigated the effect of glycerol, a polyhydric alcohol known to stabilize protein conformation, on the folding of CFTR and ΔF508 in vivo. Incubation of transient and stable ΔF508 tranfectants with 10% glycerol induced a significant accumulation of ΔF508 protein bearing complex N-linked oligosaccharides, indicative of their transit to a compartment distal to the endoplasmic reticulum (ER). This accumulation was accompanied by an increase in mean whole cell cAMP activated chloride conductance, suggesting that the glycerol-rescued ΔF508 polypeptides form functional plasma membrane CFTR channels. These effects were dose- and time-dependent and fully reversible. Glycerol treatment also stabilized immature (core-glycosylated) ΔF508 and CFTR molecules that are normally degraded rapidly. These effects of glycerol were not due to a general disruption of ER quality control processes but appeared to correlate with the degree of temperature sensitivity of specific CFTR mutations. These data suggest a model in which glycerol serves to stabilize an otherwise unstable intermediate in CFTR biosynthesis, maintaining it in a conformation that is competent for folding and subsequent release from the ER quality control apparatus.

Cystic fibrosis (CF), a lethal hereditary exocrinopathy affecting approximately one in two thousand live births among populations of Caucasian or northern European descent, is caused by the functional absence of a plasma membrane chloride channel, designated cystic fibrosis transmembrane conductance regulator (CFTR) (1). The vast majority of severe CF cases in these populations is linked to a single genetic lesion, deletion of a phenylalanine codon (ΔF508) (2, 3), which interferes with the folding of newly synthesized CFTR polypeptides. Nascent ΔF508 molecules fail to traffic to the plasma membrane (4) but rather are retained by the ER quality control mechanism that prevents unfolded or misfolded proteins and unassembled subunits from exiting the ER. Instead, these retained immature ΔF508 molecules are rapidly degraded (5, 6) in a pre-Golgi compartment by a process that appears to require covalent modification by ubiquitin (7). Moreover, plasma membrane CFTR-like Cl⁻ channel activity can be detected when ΔF508 is overexpressed (8) or synthesized at reduced temperature (9), suggesting that Phe508 does not play an essential role in CFTR function and raising the possibility of therapeutic intervention in CF by increasing the efficiency of ΔF508 folding.

Glycerol and other polyols are known to stabilize protein conformation (10), increase the rate of intrins protein refolding (11), and increase the kinetics of oligomeric assembly (12). We report here that treatment of ΔF508-expressing cells with glycerol dramatically stabilizes newly synthesized ΔF508 polypeptides and leads to the accumulation, in the plasma membrane, of stable, functional CFTR Cl⁻ channels.

EXPERIMENTAL PROCEDURES

Cell Culture and Transient Transfections—Cells were cultured and transfected exactly as described previously (5). Mutant cDNA constructs were engineered as described in (13).

Pulse-Chase Experiments and Immunoprecipitation and Immunoblotting—Pulse-chase and immunoblotting experiments were performed as described previously (5), with the following modifications in the presence of glycerol. Transfected HEK cells were first treated with 5% glycerol in methionine and cysteine-free DMEM supplemented with 5% dialyzed FCS for 30 min. HEK cells, which were floating after this treatment, were spun down and suspended in 10% glycerol in methionine and cysteine-free DMEM-FCS. Cells were pulse-labeled with [35S]protein labeling mix (1175 Ci/mmol, DuPont NEN) at a concentration of 0.5 μCi/ml for 15 min and chased with DMEM-FCS with 10 mM methionine and 4 mM cysteine.

Kinetics of Glycerol Uptake—Untransfected HEK cells were treated with 7.5% glycerol in DMEM-FCS for 30 min, and cells were spun down and resuspended in 3 ml of DMEM-FCS supplemented with 7.5% glycerol. Glycerol uptake was measured by incubating untransfected HEK cells at 37 °C with [3H]glycerol (0.1 μCi/ml, DuPont NEN) for various times. Cell-associated [3H]glycerol was separated from free [3H]glycerol by rapid centrifugation (25 s at 16,000 × g) through an 80-μl cushion of silicon oil (Versalube, General Electric). After removing the supernatant, the bottom of the tube was cut, and the cell pellets were extracted in 1% Triton X-100 for determination of cell-associated radioactivity.

[3H]Glycerol uptake measurements were adjusted for trapped extracellular volume by using [14C]sucrose (1.5 μCi/ml) as a fluid-phase marker (14).

