Diffusion in the Endoplasmic Reticulum of an Aquaporin-2 Mutant Causing Human Nephrogenic Diabetes Insipidus*

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Marc H. Levin‡‡, Peter M. Haggie¶, L. Vetrivel, and A. S. Verkman¶
From the Departments of Medicine and Physiology, Cardiovascular Research Institute, University of California, San Francisco, California 94143-0521

Mutations in the aquaporin-2 (AQP2) water channel cause the hereditary renal disease nephrogenic diabetes insipidus (NDI). The missense mutation AQP2-T126M causes human recessive NDI by retention at the endoplasmic reticulum (ER) of renal epithelial cells. To determine whether the ER retention of AQP2-T126M is due to relative immobilization in the ER, we measured by fluorescence recovery after photobleaching the intramembrane mobility of green fluorescent protein (GFP) chimeras containing human wild-type and mutant AQP2. In transfected LLC-PK1 renal epithelial cells, GFP-labeled AQP2-T126M was localized to the ER, and wild-type AQP2 to endosomes and the plasma membrane; both were localized to the ER after brefeldin A treatment. Photobleaching with image detection indicated that the GFP-AQP2 chimeras were freely mobile throughout the ER. Quantitative spot photobleaching revealed a diffusion-dependent irreversible process whose recovery depended on spot size and was abolished by paraformaldehyde fixation. In addition, a novel slow reversible fluorescence recovery (t½ - 2 s) was characterized whose recovery was independent of spot size and not affected by fixation. AQP2 translational diffusion in the ER was not slowed by the T126M mutation; diffusion coefficients were (in cm²/s × 10⁻¹⁰) 2.6 ± 0.5 (wild-type) and 3.0 ± 0.4 (T126M). Much faster diffusion was found for a lipid probe (diOC₃(3), 2.7 × 10⁻⁸ cm²/s) in the ER membrane and for unconjugated GFP in the aqueous ER lumen (6 × 10⁻⁸ cm²/s). ER diffusion of GFP-T126M was not significantly affected by up-regulation of molecular chaperones, cAMP activation, or actin filament disruption. ATP depletion by 2-deoxyglucose and azide resulted in comparable slowing/immobilization of wild-type and T126M AQP2. These results indicate that the ER retention of AQP2-T126M does not result from restricted or slowed mobility and suggest that the majority of AQP2-T126M is not aggregated or bound to slowly moving membrane proteins.

A number of genetic disorders are caused by defective cellu-
lar processing of proteins encoded by mutant genes. The human diseases cystic fibrosis, hereditary emphysema, and nephrogenic diabetes insipidus (NDI) are caused by retention of mutant plasma membrane proteins at the endoplasmic reticulum (ER) and their consequent failure to function at the plasma membrane (1). Humans with NDI are unable to form a concentrated urine, resulting in polyuria, polydipsia, and the potential for severe dehydration if not given free access to fluids (2). Hereditary non-X-linked NDI is caused by mutations in the aquaporin-2 (AQP2) protein, a water channel that is normally expressed at the luminal plasma membrane of kidney collecting duct epithelial cells after stimulation by the antidiuretic hormone vasopressin (3). Collecting duct water permeability is regulated by a membrane cycling mechanism in which vasopressin-induced phosphorylation of the cytoplasmic AQP2 C terminus results in its exocytic transport from an intracellular vesicular pool to the cell plasma membrane (4, 5).

A number of AQP2 mutations cause autosomal recessive NDI by retention at the ER (2, 3, 6–8). The missense AQP2 mutant T126M is ER-retained in transfected mammalian cells where it is functional as a water channel but appears to be mildly misfolded (9). Chemical chaperones such as glycerol, which correct mutant protein mistrafficking in cell culture models of cystic fibrosis and other diseases (10), correct the AQP2-T126M processing defect and increase cell membrane water permeability (11). Recently, a transgenic knock-in mouse model of NDI created by AQP2-T126M gene replacement confirmed the ER retention of AQP2-T126M in the in vivo kidney (12). Homozygous AQP2-T126M mutant mice manifested severe polyuria and developed renal failure in their first week of life.

