Water Channels Encoded by Mutant Aquaporin-2 Genes in Nephrogenic Diabetes Insipidus Are Impaired in Their Cellular Routing

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Abstract

Congenital nephrogenic diabetes insipidus is a recessive hereditary disorder characterized by the inability of the kidney to concentrate urine in response to vasopressin. Recently, we reported mutations in the gene encoding the water channel of the collecting duct, aquaporin-2 (AQP-2) causing an autosomal recessive form of nephrogenic diabetes insipidus (NDI). Expression of these mutant AQP-2 proteins (Gly64Arg, Arg187Cys, Ser216Pro) in Xenopus oocytes revealed nonfunctional water channels. Here we report further studies into the inability of these missense AQP-2 proteins to facilitate water transport in Xenopus oocytes. cRNAs encoding the missense AQP-2 were translated with equal efficiency as cRNAs encoding wild-type AQP-2 and were equally stable. Arg187Cys AQP-2 was more stable and Gly64Arg and Ser216Pro AQP-2 were less stable when compared to wild-type AQP-2 protein. On immunoblots, oocytes expressing missense AQP-2 showed, besides the wild-type 29 kDa band, an endoplasmic reticulum-retarded form of AQP-2 of ~32 kDa. Immunoblots and immunocytochemistry demonstrated only intense labeling of the plasma membranes of oocytes expressing wild-type AQP-2. Therefore, we conclude that in Xenopus oocytes the inability of Gly64Arg, Arg187Cys or Ser216Pro substituted AQP-2 proteins to facilitate water transport is caused by an impaired routing to the plasma membrane. (J. Clin. Invest. 1995; 95:2291–2296.) Key words: collecting duct • oocytes • mutation • vasopressin • urine concentration

Introduction

The ability of the kidney to conserve or excrete free water independent of changes in solute excretion is essential in mammals. Physiological studies have provided evidence that in the nephron water is constitutively reabsorbed in proximal tubules and descending limbs of Henle's loop, whereas water reabsorp-

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1. Abbreviations used in this paper: AQP, aquaporin; AVP, arginine-vasopressin; CFTR, cystic fibrosis transmembrane conductance regulator; endo H, endoglycosidase H; NDI, nephrogenic diabetes insipidus; Pf, osmotic water permeability coefficient; wt, wild-type.

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Methods

Preparation of antibodies. A peptide was synthesized by standard automated solid-phase techniques (14) based on the predicted 15 COOH-
terminal amino acids of rat AQP-2 (5). Via an NH₂-terminally added acetylthioacetyl group, this peptide was conjugated to keyhole limpet haemocyanin or bovine serum albumin according to Schielen et al. (15). Rabbits were immunized with 400 μg of this conjugate mixed with Freund's complete adjuvants. Starting at three weeks after priming, rabbits were boosted at three weeks intervals with conjugate mixed with incomplete adjuvant. Test bleedings were screened by ELISA.

In vitro translation. cRNAs encoding wild-type (wt) or mutant AQP-2 proteins (12, 13) were in vitro translated in wheat germ extracts as described (16).

Water permeability measurements. The isolation of Xenopus oocytes, injection of these cells with water or cRNAs coding for wt or mutant AQP-2 proteins and analysis of oocytes for water permeability were done as described (12).

Northern blot analysis. Three days after injection, RNA was isolated from 6 oocytes according to Chomczynski and Sacchi (17). RNA equivalents of three oocytes were loaded onto a 2.2 M formaldehyde, 1% (wt/vol) agarose gel. Electrophoresis, blotting and hybridization conditions were as described (18). An 850 bp EcoRI cDNA fragment encoding human AQP-2 (12) was labeled with [α-32P]dCTP by random priming (19) and used as a probe. The relative amounts of mRNA loaded onto the gel was assessed by hybridization of the blot with a probe of a 600-bp EcoRI cDNA fragment coding for Xenopus laevis 28S rRNA (20) and subsequent scanning of the autoradiographic signals with an LKB Ultrascan XL laser densitometer.

