Am. J. Hum. Genet. 69:738–748, 2001

Three Families with Autosomal Dominant Nephrogenic Diabetes Insipidus Caused by Aquaporin-2 Mutations in the C-Terminus

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The vasopressin-regulated water channel aquaporin-2 (AQP2) is known to tetramerize in the apical membrane of the renal tubular cells and contributes to urine concentration. We identified three novel mutations, each in a single allele of exon 4 of the *AQP2* gene, in three families showing autosomal dominant nephrogenic diabetes insipidus (NDI). These mutations were found in the C-terminus of AQP2: a deletion of G at nucleotide 721 (721 delG), a deletion of 10 nucleotides starting at nucleotide 763 (763–772del), and a deletion of 7 nucleotides starting at nucleotide 812 (812–818del). The wild-type AQP2 is predicted to be a 271–amino acid protein, whereas these mutant genes are predicted to encode proteins that are 330–333 amino acids in length, because of the frameshift mutations. Interestingly, these three mutant AQP2s shared the same C-terminal tail of 61 amino acids. In *Xenopus* oocytes injected with mutant AQP2 cRNAs, the osmotic water permeability (Pf) was much smaller than that of oocytes with the AQP2 wild-type (14%–17%). Immunoblot analysis of the lysates of the oocytes expressing the mutant AQP2s detected a band at 34 kD, whereas the immunoblot of the plasma-membrane fractions of the oocytes and immunocytochemistry failed to show a significant surface expression, suggesting a defect in trafficking of these mutant proteins. Furthermore, coinjection of wild-type cRNAs with mutant cRNAs markedly decreased the oocyte Pf in parallel with the surface expression of the wild-type AQP2. Immunoprecipitation with antibodies against wild-type and mutant AQP2 indicated the formation of mixed oligomers composed of wild-type and mutant AQP2 monomers. Our results suggest that the trafficking of mutant AQP2 is impaired because of elongation of the C-terminal tail, and the dominant-negative effect is attributed to oligomerization of the wild-type and mutant AQP2s. Segregation of the mutations in the C-terminus of AQP2 with dominant-type NDI underlies the importance of this domain in the intracellular trafficking of AQP2.

Introduction

Water movement across the cell membrane is a fundamental process in the maintenance of intracellular homeostasis. Water molecules move through a pore of a water channel, which has now been designated as "aquaporin" (AQP). After the discovery of AQP1 (MIM 107776) in 1992, AQPs have been detected in many organisms, including bacteria, yeast, plants, and animals (Park and Saier 1996; Ishibashi and Sasaki 1998). A cDNA for AQP2 (MIM 107777) (Fushimi et al. 1993), a vasopressin-regulated water channel, was isolated in 1993. AQP2 is localized predominantly at the subapical region of the principal cells of the collecting tubule and the inner medullary collecting duct cells (Marples et al. 1995; Nielsen et al. 1995*a;* Yamamoto et al. 1995). In response to binding of vasopressin to the V2 receptor at the basolateral membrane, cAMP-dependent processes are activated, leading to an increase in the osmotic water permeability of the apical membrane by triggering exocytosis of AQP2-containing vesicles to the apical membrane (Marples et al. 1995; Nielsen et al. 1995*a;* Yamamoto et al. 1995).

Congenital nephrogenic diabetes insipidus (NDI) is a hereditary disease characterized by a lack of responsiveness to arginine vasopressin (AVP) in the renal collecting tubule. The clinical manifestations are polyuria and polydipsia. In the majority of patients with inherited NDI, the mode of inheritance is X-linked recessive (NDI, X-linked [MIM 304800]). The mutations that are

Received July 2, 2001; accepted for publication August 6, 2001; electronically published August 30, 2001.

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0002-9297/2001/6904-0008$02.00

responsible were found in the V2 receptor gene, located in the Xq28 chromosomal region (Pan et al. 1992); subsequently, >150 mutations have been reported (Arthus et al. 2000). Less frequently, patients with NDI show an autosomal recessive form (NDI, autosomal recessive [MIM 222000]). After isolation of the cDNA for human AQP2 (Sasaki et al. 1994), mutations were identified in the *AQP2* gene in patients with this form of NDI (Deen et al. 1994). Presently, 30 AQP2 mutations are known (Morello and Bichet 2001; D.G.B., unpublished data). More recently, in 1998, an autosomal dominant form of NDI was reported (Mulders et al. 1998), in which a mutation in one allele of the *AQP2* gene caused an E258K substitution (NDI, autosomal dominant [MIM 125800]). In the present study, we report three cases of autosomal dominant NDI. Three different novel *AQP2* mutations were identified, which are predicted to result in elongation of the C-terminal tail of AQP2. To our knowledge, this is the second report of autosomal dominant NDI due to *AQP2* gene mutations.

Subjects and Methods

Subjects

Three Japanese families with NDI were investigated. The pedigrees of these families are depicted in figure 1. Clinical pictures of the two members (III-3 and IV-5) of family A were reported 17 years ago (Ohzeki et al. 1984). In brief, a 17-mo-old male patient (IV-5) was admitted to a university hospital because of recurrence of polyuria and polydipsia, which had first been noticed 1 year after birth. A water deprivation test for 5 h (causing a 6.4% body-weight reduction) decreased urine volume from 140 to 35 ml/hr and increased urine osmolality from 91 to 333 milliosmoles (mOsm)/kg H_2O. The mother (III-3) of the patient was also examined, since she had had polyuria and polydipsia from childhood. Her blood chemistry data were normal, but a large volume (10.6 L/day) and low osmolality (127 mOsm/kg H_2O) of urine were noticed. Urine volume decreased from 460 to 250 ml/hr, and urine osmolality increased from 139 to 264 mOsm/kg H_2O, after 5-h water deprivation, which caused a 4.0% body-weight reduction. Furthermore, these two patients were measured for urinary cAMP excretion, after nasal administration of 1-deamino-8-D-arginine vasopressin (DDAVP). Urine volume and osmolality did not appreciably respond to DDAVP, but cAMP excretion increased 6- and 20-fold in the son and mother, respectively. Type II NDI (in which defects are located distal to cAMP generation) was diagnosed in these two patients. The individuals shaded in black or gray in the expanded family tree shown in figure 1*A* were known to have polyuria and polydipsia. This pedigree figure clearly indicates an autosomal dominant inheritance of the disease.

