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New Mutations in the AQP2 Gene in Nephrogenic Diabetes Insipidus Resulting in Functional but Misrouted Water Channels

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Abstract. Nephrogenic diabetes insipidus (NDI) is characterized by the inability of the kidney to concentrate urine in response to vasopressin. The autosomal recessive form of NDI is caused by mutations in the AQP2 gene, encoding the vasopressin-regulated water channel of the kidney collecting duct. This report presents three new mutations in the AQP2 gene that cause NDI, resulting in A147T-, T126M-, or N68S-substituted AQP2 proteins. Expression of the A147T and T126M mutant AQP2 proteins in Xenopus oocytes revealed a relatively small, but significant increase in water permeability, whereas the water permeability of N68S expressing oocytes was not increased. cRNA encoding missense and wild-type AQP2 were equally stable in oocytes. Immunoblots of oocyte lysates revealed that only the A147T mutant protein was less stable than wild-type AQP2. The mutant AQP2 proteins showed, in addition to the wild-type 29-kd band, an endoplasmic reticulum-retarded form of AQP2 of approximately 32 kd. Immunoblotting and immunocytochemistry demonstrated only intense labeling of the plasma membranes of oocytes expressing wild-type AQP2. In summary, two mutant AQP2 proteins encoded in NDI are functional water channels. Therefore, the major cause underlying autosomal recessive NDI is the misrouting of AQP2 mutant proteins. (J Am Soc Nephrol 8: 242–248, 1997)

Aquaporins are selective water channels and form a subset of the MIP family of intrinsic membrane proteins. In the kidney, four aquaporins (AQP 1 through 4) (1–6) have been identified and are postulated to be involved in reabsorption and concentration of the glomerular filtrate. AQP1 is constitutively expressed in the proximal tubule and descending limb of Henle, and is localized to apical and basolateral membranes (7). AQP2 is the vasopressin-regulated water channel of principal cells of the collecting duct (2). In the absence of vasopressin, AQP2 is localized in vesicles in the subapical region of the cell. Upon binding of vasopressin to its V2 receptor, AQP2 water channels are inserted into the apical membrane, conferring a high water permeability to this membrane. Upon removal of vasopressin, the channels are retrieved by endocytosis (8–12). AQP3 and AQP4 are localized to the basolateral membrane of principal cells of the collecting duct, and are suggested to function as an exit pathway for water (13). So far, only AQP2 has been shown to be involved in diseases. Individuals who lack functional AQP1 do not exhibit clinical symptoms, which raised questions about the physiological significance of AQP1 (14). Mutations in AQP3 and AQP4 have not been identified so far. Mutations in the AQP2 gene, however, have been shown to be the cause of the autosomal recessive form of nephrogenic diabetes insipidus (NDI), a severe disease that is characterized by the inability of the kidney to concentrate urine in response to vasopressin (15,16). In the majority of patients, NDI is caused by a mutation in the V2 receptor gene, and is inherited as a X-linked recessive trait. In approximately 10% of the families, NDI has shown a non-X-linked pattern of inheritance. So far, one-nucleotide deletion and three mutations coding for missense mutations in AQP2 have been reported as a cause of NDI in some of these families (15,16). Upon expression in Xenopus oocytes, the missense AQP2 proteins with a G64R, R187C, or S216P substitution were unable to increase the water permeability (Pf) of oocytes, whereas expression of wild type AQP2, increased Pf values more than 10-fold. Further studies revealed that the mutant AQP2 proteins were impaired in their cellular routing (17). Consequently, it remained undecided whether these mutations resulted in non-functional water channels, since they did not reach the plasma membrane.

In the study presented here, we report three additional NDI patients who are homozygous for mutations in the AQP2 gene. In addition, we performed functional analyses of the mutant AQP2 proteins in oocytes and concluded that two mutations result in functional but misrouted water channels.
Materials and Methods

Patients

The three patients investigated in this study come from three separate families from different ethnic origin. In all three families, the parents of the patients are consanguineous.