Immunoblotting. After analysis of water permeability, oocytes injected with wt, mutant AQP-2 cRNAs, or water (40 per sample) were washed twice in homogenization buffer A (20 mM Tris (pH 7.4), 5 mM MgCl₂, 5 mM NaH₂PO₄, 1 mM EDTA, 1 mM DTT, 100 μM Oubain, 1 mM PMFS, 5 μg/ml leupeptin and pepstatin, 80 mM sucrose) at 4°C. Subsequently, the oocytes were homogenized in 5 μl homogenization buffer A per oocyte and centrifuged twice for 10 min at 125 g to remove yolk proteins. Equivalents of one oocyte were digested with endoglycosidase H₁ (endo H; New England BioLabs, Beverly, MA) or α-D-glycosidase F (Boehringer, Mannheim, Germany) according to the manufacturer, except that the protein samples were digested for 18 h after denaturation for 30 min at 37°C. For SDS-PAGE, 5 μl of oocyte lysates or 2 μl of the in vitro translation mixture were denatured by incubation for 30 min at 37°C in 1X SDS-sample buffer (2% SDS, 50 mM Tris (pH 6.8), 12% glycerol, 0.01% Connaissce Brilliant Blue R, 25 mM DTT). Proteins of the samples were separated on a 12% SDS-polyacrylamide gel of 0.1 X 7 X 9 cm using laemmli buffers (21). Immunoblotting was done essentially as described (22) with enhanced chemiluminescence to visualize sites of antigen-antibody reactions (Amerham, Buckinghamshire, England). Efficiency of protein transfer was checked by staining the membrane with Ponceau Red. With immunoblotting, the primary antibody (AQP-2 antiserum) was diluted 1:10,000. As a secondary antibody, a 1:5,000 dilution of affinity-purified goat anti-rabbit IgG conjugated to horseradish peroxidase (Sigma Immuno Chemicals, St. Louis, MO) was used. Scanning of immunoobslots was done as described above.

Protein stability. To determine the stability of mutant and wild-type AQP2 proteins, Xenopus oocytes were injected with equal amounts of the corresponding cRNAs. Next, at day 1, 2, 3, and 4 after injection, lysates were prepared from 8 oocytes per sample and 0.5 oocyte equivalents were immunoblotted as described above. Densitometric scanning of 29 and 32 kDa bands together (when appropriate) of the third day was done as described above.

Oocyte plasma membrane isolation. From 25 oocytes, injected with water or equal amounts of cRNAs, plasma membranes were isolated according to Wall and Patel (23). Subsequently, lysates or plasma membranes equivalent to 0.1 oocyte or 8 oocytes, respectively, were subjected to immunoblotting as described above. Densitometric scanning of 29 and 32 kDa bands together (when appropriate) was done as described above.

Immunocytochemistry. Unfixed oocytes were embedded in OCT compound (Tissue Tek Products, Ames Division, Miles Laboratories, Inc., Elkhart, IN), rapidly frozen in liquid nitrogen and stored at −80°C. Six μm sections were cut in a cryostat at −20°C and were fixed for 3 min in cold acetone of −20°C. After blocking with 1% normal swine serum for 30 min, the sections were incubated for 1 hour with anti-AQP-2 antiserum diluted 1:2000 with PBS-T (0.05% Tween-20 in PBS; pH 7.3). After three washings in PBS-T for 5 min, the sections were incubated for an hour with a 1:100 dilution of swine anti-rabbit antibodies coupled to FITC (DAKO). The sections were again washed 3 times for 5 min and mounted in mowiol 4-88, 2.5% Na₃. Photographs were taken with a Zeiss Axioskop with epifluorescent illumination with an automatic camera using Kodak TMY films.

Results

In the AQP-2 genes of our NDI patients three different missense mutations were detected that coded for Gly64Arg, Arg187Cys, or Ser216Pro substituted AQP-2 proteins (Fig. 1) (12, 13). Expression studies in Xenopus oocytes revealed that these mutant AQP-2 proteins did not increase the osmotic water permeability (Pᵢ) above that of water-injected controls, whereas the Pᵢ of oocytes injected with cRNAs encoding wt AQP-2 was at least ten times higher (12, 13). The absence of facilitated water transport in mutant AQPs could in principle be due to (A) a low efficiency of translation of mutant cRNAs, (B) a low stability of mutant cRNAs in Xenopus oocytes, (C) a high turnover of mutant AQPs in the oocyte, (D) a failure in the transport of mutant AQPs to the plasma membrane of the oocyte, and/or (E) a nonfunctional AQP protein.

Translational efficiency. To test whether the cRNAs were translated at a different efficiency, equal amounts of cRNAs encoding wt or mutant AQPs were in vitro translated and separated by polyacrylamide gel electrophoresis. Autoradiography of the gel revealed that each cRNA was translated with similar efficiency (Fig. 2).

