The Proteasome Is Involved in the Degradation of Different Aquaporin-2 Mutants Causing Nephrogenic Diabetes Insipidus

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Mutations in the water channel aquaporin-2 (AQP2) can cause congenital nephrogenic diabetes insipidus. To reveal the possible involvement of the protein quality control system in processing AQP2 mutants, we created an *in vitro* system of clone 9 hepatocytes stably expressing endoplasmic reticulum-retained T126M AQP2 and misrouted E258K AQP2 as well as wild-type AQP2 and studied their biosynthesis, degradation, and intracellular distribution. Mutant and wild-type AQP2 were synthesized as 29-kd nonglycosylated and 32-kd core-glycosylated forms in the endoplasmic reticulum. The wild-type AQP2 had a $t_{1/2}$ of 4.6 hours. Remarkable differences in the degradation kinetics were observed for the glycosylated and nonglycosylated T126M AQP2 ($t_{1/2}$ = 2.0 hours *versus* 0.9 hours). Moreover, their degradation was depending on proteasomal activity as demonstrated in inhibition studies. Degradation of E258K AQP2 also occurred rapidly ($t_{1/2}$ = 1.8 hours) but in a proteasome- and lysosome-dependent manner. By triple confocal immunofluorescence microscopy misrouting of E258K to lysosomes via the Golgi apparatus could be demonstrated. Notwithstanding the differences in degradation kinetics and subcellular distribution such as endoplasmic reticulum-retention and misrouting to lysosomes, both T126M and E258K AQP2 were efficiently degraded. This implies the involvement of different protein quality control processes in the processing of these AQP2 mutants. (*Am J Pathol* 2003, 163:111–120)

The endoplasmic reticulum (ER) represents a site of quality control of glycoprotein folding.^{1,2} Misfolded glycoproteins are recognized and retained by the concerted action of chaperones, lectins, and modifying enzymes such as UDP-glucose:glycoprotein glucosyltransferase and glucosidase II.³ Glycoproteins failing to achieve their correct conformation might become retrotranslocated to the cytosol⁴ and degraded by the ubiquitin-proteasome

pathway, a process referred to as ER-associated protein degradation.^{5–7}

Quality control of protein folding is of importance in congenital diseases caused by point mutations that result in the synthesis of misfolded glycoproteins.⁸ Mutations in the water channel aquaporin-2 (AQP2) can cause nephrogenic diabetes insipidus (NDI), in which patients are unable to concentrate urine in response to the anti-diuretic hormone arginine-vasopressin.^{9–11} If not corrected, this defect results in a deregulated whole-body water homeostasis that is accompanied by various symptoms of dehydration.¹²

AQP2 belongs to the large family of AQPs,^{13,14} and is a 29-kd polytope membrane protein that contains a single *N*-glycosylation site and two phosphorylation sites, and is present in the principal cells of renal collecting ducts.^{15,16} In states of hypernatremia or hypovolemia, translocation of phosphorylated AQP2 homotetramers from vesicles to the apical plasma membrane of the principal cells is triggered by a signal transduction cascade induced by arginine-vasopressin.^{16–18} Alike to normal human kidney,¹⁹ wild-type (wt) AQP2, when expressed in *Xenopus* oocytes^{20,21} or various mammalian cell lines,^{18,22} existed as a nonglycosylated 29-kd form. Autosomal recessive NDI-causing mutants expressed in *Xenopus* oocytes were detected as a nonglycosylated 29-kd and an endo H-sensitive, high-mannose 32-kd form.²⁰ But only a 29-kd form was observed in transiently transfected Chinese hamster ovary (CHO)²² or LLC-PK1 cells.²³ The autosomal dominant NDI-causing E258K AQP2 existed as a 29-kd form both in *Xenopus* oocytes²⁴ and in transiently transfected CHO cells.²²

Heterologous expression has provided evidence for an impaired routing of mutant AQP2 proteins. In many of the autosomal recessive NDI-causing AQP2 mutations, retention of the mutant protein in the ER was reported^{20,23,25} whereas the autosomal dominant NDI-causing E258K AQP2 was localized in the Golgi apparatus.²⁴ Expression of the ER-retained T126M AQP2 in clone 9

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hepatocytes caused the formation of Mallory body-type inclusion bodies probably because of ER stress.²⁶

Despite the progress made in the genetic, molecular, and functional characterization of NDI-causing AQP2 mutants and in the elucidation of the pathophysiology of this disease, specific aspects of the subcellular and molecular pathology remain to be investigated. In particular, the degradation pathway of mutant AQP2 is unknown. In a single study using the proteasome inhibitor MG132 and the lysosomotropic agent NH₄Cl, no influence of these two reagents was observed on the degradation kinetics of ER-retained AQP2 mutants when transiently expressed in CHO cells.²² In the present study we have established an *in vitro* system by transfection of clone 9 rat hepatocytes to stably express wt AQP2 as well as the ER-retained T126M AQP2 and the Golgi apparatus localized E258K AQP2 to investigate their intracellular distribution, turnover, and mode of degradation.