Family 1 is of Austrian descent. The male proband (Patient 1) was admitted to the hospital at the age of 3 months with signs of dehydration, including recurrent fever and hypernatremia (serum sodium concentration, 164 mmol/L). On admission, urinary osmolality was very low (50 mosmol/kg) and did not increase after a water-deprivation test or after the administration of arginine vasopressin. At present, at the age of 18 yr, he has a polyuria and polydipsia of approximately 13 L/day (no medication).

In his older sister, a diagnosis of diabetes insipidus was made at the age of a few months. Data on a water-deprivation test or a DDAVP (deamino-8-d-arginine vasopressin; Minrin®) test were not available in her medical record. She has been treated, however, with Minrin® without a reduction in urinary volume. Now, as an adult, she has a fluid intake of approximately 13 L/day (no medication).

Family 2 comes from Sri Lanka. The male proband (Patient 2) was referred at the age of 5 months because of intermittent high fever, weakness, irritability, and weight loss. The diagnosis of NDI was based on the presence of a high serum sodium concentration (186 mmol/L), a low urinary osmolality (173 mosmol/kg), and unresponsiveness to DDAVP. Therapy with hydrochlorothiazide proved very difficult despite tube feeding and was complicated by intermittent bulging fontanel, fever, and vomiting. The boy failed to thrive and showed muscular hypotonia and delayed psychomotor development. At the age of 13 months he was readmitted with high fever, seizures, and hypernatremia (173 mosmol/kg), followed by coma. He succumbed two weeks later. MRI and necropsy revealed severe brain lesions with necrosis of basal ganglia. Eight months later NDI was diagnosed in the younger brother at the age of 1 wk based on elevated serum sodium (147 mmol/L). Urinary osmolality was low (82 mosmol/kg).

Family 3 is Turkish by descent. In the female proband (Patient 3), the diagnosis diabetes insipidus was made at the age of 6 wk when she was admitted to the children’s hospital with failure to thrive and signs of dehydration, including fever, and hypernatremia (serum sodium concentration, 162 mosmol/L). Urinary osmolality was low (82 mosmol/kg) but increased to 236 mosmol/kg after water deprivation and to 450 mosmol/kg after administration of arginine vasopressin, suggesting a diagnosis of nephrogenic diabetes insipidus with partial resistance to vasopressin. However, at the age of 18 months, a control vasopressin test showed a rise of urine osmolality to only 239 mosmol/kg. According to the parents, two sons of the sister of the paternal grandmother, at that time 7 and 8 yr old, both suffered from a similar disease. Further investigation revealed that they both had NDI with total resistance to vasopressin. All three patients have been treated with the combination indomethacin-hydrochlorothiazide, which was replaced for amiloride and Minrin® in the proband at the age of 4 yr.

Despite treatment, she still had a fluid intake of approximately 4 L/day at that age.

DNA Amplification and Sequence Analysis of Patients

Genomic DNA was isolated by the salt-extraction technique. Primers used for amplification of the AQP2 coding regions and for cycle sequencing were as described elsewhere (15). PCR conditions were 1 min at 92°C, 1.5 min at 60°C, and 1.5 min at 72°C for 30 cycles. Cycle sequencing reactions were performed on both DNA strands, and sequences were analyzed on an automated fluorescence-based Applied Biosystems model 373A DNA sequencing system.

DNA Constructs and Transcription

To introduce the A147T mutation in our AQP2 expression construct (pT7TAsAQP2), exon 2 of the AQP2 gene was amplified from genomic DNA of Patient 1. A 66-base fragment containing the G to A transition at position 533 was isolated by gel electrophoresis. For the T126M mutation, genomic DNA of Patient 2 was amplified using primers flanking the coding region of exon 2, and a 43-bp Ddel-SacI fragment was isolated, containing the C to T transition at position 297. For the N68S mutation, exon 1 of the AQP2 gene was amplified from genomic DNA of Patient 3, and a 144-bp Apal-SacII fragment containing the A to G transition at position 297 was isolated. These fragments were inserted into the corresponding sites of pT7TAsAQP2 and clones that were identical to the wild-type (wt) AQP2 cDNA sequence, except for the described mutations, were selected by sequence analysis (18). These constructs were linearized with SalI and capped RNA transcripts were synthesized in vitro using T7 RNA polymerase according to Promega’s (1991) Protocols and Principles guide, except that 1-mM final concentrations of NTP and 7-methyl-di-guanosine triphosphate were used. The cRNA were purified and dissolved in diethyl pyrocarbonate-treated water. The integrity of the RNA was checked by agarose gel electrophoresis and the concentration was determined spectrophotometrically.

