Misfolding of Mutant Aquaporin-2 Water Channels in Nephrogenic Diabetes Insipidus*

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We reported that several aquaporin-2 (AQP2) point mutants that cause nephrogenic diabetes insipidus (NDI) are retained in the endoplasmic reticulum (ER) of transfected mammalian cells and degraded but can be rescued by chemical chaperones to function as plasma membrane water channels (Tamarappoo, B. K., and Verkman, A. S. (1998) J. Clin. Invest. 101, 2257–2267). To test whether mutant AQP2 proteins are misfolded, AQP2 folding was assessed by comparative detergent extractability and limited proteolysis, and AQP2 degradation kinetics was measured by label-pulse-chase and immunoprecipitation. In ER membranes from transfected CHO cells containing [35S]methionine-labeled AQP2, mutants T126M and A147T were remarkably detergent-resistant; for example wild-type AQP2 was >95% solubilized by 0.5% CHAPS whereas T126M was <10% solubilized. E258K, an NDI-causing AQP2 mutant which is retained in the Golgi, is highly detergent soluble like wild-type AQP2. The mutants and wild-type AQP2 were equally susceptible to digestion by trypsin, thermolysin, and proteinase K. Stopped-flow light scattering measurements indicated that T126M AQP2 at the ER was fully functional as a water channel. Pulse-chase studies indicated that the increased degradation rates for T126M (t½ 2.5 h) and A147T (2 h) compared with wild-type AQP2 (4 h) involve a brefeldin A-resistant, ER-dependent degradation mechanism. After growth of cells for 48 h in the chemical chaperone glycerol, AQP2 mutants T126M and A147T became properly targeted and relatively detergent-soluble. These results provide evidence that NDI-causing mutant AQP2 proteins are misfolded, but functional, and that chemical chaperones both correct the trafficking and folding defects. Strategies to facilitate protein folding might thus have therapeutic efficacy in NDI.

The kidney collecting duct epithelium plays a key role in the regulation of urine osmolality. In the unstimulated state, water permeability across the collecting duct is low, permitting the passage of a dilute urine. After stimulation by the antidiuretic hormone vasopressin, collecting duct water permeability increases by 10–20-fold, resulting in osmotically driven water transport from the tubule lumen to the interstitium and formation of a concentrated urine (1–3). The molecular basis for the increase in collecting duct water permeability is the cAMP-regulated trafficking of vesicles containing water channel aquaporin-2 (AQP2) from an intracellular compartment to the cell apical plasma membrane. More than 20 mutations in the human AQP2 protein have been identified that cause the autosomal form of hereditary nephrogenic diabetes insipidus (NDI) (4–9). Patients with NDI are unable to produce a concentrated urine and are subject to serious dehydration if not given access to large quantities of fluids (4, 9).

Several mechanisms have been proposed to explain how an abnormal AQP2 genotype produces phenotypically defective cells with low water permeability. Some mutations in aquaporins, particularly at residues near the conserved NPA motifs (10), produce non-functional water channels; human NDI mutations R187C and C181W are in this category (5, 7). Heterologous expression experiments in Xenopus oocytes suggested that misrouting to the plasma membrane may account for the abnormal phenotype of some NDI-causing AQP2 mutants (5–8). The only dominant negative AQP2 mutation, E258K, is retained in the Golgi (8, 11). When wild-type AQP2 is coexpressed with E258K, oligomerization of the mutant with wild-type AQP2 in the Golgi prevents trafficking of functional water channels to the plasma membrane (11). We recently analyzed Xenopus oocytes and transfected mammalian cells expressing wild-type AQP2 and a series of NDI-causing AQP2 mutants (12). In Xenopus oocytes, AQP2 mutants T126M and A147T had approximately the same intrinsic water permeability as wild-type AQP2. However in transfected mammalian cells, these mutants were retained at the endoplasmic reticulum (ER) and degraded more rapidly than wild-type AQP2. Because these results suggest that AQP2 mutants might be functional if expressed at the plasma membrane, several maneuvers were tested to correct the defective trafficking phenotype. Although low temperature, as done to correct F508 CFTR mistrafficking in cystic fibrosis (13), was unable to alter the cellular distribution of mutant AQP2 proteins, treatment with the “chemical chaperones” glycerol and trimethylamine-N-oxide (TMAO) corrected the ER retention and produced water-permeable cells (12). The use of chemical chaperones for pharmacotherapy of some forms of NDI was proposed. However, no information is available on the physical state of the ER-retained AQP2 protein in NDI, nor is it understood how chemical chaperones rescue mutant AQP2 proteins.