Materials and Methods

Reagents and Antibodies

Protease inhibitor cocktail tablets, restriction enzymes, T4 DNA ligase, and *N*-glycosidase F (recombinant in *Escherichia coli*) were purchased from Roche Diagnostics (Rotkreuz, Switzerland), endoglycosidase H (endo H) from New England Biolabs (Beverly, MA), ALLN and MG132 from Calbiochem-Novabiochem (San Diego, CA), and lactacystin from Sigma (Buchs, Switzerland). The expression vector pcDNA3.1 was from Invitrogen (San Diego, CA), lipofectamine 2000, competent *E. coli* DH5 α , TaqDNA polymerase, and all cell culture media including fetal bovine serum from Life Technologies (Basel, Switzerland), reverse transcription system from Promega (Wallisellen, Switzerland), and the plasmid purification kit from Qiagen (Basel, Switzerland). Protein A-magnetic beads were from Dynal Biotech (Oslo, Norway). Fluorescein isothiocyanate-labeled polylysine transferrin and all other chemicals of analytical grade were purchased from Sigma. ³⁵S-labeled methionine and cysteine were from Amersham Biosciences (Dübendorf, Switzerland).

The following antibodies were used: affinity-purified rabbit polyclonal anti-rat AQP2 antibody raised against a synthetic peptide comprising the carboxy terminal residues 254 to 271 of rat AQP2 cross-reactive with human AQP2 (Alomone Labs, Jerusalem, Israel); mouse monoclonal anti-Golgi mannosidase II antibody (Babco, Richmond, CA); mouse monoclonal anti-lysosomal associated membrane protein 1 (LAMP1, clone 1D4B; RDI, Flanders, NJ); Alexa 488-conjugated (Fab)₂ fragments of goat anti-mouse IgG and Alexa 546-conjugated (Fab)₂ fragments of goat anti-rabbit IgG (Molecular Probes, Eugene, OR); Cy-5-conjugated Fab fragments of goat anti-mouse IgG and alkaline phosphatase-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA).

Plasmid Constructs, Site-Directed Mutagenesis, and Stable Transfections

Polymerase chain reaction (PCR) was performed using the cDNA coding for the human AQP2 (kindly provided by Dr. A. S. Verkman, University of California, San Francisco, CA) as template and two specific oligonucleotides (forward: 5'-AAG CTT AAG CTT AGC ATG TGG GAG CTC CGC TCC A-3'; reverse: 5'-TCT AGA TCT AGA TCA GGC CTT GGT ACC C-3'). The mutant AQP2 forms were generated by a PCR-based site-directed mutagenesis strategy as described previously.²⁶

Transfection of clone 9 rat hepatocytes with the linearized AQP2-pcDNA3.1 constructs was performed using lipofectamine 2000 and clonal cell lines were established as described previously.²⁶

For the detection of rat AQP9, reverse transcriptase (RT)-PCR was performed using RNA isolated from rat liver or from clone 9 hepatocytes. Total RNA was reverse-transcribed by AMV reverse transcriptase using random primers. In the subsequent PCR amplification, the following AQP9-specific oligonucleotides were used: forward: 5'-CCA AGA TGC CTT CTG AGA AG-3'; reverse: 5'-CCA CTA CAT GAT GAC ACT GAG C-3'.