The female patient (II-2) of family B was born to unrelated parents; both the pregnancy and the delivery were uneventful. Polyuria and polydipsia were not recognized until age 3 years, when she was referred to a university hospital because of dehydration with growth and mental retardation. Her urine osmolality remained low (120 mOsm/kg H_2O), despite the presence of severe hyperosmotic dehydration (serum Na^+ 169 milliequivalents/liter; urea nitrogen 36 mg/dl; creatinine 1.3 mg/dl) and a markedly elevated plasma AVP level (18.8 pg/

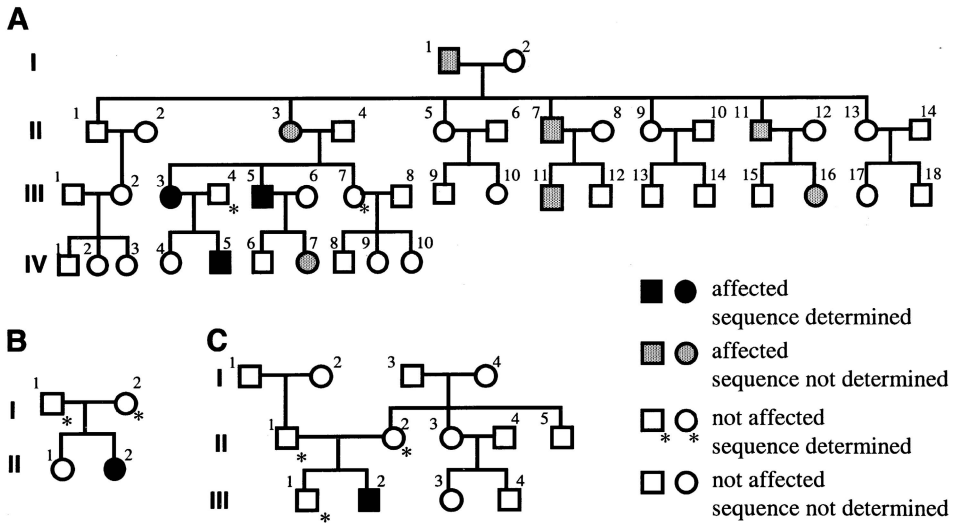

Figure 1 Pedigrees of the three families with NDI. Squares and circles represent males and females, respectively.

ml). Polyuria (4–5 liters/m^2) became apparent after hydration, and it was not improved by nasal DDAVP administration. None of her family members had polyuria or polydipsia.

The patient in family C (III-2) was born without complications. At age 5 mo, he was noticed to have frequent vomiting, and subsequent examination indicated the presence of hydronephrosis of the left kidney. After age 16 mo, he was recognized to have polyuria (>3 liters/day) and polydipsia. At age 28 mo, he was admitted to a university hospital for clinical evaluation. No abnormalities were found except polyuria. A water deprivation test showed low urine osmolality (177 mOsm/kg H_2O), despite a high blood AVP level (11.4 pg/ml) in the normal water-drinking condition, and urine osmolality gradually increased to 468 mOsm/kg H_2O after 9 h of water deprivation, with a 3.4% body weight reduction. The urine osmolality further increased to 551 mOsm/kg H_2O after administration of 10 μg of DDAVP. Partial NDI was diagnosed in this patient. There were no family members reporting similar symptoms.

Genomic DNA was obtained from the peripheral blood of the patients. Informed consent was obtained from all participants.

Construction of Mutants and In Vitro cRNA Synthesis

cDNAs of human *AQP2* (GenBank accession number NM000486) were mutated by the PCR technique, using wild-type cDNA as a template (Kuwahara et al. 1995; Goji et al. 1998). A fragment between the *Nco*I site at nucleotide 135 and the *Xba*I site in pAQP/ev1 was replaced by a PCR fragment encoding the mutants. Capped RNA transcripts of wild-type and mutated AQP2 were synthesized in vitro with T3 RNA polymerase, using *Xba*I-digested cDNA.

Measurement of Osmotic Water Permeability of Oocytes

Oocytes at stage V–VI were obtained from *Xenopus laevis*. Each oocyte was injected with 40 nl of water (as a control) or with 5 ng or 0.3 ng of either the wild-type or mutant AQP2 cRNAs. After incubation for 48 h at 20°C in Barth's buffer, osmotic water permeability (Pf) of the oocytes was measured at 20°C from the time of osmotic cell swelling (Kuwahara et al. 1995; Goji et al. 1998).

Immunoblot Analysis

Lysates and plasma-membrane fractions of oocytes were obtained as described elsewhere (Wall and Patel 1989; Kuwahara et al. 1999). After being heated at 70°C for 10 min, samples were separated by SDS-PAGE. Oocyte lysates from the equivalent of one-fifth of an oocyte, or plasma membranes from 20 oocytes, were applied in each lane. The samples were transferred to Immobilon-P filters (Millipore), using a semi-dry system. The filters were incubated for 1 h with an affinity-purified antibody against 15 C-terminal amino acids of the wild-type or mutant AQP2. Because the amino acid sequence of the C-terminal end was common among the three AQP2 mutants, as shown in figure 3, one antibody against this portion (ASAVSRRGGGCRREP) was generated to detect these mutants. The filters were further incubated for 1 h with ^{125}I-protein A solution, and autoradiography was performed.