The purpose of this study was to test the hypothesis that NDI-causing AQP2 mutants are misfolded and non-functional.

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1 The abbreviations used are: AQP2, aquaporin 2; NDI, nephrogenic diabetes insipidus; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; ER, endoplasmic reticulum; TMAO, trimethylamine-N-oxide; CHO cells, Chinese hamster ovary cells; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; ERAD, ER-associated degradation; CFTR, cystic fibrosis transmembrane conductance regulator.

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at the ER and that correction of defective trafficking by the chemical chaperone glycerol is associated with corrected folding. Two methods were adopted to assess AQP2 protein folding at the ER: extractability by detergents and comparative limited proteolysis. In addition, AQP2 degradation kinetics was analyzed by pulse-chase and immunoprecipitation, and AQP2 function in the ER was measured by stopped-flow light scattering. The principal findings were that ER-retained AQP2 mutants are remarkably more detergent-resistant than wild-type AQP2, that mutant AQP2 is fully functional at the ER, and that treatment with chemical chaperones results in apparent correction of AQP2 misfolding.

EXPERIMENTAL PROCEDURES

Construction of cDNAs Encoding AQP2 Mutants—Human AQP2 point mutants E259K, T126M, and A147T were generated by site-directed mutagenesis and confirmed by sequence analysis. Epitope-tagged constructs were prepared which encoded fusion proteins consisting of the wild-type or mutant AQP2 cDNAs with 10 amino acids of the human c-Myc epitope (EQKLISEEDL) at the N terminus. Wild-type and mutated cDNAs were cloned in plasmid pCDNA3 (Invitrogen) for transfection in mammalian cells.

CHO-K1 (University of California, San Francisco Cell Culture Facility) cells were grown in Ham’s F-12 medium containing 5% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C in 5% CO2 and 95% air. For immunostaining, cells were plated at a density of 104 per 3.8 cm2 and transfected with 0.5 μg of cDNA in the presence of 3 μg of LipofectAMINE. In some experiments, 1% glycerol was added to the medium at 4 h after transfection. For metabolic labeling and subcellular fractionation, cells were plated at a density of 5 × 104 cells per well, and transfected with 1 μg of cDNA and 6 μg of LipofectAMINE per well.

Immunofluorescence—Transfected CHO cells grown on glass coverslips were fixed in PBS containing 3% paraformaldehyde and then permeabilized with 0.1% Triton X-100. Immunostaining was done with a polyclonal AQP2 antibody as described previously (12).

Intracellular Localization of AQP2—The ability of various detergents to extract wild-type versus mutant AQP2 proteins was studied in microsomes isolated from transfected, brefeldin A-treated CHO cells. Microsomes were incubated with detergents as described under “Experimental Procedures,” supernatant (soluble) and pellet (insoluble) fractions were prepared by centrifugation of the supernatant (100,000 × g, 45 min). The membrane pellet was solubilized in PBS containing 100 mM β-oglycylglycine and incubated with protein A-Sepharose CL-4B beads (Pharmacia) for 1 h at 4 °C. The beads were pelleted (100 × g, 1 min), and the supernatant was incubated with primary antibody (AQP2 or c-Myc) bound to protein A-Sepharose CL-4B for 4 h at 4 °C. Beads were pelleted (100 × g, 1 min), and washed four times in RIPA buffer (PBS containing 1% Triton X-100, 1% deoxycholate, 0.1% SDS). Proteins were released from the beads by incubation with 30 μl of Laemmli sample buffer, resolved on a 12% SDS-polyacrylamide gel, and subjected to fluorography and autoradiography. Band intensities were quantified by densitometry and normalized to the amount of AQP2 detected by immunoblotting.