Western Blotting

Transfected cells were homogenized in 4 vol of phosphate-buffered saline (PBS) containing protease inhibitors and Triton X-100 was added in a final concentration of 1%. We found that this detergent concentration extracted all wt AQP2 as well as T126M AQP2 and E258K AQP2 (data not shown). After rotating for 1 hour on a wheel, the samples were centrifuged at 14,000 $\times g$ for 10 minutes at 4°C and the supernatant was used for Western blotting. Proteins of cell extracts (100 μ g protein/lane) were separated in 12% sodium dodecyl sulfate (SDS)-polyacrylamide gels, transferred onto nitrocellulose membranes using a semidry blotting apparatus²⁷ and probed for AQP2. The membranes were blocked with PBS containing 1% defatted milk powder and 0.05% Tween 20 for 1 hour at ambient temperature and incubated with 1 μ g/ml of affinity-purified anti-rat AQP2 antibody overnight at 4°C. The membranes were washed three times with blocking solution and incubated with alkaline phosphatase-conjugated goat anti-rabbit IgG antibodies at ambient temperature for 1 hour. After washing with TBS containing 1% defatted milk powder and 0.05% Tween 20, the membrane was equilibrated in 100 mmol/L of Tris-HCl containing 100 mmol/L NaCl and 50 mmol/L MgCl₂ (pH 9.5), and the color reaction was performed using nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate as substrates.

Confocal Immunofluorescence Microscopy

Clonal lines of clone 9 rat hepatocytes stably expressing wt and mutant AQP2 were formaldehyde-fixed and saponin-permeabilized as described.²⁶ For double-immunofluorescence microscopy, cells were simultaneously incubated with anti-AQP2 antibodies in combination with

either anti-Golgi mannosidase II or anti-LAMP 1 antibodies for 2 hours at ambient temperature, washed three times with PBS containing 1% bovine serum albumin followed by simultaneous incubation with the respective fluorescent secondary antibodies for 1 hour. Finally, coverslips were rinsed with PBS and embedded in Moviol (Harco, Harlow, UK).

To relate endosomes with immunostaining for AQP2 and LAMP 1, a triple-staining protocol was performed. Fluorescein isothiocyanate-transferrin was incubated in 100 mmol/L of Fe^{3+} -citrate buffer (pH 7.0) at 37°C for 1 hour and the iron-loaded transferrin was added to cell cultures for 1.5 hours for endocytotic uptake. After two rinses with the medium, the cells were fixed, permeabilized, and incubated with antibodies against AQP2 and LAMP 1 as described above.

Clonal cells stably expressing wt AQP2 or E258K AQP2 were treated with forskolin (10 $\mu\text{mol/L}$) for 30 minutes at 37°C and processed for AQP2 immunofluorescence. Complete series of 200-nm optical sections through entire cells were taken and rendered to shadow projection images using Imaris software (Bitplan AG, Zurich, Switzerland). The settings for the shadow projections were 0.8 for absorption and 0.5 for emission.

Immunofluorescence was observed and recorded with a Leica confocal laser-scanning microscope TSC PS2 (Wetzlar, Germany) using the 100 $\times$ objective (1.4). In the double- and triple-fluorescence overlays, effects of pixel shift were excluded. The z axis resolution of this equipment was at maximum 300 nm per voxel and the x and y settings were between 50 and 250 nm per voxel.

Metabolic Labeling, Immunoprecipitation, SDS-Polyacrylamide Gel Electrophoresis (PAGE), and Quantification

Clonal cell lines grown in Petri dishes (35 mm diameter) to 70 to 80% confluence were incubated in cysteine and methionine-free Dulbecco's modified Eagle's medium containing dialyzed fetal bovine serum for 30 minutes at 37°C. For pulse labeling, the cells were incubated in fresh medium containing 100 $\mu\text{Ci/ml}$ of ^{35}S -cysteine and ^{35}S -methionine for periods of time ranging from 10 to 120 minutes at 37°C. For the chase, the radioactive medium was removed and cells were washed twice with Ham's F12 medium containing nonradioactive methionine and cultured in fresh medium for periods ranging between 10 minutes and 8 hours. Afterward, cells were washed with ice-cold PBS and mechanically removed using a rubber policeman. Cells were sedimented by centrifugation at $3000 \times g$ for 10 minutes, resuspended in 100 μl of PBS containing protease inhibitors and proteins extracted as described above for Western blotting. The supernatant was added to protein A magnetic beads conjugated with anti-rat AQP2 antibody and incubated overnight at 4°C. Beads were collected using a magnetic stand, washed three times with PBS containing 0.1% Triton X-100, and washed again once with PBS.

Immunoprecipitated proteins were released by boiling for 10 minutes at 100°C in Laemmli buffer and separated in a

12% SDS-polyacrylamide gel. The bands were visualized either by autoradiography or by using a phosphorimager (Fuji Film Corp., Minami-Ashigara, Japan). Densitometric evaluation of the bands was performed using Wincam software (version 2.1; Cybertech, Berlin, Germany).