Water Permeability

Xenopus Laevis oocytes were isolated, injected with 10 ng cRNA and analyzed after 3 days in a swelling assay as described before (15). Oocyte swelling was performed at 22°C after transfer from 200 mosM to 70 mosM (wt AQP2 expressing oocytes) or 200 mosM to 20 mosM (water-injected control oocytes and mutant AQP2 expressing oocytes).

Northern Blot Analysis

At the day of injection and 3 days after injection, RNA was isolated from six oocytes according to Chomczynski and Sacchi (19). 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 (20). A 850-bp EcoRI cDNA fragment encoding human AQP2 (15) was labeled with [α-32P]dCTP by random priming (21) and was used as a probe. The relative amount of mRNA loaded onto the gel was assessed by hybridization of the same blot with a probe of a 780-bp EcoRI-BamHI cDNA fragment coding for Xenopus laevis Histon H3 (22) and subsequent scanning of the autoradiographic signals with an LKB Ultrascan XL laser densitometer (Pharmacia Biotech, Uppsala, Sweden).

Immunoblotting

To determine the stability of mutant and wt AQP2 proteins, eight oocytes were homogenized in 20 μL buffer A per oocyte (20 mM Tris [pH 7.4], 5 mM MgCl2, 5 mM NaHPO4, 1 mM EDTA, 1 mM dithiothreitol [DTT], 1 mM phenylmethyl sulfonyl fluoride [PMSF], 5 μg/mL leupeptin and pepstatin, 80 mM sucrose) at 4°C at 1, 2, and 3 days after injection. Subsequently, the lysates were centrifuged twice for 10 min at 125 g to remove yolk proteins. At the third day after injection, a fraction enriched for plasma membranes was isolated from 25 oocytes according to Wall and Patel (23).

Lysates or plasma membranes equivalent to 0.1 oocyte or eight oocytes, respectively, were denatured for 30 min at 37°C in sample buffer (2% sodium dodecyl sulfate [SDS], 50 mM Tris [pH 6.8], 12% glycerol, 0.01% Coomassie Brilliant Blue, 100 mM DTT), electrophoresed through a 12% SDS-polyacrylamide gel (24) and transferred to a nitrocellulose membrane as described (15). Efficiency of protein transfer was checked by staining the membrane with Ponceau Red. For immunodetection, the membrane was incubated with a 1:10,000 dilution of an
affinity-purified polyclonal antibody directed against the 15 C-terminal amino acids of rat AQP2 (17). 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. Proteins were visualized using enhanced chemiluminescence (Boehringer Mannheim). When appropriate, the 29- and 32-kd AQP2 bands of the third day were scanned as described above.

Immunocytochemistry

At 3 days after injection, remaining vitelline membranes were removed and oocytes were incubated for 1 h in 1% wt/vol paraformaldehyde fixative (PLP) (25), dehydrated, and embedded in paraffin. After being blocked with 10% goat serum in Tris-buffered saline (TBS), the sections were incubated O/N at 4°C with the polyclonal AQP2 diluted 1:500 in 10% goat serum in TBS. After three washes for 10 min in TBS, the sections were incubated for 1 h in a 1:100 dilution of goat-anti-rabbit IgG coupled to fluorescein isothiocyanate (Sigma Immuno Chemicals). The sections were again washed three times for 10 min, dehydrated by washing in 70% to 100% ethanol, and mounted in mowiol 4-88, containing 2.5% NaN3. Photographs were taken with a Zeiss Axiioskop (Zeiss, Oberkochen, Germany) with epifluorescent illumination with an automatic camera using Kodak EPH P1600X films (Eastman Kodak, Rochester, NY).