For native PAGE, a nondissociative detergent, perfluorooctanoic acid (PFO) (Wako Pure Chemical Industries) was used. PFO has been shown to be particularly effective in the solubilization of intractable membrane proteins, such as the membrane-spanning V_0 subunit of the V-type ATPase, the cystic fibrosis transmembrane regulator, the band 3 protein, and the Kir 2.1 K^+ channel (Shepherd and Holzenburg 1995; Ramjeesingh et al. 1997, 1999). PFO-PAGE was performed as described in detail elsewhere (Shepherd and Holzenburg 1995; Ramjeesingh et al. 1997, 1999). Membrane fractions of 90–100 oocytes were dissolved in 500 μl of a solubilization buffer (50 mM Tris base, 1 mM dithiothreitol, 5 mM EDTA, 4% [w/v] PFO, 10% [v/v] glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 5μg of leupeptin and pepstatin; the pH was adjusted to 8.0 with NaOH). After a 12-h incubation at 4°C, the samples were concentrated to 50 μl through use of centrifugal filter devices (Microcon YM-10, Millipore). In one protocol, the samples were further incubated with 4 M urea for 30 min at room temperature. The running buffer contained 25 mM Tris, 192 mM glycine, and 0.5% (w/v) PFO, and the pH was adjusted to 8.5 with NaOH. The samples were separated by PFO-PAGE on precast 12.5% Tris/glycine gel without SDS (Multi Gel, Daiichi Pure Chemicals), in an ice-cold running buffer, followed by immunoblot analysis.

Immunocytochemistry

Oocytes were fixed in 4% paraformaldehyde for 4 h and were cryoprotected overnight in phosphate-buffered saline (PBS) containing 30% sucrose (Kuwahara et al. 1999). The samples were embedded in OCT compound (Tissue-Tek) and were frozen in liquid nitrogen. Cryostat sections were incubated for 30 min in PBS containing 1% BSA. After three washes in PBS, the sections were incubated for 60 min with an antibody against the wild-type or mutant AQP2 diluted at 1:500, rinsed with PBS, and further incubated for 30 min with fluorescein-5-isothiocyanate (FITC)–labeled goat anti-rabbit IgG (1:200, Sigma). The oocytes were imaged with a fluorescent confocal microscope at 400 × magnification (Carl Zeiss LSM 510).

Immunoprecipitation

Membrane fractions of 60–70 oocytes were prepared as described elsewhere (Preston et al. 1993). Each sample was dissolved in 250 μl of a solubilization buffer (20 mM Hepes [pH 7.4], 1 mM dithiothreitol, 1 mM EDTA, 5 mM ethyleneglycoltetracetic acid, 5 mM β-glycerophosphate, 0.5% [v/v] NP-40, 0.5 [v/v] deoxycholate, 1 mM PMSF, and 5μg/ml of leupeptin and pepstatin). Twenty microliters of an antibody-bound protein G-agarose (Protein G PLUS-Agarose, Oncogene Science) was added to each sample and incubated for 12 h at 4°C. The samples were washed three times, were dissolved in 30 μl of Laemmli buffer, and were analyzed by SDS-PAGE (Laemmli 1970). Fifteen microliters of sample was applied in each lane. The samples that were immunoprecipitated with an antibody against the wild-type or mutant AQP2 were detected by both antibodies.

Preparation and Pf Measurement of Yeast Vesicles

The wild-type and mutant AQP2s were expressed in a protease-deficient BJ3505 strain of *Saccharomyces cerevisiae* yeast cells, as described elsewhere (Kuwahara et al. 1999; Shinbo et al. 1999). The Pf of the yeast vesicles was measured by a light-scattering method, using an SX-18MV stopped-flow apparatus (Applied Photophysics Ltd.).

Results

Direct sequencing of DNA strands from the patients in these three families detected no mutations in the *AVPR2* gene but found three different mutations (one mutation for each family) in a single allele in exon 4 of the *AQP2* gene. The mutations were confirmed by sequencing of subcloned PCR-amplified DNA fragments of the patients (fig. 2). In family A, a deletion of one of two Gs at nucleotides 720 and 721 (721delG) was found, and this mutation cosegregated with clinical symptoms of NDI (fig. 1). A 10-bp deletion starting at 763 (763–772del), as well as a deletion of 7 bp starting at 812 and encompassing the stop codon (812–818del), were found in families B and C, respectively (fig. 2). The mutations in families B and C were not observed in other members of the families (fig. 1), indicating that these were de novo mutations. Interestingly, all three mutations were found in the C-terminus of AQP2 (fig. 3*A*). The 3′-most distal mutation found in exon 4, 812–818del (family C), causes a frameshift, and a new stop codon is created 180 nucleotides downstream (fig. 3*B*). As a result, this mutant AQP2 protein consists of a wild-type AQP2 lacking the last amino acid (alanine 271) and having a new polypeptide of 61 amino acids (fig. 3*C*). The other mutations, 763–772del and 721delG, also cause frameshifts that result in the addition of 76 and 93 amino acids,

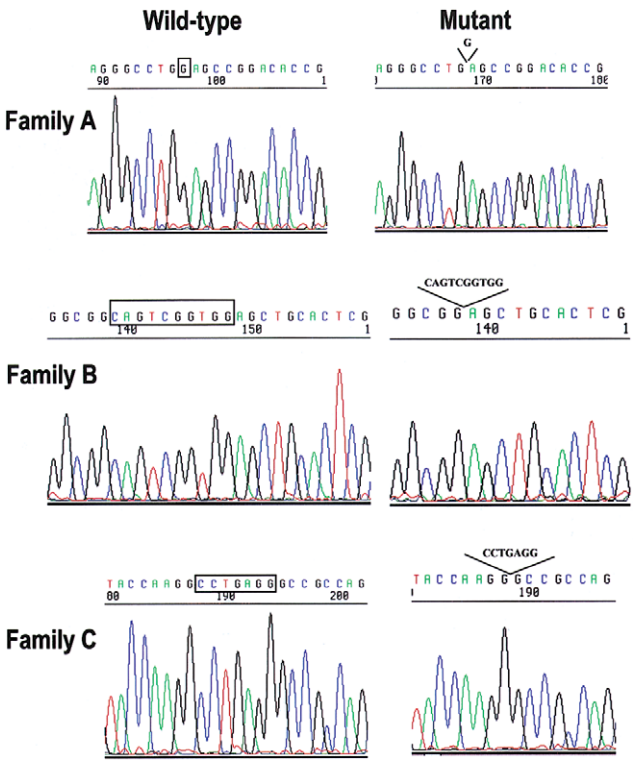

Figure 2 DNA sequencing of the mutated regions of the *AQP2* gene found in three families with NDI.

respectively (fig. 3*C*). These three mutant AQPs consist of 330–333 amino acids and share the most terminal 61 amino acids, as shown in figure 3*B*. Because the mutations were found only in one allele, NDI was considered to follow an autosomal dominant pattern in these patients; the results for family A are consistent with this inheritance pattern (fig. 1).

