Functional analysis of aquaporin-2 mutants associated with nephrogenic diabetes insipidus by yeast expression

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Abstract

Mutations of aquaporin-2 (AQP2) vasopressin water channel cause nephrogenic diabetes insipidus (NDI). It has been suggested that impaired routing of AQP2 mutants to the plasma membrane causes the disease; however, no determinations have been made of mutation-induced alterations of AQP2 channel water permeability. To address this issue, a series of AQP2 mutants were expressed in yeast, and the osmotic water permeability (Pf) of the isolated vesicles was measured. Wild-type and mutant AQP2 were expressed equally well in vesicles. Pf of the vesicles containing wild-type AQP2 was 22 times greater than that of the control, which was sensitive to mercury and weakly dependent on the temperature. Pf measurements and mercury inhibition examinations suggested that mutants L22V and P262L are fully functional. In contrast, mutants N123D, T125M, T126M, A147T, and C181W had very low water permeability. Our results suggest that the structure between the third and fifth hydrophilic loops is critical for the functional integrity of the AQP2 water channel and that disruption of AQP2 water permeability by mutations may cause NDI.

AQUAPORIN-2 (AQP2) is a member of the aquaporin water channel family (1), which is a group of ancient membrane intrinsic proteins (10) involved in water transport (16, 19). AQP2 is designated as the vasopressin water channel, because the vasopressin-stimulated translocation of AQP2 to the apical membrane is needed for regulatory water reabsorption and urine concentration (36, 45). The importance of this protein has been highlighted by the identification of mutations in the AQP2 gene in patients with nephrogenic diabetes insipidus (NDI), a rare hereditary disease in which the kidney cannot produce concentrated urine in response to vasopressin (14). However, the relationship between mutation-induced alterations in AQP2 structure and the pathogenesis of NDI is not clear. Accelerated degradation, misrouting, and the loss of water permeability of the AQP2 protein by mutation-induced structural changes have been postulated as the possible causes of NDI (41). Expression of various AQP2 mutants in Xenopus oocytes has suggested that many AQP2 mutations inhibit its proper routing to the plasma membrane of the channel; however, the effects of these mutations on the intrinsic AQP2 water permeability are not clear (13, 31, 32). Thus the biophysical characterization of AQP2 mutants is important for understanding the pathogenesis of NDI.

Furthermore, functional analyses of AQP2 mutants will provide significant insights into a classic and fundamental issue in membrane biology, namely, how water molecules pass through a protein aqueous pore. Many groups have investigated the structure of the aquaporins by expressing site-directed mutants (3, 23) or NDI mutants in Xenopus oocytes (13, 32, 41). Aquaporins are predicted to have six transmembrane segments connected by five hydrophilic loops. The hourglass model proposed for AQP1 structure suggested that an aqueous pore is assembled with the second and fifth hydrophilic loops, the amino acid sequences of which are highly conserved among the aquaporin family (23). From analyses of AQP2 structure, importance of the third and fourth hydrophilic loops for water permeability was suggested (3). Although structural models proposed by these studies are roughly in agreement with recent findings by electron crystallography (9, 44), details on the structure of the aqueous pore have been controversial and left unresolved. It has been difficult to evaluate the channel water permeability of some of the AQP mutants using Xenopus oocytes because of the reduced plasma membrane expression of some mutants (26, 33). On the other hand, a yeast expression system has been used for directly analyzing function of many transporters and their mutants, because this system does not require intact plasma membrane expression of transporters, which are often disturbed by protein mutation (24, 34, 39). Recently, this system has been successfully applied to study aquaporins (12, 27) and those with mutations (26). In this study, the AQP2 mutants were expressed sufficiently well in yeast intracellular vesicles, and the intrinsic channel function of a series of AQP2 mutants was determined.