Results

From three NDI patients, in whom a V2 receptor defect was either excluded or unlikely, we amplified and sequenced the four exons of the AQP2 gene. All patients were found to be homozygous for three different missense mutations. In Patient 1, a G533A transition was found in exon 2, leading to a substitution of an alanine for threonine (A147T). In Patient 2, a C47IT transition in exon 2 leads to a threonine to methionine substitution (T126M). The AQP2 gene of patient 3 showed a A297G transition in exon 1, which results in a substitution of an asparagine for a serine (N68S) in one of the most conserved regions of the MIP family proteins (26) (Figure 1).

The asymptomatic parents and a healthy brother and sister of Patient 1 were shown to be heterozygous for the A147T mutation; another asymptomatic sister appeared to be homozygous for the normal allele. As expected, the affected brother of Patient 2 appeared homozygous for the T126M mutation as well, whereas their asymptomatic parents were both shown to be heterozygous for that mutation. DNA of an elder healthy brother was not available for testing. Sequencing of exon 1 in the two affected male family members of Patient 3 revealed that they both were homozygous for the N68S mutation. The asymptomatic parents, an asymptomatic sister of the father and the paternal grandparents of Patient 3, as well as the mother of the two other patients in family 3, were all shown to be heterozygous for the N68S mutation. The data are consistent with co-segregation of the mutant AQP2 allele with the disease and with autosomal recessive inheritance of NDI in these families.

To test whether these mutant AQP2 proteins are functional water channels, PCR fragments containing the mutations were cloned into the AQP2 expression vector and transcripts were injected into Xenopus oocytes. Three days later, water permeability measurements revealed that the water permeability (Pf ± SE) of oocytes expressing the N68S mutant (8.3 ± 1.9 μm/s) was not different from water-injected control oocytes (8.9 ± 2.2 μm/s), whereas oocytes expressing the T126M (41.4 ± 3.8 μm/s) or A147T (46.2 ± 2.7 μm/s) AQP2 proteins showed a significantly increased water permeability when compared with water-injected control oocytes.

The water permeability of wild-type (wt) AQP2 injected oocytes was 275 ± 9.7 μm/s (Figure 2). The low or absent water permeability of mutant AQP2 proteins in Xenopus oocytes could be caused by (1) a low stability of the mutant cRNAs in Xenopus oocytes; (2) a low stability of the mutant AQP2 proteins in oocytes; (3) an impairment of the routing of the mutant AQP2 proteins to the plasma membrane; and/or (4) a mutant AQP2 protein that is a non-functional water channel.

To test for differences in stability of wt and mutant cRNA, RNA isolated from oocytes directly after, and 3 days after...
injection was subjected to Northern blot analysis. A specific signal with our human AQP2 cDNA probe was only obtained in lanes loaded with RNA isolated from AQP2 cRNA-injected oocytes (Figure 3). After normalization for the amounts of RNA loaded by hybridization with a Xenopus Histon H3 probe, the amounts of wt and mutant AQP2 cRNAs were comparable.

To compare the size and stability of mutant and wt AQP2 proteins, oocyte lysates were prepared 1, 2, and 3 days after injection and were subjected to immunoblotting using AQP2 antibodies (Figure 4). Ponceau Red staining of the immunoblot showed that equal amounts of protein were loaded (not shown). Chemiluminescence detection revealed a band of 29-kd present in all lanes loaded with AQP2 protein, except in the lane loaded with water-injected control oocytes. In the lanes loaded with mutant AQP2 protein an additional band of approximately 32 kd was present. Densitometric scanning of the bands from the third day samples revealed that the stability of the T126M and N68S mutants and wt AQP2 were equal, whereas the stability of the A147T mutant was less than 10% of wt AQP2.