To examine the effect of proteasome and lysosome inhibitors on the turnover of AQP2, the cells were pulsed for 45 minutes with 300 $\mu\text{Ci/ml}$ of ^{35}S -cysteine and ^{35}S -methionine followed by a 2-hour chase in the presence of one of the inhibitors, immunoprecipitated with the AQP2 antibody, and subjected to SDS-PAGE followed by quantification as described above. The inhibitor concentrations were as follows: lactacystin, 50 $\mu\text{mol/L}$; MG132, 10 and 100 $\mu\text{mol/L}$; ALLN 10, 100 $\mu\text{mol/L}$; chloroquine, 50 $\mu\text{mol/L}$; and NH_4Cl , 10 mmol/L.

To inhibit *N*-glycosylation, clone 9 cells expressing T126M AQP2 were incubated in cysteine and methionine-free Dulbecco's modified Eagle's medium containing tunicamycin (1 $\mu\text{g/ml}$) for 30 minutes at 37°C and then pulsed for 40 minutes with 100 $\mu\text{Ci/ml}$ of ^{35}S -cysteine and ^{35}S -methionine in the presence of tunicamycin (1 $\mu\text{g/ml}$). Afterward, immunoprecipitation and SDS-PAGE was performed as described above.

Endoglycosidase Treatment of Immunoprecipitated AQP2

Immunoprecipitated AQP2 was digested with 5 U endo H in 0.1 mol/L of sodium acetate buffer (pH 5.6) for 17 hours at 37°C. For *N*-glycosidase F digestion, samples were incubated with 5 U of *N*-glycosidase F in 0.1 mol/L of phosphate buffer (pH 8.4) containing 1% Nonidet P-40 detergent for 17 hours at 37°C. The samples were denatured in Laemmli buffer and subjected to 12% SDS-PAGE. The bands were visualized using a phosphorimager.

Results

Stable Expression of Wild-Type and Mutant AQP2 in Clone 9 Rat Hepatocytes

We have analyzed two mutant AQP2 as compared to wt AQP2, namely the T126M point mutation located in the second extracellular loop and the E258K point mutation present in the carboxy terminus of AQP2 (Figure 1). The presence of wt as well as T126M AQP2 and E258K AQP2 in clonal cell lines derived from transfected clone 9 rat hepatocytes was verified by RT-PCR, Western blot analysis, and immunoprecipitation of metabolically labeled proteins. An 840-bp PCR fragment representing full-length coding cDNA of human AQP2 was detected in all AQP2-transfected cell lines but the mock-transfected ones (Figure 2A). Because it has been reported that AQP9 exists in liver,^{13,28} we examined its possible presence in clone 9 hepatocytes by RT-PCR. The reason was that mixed tetramer formation between wt and E258K AQP2 has been reported that resulted in the retention of the wt protein and causing dominant NDI.^{24,29} As shown in Figure 2B, AQP9 RNA was undetectable in clone 9

Figure 1. Schematic presentation of the membrane topology of AQP2. The locations of the amino acid substitutions of the two studied mutant AQP2 as well as the single *N*-glycosylation site at N123 and the phosphorylation site at S256 are indicated.

hepatocytes in contrast to rat liver. Thus, the possible formation of mixed tetramers composed of wt AQP9 and E258K AQP2 can be excluded. Western blot analysis revealed one immunoreactive band with an apparent molecular mass of 29 kd in cells expressing either wt AQP2 or E258K AQP2 and an additional immunoreactive band migrating at 32 kd in T126M AQP2-expressing cells (data not shown). When AQP2 synthesis was analyzed by metabolic labeling and immunoprecipitation, two bands with an apparent molecular mass of 29 kd and of 32 kd were observed in the wt AQP2-, T126M AQP2-, and E258K AQP2-expressing cells (Figure 3A). In wt and E258K AQP2-expressing cells, the 32-kd form in contrast to the 29-kd form became detectable as a faint band only after a 30-minute pulse and its intensity increased in cells pulsed for 60 minutes. To test whether the 32-kd band corresponds to glycosylated AQP2, endo H treatment of the immunoprecipitate was performed. This resulted in the disappearance of the 32-kd band of the wt and the two mutant AQP2 and in a concomitant increase in intensity of the 29-kd band (Figure 3A). In line with this, *N*-glycosidase F treatment of the immunoprecipitate resulted in an increased 29-kd band and disappearance of the 32-kd band in wt and both mutant AQP2 (Figure 3B). However, the increase in intensity of the 29-kd band of wt and E258K AQP2 after *N*-glycosidase F treatment was

Figure 2. Demonstration of AQP2 expression in transfected clone 9 hepatocytes. **A:** RT-PCR amplification was performed using isolated RNA from mock- and AQP2-expressing clone 9 hepatocytes and specific AQP2 and rat β -actin primers. **B:** RT-PCR of aquaporin-9 in nontransfected clone 9 hepatocytes and rat liver.