In oocytes injected with water (controls) or with 5 ng of cRNA encoding the wild-type or mutant AQP2, Pf values (± standard error [SE]; in 10^{-4} cm/s) were 12 ± 1 (control), 179 ± 12 (wild type), 27 ± 3 (721delG), 25 ± 2 (763–772del), and 30 ± 2 (812–818del) (fig. 4*A*). The relative Pf value for wild-type AQP2 versus the control was 14.9, whereas those for the mutants were 2.1–2.5. To see whether these mutants show a "dominant-negative effect," 5 ng of one of the mutant cRNAs was coinjected with 5 ng of the wild-type cRNA. Pf was lowered by 39%–46% but was still 8.1–9.0 times higher than that of the control. Pf was also measured in oocytes injected with 0.3 ng of cRNA (fig. 4*B*). The wild-type AQP2 increased Pf 6.0-fold, whereas the mutant AQP2s elevated Pf only 1.3–1.8-fold. A large reduction of Pf (58%–65%) was observed upon coinjection of one of the mutant cRNAs with the wild-type cRNA, which provided support for a dominant-negative effect. All of these three mutants lack the

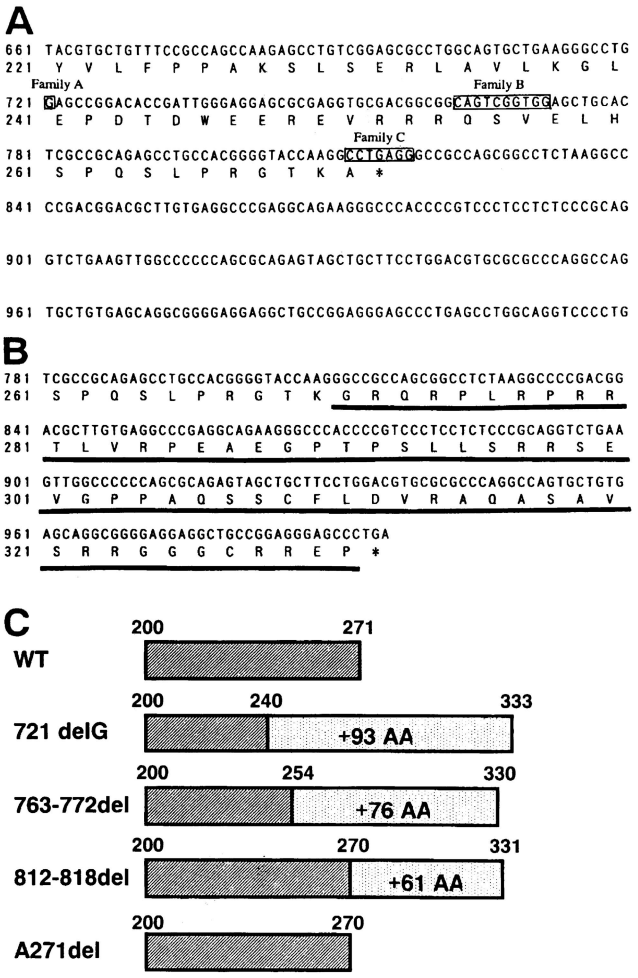

Figure 3 Sequence and structure of the wild-type (WT) and mutant AQP2 cDNAs. Nucleotide and amino acid residue numbers correspond to the cDNA; the A of the initiator Met codon is nucleotide 1. *A,* Nucleotide and amino acid sequence at the C-terminal site of wild-type AQP2. The boxed bases are the deleted bases detected in patients with NDI who are included in the present study. One, ten, and seven nucleotides were deleted, starting at 721 (721delG), 763 (763–772del), and 812 (812–818del), respectively. *B,* Sequences of the C-terminus in 812–818del. This mutation shifted the stop codon 180 nucleotides downstream, resulting in an addition of 61 new amino acids (*underlined*) to the terminal end. *C,* Schematic structure of mutant AQP2s. The frameshift mutations 721delG, 763–772del, and 812–818del result in the addition of 93, 76 and 61 new amino acids, respectively. Because the number of deleted bases were $3 \times n + 1$ ($n = 0$, 2, or 3), 61 C-terminal amino acids are common in these three mutants. All of the three AQP2 mutants lack the last amino acid of the wild-type AQP2 (alanine at codon 271).

alanine at position 271 that is present in the wild-type AQP2. The mutant lacking A271 (A271del) was constructed to evaluate a possible role of this residue. The Pf of A271del was comparable to that of the wild type, suggesting that the loss of A271 was not responsible for the decreases of Pf in the mutants.

The expression of the wild-type and mutant AQP2 proteins were examined by immunoblotting of the lysates and plasma-membrane fractions of the oocytes injected with 5 ng of cRNA. An antibody against the wild-type AQP2 identified a 29-kD band in both the lysates and plasma membranes of oocytes expressing the wild-type or A271del mutant AQP2 (A271), but no bands in oocytes injected with cRNA of 721delG, 763–772del, or 812–818del mutant AQP2 (fig. 5*A*). This would be due to reconstruction of the C-terminus of AQP2, which is the site that the antibody recognizes. An antibody against the mutant AQP2 detected bands at 34 kD in the lysates of mutant-protein–expressing oocytes but not in the plasma membranes (fig. 5*B*), suggesting that the mutant AQP2 proteins were synthesized but not translocated to the cell surface. In oocytes coinjected with cRNA of one of the mutant AQP2s and wild-type AQP2, the mixture of the two antibodies recognized both 29-kD and 34-kD bands in the lysates, at similar expression levels (fig. 5*C*). Comparison of 29-kD bands suggested that the coinjection slightly decreased the expression of the wild-type AQP2 proteins. In the plasma membranes, only 29-kD bands were seen, and the band intensity of the coinjected oocytes was weaker than that of the wild-type oocytes, suggesting that the coexpression of the AQP2 mutants attenuated the cell-surface expression of the wild-type AQP2.