To determine the plasma membrane expression of wt and mutant AQP2 proteins, a fraction enriched for plasma membranes was subjected to immunoblotting (Figure 5). Only in the membrane fraction of oocytes expressing wt AQP2 was a clear 29-kd band visible, whereas no AQP2 protein could be detected in the membrane fractions of AQP2 mutants. To visualize the location of AQP2 proteins, immunocytochemistry was performed on injected oocytes (Figure 6). Staining with the AQP2 antibody revealed a clear, intense staining of the plasma membrane of oocytes expressing wt AQP2 (Figure 6A), whereas oocytes expressing mutant AQP2 proteins showed a very weak staining of the plasma membrane, with a more pronounced labeling of the cytoplasm (Figure 6B-D). The water-injected control oocytes showed no staining (Figure 6E).

Discussion

The discovery of mutations in the V2 receptor gene, located on the X-chromosome, explained the cause of NDI in a majority of the patients (27), but not in all. Some of these unexplained NDI cases appeared to segregate as an autosomal recessive trait, and mutations in the V2 receptor coding region could often be excluded in these cases. Therefore, the involvement of a second gene causing NDI was likely. In search for proteins involved in the cascade of events between the binding of vasopressin to the V2 receptor at the basolateral membrane and the reabsorption of water at the apical membrane of the collecting duct cell, the cloning of the rat AQP2 water channel, which is exclusively expressed in the collecting duct (2), attracted attention. The AQP2 gene was assigned to chromosome 12, region q12-q13, and was therefore a likely candidate (28). The subsequent identification of mutations in the AQP2 gene in some of these NDI patients provided a definitive proof for a second gene defect in NDI (15,16).

Here we report three new missense mutations in the AQP2 gene of three NDI patients coding for a A147T-, T126M-, or N68S-substituted AQP2 protein. Expressed in Xenopus oo-
The sections were incubated with AQP2 antibodies and visualized by FITC-conjugated anti-rabbit immunoglobulins.

Figure 6. Sections of oocytes injected with cRNA encoding wt (A), A147T (B), T126M (C), or N68S (D) AQP2. As a negative control, water-injected control oocytes were used (E). The sections were incubated with AQP2 antibodies and visualized by FITC-conjugated anti-rabbit immunoglobulins.

cytes, the N68S mutant AQP2 was non-functional (Figure 2). This could be anticipated, because the substituted amino acid is part of the NPA box in loop B, which forms, together with a second NPA box in loop E, the most conserved amino acid sequence of the MIP-family (26). Unlike the N68S-substituted protein and previously reported AQP2 mutants (G64R, R187C, and S216P) (15,16), the A147T and T126M AQP2 mutant proteins were functional. The alanine at position 147 is also well-conserved among the MIP family members (26). The observed water permeability of the oocytes expressing the A147T and T126M AQP2 proteins was, however, much lower than that of oocytes expressing wt AQP2.

To find an explanation for the reduced Pf of oocytes expressing NDI-related AQP2 proteins, these oocytes were analyzed in detail and were compared with wt AQP2-expressing oocytes. The stability of injected cRNA was equal for mutant
and wt AQP2 (Figure 3). In contrast, differences with wt AQP2 were observed on the protein level. Immunoblot analysis revealed that wt AQP2 was only expressed as a 29-kd protein, whereas the three mutants showed an additional 32-kd form (Figure 4). In a previous study on mutant AQP2 proteins similar 32-kd bands were detected, and they were shown to be endoglycosidase H-sensitive (17). Because endoglycosidase H hydrolyses endoplasmic reticulum-specific high-mannose glycosylation groups, the 32-kd bands presumably represent ER-retarded forms of mutant AQP2 proteins. The mutant AQP2 proteins are apparently retained in the endoplasmic reticulum and are thus impaired in their routing to the plasma membrane. The indication that the A147T-, T126M-, and N68S-substituted AQP2 proteins were impaired in their transport was further substantiated by the absence of these proteins in an immunoblotted oocyte fraction enriched for plasma membranes, whereas the wt AQP2 protein was clearly present (Figure 5). In addition, immunocytochemistry showed a clear AQP2 labeling in the plasma membrane of oocytes expressing wt AQP2, whereas the mutant AQP2 proteins were abundantly expressed in the cytoplasm, but were hardly detectable in the plasma membrane, which confirms the impaired transport of the mutant AQP2 proteins (Figure 6). In the ER, newly synthesized proteins undergo various post-translational modifications, including folding, oligomerization, and glycosylation (29,30). Proteins that are not properly processed do not pass the "quality control" of the ER and are usually retained. The processing of the new protein depends in part on the structural motifs displayed during folding and assembly, and by the molecular interactions with chaperones and folding factors. The quality control of the ER recognizes certain conformational features of the misfolded protein, such as hydrophobic peptide elements exposed on the surface of the molecule, and, in most cases, misprocessed proteins are subsequently degraded (29–31). In Xenopus oocytes, the stability of the T126M and N68S mutants was comparable with that of wt AQP2, but the A147T mutant was considerably less stable, a phenomenon previously shown for the S216P mutant also (17). Because the A147T and S216P mutations are both located in a transmembrane domain, misfolding may cause the exposure of hydrophobic regions on the surface of the molecule, which could make these mutants more accessible to ER-resident proteases. The other mutations (G64R, N68S, T126M, and R187C) are located in the more hydrophilic extramembranous loops, which may explain their stability in oocytes.