Detergent Extraction—[35S]Labeled proteins from transfected CHO cells treated with 10 μg/ml brefeldin A were incubated with PBS containing 50–100 mM β-oglycylglycine, 0.05–1% Triton X-100, or 0.1–5.0% CHAPS at 4 °C for 30 min. Detergent-insoluble proteins were pelleted at 100,000 × g for 45 min and resuspended in RIPA buffer containing 0.1% SDS, 1% deoxycholate, and 1% Triton X-100. RIPA buffer was also added to the supernatant to give a final concentration of 0.1% SDS, 1% deoxycholate, and 1% Triton X-100. AQP2 was immunoprecipitated from both the supernatant (detergent-soluble) and the pellet (detergent-insoluble) fractions with a c-Myc monoclonal antibody as described above. Immunoprecipitated proteins were resolved on a 12% SDS-polyacrylamide gel and subjected to fluorography, autoradiography, and quantitative densitometry.

Limited Proteolytic Digestion—Microsomes and ER membranes obtained from transfected CHO cells were resuspended at a concentration of 0.5 mg/ml in PBS. Membranes were incubated with pepsin, trypsin, thermolysin, or proteinase K (0–10 μg/ml) for 10 min on ice. In the case of thermolysin, proteolysis was carried out in the presence of 4 mM CaCl2. Proteolysis was terminated by addition of 1 mM 4-aminobenzensulfonyl fluoride (Pefabloc), 5 mM EDTA, and samples were denatured with an equal volume of 2× Laemmli sample buffer at 50 °C for 5 min. Proteins were resolved on a 16% SDS-polyacrylamide gel and subjected to fluorography and autoradiography. Alternatively, proteins resolved by SDS-polyacrylamide gel electrophoresis were transferred to nitrocellulose membrane, and proteolytic fragments were detected by immunoblotting with an AQP2 polyclonal antibody or a monoclonal c-Myc antibody.

Subcellular Fractionation—CHO cells (control or transfected) grown on 10-cm plates were washed twice with ice-cold PBS, removed using a rubber cell scraper, and pelleted in PBS (100 × g, 10 min). Cells were resuspended in 250 mM sucrose, 100 mM Hepes, 1 mM EDTA, 20 mM sodium azide, 1 mM EDTA, 20 μg/ml PMSF, 1 μg/ml pepstatin A, 1 μg/ml leupeptin, pH 7.4) and homogenized as described above. The post-nuclear supernatant was fractionated by sedimentation on a discontinuous sucrose density gradient as described previously (12). Each fraction was mixed with 4.5 ml of resuspension buffer (3 mM imidazole, 2 mM EDTA, 20 μg/ml PMSF, 1 μg/ml pepstatin A, 1 μg/ml leupeptin, pH 7.4), and membranes were pelleted (100,000 × g, 30 min) and resuspended in a minimal volume of resuspension buffer. Protein concentrations were determined using the BCA colorimetric assay (Pierce).

Immunoblot Analysis—Equal amounts of protein (2.0 μg) were electrophoresed on a 12% or 16% SDS-polyacrylamide gel and electrophoresed into a nitrocellulose membrane. Membranes were blocked with 5% non-fat dry milk in PBS, incubated for 150 mM NaCl, pH 7.4 for 2 h, followed by a 1-h incubation in AQP2 polyclonal antibody (1:500), c-Myc monoclonal antibody (1:300). Membranes were washed in the blocking solution containing 0.05% Tween, and incubated with HR-conjugated goat anti-rabbit IgG. Bands were visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech).

Osmotic Water Permeability—Osmotic water permeability in ER membranes was determined by stopped-flow light scattering (14). Fractionated ER vesicles (0.25 mg protein/ml) were suspended in 150 mM sucrose, 3 mM imidazole, pH 7.4, and subjected to a 150 mM inwardly directed sucrose gradient at 10 °C. The time course of scattered light intensity at 530 nm was measured.