Stability of cRNAs in oocytes. To circumvent the possibility that mutant cRNAs and/or peptides would be hardly detectable because of instability, the amounts of mutant cRNAs injected into Xenopus oocytes were three times higher than that of wt AQP-2 cRNA. Three days after injection, the Pᵢ of oocytes injected with mutant cRNAs or water were unchanged, whereas wt cRNA-injected oocytes showed a high Pᵢ (Fig. 3). Northern blot analysis of oocytes from the same injection demonstrated that an RNA species of ~1.2 kb was recognized by the human AQP-2 cDNA probe in the lanes loaded with RNA isolated from cRNA-injected oocytes (Fig. 4). Normalized for the amount of RNA loaded through hybridization with a Xenopus 28S-rRNA
cDNA probe, the amounts of mutant AQP-2 cRNAs were about three times higher than that of wild-type AQP-2 cRNA.

Expression of AQP-2 in Xenopus oocytes. To compare the sizes of wild-type and mutant AQP-2 cRNAs, oocytes were injected with wild-type (wt), Gly64Arg (GR), Arg187Cys (RC), and Ser216Pro (SP) AQP-2 proteins obtained by in vitro translation of equal amounts of their corresponding cRNAs. Molecular weights of marker proteins (Bio-Rad) are indicated.

Figure 2. SDS-PAGE analysis of wild-type (wt), Gly64Arg (GR), Arg187Cys (RC), and Ser216Pro (SP) AQP-2 proteins obtained by in vitro translation of equal amounts of their corresponding cRNAs. Molecular weights of marker proteins (Bio-Rad) are indicated.

AQP-2 cDNA probe (upper panel). In comparison to wild-type AQP-2, threefold amounts of cRNA of missense AQP-2 was injected. For normalization of the amount of RNA loaded, the blot was hybridized with a 28S RNA cDNA probe (lower panel). Sizes of RNA marker bands (GIBCO-BRL) are indicated on the right.

2. respectively. Immunoprecipitation of in vitro translated wild-type and mutant AQP-2 proteins followed by SDS-PAGE showed that the affinity of our AQP-2 antiserum for wild-type and mutant AQP-2 was similar (data not shown).

Figure 3. Osmotic water permeabilities ($P_f$) of Xenopus oocytes injected with wild-type, Gly64Arg, Arg187Cys or Ser216Pro AQP-2 protein. Mean and SEM values of at least 10 oocytes are shown.

Figure 4. Northern blot analysis of the stabilities of AQP-2 cRNAs in Xenopus oocytes. Three days after injection with water ($H_2O$) or cRNAs encoding wild-type (wt), Gly64Arg (GR), Arg187Cys (RC), or Ser216Pro (SP) AQP-2 proteins, RNA was extracted from six oocytes per sample. Equivalents of three oocytes were blotted and hybridized with a human AQP-2 cDNA probe (upper panel). In comparison to wild-type AQP-2, threefold amounts of cRNA of missense AQP-2 was injected. For normalization of the amount of RNA loaded, the blot was hybridized with a 28S RNA cDNA probe (lower panel). Sizes of RNA marker bands (GIBCO-BRL) are indicated on the right.

Figure 5. Immunoblot analysis of AQP-2 proteins expressed in cRNA-injected Xenopus oocytes. Three days after injection with water ($H_2O$) or cRNAs encoding wild-type (wt), Gly64Arg (GR), Arg187Cys (RC), or Ser216Pro (SP) AQP-2, lysates were prepared of 40 oocytes each. Compared with wild-type AQP-2, three times more cRNA of missense AQP-2 was injected. Proteins of an equivalent of one oocyte were separated by SDS-PAGE and blotted (A) or digested with (+) or without (−) endo H, before loading (B). AQP-2 proteins were visualized by chemiluminescence autoradiography using rabbit polyclonal antibodies raised against the 15 COOH-terminal amino acids of rat AQP-2 (5) as a first antibody and goat anti−rabbit IgG, conjugated to horse radish peroxidase, as a second antibody.