Figure 3. The wild-type and mutant AQP2 expressed in clone 9 hepatocytes exist as a core-glycosylated and nonglycosylated form. **A:** The 32-kd form of metabolically labeled and immunoprecipitated wild-type, T126M, and E258K AQP2 is endo H-sensitive and becomes converted to a 29-kd form. **B:** Likewise the 32-kd form of wild-type, T126M, and E258K AQP2 is *N*-glycosidase F-sensitive. **C:** Tunicamycin treatment results in absence of the 32-kd form of T126M AQP2.

stronger as compared to that observed after endo H treatment. This difference was not observed with T126M AQP2. This demonstrates that the wt and E258K AQP2 oligosaccharide has been processed in the Golgi apparatus. In addition, T126M AQP2 synthesized in the presence of tunicamycin to block its *N*-linked glycosylation gave only a single 29-kd band (Figure 3C). Taken together, these data demonstrate that in clone 9 hepatocytes both the wt and the studied AQP2 mutants are synthesized in the ER as a nonglycosylated 29-kd form and a core glycosylated high mannose-type 32-kd form.

Intracellular Distribution of Wild-Type and Mutant AQP2

By confocal laser-scanning immunofluorescence, the wt AQP2 expressed in clone 9 hepatocytes exhibited predominantly a punctate supranuclear and faint cell surface staining (Figure 4A) alike to its inherent distribution in renal collecting duct cells.^{15,16} When living cells were incubated with fluorescently labeled transferrin to label endosomes (Figure 4B) and were subsequently subjected to confocal immunofluorescence microscopy to detect AQP2 and late endosomes/lysosomes with an antibody against LAMP 1 (Figure 4D), this triple labeling protocol provided evidence for co-distribution of wt AQP2 with a limited number (~20 to 30%) of transferrin-loaded endosomes (Figure 4C). However, the limited x-y resolution of confocal immunofluorescence (≤ 200 nm) allows no statement that wt AQP2 actually co-localizes with endosomes. On the other hand we obtained no evidence for co-distribution with lysosomes under our conditions (Figure 4, E and F). Because it has been demonstrated that relocation of wt AQP2 from intracellular vesicles to the plasma membrane can be experimentally induced by forskolin treatment,¹⁸ we investigated whether this cyclic AMP-dependent shuttling can be observed in clone 9 cells. As shown in Figure 5, forskolin treatment efficiently triggered relocation of wt AQP2 to the apical cell surface.

Confocal immunofluorescence microscopy for the detection of AQP2 in clone 9 cells expressing the T126M AQP2 revealed an ER-like pattern (Figure 6) and no evidence for co-distribution with Golgi mannosidase II (data not shown). In contrast, the E258K AQP2 immunofluorescence co-distributed with Golgi mannosidase II (Figure 7;

Figure 4. Localization of wild-type AQP2 stably expressed in clone 9 hepatocytes by confocal immunofluorescence microscopy. Triple labeling for the simultaneous detection of AQP2 (**A**), endocytosed transferrin (**B**), and LAMP1 (**D**) of late endosomes/lysosomes. In a single confocal section, AQP2 predominantly exhibits a punctate cytoplasmic staining (**A**) that partially co-distributes with endocytosed transferrin (**B**) as demonstrated by the yellow color in the merged fluorescence image (**C**). No co-distribution of AQP2 (**A**) with LAMP1 (**D**), which would have resulted in a purple color, could be detected in the triple merge image (**E**). In **F**, a high-resolution detail from this triple merge image (marked by a square in **E**) demonstrates co-distribution of AQP2 and transferrin as indicated by the yellow color and none with LAMP1 as indicated by the absence of purple color. Furthermore, the absence of white-colored elements implies that AQP2, transferrin, and LAMP1 are not present in the same structures. Scale bar, 10 μ m.