RESULTS

The ability of various detergents to extract wild-type versus mutant AQP2 proteins was studied in microsomes isolated from transfected, brefeldin A-treated CHO cells. Microsomes were incubated with detergents as described under “Experimental Procedures,” supernatant (soluble) and pellet (insoluble) fractions were prepared by centrifugation, and total AQP2 was immunoprecipitated. Fig. 1A shows that under identical detergent incubation conditions, wild-type AQP2 was remarkably more susceptible to detergent extraction than the AQP2 mutants T126M and A147T. In contrast, E258K was fully detergent-soluble. Greater than 90% of wild-type AQP2 and E258K was solubilized under the conditions shown in Fig. 1A, whereas the mutants were poorly soluble particularly in CHAPS. Fig. 1B shows a detergent concentration dependence of AQP2 solubilization. Whereas solubility was nearly 100% at high detergent concentrations, the detergent potencies differed considerably for wild-type versus mutant T126M AQP2. T126M was solubilized at 40% detergent concentration in CHAPS. The detergent concentration dependence of AQP2 solubilization was also examined in micelles of digitonin and CHAPS. AQP2 solubility was not related to differences in lipid environment or membrane integration. Fig. 2A shows immunofluorescence micrographs of transfected CHO cells expressing wild-type, T126M, and E258K AQP2. As reported previously (12), without brefeldin A treatment, wild-type AQP2
was distributed in a recycling endosome/plasma membrane pattern, whereas T126M and A147T (not shown) were localized to the ER. E258K expression showed a Golgi distribution. After brefeldin A treatment, which prevents vesicle traffic from the ER, the pattern of AQP2 localization was indistinguishable for the wild-type and mutant proteins. Fig. 2B shows differential detergent extractability, as in Fig. 1A, but for purified ER membranes isolated from the transfected CHO cells by sucrose gradient centrifugation. Again, wild-type AQP2 was remarkably more solubilized by detergents than the mutants. Fig. 2C shows that neither wild-type nor mutant AQP2 could be extracted from microsomes by high pH, indicating that both proteins were fully integrated in the membrane.

Comparative limited proteolysis was used to test for differences in the folding state of wild-type versus mutant AQP2 proteins in the ER. Experiments were carried out on ER-deprived microsomes from transfected CHO cells expressing AQP2 proteins encoding a c-Myc tag at the N terminus. The N and C termini peptides were visualized by immunoblot analysis using c-Myc and AQP2 antibodies, respectively. Fig. 3, top, shows representative immunoblots using an AQP2 antibody. Progressive proteolysis of wild-type and mutant AQP2 with thermolysin gave a decrease in the amount of the full-length protein. Proteolysis of wild-type and mutant AQP2 with proteinase K resulted in a decrease in the amount of the full-length protein with the appearance of smaller fragments. Quantitative concentration-dependent proteolysis data in Fig. 3B indicated that wild-type, E258K and T126M AQP2 mutants are equally susceptible to digestion. The concentration-dependent appearance of smaller fragments with proteinase K incubation showed a similar profile for wild-type and mutant AQP2. Multiple additional studies, which included c-Myc and AQP2 immunoblot analysis of proteolytic fragmentation patterns by trypsin and pepsin (data not shown), supported the conclusion that wild-type, E258K and T126M AQP2 mutants are equally susceptible to digestion by proteases.

[35S]methionine pulse-chase experiments were carried out to quantify the kinetics of AQP2 degradation in CHO cells. Cells were depleted of methionine and labeled for 30 min with [35S]methionine followed by chase in media containing excess unlabeled methionine. Fig. 4A shows representative autoradiograms, and Fig. 4B gives a summary of the quantitative densitometry data. AQP2 mutants were degraded significantly more rapidly than wild-type AQP2. In brefeldin A-treated CHO cells, the degradation of wild-type AQP2 was significantly slowed, whereas the degradation of mutant AQP2 proteins was

**Fig. 1.** Detergent extractability of AQP2 from ER of CHO cells. CHO cells were transiently transfected with AQP2 and labeled with [35S]methionine for 8 h in the presence of 10 μg/ml brefeldin A. AQP2 was solubilized from cell microsomes by incubation with the indicated non-ionic detergents. AQP2 was immunoprecipitated from the detergent-soluble supernatant and the detergent-insoluble pellet fractions (see “Experimental Procedures”). A, representative autoradiogram of immunoprecipitated AQP2 proteins from supernatant (S) and pellet (P) fractions. B, top, autoradiogram showing dependence of AQP2 solubilization on detergent concentration. B, bottom, summary of quantitative detergent extraction experiments (average of three sets of experiments ± S.E.).