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Localization of AQP s in oocytes. To determine whether mutant AQPs are localized in the plasma membrane of the oocyte, immunocytochemistry was performed on oocytes injected with water or cRNAs (Fig. 7). All oocytes injected with mutant or wt cRNA demonstrated intense intracellular labeling, while staining was absent in water-injected controls. Clear immunofluorescence staining of the plasma membrane was only found in oocytes injected with wt AQP-2 cRNAs, while labelling of the plasma membranes of oocytes expressing mutant AQPs ranged between weak to hardly detectable in the order R187C, G64R, S216P, AQP2. Oolemmas of oocytes injected with water were negative. Expression of wt and mutant AQPs in oolemmas was also determined in fractions enriched in plasma membranes (Fig. 8). Densitometric scanning revealed that AQP2 in plasma membranes was 4.65, 0.25, or 0.12 times enriched for wt, Gly64Arg or Arg187Cys AQP2, respectively, when compared with their corresponding lysates. For oocytes expressing Ser216Pro AQP2, only after prolonged exposure bands could be detected in the lysate (data not shown).

**Discussion**

Gene mutations, such as promoter defects, nonsense mutations and defects related to mRNA splicing, can cause genetic diseases. Furthermore, mutations that lead to a changed mRNA stability or an impairment in the intracellular routing of the protein can produce a diseased phenotype (24, 25). Integral membrane proteins, such as AQP-2, travel through the secretory pathway to a variety of destinations. After folding and glycosylation in the endoplasmic reticulum (ER), proteins traverse the Golgi complex, where modifications such as N-linked oligosaccharide processing and O-linked glycosylation occur (25). Proteins leave the Golgi complex in vesicles destined to fuse with several different targets, such as the plasma membrane and secretory granules (24). Given the complexity and importance of the intracellular trafficking pathways, it is not surprising that mutations that affect the intracellular routing are the cause of many genetic diseases (24). In previous studies, we reported a one nucleotide deletion and three missense mutations in the gene encoding water channel AQP-2 in four NDI patients (12, 13). Expression of the encoded Gly64Arg, Arg187Cys or Ser2-
16Pro substituted AQPs (Fig. 1) in *Xenopus* oocytes, revealed that none was functional and thus it was concluded that the mutations in the AQP-2 genes caused the autosomally inherited NDI in the four patients (12, 13).

In the present study we addressed the failure of the missense AQPs to function as a water channel in *Xenopus* oocytes. Independent in vitro translations of equal amounts of cRNAs encoding wt or missense AQP-2 revealed that the cRNAs were translated with equal efficiency into a protein of ~ 29 kD (Fig. 2). This size is similar to that deduced from the cDNA sequence (28839 D) and the endogenous, nonglycosylated form of human AQP-2 (26). Since in vitro translation is regarded as a reliable test for translation in *Xenopus* oocytes, we assume that all cRNAs will be translated with similar efficiency in these cells.

The vast majority of Tay-Sachs cases in the past were caused by mutations in the gene encoding lysosomal enzyme β-hexosaminidase resulting in defects in maturation and stability of its mRNA (27). To test whether cRNAs of mutant AQP-2 proteins were less stable than wt cRNAs in *Xenopus* oocytes, the amounts of mutant and wt cRNAs present in oocytes after an incubation period of three days were compared by Northern blot analysis (Fig. 4). Albeit the relative abundance of mutant and wt cRNAs was similar after three days, only oocytes injected with wt cRNA showed increased Pf values (Fig. 3).

Therefore, the absence of increased osmotic water permeability in oocytes expressing mutant AQPs is not due to a decrease in cRNA stability. Immunoblotting and immunocytochemistry were performed to investigate whether the stability and/or the intracellular routing of mutant AQPs was affected in oocytes. On immunoblots, oocytes expressing mutant AQPs showed, besides the wt AQP-2 band of 29 kD, additional bands of ~ 32, 43, and 67 kD (Fig. 5A). The appearance of these additional bands is inherent to the mutant AQP2 proteins and is not a result of overexpression per se, since after injection of increasing amounts of wt AQP-2 cRNA the 29-kD band is still the only detectable form (result not shown). In addition, injection of equal amounts of cRNAs revealed the 32-, 43-, and 67-kD bands in lanes of oocytes expressing mutant AQP2 proteins only (for 32-kD band see Fig. 6). In general, plasma membrane integral proteins are synthesized and glycosylated to high-mannose glycoproteins in the ER, rapidly processed in the Golgi apparatus, where they are deglycosylated and sometimes reglycosylated to complex glycoproteins, and transported to the cell surface. Glycoproteins containing ER-specific glycosylation chains are endo H sensitive, but will become endo H resistant during processing in the Golgi complex (28, 29 and references therein). The 32-kD band was shown to be sensitive to endo H (Fig. 5B), and therefore represents a high-mannose, ER-retarded form of AQP-2. The higher bands did not disappear upon endo H or endo F digestion and are presumably aggregates of the mutant AQP-2 proteins. Resident components of the ER govern numerous post-translational modifications of proteins including folding, glycosylation and oligomerization. These processes determine in part the fate of newly synthesized proteins, as proteins that fold incorrectly or that are not assembled properly will in most cases be retained in the ER and are typically degraded in a pre-Golgi compartment (28, 29 and references therein). The extent to which degradation occurs might be determined by structural motifs in the protein (28).