The purpose of this study was to determine whether the ER retention of AQP2 mutants results from restricted or slowed intramembrane mobility caused by protein aggregation or interaction with components of the quality control machinery such as molecular chaperones. AQP2 mobility in living cells was measured by fluorescence recovery after photobleaching (FRAP) using renal epithelial cells expressing green fluorescent protein (GFP)-labeled wild-type and mutant AQP2. We previously established photobleaching methods and analysis procedures to quantify solute mobility in organelles including the ER and mitochondria (13–15). The GFP label here did not affect AQP2 cellular localization, and it was found previously that GFP-labeled wild-type AQP2 had native tetrameric structure, water channel function, and regulated cellular trafficking (16). Diffusion of wild-type and mutant AQP2 were compared quantitatively, and the effects were characterized of a series of maneuvers to modify putative AQP2 interactions.

The results here provide the first information about the ER

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‡ Both authors contributed equally to this work.

¶ Summer student scholar supported by the American Heart Association, Western States Affiliate.

To whom correspondence should be addressed: 1246 Health Sciences East Tower, Cardiovascular Research Institute, University of California, San Francisco, CA 94143-0521, Tel.: 415-476-8530; Fax: 415-665-3847; E-mail: verkman@itsa.ucsf.edu.

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retention mechanism of a naturally occurring mutation in a membrane protein associated with human disease. During the course of our experiments, a paper by Nehls et al. (17) was published on the ER mobility of a temperature-sensitive folding mutant of the vesicular stomatitis virus G (VSVG) protein. Although some conclusions about VSVG and AQ2P2 mobility at the ER are similar, there are substantial differences in ER diffusion rates and interactions with molecular chaperones (see “Discussion”).

EXPERIMENTAL PROCEDURES

Cell Culture and Transfections—Missense point mutant T126M of human AQ2P2 was generated by site-directed mutagenesis and confirmed by sequence analysis. The cDNAs encoding wild-type and mutant AQ2P2 were fused downstream from EGFP (CLONTECH) as described previously for wild-type AQ2P2 (16). LLC-PK1 renal epithelial cells (obtained from the University of California, San Francisco Cell Culture Facility) were grown at 37 °C in a 5% CO2/95% air atmosphere using DMEH-21 medium. Cells were cultured on 18 mm-diameter round coverslips in 12-well dishes in DMEH-21 medium containing 500 μg/ml G418. For some studies cells were transiently transfected with the GFP-labeled AQ2P2-T126M mutant by overnight incubation with DMEH-21 medium containing 1 μg of plasmid and 6 μl of Effectene (Qiagen) per well. LLC-PK1 cells were also transfected with unconjugated EGFP as described by Kneen et al. (9) and in the ER as described by Dayel et al. (14). In some experiments ER membranes were stained with 3,3′-dibutylxocarbocyanine iodide (diOC4(3), Molecular Probes) by incubation for 10 min at 37 °C using 10 μM dye concentration.

Cell Treatments—Transfected cells were cultured on glass coverslips until near confluence and mounted in a custom-built closed perfusion chamber designed to fit in a PDMI-2 microincubator (Harvard Apparatus) until near confluence and mounted in a custom-built closed perfusion chamber designed to fit in a PDMI-2 microincubator (Harvard Apparatus). Perfusion chamber temperature was controlled by a TC-201A temperature regulator (Harvard Apparatus) and objective temperature by a lens thermodregulator (Biophtechs, Inc., Butler, PA). Photobleaching measurements were performed at 37 °C unless otherwise indicated. Cell cultures were generally perfused with phosphate-buffered saline containing 60 mM glucose at 1 ml/min and photobleaching measurements were made within 1 h of mounting the cells in the perfusion chamber. In some experiments, cells were preincubated with brefeldin A (1 μg/ml, 16 h, 37 °C) to give AQ2P2 localization in the ER. In some experiments cells were incubated with the fixative paraformaldehyde (4%, 30 min, 23 °C), the metabolic inhibitors 2-deoxyglucose (50 mM, 1 h, 37 °C) and sodium azide (0.05%) (for ATP depletion), the cAMP agonist forskolin (50 μM, 15 min, 37 °C), or the cytoskeleton-disrupting agent cytochalasin D (5 μg/ml, 45 min, 37 °C). Some cell cultures were heat-shocked by incubation at 42 °C for 1 h followed by 16-h incubation at 37 °C.