Electrophysiological Recording—After treatment with 10% glycerol for 24 h, glycerol was removed from HEK cells expressing CFTR or ΔF508 by diluting the glycerol-containing medium slowly with fresh medium over the course of 1 h. After removal of the glycerol, cells were allowed to recover for at least 1 h prior to analysis by whole cell patch clamp recording. The electrodes were made from Dagan LA-16 glass, Sylgard™ coated and fire polished to give a final resistance of 2–5 MΩ when filled with pipette solution. The bath solution contained in mM: 150 NaCl, 5 KCl, 2.5 CaCl2, 2.5 MgCl2, and 10 HEPES.
Glycerol and CFTR Folding

RESULTS AND DISCUSSION

The effect of glycerol on steady-state expression of ΔF508 was initially evaluated by immunoblot analysis of detergent extracts of HEK 293 cells transfected with ΔF508 cDNA (Fig. 1). In untreated cells, only the immature (core glycosylated, 140 kDa) form was detected in cells incubated at 37 °C (Fig. 1A), as previously observed (5). However, a diffuse immunoreactive band, corresponding to the mobility of mature (complex glycosylated, 165 kDa) CFTR, was apparent in extracts of cells treated with glycerol for 24 h. The steady-state levels of mature ΔF508 induced by glycerol or by incubation at reduced temperature in these cells were similar, but the effects appear to be additive. The small difference in mobility between mature ΔF508 rescued by reduced temperature in these cell was not significant (16); similar results were obtained with other structurally related polyols (1,2-propanediol or 1,3-propanediol), perhaps because of lower permeability of cell membranes to these agents. Similarly, dimethyl sulfoxide (2%) did not support ΔF508 maturation; higher concentrations were not tested because of its toxicity. Glycerol, a small (M, = 98) polyhydric alcohol, is highly permeant across the plasma membrane of animal cells (17, 18). In HEK cells incubated at 7.5% glycerol, [3H]glycerol equilibrated rapidly (t½ = 5 min) across the plasma membrane (data not shown). As glycerol is uncharged, its distribution across the plasma membrane is independent of membrane potential. Thus, it is likely that intracellular and extracellular glycerol concentrations rapidly equilibrate in HEK cells and that glycerol’s effect on ΔF508 processing is due to its high intracellular concentration.

At least two mechanisms could account for the effect of glycerol on the accumulation of mature ΔF508. One possibility is that glycerol stabilizes a ΔF508 folding intermediate, which is normally rapidly diverted to the degradation apparatus. The stabilized folding intermediate would remain competent to fold into a conformation resistant to proteolysis and permissive for maturation beyond the ER. A second possibility is that glycerol acts by increasing the stability of a mature but unstable ΔF508 polypeptide that has escaped ER retention. To discriminate between these models, the kinetics of ΔF508 maturation and degradation were evaluated by pulse-chase labeling and immunoprecipitation in ΔF508-transfected HEK cells (Fig. 2). In control cells not treated with glycerol, label in the band corresponding to immature ΔF508 decayed rapidly and was nearly undetectable after 6 h of chase (Fig. 2A). The t½ of this decay was estimated to be 45 min (Fig. 2, A and D), similar to previously reported values (5). No label was detected at the mobility corresponding to the mature protein. By contrast, in the presence of glycerol, the kinetics of immature CFTR degradation were significantly slowed (t½ = 87 min); some of this label was clearly chased into mature ΔF508 (Fig. 2, A and D). The fractional conversion of immature ΔF508 in glycerol-treated (Fig. 2E) cells ranged between 3 and 8% in separate experiments, which is considerable when compared with the ~20–25% efficiency of wild-type CFTR processing. The kinetics of wild-type CFTR degradation were also slowed by glycerol, suggesting that the effect of glycerol is not unique to the folding of ΔF508 molecules (Fig. 2, C and D). Glycerol had no measurable effect on the stability of mature ΔF508 or CFT (Fig. 2, B and E). These data suggest that accumulation of mature ΔF508 in glycerol-treated cells is not the result of an effect on the mature protein and that glycerol stabilizes the immature form of ΔF508. However, inhibition of ΔF508 degradation, either...
directly with protease inhibitors or indirectly by blocking ubiquitination, also stabilizes the immature form of the protein but, unlike glycerol treatment, does not result in any accumulation of mature forms (7). This disparity in the fate of stabilized immature ΔF508 molecules suggests that glycerol maintains immature ΔF508 in a maturation-competent state, either by inhibiting reactions that are off pathway or by enhancing reactions that are on the folding pathway.

To rule out the possibility that the effects of glycerol on ΔF508 processing and degradation are due to a general disruption of the ER quality control machinery, we examined the effect of glycerol on the maturation and degradation of other CFTR mutants that, like ΔF508, are unable to escape the ER (Fig. 3). Cells expressing the missense mutants D572A and S1251A and a mutant harboring a deletion of exon 13 (ΔEX13) were pulse-labeled with [35S]Met and chased for 5 h (Fig. 3A). These mutants were synthesized as immature polypeptides that were degraded and, unlike ΔF508, failed to mature even in the presence of glycerol. Thus, some mutations in all three major cytoplasmic domains of CFTR, including the first and second nucleotide binding domains (D572A and S1251A, respectively) as well as the “R” domain, can lead to a glycerol-insensitive ER retention phenotype, suggesting that glycerol rescue of CFTR maturation is not the result of a general suppression of ER retention mechanisms. The efficiency of processing and the ability to be rescued by glycerol are also highly dependent upon the nature of the substituted amino acid in CFTR Lys464 missense mutants (Fig. 3B). Processing of mutants K464R and K464A was inefficient by comparison with wild type and was enhanced by incubation in the presence of 10% glycerol, even after accounting for the unequal label present in the immature precursor in the presence of glycerol (Fig. 3B). By contrast, no maturation was detectable for the mutant K464W in the presence or absence of glycerol. These data support the argument that glycerol rescue of CFTR maturation is not the result of a general suppression of ER quality control and suggest a correlation between the “leakiness” of the mutation and its ability to be remediated by glycerol.