The presence of ER-retarded mutant AQP2 proteins (Fig. 5) and the lower stability of the Gly64Arg and Ser216Pro AQPs, as compared with wt AQP2 (Fig. 6), clearly indicate an impaired intracellular transport of mutant AQP2 proteins. This was further corroborated by immunocytochemistry on oocytes and immunoblot analysis of their plasma membranes. Oocytes injected with cRNAs encoding wt or mutant AQPs exhibited an intense intracellular staining. It is known that in *Xenopus* oocytes that overexpress membrane proteins, only a small percentage of the expressed proteins is found in the plasma membrane (30). However, the plasma membrane of oocytes expressing wt AQP2 was distinctly stained, while labeling of oolemmas of other injected oocytes was either weak (R187C, G64R, S214P) or absent (water). In fact, the order of staining intensity of oolemmas from oocytes expressing mutant AQP2 proteins seems to reflect their relative stability. Immunoblot analysis of plasma membranes of oocytes injected with the different cRNAs only revealed a strong increase in the AQP2 signal in membranes isolated from oocytes expressing wt AQP2 (Fig. 8). The weak 29 and 32 kDa signals visible in membrane fractions of oocytes expressing Arg187Cys or Gly64Arg AQP2 proteins are most likely due to a minor contamination with intracellular organelles. Furthermore, it seems that the weak oolemma staining detected with immunocytochemistry is not retrieved or detected after biochemical fractionation of the oocyte. On the other hand, the injection of 25 ng cRNA encoding mutant AQPs into each oocyte used for immunocytochemistry and 10 ng cRNA into each oocyte used for plasma membrane isolation can explain the difference in detection of AQP2 in their plasma membranes.

In conclusion, our results clearly demonstrate that, except for the Cys187Arg AQP2 which is more stable, all mutant AQP2 proteins are less stable than wt AQP2 and that all three missense AQPs are impaired in their trafficking to the plasma membrane. Since defective folding is likely to cause the defective maturation of the AQP-2 mutants and, consequently, their membrane distribution, it can at present not be resolved whether these mutants are functionally inactive water channels. In fact, our observations are comparable with oocyte expression studies in which AQP-1 was used (31). These authors showed that oocytes expressing non-functional mutant AQP-1 proteins, synthesized the native 28-kD AQP-1 protein and a slightly larger protein than the 28-kD subunit. This latter band was also ascribed to incomplete carbohydrate processing as a consequence of disturbed intracellular routing. Based on functional tests of many AQP-1 mutants, Jung et al. (32) proposed a three-dimensional hour-glass model for AQP-1, which might also be valid for AQP-2. Since in this model the integrity of loops B and E are thought to be essential, the disturbed intracellular transport of AQP-2 proteins with G64R or R187C substitutions, that reside in loops B and E (Fig. 1), respectively, substantiate this model.

*Xenopus* oocytes have been shown to be ideal for functional expression of many plasma membrane proteins (33), including water channels. However, differences in protein routing and glycosylation have been described between oocytes and mammalian cells. Although in *Xenopus* oocytes wt AQP-1 is detected in its native and complex glycosylated form (34), we could only detect the native form of wt AQP-2 in these cells (Fig. 5). In human and rat kidney, AQP-2 is present as a sharp 29-kD and a diffuse 40–50-kD band, resembling the native and complexly glycosylated form of AQP-2 (26). Whether AQP-2 in oocytes is not complex glycosylated or whether this form of AQP-2 is not detected by our antisera is presently unclear. Functional differences between oocytes and mammalian cells were reported in CFTTR expression studies. While expression of mutant CFTRs in mammalian cells did not reveal an activated Cl− conductance (35), expression of the same mutants in oo-
cytes resulted in functional CFTRs, although with a reduced sensitivity to cAMP (36). Therefore, further studies on water transport and on subcellular localization of wt and mutant AQP-2 proteins expressed in mammalian cells are warranted in order to elucidate the molecular and cellular defects in NDI caused by mutations in the AQP-2 gene.

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