Fluorescence Recovery after Photobleaching—Photobleaching measurements were carried out on an apparatus described previously (19) with modification. For spot photobleaching, an argon ion laser beam (488 nm, Innova 70–4; Coherent) was modulated by an acousto-optic modulator (Primrose) and directed onto the transfected cells through an objective lens designed to focus in a PDMI-2 microincubator (Harvard Apparatus). Photobleaching experiments were done on transfected LLC-PK1 cells expressing wild-type and T126M AQ2P2 proteins fused downstream from EGFP reporter. 5 mCi of [32P]orthophosphate was added to the perfusion medium 2 h before observation.

RESULTS

Measurements were done on transfected LLC-PK1 cells expressing wild-type and T126M AQ2P2 proteins fused downstream from a GFP reporter. Fig. 1A shows the cDNA constructs, indicating the amino acid sequence at the GFP-AQ2P2 fusion site and the location of the T126M mutation. Fig. 1B shows confocal fluorescence micrographs of cells expressing GFP-AQ2P2 constructs. Without brefeldin A treatment, GFP-labeled AQ2P2-T126M was localized in the ER (left) and wild-type AQ2P2 in endosomes and the plasma membrane (middle). After incubation with forskolin for 15 min, GFP-labeled wild-type AQ2P2 was seen mainly at the plasma membrane, whereas the localization of AQ2P2-T126M was unchanged (data not shown). After treatment with brefeldin-A, which partially collapses the Golgi and prevents ER-to-Golgi vesicle traffic, both GFP-AQ2P2 chimeras showed the characteristic ER staining pattern (wild-type AQ2P2 shown at right), as also seen in cells expressing KDEL-tagged unconjugated GFP or stained with cyanine diOC4(3) (not shown). Photobleaching studies of GFP-AQ2P2 diffusion in the ER were carried out on brefeldin A-treated cells or cells expressing AQ2P2-T126M without brefeldin A treatment.

Photobleaching imaging experiments were done to examine the continuity of the ER membrane and to determine whether GFP-labeled AQ2P2-T126M is restricted in its ER mobility. A large region of the cell was bleached by the laser pulse. Fig. 1C shows pre-bleach and a series of post-bleach images of brefeldin A-treated LLC-PK1 cells expressing wild-type GFP-AQ2P2. The post-bleach image showed a darkened region corresponding to the site of the bleach pulse. There was progressive recovery of fluorescence over the first ~50 s. At 200 s, the ER was similar in appearance to the pre-bleach image, except for reduced intensity as a consequence of the substantial fraction of total cell GFP-AQ2P2 that was irreversibly bleached. Similar results for many different cells, different laser spot sizes, and different bleached regions of the cell indicated that with respect to diffusion of wild-type AQ2P2 the ER membrane is continuously connected throughout the ER membrane. Similar findings were reported previously for unconjugated GFP in the aqueous lumen of ER, albeit with a substantially faster rate of diffusion (14).

Spot photobleaching experiments were also done on cells expressing AQ2P2-T126M. Examination of serial images from many cells (representative example in Fig. 1D) showed that the GFP-labeled AQ2P2-T126M protein was able to move freely throughout the ER, with qualitatively comparable fluorescence recovery kinetics to wild-type AQP2.

Spot photobleaching measurements were done to character-
FIG. 1. Photobleaching of GFP-labeled AQP2 proteins in the ER membrane of transfected LLC-PK1 cells at 37 °C. A, cDNA construct showing fusion of AQP2-T126M downstream from the GFP reporter. B, fluorescence micrographs of transfected LLC-PK1 cells expressing the indicated GFP-AQP2 proteins. C and D, series of fluorescence micrographs are shown for brefeldin A-treated cells expressing wild-type GFP-AQP2 (C) and cells expressing the AQP2-T126M mutant (D). Images are shown before bleaching and at indicated times after bleaching. See “Results” for explanations. Temperature was maintained at 37 °C. Cross-hairs indicate center of bleach spot. Scale bar: 10 μm.

ize quantitatively the fluorescence recovery processes and to compare the diffusion of GFP-labeled wild-type AQP2 and AQP2-T126M. GFP fluorescence in a small spot was bleached: ~0.7-μm diameter produced by a 100× oil immersion objective, or ~2-μm diameter produced by a 40× objective. Bleach times were selected to reduce fluorescence intensity just after the bleach to ~60% of initial fluorescence.