In summary, all six NDI-related missense AQP2 proteins (G64R, N68S, T126M, A147T, R187C, and S216P) are impaired in their transport to the plasma membrane when expressed in Xenopus oocytes (17; this study). Despite the impairment in routing, the A147T and T126M mutants significantly increased the Pf of oocytes. In view of the fact that the A147T mutant is very unstable in oocytes, the mutants that are stable in oocytes (G64R, N68S, and R187C) should also confer water permeability to oocytes, if they were functional water channels. Therefore, it is likely that the G64R, N68S, and R187C AQP2 mutants are nonfunctional.

All of our data regarding AQP2 are in line with the hourglass model, which is a structural-functional model proposed for AQP1. In this model, loops B and E are essential for the formation of the pore through which water transport takes place (32). The hourglass model is based on site-directed mutagenesis studies in which mutations in the B and E loops of AQP1, containing the conserved NPA boxes, resulted in loss of water permeability. Mutations in the A, C, and D loops and the N- and C-terminal regions, however, affected the water transporting properties of AQP1 to a lesser degree (33). All AQP2 proteins in NDI with mutations in the B loop (G64R, N68S) or E loop (R187C) are nonfunctional, whereas AQP2 proteins with mutations in the C loop (T126M) or near the D loop (A147T) are functional. Recently, Bai et al. (34) proposed that in AQP2 loops C and D are closely located to the aqueous pathway, instead of loops B and E. This is not in line with our data from NDI-related mutant AQP2 proteins. Bai et al. (34) reported that mutations near the NPA boxes in loops B and E did not alter water channel function, which contrasts sharply with our data.

In conclusion, two mutant AQP2 proteins, encoded by AQP2 genes of patients suffering from autosomal recessive NDI, appeared to be functional water channels. Therefore, the major cause underlying this disease is the misrouting of the mutant AQP2 proteins, and not the dysfunction of the water channels. As shown for the most common mutant form of the cystic fibrosis transmembrane conductance regulator (CFTR), which has a deletion of the phenylalanine at position 508, the primary effect is not a functional impairment, but rather an impairment in the routing of the mutant protein to the plasma membrane. In CFTRΔF508-expressing cells it has been shown that culturing cells at lower temperatures relieved the impairment in routing of the ΔF508 mutant, which resulted in the appearance of functional Cl– channels in the plasma membrane (35). Furthermore, elevated levels of molecular chaperones appeared to increase proper folding of the K304E mutant of the medium chain acyl-CoA dehydrogenase (MCAD) (36) and the Y393N mutation of the E1α subunit of the mitochondrial branched chain α-ketoadic dehydrogenase complex (37). In future studies, attempts should be undertaken to overcome the biosynthetic arrest and to promote trafficking of the T126M and A147T AQP2 mutants to subapical vesicles or the plasma membrane. This will require knowledge of the mechanism that causes retention. The opportunity to manipulate the cellular machinery associated with protein folding and trafficking may provide the tools for novel pharmacotherapeutic strategies that may be used in the treatment of this form of nephrogenic diabetes insipidus.

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