METHODS

Yeast strain and plasmid construction. The protease-deficient BJ 3505 strain (30) of Saccharomyces cerevisiae was used in this study (24). The rat AQP2 mutants L22V, N68S, N123D, T125M, T126M, A147T, C181W, R187C, S216P, and P262L were generated by PCR-based site-directed mutagenesis. N123D and C181W have been shown to be nonfunctional...
by oocyte expression study (3). All the mutants except N123D have been shown to be related to NDI (8). In this study, a series of rat AQP2 mutations were chosen to determine the structure of the aqueous pore in conjunction with our previous report (3). It is conceivable that the difference between rat and human AQP2 is not significant for mutation analyses, because the primary structures of rat and human AQP2 are more than 90% identical, and because rat and human AQP2 have similar channel water permeability and mercury sensitivity (25). The nucleotide sequences of both strands of the mutants were verified by DNA sequence analysis using an automated fluorescence sequencer (model 373A, Applied Biosystems). The PCR fragments generated with two primers, 5’-CGC GGA TCC AGC ATG TGG GAA CTC AGA TCC-3’ and 5’-CGC GAA TTC TCA GGC CTT GCT GCC GCG-3’, encoding the open reading frames of wild-type rat AQP2 (WT-AQP2) and the mutants were subcloned between a GAL10-CYC1 promoter and a phosphoglycerate kinase terminator using the BamH I and EcoR I sites of a yeast expression vector, pYeDP10, which was kindly provided by Dr. Philippe (21, 37). For a yeast expression vector, pYES2 (Invitrogen), PCR fragments generated with two primers, 5’-CCC AAG CTT ATT GCG TTT GGA GAA TCC AAG AGT CAC GCT TCC-3’ and 5’-CCC TCT AGT CCA GCA GCA TGA CAG TGA TCC TCT GCG GCG-3’, were subcloned using Hind III and Xba I sites downstream of a GAL1 promoter. pYeDP10 and pYES2 encode URA3 as a selectable marker in yeast cells. The yeast cells were transformed with the recombinant plasmids using a lithium acetate protocol (22). The transformants were selected by growth on plates containing synthetic minimal medium (SD) (40 supplemented with 2% glucose and 0.08% amino acid mixture but lacking uracil (Clontech). To induce the expression of the AQP2 protein, single yeast colonies were picked and grown at 30°C until they reached an optical density at 650 nm (OD650) of ~2–3 in SD supplemented with 2% galactose and 0.08% amino acid mixture lacking uracil (SD-galactose medium). From this stock, the yeast cells were diluted to an OD650 of 0.05 into 400 ml of SD-galactose media and grown at 30°C until they reached an OD650 of ~1.2–1.8. Immunostaining of AQP2-expressing yeast cells indicated intracellular distribution of AQP2 protein was similar for WT and mutants.

Vesicle preparation. A previously described procedure (39, 43) was modified to isolate intracellular vesicles from yeast cells. The cells were collected by centrifugation, washed with cold 10 mM NaNO3, resuspended in 50 ml of spheroplasting media (1.4 M sucrose, 50 mM K2HPO4, pH 7.4, 10 mM NaNO3, and 40 mM β-mercaptoethanol) containing 217 μg/ml of zymolyase 100T (Seikagaku), and incubated at 37°C for 1 h. The spheroplasts were collected by centrifugation (5,000 g, 5 min), suspended in 8 ml of cold hypotonic lysis buffer [0.8 M sucrose, 10 mM triethanolamine, pH 7.2, 1 mM EDTA supplemented with protease inhibitor cocktail tablets (Boehringer (Mannheim)) and lysed with 20–30 strokes of a glass Dounce homogenizer on ice. The homogenate was centrifuged at 3,000 g for 10 min at 4°C, and the sucrose concentration of the supernatant was adjusted to 1.4 M. A discontinuous sucrose gradient [2 M (1.5 ml), 1.6 M (3 ml), 1.4 M (6 ml, containing homogenate), and 0.8 M sucrose (1.5 ml)] was centrifuged for 3 h at 100,000 g in an SW41 rotor (Beckman) at 4°C. Following centrifugation, three interfacial layer fractions were collected, and each was washed twice (95,000 g, 20 min) with 2 ml of ice-cold vesicle buffer (50 mM mannitol, 90 mM KCl, 1 mM EDTA, and 20 mM Tris-HCl, pH 7.4) and stored on ice until further use.

