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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 to facilitate water transport 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-2s were translated with equal efficiency as cRNAs encoding wild-type AQP-2 and were equally stable. Arg187Cys AQP2 was more stable and Gly64Arg and Ser216Pro AQP2 were less stable when compared to wild-type AQP2 protein.

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 reabsorption in collecting ducts is regulated by the hormone arginine-vasopressin (AVP). Although water can pass membranes by diffusion, it has now been shown unambiguously that water transport in the nephron is facilitated by water channels. The first-identified water-selective channel aquaporin-1 (AQP-1) or CHIP28 (3, 4) is abundantly expressed in proximal tubule and descending limb of Henle’s loop, where the water recovery is entirely attributed to this channel. AQP-1 is a member of a family of transporting proteins with the major intrinsic protein (MIP) of lens fiber cells as its prototype (1). Based on conserved sequences within this family, cDNAs encoding new putative water channels were isolated from kidney tissue. One such a cDNA clone encoded a protein with 43% amino acid identity to AQP-1. Since this AQP-2 or WCH-CD, showed all the biochemical characteristics of water channels and since it was exclusively expressed in principal cells and inner medullary collecting duct cells, this protein was suggested to be the AVP-regulated water channel (5).

The apical membrane of principal cells and inner medullary collecting duct cells is considered to be rate-limiting in AVP-regulated trans-cellular water flow. When AVP binds to V2-receptors in the basolateral membrane of these cells, the intracellular cAMP level increases and triggers, by an unknown mechanism, fusion of intracellular vesicles containing water channels with the apical membrane. Upon removal of AVP, water channels are withdrawn from the apical membrane by endocytosis (6). Recent immunocytochemical studies on collecting duct cells revealed that AQP-2 was indeed present in a subcellular compartment, expected to contain the AVP-regulated water channel (7).

Congenital nephrogenic diabetes insipidus (NDI) is an inherited disease in which the kidney fails to concentrate urine in response to AVP. In most families, NDI is caused by a mutation in the V2-receptor gene, located on the X-chromosome (8–10). Some families, however, show an autosomal recessive mode of inheritance (11). Analysis of genomic DNAs of four NDI patients, in whom mutations in the V2-receptor gene was excluded or unlikely, have revealed a one nucleotide deletion and three missense mutations in their AQP-2 genes (12, 13).

The missense mutations result in AQP-2 proteins with a Gly64Arg, Argl87Cys, or Ser216Pro substitution and appeared to be non-functional when expressed in Xenopus oocytes. These results proved that AQP-2 is essential in AVP-regulated antidiuresis. The identification of NDI patients, whose AQP-2 genes are mutated, offer the opportunity to study the structure–function relationship of naturally occurring non-functional AQP-2 proteins. In the present study we carried out Northern blotting, immunoblotting and immunocytochemistry in order to address the inability of the mutant AQP-2 proteins to facilitate water transport.

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 M sucrose gel. Electrophoresis, blotting and hybridization conditions were as described (18). An 850 bp EcoRI cDNA fragment encoding human AQP-2 (12) was labeled with [c-32p]dCTP by random priming (19) and was 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 two times 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 PMSE, 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 N-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 mixtures 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% Tsennion 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 laemmlı buffers (21). Immunoblotting was done essentially as described (22) with enhanced chemiluminescence to visualize sites of antigen-antibody reactions (Amersham, 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 horse radish peroxidase (Sigma Immuno Chemicals, St. Louis, MO) was used. Scanning of immunoblots 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 kD 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 kD bands together (when appropriate) was done as described above.

Immunocytochemistry. Fixed oocytes were embedded in OCT compound (Tissue Tek Products, Arteson 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 h 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% NaN₃. 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 (Pₑ) above that of water-injected controls, whereas the Pₑ 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 AQP3 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 AQP3s in the oocyte, (D) a failure in the transport of mutant AQP3s 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 AQP3s 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 Pₑ of oocytes injected with mutant cRNAs or water were unchanged, whereas wt cRNA-injected oocytes showed a high Pₑ (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 wt AQP-2 cRNA.

Expression of AQP s in Xenopus oocytes. To compare the sizes of wt and mutant AQP s expressed in Xenopus oocytes, lysates were prepared and immunoblotted using an antiserum raised against the 15 COOH-terminal amino acids of the rat AQP-2 protein. Reversible staining with Ponceau Red showed that equal amounts of protein were loaded (not shown). Chemiluminescence detection demonstrated a protein of ~29 kD in all lanes of oocytes injected with AQP-2 cRNAs, but not in the lane of water-injected controls (Fig. 5 A). Furthermore, in the lanes of oocytes injected with mutant cRNAs a discrete band of ~32 kD and diffuse bands around 43 and 67 kD were observed. When the antiserum was preincubated with excess synthetic peptide no bands were detectable anymore (results not shown). The 32-kD band was sensitive to endo H digestion (Fig. 5 B), but the sizes of the higher bands were not changed after endo H or F digestions (not shown). To determine the stability of wt and mutant AQP2 proteins, oocytes were injected with equal quantities of cRNA. Next, at 1, 2, 3, and 4 d after injection, lysates were prepared and immunoblotted (Fig. 6). Densitometric scanning of the exposed film revealed that the levels of expression of Gly64Arg, Arg187Cys, and Ser216Pro substituted AQP s was 0.8, 1.1 and 0.1 times that of wt AQP-

Figure 3. Osmotic water permeabilities (P1) of Xenopus oocytes injected with water or cRNAs encoding 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 (H2O) or cRNAs encoding wild-type (wt), Gly-64Arg (GR), Arg187Cys (RC), or Ser216Pro (SP) aquaporin-2, 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 s 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 (H2O) or cRNAs encoding wild-type (wt), Gly64Arg (GR), Arg187Cys (RC), or Ser216Pro (SP) aquaporin-2, lysates were prepared of 40 oocytes each. Compared with wild-type AQP-2, three times more cRNA of missense AQP s 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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*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 of 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 Ser216Pro AQP2 proteins in oocytes injected with corresponding cRNAs. As negative control, water-injected oocytes were used. Compared to wild-type AQP2, three times more cRNA of missense AQP2 was injected. Thin sections of injected oocytes were incubated with rabbit polyclonal antibody raised against the 15 C-terminal amino acids of rat AQP-2 (5) and visualized by FITC-conjugated swine anti-rabbit immunoglobulins. ×40.
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. 5 A). 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. 5 B), 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 retarded 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. 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 not 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 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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