**Fig. 2.** Intracellular localization of AQP2 protein and detergent extractability of AQP2 from ER membranes. A, panel of representative cells expressing wild-type AQP2, E258K, and T126M in the absence (left) and presence (right) of 10 μg/ml brefeldin A for 8 h. Cells were fixed, permeabilized, and stained with AQP2 polyclonal antibody. B, immunoblot of detergent-soluble (S) and insoluble (P) fractions of ER membranes from AQP2 transfected cells incubated in the indicated detergents. ER membranes were isolated from microsomes by sucrose density gradient centrifugation. C, membrane integration of AQP2. ER microsomes were incubated in 1 M Na2CO3 for 15 min at room temperature prior to separation of soluble (S) and pellet (P) fractions.
not affected. These results suggest an ER-dependent degradation mechanism for mutant AQP2 and a post-ER degradation mechanism for wild-type AQP2. Averaged $t_{1/2}$ values (time for 50% degradation) are summarized in Fig. 2B, right. The degradation kinetics of mutant AQP2 was not affected by brefeldin A, lysosomotropic agents (NH$_4$Cl), and an inhibitor of the ubiquitin-proteasome degradation pathway (MG132).

Stopped-flow light scattering was done to determine whether the ER-retained AQP2 mutant T126M was functional as a water channel. Non-transfected (control) and transfected CHO cells were treated with brefeldin A, and ER membrane vesicles were isolated by sucrose density gradient centrifugation. Osmotic water permeability was determined from the time course of vesicle shrinkage, as measured by light scattering, in response to a rapidly imposed 150 mM gradient of sucrose. The light scattering kinetics are shown on three contiguous time scales to visualize the full time course. ER membranes from cells transfected with wild-type AQP2 (Fig. 5, top curve) showed a highly water-permeable fraction of vesicles with a half-time for shrinking of $\sim$100 ms (curved time course seen on 0.3-s time scale). The corresponding osmotic water permeability coefficient ($P_o$) for these vesicles is $\sim 0.02$ cm/s. Highly water permeable vesicles were not seen in ER membranes from control cells (Fig. 5, middle curve), which had a remarkably slower rate of shrinking. In ER membranes from T126M transfected cells, rapid shrinking was found as seen in the ER membranes containing wild-type AQP2. The similar amount of AQP2 protein in ER containing wild-type versus T126M AQP2 (Fig. 5, inset) suggests that the intrinsic water permeabilities of wild-type and T126M AQP2 are comparable. Thus, T126M AQP2 is fully functional even though it appears to be folded differently from wild-type AQP2 at the ER membrane (see “Discussion”).

Detergent extraction experiments were carried out to test whether correction of mutant AQP2 mistrafficking is associated with correction of the apparent protein folding defect. Cells were grown in glycerol for 48 h prior to isolation of microsomes and detergent solubilization. As reported previously (12), growth of transfected CHO cells in glycerol for 48 h resulted in a redistribution of mutant AQP2 proteins from the ER to a recycling endosome/plasma membrane compartment. Fig. 6A, top-panel, shows an ER intracellular distribution of AQP2 T126M. After glycerol treatment, the pattern became primarily endosomal (Fig. 6A, panel 2); incubation of glycerol-treated cells with brefledin A gave an ER staining pattern as expected (Fig. 6A, middle panel). The Golgi distribution of E258K AQP2 was not changed by incubating cells in glycerol (Fig. 6A, panels 4 and 5). There was no detectable effect of glycerol on the localization of wild-type AQP2, nor was there significant correction of the T126M AQP2 trafficking defect by a 6-h incubation in glycerol (not shown). Fig. 6B shows representative autoradiograms from the detergent extraction experiments using 1% CHAPS, a concentration in which there was a large difference in the nearly complete solubilization of wild-type AQP2 versus mutant AQP2 (see Fig. 1B). After growth of cells in glycerol, there was no difference in the nearly complete solubilization of wild-type AQP2 by 1% CHAPS. However, a remarkably greater fraction of T126M and A147T became soluble in the detergent and were immunoprecipitated from the supernatant. Fig. 6C summarizes averaged solubilization data.