Immunoblot analysis. SDS-PAGE was performed as described (18). The total cell lysates and vesicle fractions were incubated with SDS sample buffer [1.5% SDS, 30 mM Tris·HCl, pH 6.8, 2.5% β-mercaptoethanol, and 5% (vol/vol) glycerol] at 37°C for 1 h. A 20-μl sample was loaded in each lane, separated by a 10–20% continuous-gradient gel for 1 h with a 40-mA current, and electrotransferred to an enhanced chemiluminescence (ECL) nitrocellulose membrane (Amersham Pharmacia Biotech). After the membrane was blocked with Superblock (Promega) for 1 h at 4°C and washed once with TBS-T (20 mM Tris, 150 mM NaCl, and 0.05% Tween-20, pH 7.4), it was incubated with affinity-purified anti-AQP2 antibody against a synthetic peptide corresponding to 15 carboxy-terminal amino acid residues of rat AQP2 (19) diluted at 1:1,000 in TBS-T at room temperature for 1 h, then washed three times with TBS-T. Next, the membrane was incubated with biotin-labeled anti-rabbit IgG antibody (Vector, Burlingame, CA) diluted 1:500 in TBS-T at room temperature for 1 h, washed three times, and incubated with a 1:500 dilution of the ABC mixture (Vector) at room temperature for 30 min. After the blot was washed with TBS-T three times, the blot was visualized by ECL using an ECL mini camera (Amersham Pharmacia Biotech). For 125I detection, after the incubation with primary antibody, the washed membrane was incubated with 3 μCi of 125I-labeled protein A (Amersham Pharmacia Biotech) in TBS-T for 1 h, washed again, exposed to X-ray film for 4 h, and developed (24).

Vesicle osmotic water permeability measurement. The osmotic water permeability (P0) of the yeast vesicles was measured by a light-scattering method using a SX-18MV stop-flow apparatus (Applied Photophysics, Leatherhead, UK). The instrument dead time was 1.6 ms, and sample temperature was controlled by circulating water bath. The vesicles were diluted at 1:30 with the vesicle buffer. The vesicle suspension was mixed abruptly with the same volume of the vesicle buffer containing 360 mM mannitol, and 180 mM inwardly directed osmotic gradient was imposed. A decrease in the cell volume due to osmotic water efflux driven by the osmotic gradient was monitored as the time-dependent increase in the 90° scattered light intensity monitored at 466 nm. A light intensity trace was obtained for each sample by averaging four to seven measurements. The light scattering intensities were fitted to biexponential curves, and P0 was calculated as described (2, 17) by iteratively solving the following equation with Mathematica software (Wolfram Research, Champaign, IL)

\[
\frac{dv(t)}{dt} = P_0 \times SAV \times \frac{vw}{\text{Osm}} \times (\text{Osm}_{\text{in}}/\text{Osm}_{\text{out}})
\]

where V(t) is the volume of the cell at time t, SAV is the surface area-to-volume ratio at t = 0 (2.58 × 10^{-8} cm^{-3}), calculated from the average vesicle diameter of 233 nm measured by a Coulter counter, vw is the molecular volume of water (18 cm^3/mol), Osm is the osmolarity inside the vesicles, and Osm_{out} is the osmolarity outside the vesicles.