A to C), which is consistent with its reported Golgi apparatus localization in transfected *Xenopus* oocytes²⁴ and CHO cells.²² However, additional prominent dot-like immunostaining for AQP2 was evident in trans-

fected clone 9 hepatocytes. By using the triple-staining protocol to visualize AQP2 (Figure 7D), endocytosed transferrin (Figure 7E) and lysosomes (Figure 7G), this AQP2 staining was shown to be due to the co-distribution of E258K AQP2 with late endosomes and lysosomes (Figure 7, H and I). Co-distribution of E258K

Figure 5. Forskolin treatment results in cell surface relocation of wild-type AQP2 stably expressed in clone 9 hepatocytes. **A:** In untreated cells, immunofluorescence is predominantly associated with cytoplasmic vesicles and only faint staining is related to the cell surface. **B:** After forskolin treatment, the apical cell surface exhibits strong immunofluorescence and only a few positive cytoplasmic vesicles can be seen. Three-dimensional rendering to shadow projection images based on complete series of 200-nm optical sections through entire cells (for details, see Materials and Methods). Scale bar, 10 μ m.

Figure 6. The T126M AQP2 stably expressed in clone 9 hepatocytes exhibits an ER-like immunofluorescence. Single confocal section. Scale bar, 10 μ m.

Figure 7. Mutant E258K AQP2 stably expressed in clone 9 hepatocytes exhibits a dual Golgi apparatus and late endosome/lysosome distribution. A single confocal section reveals a perinuclear crescent-shaped and widespread dot-like immunofluorescence for E258K AQP2 (**A**) with the former overlapping with Golgi mannosidase II immunofluorescence (**B**) as indicated by the yellow color in the merged double-immunofluorescence image (**C**). Triple labeling for the simultaneous detection of E258K AQP2 (**D**), endocytosed transferrin (**E**), and LAMP1 (**G**). A single confocal section reveals the above-described dual distribution of E258K AQP2 (**D**) and an extensive dot-like distribution of transferrin (**E**). In the merged image (**F**), co-distribution is evident by the appearance of the yellow color and some of these double-labeled elements are marked by **arrows** in **D**, **E**, and **F**. The merged triple-labeling image shown in **H** reveals co-distribution of E258K AQP2 with transferrin and LAMP1 as indicated by the white color. A high-resolution image of the squared area in **H** is shown in **I**. The **arrowheads** in **D**, **G**, and **H** point to some of the elements that are positive for E258K AQP2, transferrin, and LAMP1 that are reflected by the white color. The purple color visible in **I** indicates co-distribution of E258K AQP2 and LAMP1 in lysosomes. It should be noted that in the merged image in **H**, most lysosomes are actually labeled for AQP2 as indicated by the **small purple dots**. Scale bars, 10 μ m.

AQP2 with endocytosed fluorescent transferrin was evident in double overlays (Figure 7F), which was apparently due to the presence of transferrin in early and late endosomes as could be concluded from the results

of triple overlays (Figure 7, H and I). When cells expressing E258K AQP2 were incubated with forskolin, no change in the pattern of AQP2 immunofluorescence could be observed (data not shown).

Figure 8. Turnover kinetics of wild-type and mutant AQP2 stably expressed in clone 9 hepatocytes. **A:** Autoradiogram of metabolically labeled and immunoprecipitated AQP2. **B:** Averaged turnover kinetics from three independent sets of experiments. The cells were pulsed with ^{35}S -cysteine and ^{35}S -methionine and chased for the time periods indicated (for details, see Materials and Methods).

Turnover of Wild-Type and Mutant AQP2 and the Effect of Proteasome and Lysosome Inhibitors

We studied the turnover of wt and AQP2 mutants in clone 9 rat hepatocytes by metabolic labeling. As illustrated in Figure 8, the T126M AQP2 and E258K AQP2 were more rapidly degraded than the wt AQP2. The average $t_{1/2}$ of degradation of wt AQP2 was 4.6 ± 0.4 hours (Figure 8B). When the degradation of the glycosylated 32-kd and the nonglycosylated 29-kd forms of T126M AQP2 were determined, their average $t_{1/2}$ were 2.0 ± 0.5 hours and 0.9 ± 0.3 hours, respectively (Figure 8B). Although the glycosylated 32-kd form was detectable at the end of the pulse in both the wt AQP2 and E258K AQP2, it became undetectable after a 1-hour chase, in contrast to the