Initial experiments done using a 100× objective showed nearly complete fluorescence recovery in a few seconds in brefeldin A-treated cells expressing GFP-labeled wild-type AQP2 (Fig. 2A, top curve). “No bleach” control experiments were done for each set of studies to prove that the attenuated probe beam did not produce bleaching. To confirm that the recovery corresponded to diffusion, measurements were done after paraformaldehyde treatment (to immobilize GFP-AQP2) and using a 40× lens (to increase spot diameter and slow diffusion-related recovery – 100/40² – 6-fold). Interestingly, partial recovery was seen in paraformaldehyde-fixed cells with an exponential time constant, τ, of 3.4 ± 0.8 s (second curve). Fluorescence recovery in the absence of diffusion defines a reversible recovery process, as created by triplet state or other photophysical relaxation processes. For example, the decay of a GFP molecule from an excited triplet state to the ground state would produce fluorescence recovery because of restoration of the population of excitable GFP molecules. Reversible GFP fluorescence recovery has been described (14, 22), albeit at a much faster rate (1–5 ms, depending on construct), and slow GFP flickering has been reported (23). Examination of the early fluorescence time course after bleaching in the paraformaldehyde-treated cells revealed a second apparent reversible recovery process (τ ~ 4 ms) of low amplitude (inset). In addition to insensitivity to fixation, diffusion-independent reversible fluorescence recovery processes do not depend on spot size. The recovery of the slow reversible process was not affected by increasing objective spot diameter ~ 2.5-fold (third curve) in the paraformaldehyde-treated cells (3.0 ± 0.5 s), confirming reversible recovery. Similar results were obtained using a 10× objective to illuminate whole cells (not shown). The remaining recovery after the reversible process seen for the first curve in Fig. 2A was diffusion-dependent, as shown by its disappearance after paraformaldehyde treatment (second curve) and its significant slowing when bleached and viewed with a larger spot size (τ, ~ 12 s, fourth curve).

The slow reversible fluorescence recovery processes for GFP-AQP2 were not noted previously for GFP in cytoplasm, where recovery was measured over much shorter times (22). Fig. 2B shows a series of photobleaching measurements similar to those in Fig. 2A, but for unconjugated GFP in the cytoplasm of LLC-PK1 cells. The top curve shows substantially faster fluorescence recovery of the free GFP in aqueous-phase cytoplasm compared with membrane diffusion of GFP-AQP2. The second and third curves, where GFP mobility was abolished by paraformaldehyde fixation, shows recovery similar to that seen for GFP-AQP2 in paraformaldehyde-fixed cells in Fig. 2A (τ, 3–5 s). The fourth curve shows that the irreversible (diffusion-dependent) fluorescence recovery process was strongly depend-
ent on spot size as anticipated ($t_{1/2}$, 10 ms, 100× *versus* 57 ms, 40×). Thus the slow (3–5 s) reversible recovery process seen for GFP-AQP2 does not appear to be context-specific.

Quantitative spot photobleaching experiments were done to compare the ER membrane diffusion of GFP-labeled wild-type AQP2 and AQP2-T126M. It was postulated that interactions of the mutant ER-retained protein with components of the quality control machinery might result in slowed diffusion. Fig. 3A shows representative fluorescence recovery data, and Fig. 3B summarizes averaged diffusion coefficients. Qualitatively the recovery curves for the brefeldin A-treated GFP-labeled wild-type and mutant AQP2 were similar, as was the recovery curve for AQP2-T126M in non-brefeldin A-treated cells. The averaged diffusion coefficients did not differ significantly. Fitted percentage recoveries were generally >90%, indicating that nearly all AQP2 is mobile, in agreement with the conclusion from the image photobleaching studies in Fig. 1, C and D. In addition a series of experiments were done at 23 °C. Although the absolute diffusion coefficients were lower than those at 37 °C, there was no significant effect of the T126M mutation.