Next, immunocytochemistry was performed to further determine the expression of the wild-type and mutant AQP2s. The plasma membrane of water-injected oocytes was not stained with an anti–wild-type AQP2

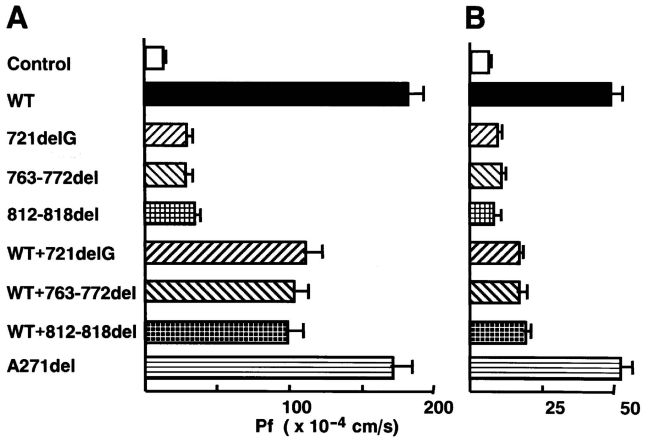

Figure 4 Pf of oocytes expressing wild-type and mutant AQP2s. Oocytes were injected with 40 nl of water (control), or cRNA of the wild-type (WT) or one of the mutant AQP2s (721delG, 763–772del, 812–818del, and A271del). Either 5 ng (*A*) or 0.3 ng (*B*) of cRNA was injected. For coexpression, one of the mutated cRNAs was coinjected with the wild-type cRNA. Pf was calculated from the time of osmotic cell swelling of the oocytes. Each bar represents the mean ± SE of 21–24 measurements.

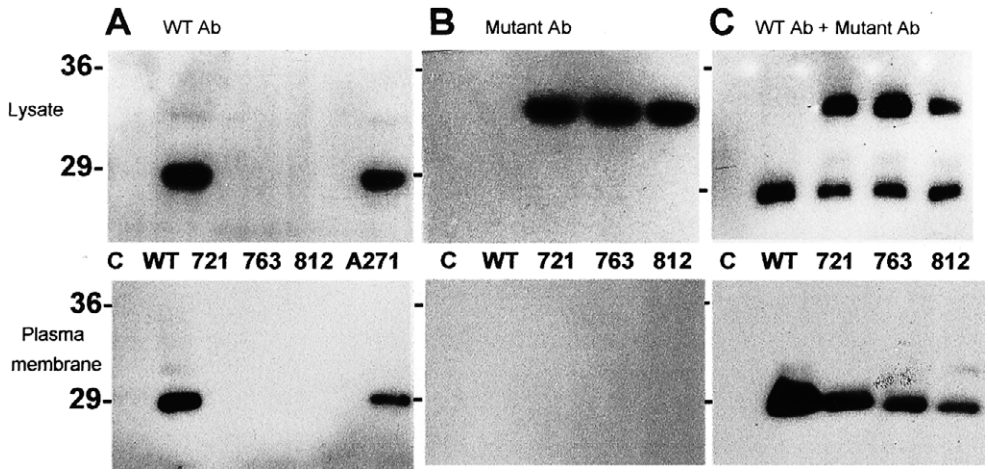

Figure 5 Immunoblot of the oocyte lysates and plasma-membrane fractions probed with an affinity-purified antibody (Ab) against wild-type or mutant AQP2. Oocytes were injected with water (C) or with 5 ng cRNA of wild-type (WT) or mutant AQP2s ("721" denoes 721delG; "763" denotes 763–772del; "812" denotes 812–818del; and "A271" denotes A271del). The lysates from the equivalent of one-fifth of an oocyte (*upper panels*) or the membranes from the equivalent of 20 oocytes (*lower panels*) were loaded in each lane. The protein expression was detected with an anti–wild-type-AQP2 antibody (*A*), an anti–mutant AQP2 antibody (*B*), or a mixture of both (*C*).

antibody (fig. 6*A*), whereas a bright surface staining was observed in oocytes expressing the wild-type AQP2 (fig. 6*B*). Immunofluorescence of the plasma membranes stained with anti–mutant AQP2 antibody was trivial in oocytes injected with 5 ng of cRNA of either 721delG, 763–772del, or 812–818del (fig. 6*C–E*). In oocytes coinjected with 5 ng each of the wild-type and 721delG cRNA, a clear membrane labeling was visible with an anti–wild-type AQP2 antibody (fig. 6*F*) but not with an anti–mutant AQP2 antibody (fig. 6*G*). These results suggested that the surface expression of the wild-type AQP2 is responsible for the significant Pf of the oocytes coinjected with the wild-type and mutant cRNAs, as shown in figure 4*A*. An anti–wild-type AQP2 antibody detected a sharp staining in oocytes injected with 0.3 ng cRNA of the wild-type AQP2 (fig. 6*H*) but did not show the surface staining in oocytes coinjected with 0.3 ng each of the mutant cRNAs (fig. 6*I–K*).

Dominant-negative effects of these mutants may be due to the oligomerization of the wild-type and mutated AQP2 proteins. To examine whether the wild-type and mutant AQP2s form oligomers, immunoprecipitation was performed with the oocyte membrane fractions injected with cRNA of the wild-type AQP2, 721delG, or both. When immunoprecipitated with the anti–wild-type AQP2 antibody, the same antibody identified a 29-kD band in the samples of the wild-type and coinjected oocytes (fig. 7*A, upper panel*). In the same samples, a 34-kD band was detected by the antibody against the mutant AQP2 (fig. 7*A, lower panel*), suggesting that the wild-type AQP2 and 721delG mutants form mixed oligomers. In the samples immunoprecipitated by the antibody against the mutant AQP2, an anti–wild-type AQP2 antibody recognized a 29-kD band (fig. 7*B, upper panel*), again suggesting the formation of mixed oligomers. Similar results of oligomerization were seen in oocytes coinjected with cRNA of the wild-type AQP2 and either the 763–772del or 812–818del mutant (data not shown). It has been demonstrated that the wild-type AQP2 exists as tetramers (Jung et al. 1994; Kamsteeg et al. 1999). To analyze oligomeric states of the wild-type and mutant AQP2s, the oocyte membrane samples were separated by PFO-PAGE. The wild-type AQP2 and 721delG migrated as bands with apparent molecular masses of 112 kD (fig. 7*C, lane 1*) and 126 kD (fig. 7*C, lane 2*), respectively—findings consistent with tetramerization of both the wild-type and mutant AQP2 proteins. When the same sample of 721delG was further incubated with 4 M urea before loading, a clear band and a faint band were detected at 34 kD and 65 kD, respectively (fig. 7*C,* lane 3). These apparent molecular masses corresponded to those of the monomer and dimer of the mutant AQP2, suggesting that the treatment with 4 M urea led to the dissociation of the tetramer of the mutant AQP2. Similar results of native PAGE were obtained in oocytes expressing 763–772del or 812–818del (data not shown).