Figure 9. Effect of proteasome inhibitors and lysosomotropic agents on turnover of T126M and E258K-AQP2 in clone 9 hepatocytes. **A:** Autoradiogram of metabolically labeled and immunoprecipitated T126M AQP2 chased in the presence of the indicated proteasome inhibitors or chloroquine. **B:** Averaged turnover kinetics from three independent sets of experiments (mean $\pm$ SE of densitometric evaluation). **Filled columns** represent the glycosylated 32-kd form and **open columns** the nonglycosylated 29-kd form of T126M AQP2. **C:** Autoradiogram of metabolically labeled and immunoprecipitated E258K AQP2 chased in the presence of lactacystin, MG132, chloroquine, or ammonium chloride. **D:** Averaged turnover kinetics of the 29-kd form (mean $\pm$ SE of densitometric evaluation of three independent sets of experiments).

nonglycosylated 29-kd form (Figure 8A). The average $t_{1/2}$ of degradation of E258K AQP2 was determined to be 1.8 ± 0.1 hours (Figure 8B).

The effect of the proteasome inhibitors lactacystin, MG132, and ALLN on the turnover of T126M AQP2 was studied in cells pulsed for 45 minutes in the absence of the inhibitors and chased for 2 hours in the presence of the respective proteasome inhibitor. The effect of the lysosomotropic agent chloroquine was investigated under the same conditions. As shown in Figure 9, A and B, all three inhibitors had a pronounced inhibitory effect on both the 32-kd and 29-kd form of the ER-retained T126M AQP2, whereas the lysosomotropic agent chloroquine had none. The densitometric evaluation demonstrated a stronger effect of the different proteasome inhibitors on the glycosylated 32-kd T126M AQP2 compared to the nonglycosylated form (Figure 9B). If this reflects only the different turnover kinetics of these two mutant AQP2 forms will require further studies. It should be noted that the more pronounced differential inhibitory effect of lactacystin may reflect its slower mode of action as compared to MG132 and ALLN. We applied the same protocol to study the effect of proteasome inhibitors and lysosomotropic agents on the turnover of E258K AQP2. As illustrated in Figure 9, C and D, both the used proteasome inhibitors and the lysosomotropic agents retarded the degradation of E258K AQP2 to a similar degree.

Discussion

In the present study we have investigated aspects of the biosynthesis and degradation of the ER-retained T126M AQP2 and the E258K AQP2 reported to be retained in the Golgi apparatus. Our results indicate that the T126M AQP2 is synthesized as a glycosylated 32-kd as well as a nonglycosylated 29-kd form, both of which are rapidly degraded by the proteasome, albeit at different kinetics. Furthermore, our results demonstrate that the E258K AQP2 is not exclusively retained in the Golgi apparatus but becomes misrouted to late endosomes/lysosomes and that its degradation involves both proteasomes and lysosomes.

Previous studies have shown that the T126M AQP2 represents a functional water channel that is retained in the ER.³⁰ In our stable expression system, T126M AQP2 was synthesized as a high mannose-type 32-kd and a nonglycosylated 29-kd form, alike observed in kidney extracts of T126M AQP2 knock-in mice.³¹ The two T126M AQP2 forms exhibited significant differences in their turnover rates. Although larger amounts of the nonglycosylated 29-kd form were initially synthesized, their average half-life was considerably shorter ($t_{1/2} = 0.9$ hours) than that of the glycosylated 32-kd form ($t_{1/2} = 2.0$ hours). Glycosylation-related differences in the degradation kinetics have been reported for a truncated soluble variant of the type I ER transmembrane glycoprotein ribophorin I.³² The glycosylated fragment of ribophorin I was more slowly degraded than the nonglycosylated mutated one. Therefore, such differences seem to occur regardless if these are naturally existing glycosylated and nonglycosylated forms of the same protein (our study) or experimentally generated ones.³² Our observations may be also of relevance for strategies to induce cell surface expression of ER-retained misfolded but functional glycoproteins in which chemical chaperones,^{21,33,34} calcium pump inhibitors³⁵ and low-molecular weight drugs and compounds^{36–38} have been applied to correct the traffic defect. In the case of the T126M AQP2, strategies to preferentially target the glycosylated form should be envisaged.