RESULTS

Expression of AQP2 in yeast cells. The expression of AQP2 in a protease-deficient yeast strain BJ 3505 was induced by switching cells from glucose- to galactose-containing media. No immunoreactive band was observed in native yeast vesicles or in vesicles from mock-transfected yeast cells, indicating that no protein that cross-reacts with AQP2 antibody is present in BJ 3505 cells. In addition, no band was observed in total cell lysate prepared from yeast cells transfected with the AQP2 vector incubated in glucose medium (Fig. 1A). After stimulation by glucose depletion and galactose supplementation, AQP2 expression was induced, including the production of antibodies that cross-react with AQP2.
AQP2 mutants in yeast vesicles

Fig. 1. A: effects of induction conditions on heterologous aquaporin-2 (AQP2) expression in yeast cells. Total cell lysates prepared as described (24) were isolated from yeast cells, and 15 µg of protein was applied to each lane, separated by SDS-PAGE, and blotted. Lane 1, 24-h glucose culture; lane 2, 12-h galactose culture after 24-h glucose culture; lane 3, 24-h galactose culture; lane 4, 12-h galactose culture; and lane 5, 18-h galactose culture after preculture in galactose. Molecular mass standards (kDa) are indicated on the left. B: expression of AQP2 protein in yeast cells using two different yeast expression vectors, pYE52 and pYeDP10. Fifteen micrograms of protein was obtained from total cell lysates of LLC-PK1 cells (lane 1) and yeast cells (lanes 2–5), separated by SDS-PAGE, and blotted. Lane 1, wild-type (WT) AQP2-transfected LLC-PK1 cells; lane 2, pYE52 vector; lane 3, pYeDP10 vector; lane 4, pYE-AQP2; and lane 5, pYeDP10-AQP2. Molecular mass standards (kDa) are indicated on the left.

and it accumulated in yeast cells in a time-dependent manner. A large amount of AQP2 had accumulated by mid- and late-exponential growth phase between 12 and 18 h galactose induction after preincubation in the galactose medium (Fig. 1A, lanes 4 and 5). The apparent molecular mass of the AQP2 expressed in the yeast cells was $\sim 29$ kDa, identical to that in LLC-PK1 cells (Fig. 1B). The bands with a molecular mass of $\sim 60$ kDa, which were occasionally observed in both yeast cells and LLC-PK1 cells, are likely to be a dimer of AQP2. These are unlikely to be N-glycosylated forms of AQP2, since AQP2 is not N-glycosylated in yeast cells (12) or in LLC-PK1 cells (17). Although native AQP2 is partly N-glycosylated (18), N-glycosylation is not necessary for the integrity of the water permeability function of AQP2 (3). These results indicate that the AQP2 protein is properly processed and expressed in yeast cells, similar to that in mammalian cells. Note that no significant degradation products of expressed AQP2 were observed in yeast cells, which is helpful for generating the maximum amount of functional product and for analyzing channel water permeability of AQP2 and the mutants without potential interference from the degradation products. No significant difference was found between the two yeast expression vectors, pYE52 and pYeDP10. In the subsequent studies, pYeDP10 was used as the yeast expression vector, and the cells were harvested at mid- and late-exponential growth phase after 12–18 h of galactose incubation.

Osmotic water permeability of yeast vesicles expressing AQP2. Yeast cell homogenates were fractionated by a discontinuous sucrose-gradient centrifugation to obtain fractions rich in vesicles expressing AQP2 (29, 47). The pellet was discarded, and three interfacial layer fractions were collected. Subsequently, immunoblot analyses and measurements of the osmotic water permeability of each fraction were performed. For the vesicles of all fractions from mock-transfected cells, no immunoreactive band was detected, and the time-dependent increases in the scattered-light intensity by stop-flow analysis were very slow (see Fig. 3B). For yeast cells expressing WT-AQP2, immunoblot analyses indicated that AQP2 is most abundant in the middle interfacial layer fraction between 1.4 M and 1.6 M. Vesicles from this fraction produced rapid changes in the scattered-light intensity by stop-flow analysis, indicating that these vesicles have high water permeability. In the subsequent experiments, vesicles from the middle fraction were used. Size distribution analysis of the vesicles determined by quasi-elastic light scattering indicated that the vesicles consisted of a single population with a mean diameter of $233 \pm 15$ nm (Fig. 2). The diameters of the vesicles from the yeast transfected with mock plasmid, WT-AQP2, and AQP2 mutants were not significantly different.