The oocytes injected with 5 ng of mutant cRNA showed faint expression of the mutants in the plasma membrane and some water permeability (figs. 4 and 6), suggesting that these mutants may be functional water channels. Since the yeast expression system is free from trafficking problems (Shinbo et al. 1999), we expressed the wild-type and mutant AQP2s in yeast cells and mea-

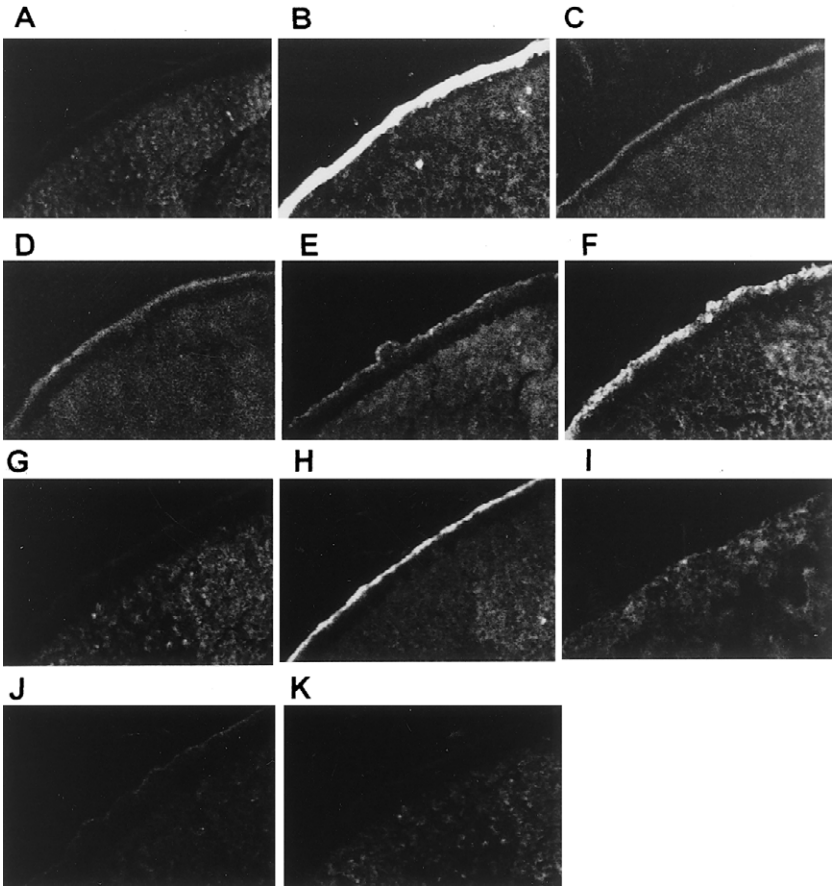

Figure 6 Immunocytochemistry of oocytes injected with cRNA encoding the wild-type or mutant AQP2s. Oocyte sections were incubated with an affinity-purified antibody against the wild-type or mutant AQP2 and immunostained with goat anti-rabbit IgG conjugated with FITC. The sections were viewed with a confocal microscope at 400× magnification. *A* and *B*, Water-injected oocyte (*A*) and oocyte injected with 5 ng of the wild-type cRNA (*B*), probed with an anti–wild-type AQP2 antibody. *C–E*, Oocyte injected with 5 ng cRNA of 721delG (*C*), 763–772del (*D*), or 812–818del (*E*), detected with an anti–mutant AQP2 antibody. *F–G*, Oocyte coinjected with 5 ng each of cRNA of the wild-type and 721delG mutant incubated with an anti–wild-type AQP2 antibody (*F*) or an anti–mutant AQP2 antibody (*G*). *H–K*, Oocyte injected with 0.3 ng cRNA of the wild-type (*H*) or coinjected with 0.3 ng each of cRNA of the wild-type and one of 721delG (*I*), 763–772del (*J*), or 812–818del (*K*), incubated with an antibody against the wild-type AQP2.

sured their Pf. The rate of initial volume decrease provides a quantitative measure of Pf. Pf values of the wild-type AQP2 vesicle ($338 \pm 40 \times 10^{-4}$ cm/s, $n = 7$) were 8.7 times higher than those of the control (39 ± 8, $n = 8$). The Pf of 721delG (273 ± 22, $n = 7$) and of 812–818del (298 ± 36, $n = 6$) were as high as that of wild-type AQP2, suggesting that water channel function is maintained in these AQP2 mutants. These results may be explained by the fact that the water-transporting pore of AQP monomers is dependent on the six transmembrane spanning domains and five connecting loops that are identical in these mutants (Jung et al. 1994; Cheng et al. 1997; Walz et al. 1997; Murata et al. 2000).