The higher stability of the glycosylated 32-kd T126M AQP2 form is apparently due to the recognition of its oligosaccharide by the protein quality control system.¹ Although further studies will be required to elucidate the details of this interaction, we assume bona fide that the mechanism we established for misfolded carboxypeptidase Y in yeast^{39,40} and which has been shown to be functioning for misfolded $\alpha(1)$ -antitrypsin, ribophorin I and Ig subunits expressed in mammalian cells^{32,41,42} is also operative for the glycosylated 32-kd T126M AQP2 form. In the case of misfolded carboxypeptidase Y, the presence of mono-glucosylated oligosaccharides provided a positive signal for protein folding³⁹ and that of the Man₈GlcNAc₂-B isomer generated by ER mannosidase I a signal for ERAD.⁴⁰ Naturally, this mechanism cannot be functioning in the disposal of the nonglycosylated 29-kd T126M AQP2 form and to the best of our knowledge, no data regarding the recognition mechanism acting on

nonglycosylated ER-retained mutated proteins are available.

The other novel observation is that the proteasome is involved in the degradation of T126M AQP2 whereas no evidence for the involvement of lysosomes in the degradation process was obtained. Although the three different proteasome inhibitors exhibited a similar stabilization effect on the glycosylated T126M AQP2 form, the effect on the nonglycosylated form was less pronounced. Thus, for the nonglycosylated form of T126M AQP2 mutant, we cannot rule out that other proteolytic activities in addition to the proteasome may be involved. The differential stabilization effect of the proteasome inhibitors on the glycosylated *versus* the nonglycosylated T126M AQP2 form implies that in our system no significant deglycosylation of the retained 32-kd T126M AQP2 occurs under these conditions and that deglycosylation is apparently coupled to its proteasomal degradation.

In contrast to the recessive NDI-causing ER-retained T126M AQP2, the E258K AQP2 has been reported to be retained in the Golgi apparatus of *Xenopus* oocytes²⁴ and transiently transfected CHO cells²² and to form mixed oligomers with wt AQP2, thereby causing dominant NDI.²⁹ In the present study we confirm the Golgi localization of E258K AQP2 by using double confocal immunofluorescence to detect the Golgi marker mannosidase II. However, by using additional double confocal immunofluorescence microscopy to detect the late endosome/lysosome marker LAMP 1, co-distribution of E258K AQP2 with LAMP 1 could be unequivocally demonstrated in the clone 9 hepatocytes. A triple fluorescence labeling experiment additionally visualizing endocytosed transferrin fully supported the post-Golgi localization of E258K AQP2. If the misrouting of E258K AQP2 via the Golgi apparatus to lysosomes is related to specific features of clone 9 hepatocytes as compared to *Xenopus* oocytes or CHO cells is unknown. Interestingly, an additional dominant NDI-causing AQP2 mutant, Δ G727, was reported.⁴³ When studied by double-immunofluorescence microscopy, a co-distribution of this mutant AQP2 with LAMP 1-positive late endosomes/lysosomes was clearly evident, whereas no co-distribution with Golgi markers and VAMP2-positive recycling vesicles could be observed. Thus, although there seems to exist a common mechanism of how mutations in the carboxy terminus of AQP2 cause the autosomal dominant form of NDI,⁴³ the two studied mutants nonetheless differ in their steady state intracellular distribution as demonstrated by confocal immunofluorescence microscopy (present study).⁴³ The presence of these two dominant NDI-causing mutants in lysosomes may be indicative of their common final degradative pathway. When we investigated the degradation kinetics of the E258K AQP2, metabolically labeled protein initially existed as a 29-kd and a 32-kd form with the latter rapidly disappearing, as has been observed for the wt AQP2. The E258K AQP2 had an average half-life similar to that of the T126M AQP2. Taking into account the intracellular steady state distribution of E258K AQP2 in late endosomes and lysosomes in addition to the Golgi apparatus, it was not unexpected that we found it to be stabilized by the lysosomotropic agents chloroquine and

ammonium chloride as well as the proteasome inhibitor lactacystin and MG132. This strongly suggests that both proteasomes and lysosomes are involved in the degradation of this AQP2 mutant. Very recently, the involvement of both proteasome and lysosomes in the degradation pathway of wt AQP2 in a mouse renal collecting duct cell line was reported.⁴⁴ Thus, as observed for other membrane proteins,^{45,46} the final degradative pathway for wt AQP2 and misrouted E258K AQP2 seems to involve both the proteasome and lysosomes. However, the T126M AQP2 and the E258K AQP2 expressed in clone 9 hepatocytes seem to be recognized by different quality control mechanisms and become efficiently degraded despite the fact that one is retained in the ER and the other is being misrouted to late endosomes and lysosomes.

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