Figure 3 shows representative traces of vesicle shrinkage after the imposition of a 180 mOsm inwardly directed osmotic gradient. The vesicles were isolated from WT-AQP2- and mock-transfected yeast cells. Vesicle volume decreased $\sim 63\%$ in both vesicles, and vesicle shrinkage correlated linearly with the increase in the osmolality up to $+230$ mOsm (data not shown), indicating that the change of the vesicle volume perfectly reflects the change of osmolarity inside the vesicles under our experimental condition. Vesicles from mock-transfected yeast exhibited very low osmotic water permeability, suggesting that this yeast expression system is suitable for the heterologous expression of aquaporin water channels (Fig. 3B). Although a few members of the MIP family are present in yeast (4, 28, 38), functional water channels were not found normally present in B) 3505 yeast vesicles. The osmotic water permeability ($P_v$) of vesicles expressing WT-AQP2 was $328 \pm 19$ $\mu$m/s (mean $\pm$ SE, $n = 9$), which was 22-fold...
greater than that of mock-transfected vesicles (15 ± 2 µm/s, n = 8). Two additional characteristics of the AQP2 water channel, that is, its inhibition by mercury compounds and its weak temperature dependence, were examined. The Pf of vesicles expressing WT-AQP2 after treatment with 0.3 mM p-chloromercuribenzoic acid (pCMB) was nearly identical to that of the mock-transfected control (14 ± 1 µm/s, n = 4) (see Fig. 7A).

Figure 4 shows an Arrhenius plot for the temperature dependence of the osmotic water permeability. The activation energy (Ea), given by the slope of the Arrhenius plot, was 2.0 ± 0.2 kcal/mol for AQP2-expressing vesicles and 13.1 ± 0.5 kcal/mol for mock-transfected yeast vesicles. These data indicate that functional AQP2 proteins are efficiently expressed in yeast vesicles.

Osmotic water permeability of AQP2 mutants causing NDI. The 10 AQP2 mutants were expressed in yeast cells, and the vesicles from these cells were isolated. The expression of AQP2 mutant proteins was examined by immunoblot analysis. The quantity and the molecular weight of the expressed AQP2 mutants were almost identical to those of WT-AQP2, indicating that AQP2 mutants were synthesized and processed similar to that of WT-AQP2 in yeast cells (Fig. 5). The slightly larger apparent molecular weight of N123D and S216P may be due to residual differences in the molecular structure even with SDS denaturation or in the protein modification of these mutants as observed in LLC-PK1 cells (5, 11). Similar levels of protein expression of WT and mutant AQP2 in yeast vesicles confirmed that the yeast expression system is suitable for the direct biophysical characterization of AQP2 mutants. The Pf of vesicles expressing each type of mutant was measured (Fig. 6), and the data are summarized in Fig. 7A. Single channel water permeabilities of AQP2 mutants relative to that of WT estimated roughly by calculating Pf values per unit protein expression determined from immunoblot (Fig. 5) were 0.88 (L22V), 0.58 (N68S), 0.05 (N123D), 0.07 (T125M), 0.04 (T126M), 0.08 (A147T), 0.05 (C181W), 0.31 (R187C), 0.44 (S216P), and 0.71 (P262L). In the absence of pCMB, L22V and P262L yielded a very high Pf, similar to that of WT. The Pf of vesicles expressing N68S, R187C, and S216P was higher than that of the mock-transfected control vesicles but significantly lower than that of WT. The Pf of these mutants was inhibited by pCMB, indicating that these mutants are functional water channels. In contrast, the Pf of vesicles expressing N123D, T126M, A147T, and C181W was very low and similar to that of the control vesicles. Thus the AQP2 mutants examined in this study can be classified into three groups: nonfunctional mutants (N123D, T126M, A147T, and C181W), partially functional mutants (N68S, R187C, S216P), and fully functional mutants (L22V, P262L). A comparison of the location and the channel water permeability of these mutants suggests that the position of the missense mutation is a crucial determinant for its effects upon water channel function (Fig. 7B). Mutations between the third and fifth hydrophilic loops appear to disrupt water channel function, whereas mutations closer to the termini have less drastic effects on function.