Our results presented in figures 5 and 6 suggest an impaired intracellular trafficking of the AQP2 mutants. One possible mechanism for this effect may be that the common C-terminal tail of the AQP2 mutants (the last 61 amino acids; see fig. 3) may bind with other intracellular proteins, thereby hindering the trafficking of the AQP2 mutants. If this is the case, the following scenario would be possible: if the C-terminal polypeptide is coexpressed with the AQP2 mutants, the polypeptide would combine with the putative binding proteins, which, in turn, would stimulate the plasma membrane expression of the mutant AQP2 and increase Pf. To examine this possibility, cRNAs encoding the C-terminal portion and a mutant AQP2 (721delG) were coinjected into oocytes. The coexpression did not affect the oocyte Pf of the 721delG AQP2 (fig. 8*A*). In coinjected oocytes, the immunoblot of the lysates detected independent expression of two proteins, at 34 kD and 7 kD, that correspond to the 721delG mutant and the C-terminal peptide, respectively (fig. 8*B*), but these bands were absent in the immunoblot of plasma membranes (fig. 8*C*). Thus, the expression of the C-terminal peptide did not show a rescue effect.

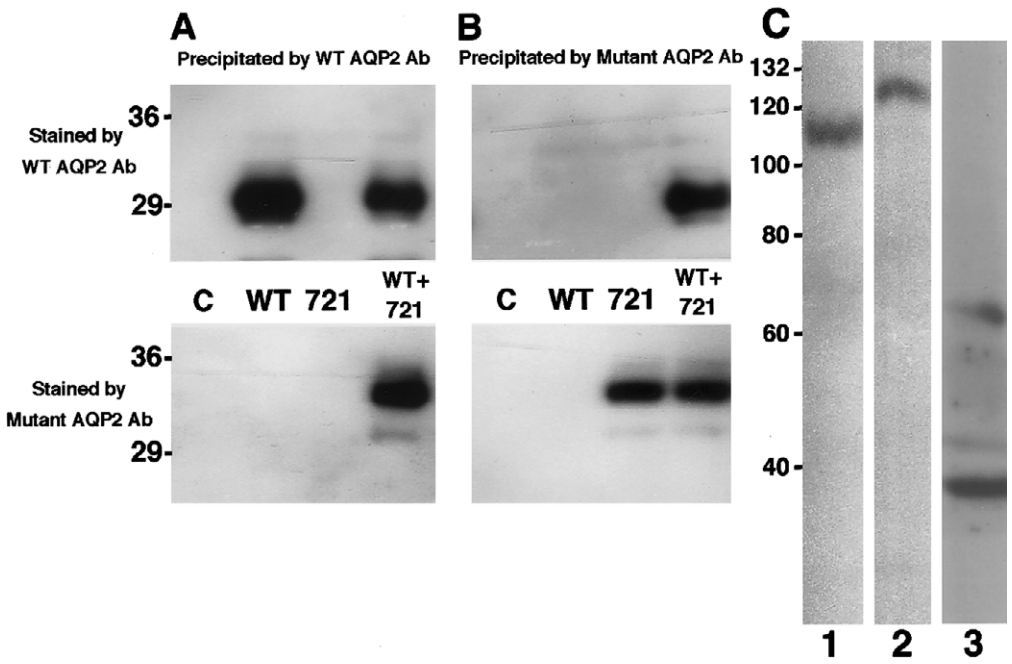

Figure 7 Oligomeric states of the wild-type and mutant AQP2 proteins. *A,* Immunoprecipitation of oocyte membrane fractions injected with water (C) or cRNA of the wild-type AQP2 (WT), 721delG (721), or both (WT + 721). Samples were immunoprecipitated with a wild-type AQP2 antibody (Ab) and were immunoblotted with the same antibody (*upper panel*) or with an antibody against mutant AQP2 (*lower panel*). *B,* Samples were immunoprecipitated with an antibody to the mutant AQP2 and were immunoblotted with an antibody to the wild-type AQP2 (*upper panel*) or the mutant AQP2 (*lower panel*). *C,* Determination of the oligomeric structure by PFO-PAGE. Membrane samples from 90–100 oocytes expressing the wild-type AQP2 and 721delG mutant were applied in each lane. *Lane 1,* Wild-type AQP2; *lane 2,* 721delG; *lane 3,* 721delG treated with urea. The sample of 721delG was further incubated with 4 M urea for 30 min at room temperature before loading.

Discussion

NDI in the three families in the present study is consistent with an autosomal dominant trait. Several clear differences exist between clinical characteristics of our patients and those of patients with autosomal recessive NDI caused by *AQP2* mutations or with X-linked NDI caused by *AVPR2* mutations (Arthus et al. 2000; Morello and Bichet 2001). Polyuria and polydipsia, the most prominent symptoms in patients with typical NDI, were not evident immediately after birth, and they appeared after some time lag in our patients (after age 1 year). This is in sharp contrast to an immediate onset of these symptoms in patients with autosomal recessive or X-linked recessive NDI. Furthermore, urine-concentrating ability was conserved to some extent in our patients; water deprivation could increase urine osmolality to above or near plasma osmolality, which, again, is different from that found in patients with recessive or X-linked NDI, in whom urine osmolality hardly exceeds 200 mOsm/kg H_2O. This residual urine-concentrating ability might prevent a clear awareness of polyuric symptoms in our patients in infancy, when milk feeding causes natural mild polyuria. Mulders et al. (1998) recently reported a family with NDI showing dominant inheritance. The mutation in that case was an *AQP2* missense mutation (E258K) located in the C-terminus. Although the type of mutation is different from that in our cases, the clinical features of the patients appear similar. Their female patient and her daughter with E258K could increase urine osmolality to 350 mOsm/kg in response to dDAVP infusion. The daughter progressively showed increased polyuric-polydipsic symptoms with age. Taken together, milder clinical presentation of NDI symptoms appear to be a common feature of dominantly inherited NDI.