**Discussion**

We showed previously that several NDI-causing AQP2 mutants are retained in the ER of transfected mammalian cells (7, 12). The present study was carried out to test the hypotheses that ER-retained AQP2 mutants are misfolded and non-functional, and that correction of defective trafficking by chemical chaperones gives the correctly folded protein. Analysis of the folding of an integral membrane protein trapped at the ER is challenging. The difficulty in expressing and purifying mutant AQP2 in its native state precludes the use of spectroscopic or crystallographic methods to study structure. In addition, analysis of wild-type versus mutant AQP2 requires a common membrane environment so that differences in results can be attributed to differences in AQP2 folding state. Our strategy was to use comparative detergent extractability and protease sensitivity as indicators of misfolding. ER-retained AQP2 mutants A147T and T126M, which were found previously to be functional as water channels in *Xenopus* oocytes and glycerol-treated CHO cells (12), were significantly more resistant to detergent solubilization than wild-type AQP2. However, E258K, a mutant that undergoes normal trafficking from the ER to the Golgi, was readily detergent-soluble like wild-type AQP2. Although we expected to find significant changes in protease sensitivity of ER-retained AQP2 mutants, it was found that wild-type and mutant AQP2 in the ER were equally sensitive to degradation by proteases. Another unanticipated observation was that an ER-retained AQP2 mutant was fully functional at the ER. As discussed below, the resistance to detergent solubilization in the absence of a change in susceptibility to protease degradation, and the preservation of function of the mistrafficked mutant AQP2, make it unique among the known examples of membrane transport proteins that are retained in the ER.

Several mutant proteins that cause human disease are thought to involve misfolding, including ΔF508 CFTR (15–17), the α subunit of unassembled T cell receptor (18, 19), the scrapie form of the prion protein, PrPsc (20, 21) and the antitrypsin Z mutant (22, 23). These proteins are retained and degraded in the ER and are poorly soluble in non-ionic detergents (17, 21–24). In the case of ΔF508 CFTR, PrPsc, and the
antitrypsin Z mutant, detergent insolubility has been thought to result from defective folding and aggregation of misfolded protein units. The data here show that, compared with wild-type AQP2, the NDI-causing ER-retained AQP2 mutants are remarkably more resistant to extraction by detergents. The simplest interpretation of this finding is that mutant AQP2 proteins are misfolded at the ER. Reduced detergent extractability could result from AQP2 misfolding, intramembrane aggregation, and/or interaction with molecular chaperones. Aggregation of the protein or binding to ER resident chaperones may result in ER retention of the protein. The similar detergent solubility of the E258K AQP2 mutant and wild-type AQP2, both of which exit the ER, suggests that binding of ER chaperones to misfolded AQP2 may contribute to detergent insolubility. It has been shown that AQP2 plasma membrane targeting is in part regulated by phosphorylation of residue Ser-256 at its polar C terminus (25). However, it is unlikely that this regulatory mechanism is involved in the ER-retention of AQP2 mutants because the phosphorylation probably controls post-ER endosome targeting and because the point mutations in AQP2 are far from its C terminus.

The similar sensitivity of AQP2 mutants to protease digestion compared with wild-type AQP2 suggests that the extent of misfolding of the ER-retained AQP2 mutants is not severe enough to expose residues that are inaccessible in wild-type

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The similar sensitivity of AQP2 mutants to protease digestion compared with wild-type AQP2 suggests that the extent of misfolding of the ER-retained AQP2 mutants is not severe enough to expose residues that are inaccessible in wild-type
AQP2. Altered protease sensitivity has been shown for ΔF508 CFTR, the unassembled T cell receptor α subunit, and Prp<sup>sc</sup> (16, 18, 21). In the case of Prp<sup>sc</sup>, the formation of misfolded Prp<sup>sc</sup> aggregates has been shown to confer protection from digestion by protease K (21). Alternatively, misfolded proteins can exhibit increased susceptibility to proteases. ΔF508 CFTR is more susceptible than wild-type CFTR to degradation by the proteases thermolysin, kallikrein, and trypsin (16). It was suggested that a protease-sensitive ER intermediate of ΔF508 CFTR is conformationally distinct from the protease-insensitive ER intermediate of wild-type CFTR and that the inability of ΔF508 CFTR to attain a mature folded conformation prevents its exit from the ER (16). In the case of the unassembled T cell receptor α subunit, trypsin susceptibility of the misfolded intermediate has been attributed to incomplete membrane insertion of the protein (18). In the case of T126M AQP2, a subtle conformational change, which is not manifested as a change in susceptibility to proteases, may promote aggregation or allow recognition by molecular chaperones and cause ER retention.