Although these data establish that glycerol treatment facilitates the maturation of ΔF508 molecules to a post-ER compartment, they do not establish that the “rescued” ΔF508 molecules actually move to and are functional at the plasma membrane. To test the functional surface expression of glycerol-rescued ΔF508 molecules, whole cell CFTR currents were measured in HEK cells expressing ΔF508 or wild type CFTR (Fig. 2). As shown in Fig. 2A, the surface expression of ΔF508 molecules was increased by approximately 2-fold in the presence of 10% glycerol compared with 0% glycerol. In contrast, CFTR currents were not significantly increased by glycerol treatment. These data suggest that glycerol rescue of ΔF508 maturation results in the functional expression of ΔF508 molecules at the plasma membrane.
Glycerol treatment induces the expression of plasma membrane ΔF508 CFTR Cl\(^{-}\) channels in the HEK cells. A, the mean whole cell maximum current density measured at −50 mV. The currents were divided by the cell capacitance in order to compare results from cell to cell. The currents in ΔF508 cells not exposed to cAMP (n = 8, column 1) or not glycerol-treated (n = 6, column 2) had maximum current at t = 0 after going whole cell. The currents in glycerol-treated ΔF508 cells exposed to protein kinase A and/or cAMP (n = 9, column 3) increased slowly and peaked at −3 min. CFTR-expressing cells responded rapidly to intracellular cAMP (n = 3, column 4), peaking at less than 1 min. pF, picofarad. B, typical whole cell currents seen in 3 cells exposed to intracellular cAMP. The currents on the left are the currents seen upon break in, and the currents on the right are currents near the peak of the response. The currents shown are in response to voltage steps from +100 mV to −75 mV in steps of −25 mV from a holding potential of −50 mV. The currents showed linear current-voltage (IV) behavior and no time dependence.

examined in HEK cells expressing CFTR or ΔF508 by the patch-clamp technique (Fig. 4). Large (56 ± 4 pA/picofarad) rapidly activated, cAMP-dependent Cl\(^{-}\) selective currents were readily observed in cells expressing CFTR but not in control (not glycerol-treated) cells expressing ΔF508 (1.54 ± 0.13 pA/picofarad). By contrast, significant (13.31 ± 2.3 pA/picofarad; p < 0.002 compared with untreated; Student’s t test) Cl\(^{-}\) currents, although slower activating than wild type, were observed in glycerol-treated cells expressing ΔF508 cDNA. These currents were not observed in the absence of cAMP, as expected of CFTR. The difference in maximal current level and activation kinetics between CFTR and glycerol-treated ΔF508 expressing cells is likely due both to the lower steady-state expression of mature ΔF508 and to the lower open probability of the mutant channels (19).

Collectively, these data suggest a model in which glycerol, a short chain polyhydric alcohol, serves to stabilize an otherwise unstable intermediate in CFTR biosynthesis, maintaining it in a conformation that is competent for folding and its subsequent release from the quality control apparatus. As degradation of immature CFTR and ΔF508 is initiated without an apparent lag following translation (5), we propose that glycerol serves to stabilize nascent ΔF508 chains soon after or during translation. In this role, glycerol would function as a chemical chaperone, much as HSP70 serves as a molecular chaperone. Our data suggest that these effects are not due to a generalized breakdown of ER quality control nor to stabilization of cell surface mature ΔF508 molecules that have escaped quality control surveillance. We hypothesize that glycerol stabilizes an early intermediate in CFTR folding that lies at a branch point between productive folding (on pathway) and competing non-productive (off pathway) steps. In this respect the effect of glycerol on ΔF508 is similar to “osmotic remedial” mutants previously observed in yeast (20) and Escherichia coli (21). These mutations are temperature-sensitive and can be reversed by increasing the osmotic potential of the incubation medium causing the microorganisms to synthesize and accumulate high concentrations of intracellular osmolytes such as glycerol (22). Interestingly, we observe a strong correlation between the temperature sensitivity of CFTR mutations like ΔF508, K464R, and K464A (data not shown) and their ability to be remediated by glycerol. Glycerol may provide a useful tool to manipulate the temperature-sensitive phenotypes of CFTR and perhaps other genes at non-permissive temperatures.

Our data establish the precedent that both the intracellular processing and the membrane Cl\(^{-}\) transport phenotypes of the ΔF508 mutation can be remediated by chemical means. These data should stimulate a search for other small membrane-permeant molecules, which may be more effective or more easily delivered than glycerol at enhancing ΔF508 processing. Finally, these data may have implications for the study or treatment of other diseases, including Alzheimer’s, retinitis pigmentosa, and proteinase inhibitor deficiency that are associated with protein misfolding.

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REFERENCES

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