The computation of absolute diffusion coefficients relied on a Monte-Carlo random walk model, which takes into account the reticular geometry of the ER and relates diffusion coefficients to fluorescence recovery kinetics (15). Previously this model was used to compute the diffusion coefficient of unconjugated GFP in the aqueous lumen of the ER (14). A similar experiment was done to measure the diffusion of unconjugated GFP in the ER lumen of the LLC-PK1 cells used here. GFP fluorescence recovered much faster (Fig. 3A, *bottom curve*), with a diffusion coefficient of $6.0 \pm 0.7 \times 10^{-8}$ cm$^2$/s (Fig. 3B), in agreement with that of $5–10 \times 10^{-8}$ cm$^2$/s for GFP diffusion in the ER of CHO cells. The 200-fold lower diffusion coefficient for GFP-AQP2 indicates the remarkably slowed diffusion of integral membrane proteins compared with aqueous-phase macromolecules.

ER membrane diffusion of GFP-labeled AQP2-T126M was measured in response to a series of cell treatments designed to modulate AQP2 phosphorylation state (forskolin), interactions with molecular chaperones (ATP metabolic depletion and heat shock), and interactions with cytoskeleton (cytochalasin D). Fig. 4A shows representative fluorescence recovery curves for cells expressing GFP-labeled AQP2-T126M, with averaged diffusion coefficients for a series of measurements summarized in Fig. 4C. There was no significant effect of forskolin treatment, heat shock, or cytochalasin treatment on AQP2-T126M diffusion coefficients or mobilities. However, ATP depletion by 2-deoxyglucose and azide produced a small but significant slowing of diffusion, as well as a decrease in the mobile fraction (percentage fluorescence recovery) from >90% to 64 ± 7%. To determine whether the effect of ATP depletion was specific for AQP2-T126M, similar measurements were done on GFP-labeled wild-type AQP2 (Fig. 4B). ATP depletion produced similar slowing and reduced mobility of GFP-labeled wild-type AQP2 and unconjugated GFP. ATP depletion also caused a significant reduction in the mobility of unconjugated GFP in the ER lumen (Fig. 4B, *middle curves*). To distinguish between an effect of ATP depletion on ER “crowding,” as proposed by Nehls et al. (17), versus ER restructuring and geometric differences, the diffusion of a small lipophilic ER membrane probe, diOCl$_3$, was measured. Fig. 4, B (*bottom curves*) and C, showed no effect of ATP depletion on diOCl$_3$ diffusion, indicating that ATP depletion does not affect ER geometry in a manner that influences the relationship between fluorescence recovery and molecular diffusion.

**DISCUSSION**

The principal conclusion of this study is that despite its retention at the endoplasmic reticulum, the human disease causing AQP2 mutant T126M is not slowed in its translational diffusion in the ER compared with wild-type AQP2. Both wild-type AQP2 and AQP2-T126M diffused freely throughout the ER without restrictions. Furthermore, AQP2-T126M diffusion was not changed significantly by maneuvers designed to modulate AQP2 phosphorylation and interactions with components of the cellular quality control machinery and the actin skeleton. Therefore slowed or restricted ER membrane diffusion of AQP2-T126M cannot account for its efficient retention in the ER. Formally our results indicate that the rate-limiting step for the ER diffusion of AQP2 is not sensitive to the T126M mutation. It is noted that interactions between AQP2-T126M and soluble macromolecules are unlikely to be detected by diffusion measurements, because the diffusion of intramembrane proteins is orders of magnitudes slower than that of aqueous-phase macromolecules. For example, the binding of large 30-nm diameter gold beads does not slow the diffusion of much smaller membrane receptors (24). The quantitatively comparable diffusion of wild-type AQP2 and AQP2-T126M makes an unlikely significant AQP2-T126M aggregation or interactions with intramembrane structures that have diffusive rates comparable with or slower than AQP2. However, we cannot rule out the possibility of slowing or immobilization of a small fraction (<5–10%) of AQP2-T126M molecules that cannot be resolved by the photobleaching recovery measurements.