Misfolded proteins that are retained in the ER are often degraded by ER-associated degradation (ERAD) pathways including resident ER proteases such as ER60 protease, or ubiquitin and the cytosolic proteasome (26, 27). Misfolded proteins that undergo ERAD include ΔF508 CFTR, the unassembled T cell receptor α subunit, and the antitrypsin Z mutant (18, 19, 23, 24). The rate of degradation of these proteins is unaffected by inhibitors that block vesicle traffic from the ER. It is therefore inferred that these proteins are degraded by an ER-dependent degradation pathway. In the case of the ΔF508 CFTR and the unassembled T cell receptor α subunit, it was shown that the degradation involved ubiquitination and subsequent proteolysis by the proteasome. It was shown that inhibitors of the proteasome cause an accumulation of ΔF508 CFTR and the unassembled T cell receptor α subunit in the ER (18, 19, 24). The misfolding and ER retention of AQP2 mutants A147T and T126M suggests an ERAD mechanism. The insensitivity of mutant AQP2 degradation rates to inhibitors of downstream vesicle traffic (brefeldin A) and lysosomal proteases further supports an ERAD mechanism. In contrast, the slowed degradation of wild-type AQP2 in the presence of brefeldin A indicates a post-ER degradation mechanism. These results suggest that the misfolded AQP2 mutant at the ER is recognized differently than wild-type AQP2 by resident ER proteases and components of the quality control machinery. The lack of inhibition by known ER-dependent protease inhibitors of mutant AQP2 degradation rates implicates a possible novel ER degradation mechanism that remains to be identified.

Despite the apparent misfolding of NDI-causing AQP2 mutants at the ER and the dramatically different trafficking compared with wild-type AQP2, the T126M mutant was found to be fully functional as a water channel. This unexpected finding is what has also been found for ΔF508 CFTR, where the CFTR chloride channel electrophysiological signature was found in single channel recordings of outer membranes of the nuclear envelope of CFTR-expressing CHO cells (28). To our knowledge the functionality of other ER-retained proteins has not been studied. It appears that some point mutations can alter protein conformation in a manner that affects trafficking but not function. This finding has important implications in terms of strategies to correct some of the mistrafficked AQP2 mutants in NDI. Maneuvers that permit plasma membrane targeting should be sufficient to correct the cell phenotype, even if the perfect native conformation is not achieved. It is noted that glycerol treatment was found to restore the normal phenotype of CHO cells expressing T126M AQP2 (12), despite the finding here that glycerol only partially restored the native folding state.

It was found previously that various chemical chaperones (glycerol, TMAO, Me<sub>2</sub>SO) facilitate the translocation of AQP2 mutants to the plasma membrane and endosomes, thereby restoring a normal cell phenotype of high plasma membrane water permeability. Glycerol has also been shown to correct the apparent cellular processing defect of ΔF508 CFTR (29, 30), Prp<sup>sc</sup> (21), p53 tumor suppressor protein, and temperature-sensitive mutants of E1 enzyme in the ubiquitination pathway (31). Although the mechanism by which chemical chaperones affect protein maturation in vivo has not been established, it has been proposed that these agents facilitate the correct folding of a nascent protein by physico-chemical interactions or by interfering with alternate folding pathways (32). Other proposed mechanisms include glycerol-induced slowing of protein synthesis and processing to facilitate protein folding, and glycerol-protein binding to alter the energy barrier for formation of properly folded conformers (33, 34). The results here show that correction of mutant AQP2 misprocessing by glycerol is associated with a pattern of detergent extractability that begins to resemble that of wild-type AQP2. It was found that correction of defective mutant AQP2 trafficking required more than 24 h of exposure to glycerol, a time much greater than the degradation half-time for wild-type or mutant AQP2 proteins. Therefore it is not possible to determine whether glycerol is able to rescue ER-retained AQP2 mutants that are already in an apparently misfolded state. Further, the need for prolonged glycerol exposure suggests that the correction of misfolded proteins involves slow adaptive changes in cell constituents, such as in the molecular recognition machinery; a direct effect of glycerol on mutant AQP2 folding should be manifest within the 4–6 h AQP2 turnover time. Further studies are needed to establish the cellular adaptive changes conferred by chemical chaperones.

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