Oocyte Pf increased 15-fold after expression of 5 ng cRNA encoding the wild-type AQP2 (fig. 4*A*). The wild-type AQP2 was abundantly expressed in the oocyte plasma membrane (figs. 5*A* and 6*B*), and immunoblot and immunoprecipitation analysis suggested the formation of tetramers (fig. 7*C*), findings that are consistent with previous reports (Jung et al. 1994; Kamsteeg et al. 1999). On the other hand, expression of 5 ng cRNA of either 721delG, 763–772del, or 812–818del mutant AQP2 increased Pf (2.1–2.5-fold) much less than did that of wild-type AQP2 (fig. 4*A*). The mutant proteins were expressed weakly in the plasma membrane (figs. 6*C*–6*E*), despite preserved intracellular synthesis (fig. 5*B*) and tetramerization (fig. 7*C*). Thus, impaired trafficking to the cell surface membrane is likely the primary defect of these mutant AQP2 proteins. In general, membrane proteins are synthesized on ribo-

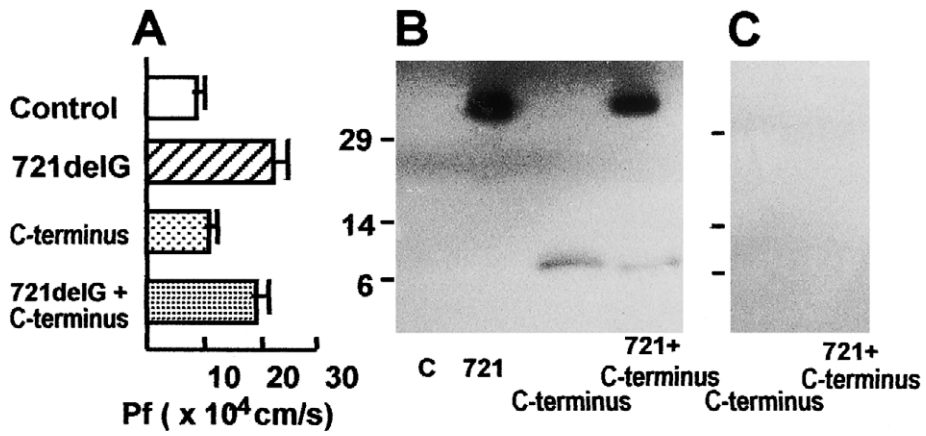

Figure 8 Coexpression of the C-terminal portion of the mutant AQP2 protein with mutant AQP2 protein in oocytes. Five nanograms of cRNAs encoding the portion of the last 61 amino acids (C-terminus), the mutant AQP2 721delG (721), or both, were injected into oocytes. *A*, Oocyte Pf. Each bar represents the mean ± SE of 17–20 measurements. *B* and *C*, Immunoblot of the oocyte lysates (*B*) and plasma-membrane fractions (*C*), probed with an antibody against the mutant AQP2.

somes and enter the endoplasmic reticulum (ER). After folding and oligomerization in the ER, proteins are transported to the Golgi apparatus (Amara et al. 1992; Halban and Irminger 1994). However, the oligomerization is suggested to take place after leaving the ER in several proteins, such as dipeptidylpeptidase IV (Jascur et al. 1991), connexin43 (Musil and Goodenough 1993), and AQP2 (Kamsteeg 1999). For the E258K mutation, Kamsteeg et al. (1999) reported that the mutated AQP2 proteins were retained in the Golgi apparatus and were inhibited from further translocation to the plasma membrane; our mutant proteins might be mishandled similarly.

Immunoprecipitation and subsequent immunoblot studies demonstrated the ability of the mutated AQP2s to form tetramers with wild-type AQP2 (fig. 7). The dominant-negative effect of the mutants can be explained by this ability. Only tetramers made of wild-type AQP2 can be translocated to the cell surface, whereas those containing at least one AQP2 mutant may be retained inside the cell. Interestingly, a dominant-negative effect was clearly shown when 0.3 ng cRNA was expressed in the oocytes, but it was not apparent when 5 ng was expressed. A similar result was reported for the E258K mutant (Mulders et al. 1998). This may be explained as follows: synthesis and surface expression of wild-type AQP2 are efficient and well saturated with 5 ng cRNA expression. A dominant-negative effect may not be apparent in this condition but would become clear when the amount of AQP2 expression is reduced (to 0.3 ng).

Several cellular mechanisms are possible for the retarded trafficking of the three AQP2 mutants found in the present study. One possibility is that the sequence deleted by the mutations might be critical for normal translocation of AQP2. Alanine at 271 (the last amino acid) was the only residue commonly eliminated in these mutations (fig. 3). However, the A271del mutant had a Pf similar to that of the wild type (fig. 4) and was well expressed in the plasma membrane (fig. 5*A*), suggesting that deletion of A271 is not involved in the misrouting of the mutants. The 721delG, 763–772del, and 812–818del mutations resulted in addition of new 93–, 76–, and 61–amino acid residues, respectively, and 61 residues at the C-terminal tail were shared among these mutants (fig. 3). Therefore, it is very likely that this C-terminal tail causes a trafficking defect of the AQP2 mutants. How can this be possible? It is unlikely that these new C-terminal tails bind to other cellular proteins (for example, cytoskeletons), thereby inhibiting translocation of the mutant AQP2s, because coexpression of the new C-terminal polypeptide did not increase the Pf or surface expression of the AQP2 mutant (fig. 8). It may be that the addition of new amino acids to the C-terminus of AQP2 inhibits normal trafficking. The new C-terminal tail may cause a conformational change of the whole C-terminus and inhibit binding of vesicle trafficking–related targeting proteins, such as VAMP2 (Nielsen et al. 1995*b*), syntaxin-4 (Mandon et al. 1996), and dynein/dynactin (Marples et al. 1998), which have been shown to colocalize with AQP2 in the collecting-duct cells. This speculation is consistent with the location of the E258K mutation in the C-terminus. This missense mutation may also elicit a conformational change in the C-terminus. Thus, mutations identified in patients with autosomal dominant NDI underscore the importance of the C-terminus of AQP2, in its trafficking to the cell surface.

Acknowledgments

This work was supported by a Grant-in-Aid from the Ministry of Education, Science, and Culture, Japan; by the Canadian Institutes of Health Research (grant MOP-8126 to D.G.B.); and by the Kidney Foundation of Canada. D.G.B. is a Chercheur de Carrière of Le Fonds de la Recherche en Santé du Québec. T.M.F. is supported, in part, by a donation from Alcan Aluminium Limitée.

Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:

GenBank, http://www.ncbi.nlm.nih.gov/Genbank/index.html (for AQP2 [accession number NM000486])

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for AQP1 [MIM 107776]; AQP2 [MIM 107777]; NDI, X-linked [MIM 304800]; NDI, autosomal recessive [MIM 222000]; and NDI, autosomal dominant [MIM 125800])

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