The approach here utilized fluorescence recovery after pho-
PK1 cells (40°C were done at 37 °C in transfected LLC-PK1 cells (40× objective). A, photobleaching of cells expressing GFP-labeled AQP2-T126M with indicated treatments: forskolin (15 min, 50 μM), heat shock (42 °C for 1 h at 16 h prior to measurement), cytochalasin D (45 min, 5 μg/ml), azide (0.05%) + 2-deoxy-o-glucose (50 mM, 1 h). Bleach times were 5–10 ms. B, photobleaching of cells expressing GFP-labeled wild-type AQP2 (top) and untagged GFP in the ER lumen (middle), and untransfected cells whose ER membrane was stained with diOC4(3). Measurements done before (left) and after (right) ATP depletion with azide and 2-deoxyglucose as done in A. C, absolute diffusion coefficients (D, mean ± S.E.) averaged from three to four sets of separate measurements. *, p < 0.05 comparing control versus ATP-depleted cells.

The data here represent the first study of the mobility of a disease-causing mutant membrane protein that produces a defective cell phenotype by ER retention. It will be interesting to determine the generality of the conclusions here to other mutant membrane proteins, such as the ΔF508-CFTR protein that is retained at the ER and causes the disease cystic fibrosis. In agreement with the conclusion here, Nehls et al. (17) reported that the diffusion of GFP-labeled VSVG was unimpeded, so that its ER retention was not the consequence of slowed mobility. However, their diffusion coefficient for VSVG was 10-fold greater than that of AQP2 found here, and the temperature-sensitive unfolded VSVG was largely immobilized after cellular ATP depletion, which allowed a stable interaction with BiP. Comparative studies of folded and unfolded VSVG required measurements at 32 °C (with brefeldin A treatment) and 40 °C, respectively. We find here that the diffusion of wild-type and mutant AQP2 in renal epithelial cells at 37 °C was not quantitatively different following ATP depletion or up-regulation of molecular chaperones by temperature shock. It is likely that fundamentally different mechanisms apply for the recognition of unfolded VSVG versus mutant AQP2. For VSVG interactions with BiP, protein disulfide isomerase, and calnexin have been reported, whereas thus far it has not been possible to detect an interaction between mutant AQP2 and known components of the quality control machinery.

The diffusion coefficients of AQP2 and AQP2-T126M at the ER, −3 × 10⁻¹⁰ cm²/s, are a few-fold greater than that of wild-type AQP1 or AQP2 at the plasma membrane (−0.9 × 10⁻¹⁰ cm²/s at 37 °C), which is consistent with the relatively low cholesterol content and high membrane fluidity of the ER. The ER diffusion of AQP2 was similar to another ER protein, cytomegrome P450 (2.6–6.2 × 10⁻¹⁰ cm²/s) (27), but much less than that reported for VSVG (4–6 × 10⁻¹⁰ cm²/s) (17). To date the diffusional mobilities of other ER proteins have not been reported. For comparison, unconjugated GFP in the aqueous-phase ER lumen diffused substantially faster (6 × 10⁻⁸ cm²/s) than membrane-bound GFP chimeras. The small lipid probe diOC4(3) in the ER membrane also diffused substantially faster (3 × 10⁻⁸ cm²/s), in agreement with previous measurements of the diffusion of fluorescently labeled phospholipids (28).

An important unresolved issue is how proteins like AQP2-T126M are retained at the ER. AQP2-T126M does not contain a classical ER retention sequence (like KKKC), and wild-type AQP2 efficiently exits the ER. No evidence was found for a population of AQP2-T126M that was discontinuous from the down-stream compartment is not the mechanism of its ER retention. The photobleaching results indicate that AQP2-T126M aggregation or interactions (transient or sustained) with slowly moving membrane components cannot account for its ER retention. The ER may contain specialized membrane domains that bud off during the formation of vesicles destined to fuse with the Golgi. The AQP2-T126M protein might be excluded from these specialized submicroscopic domains because of misfolding and...
inability to be recognized by as yet unidentified proteins responsible for accumulation in transport vesicles (29). The identification of ER retention mechanisms of disease-causing protein mutations remains a major challenge.

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