DISCUSSION

In this study, we performed the first direct characterization of channel water permeability of NDI-related AQP2 mutants by measuring the osmotic water permeability of AQP2-containing yeast vesicles. Stop-flow measurements of osmotic water permeability of vesicles isolated from AQP2-transfected yeast cells (A) and mock-transfected yeast cells (B) at 10°C. Representative traces and curves fitted to a biexponential are shown. Inset: an early portion of the time course showing rapid vesicle shrinking.

![Fig. 3. Osmotic water permeability of AQP2-containing yeast vesicles.](http://ajprenal.physiology.org/)

![Fig. 4. An Arrhenius plot for the temperature dependence of osmotic water permeability in vesicles isolated from the mock-transfected (○) and AQP2-transfected yeast cells (□). Each data point is the average of 4–7 measurements. Fitted line slopes yielded activation energies of 13.1 ± 0.5 kcal/mol for the control and 2.0 ± 0.2 kcal/mol for WT-AQP2.](http://ajprenal.physiology.org/)

Fig. 3. Osmotic water permeability of AQP2-containing yeast vesicles.

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ability of yeast intracellular vesicles that express a series of AQP2 mutants. The expression of WT-AQP2 protein in yeast vesicles induced osmotic water permeability more than 20 times greater than that of mock-transfected vesicles or native yeast vesicles, and the functional properties of the water channels generated were identical to those of AQP2 expressed in oocytes and cultured cells. We found that this yeast expression system is suitable for quantitative analysis of the effects of mutations on channel water permeability. In addition, since WT and mutant AQP2 were expressed equally well in vesicles, the channel water permeability of the expressed mutants can be compared directly without the possibly complicating effects of mutations on the intracellular distribution of the protein. It has been very difficult to exclude the effects of plasma membrane expression in the analyses of AQP2 mutations with the oocyte expression system (13, 32, 33). This yeast expression system enabled us to analyze directly and precisely the effects of various amino acid substitutions in the AQP2 protein on channel water permeability.

We found that all of the tested mutations of AQP2 between the second and sixth transmembrane segments have significant deleterious effects on water channel function. The mutations N123D, T125M, T126M, A147T, and C181W, which are between the third hydrophilic loop and the second NPA motif, seemed to have the most disruptive effects on the water channel function of AQP2. These results are compatible with previous findings by oocyte experiments that...
N123D, T125M, and C181W are nonfunctional water channels (3, 20). No function of A147T has been shown in LLC-PK1 cells (46). However, our data argue with oocyte findings that T126M and A147T have some defect in surface expression but have intact water permeability (32, 41). Possible reasons for the difference are first that water permeability of the mutants was calculated indirectly from the ratio of small increase in water permeability and small amount of surface protein in oocyte experiments. Estimation of small change by ratio calculation may often be ambiguous. In contrast, water permeabilities of the mutants can be determined directly in our yeast vesicle examination. Mutations do not alter AQP2 expression or protein routing in the yeast expression system. Second, findings in oocytes are not always relevant to other cells, as observed for CFTR Δ508, probably because low temperature for oocyte culture may affect protein folding condition (15). Thus, for mutation analyses, observations only in oocyte expression system may be misleading.

In contrast, N68S and R187C near the two NPA motifs and S216P in the sixth transmembrane segment had only a partially negative effect on the channel water permeability of AQP2. Our data are again inconsistent with previous oocyte experiments that showed that these mutants are not functional. The difference can be attributed to the difference in sensitivity of water permeability measurement. In yeast system, P_i of WT-AQP2 was as much as 20 times as large as the control but the ratio was only about 5 for the oocyte system (41). In addition, possible effects of high mannose glycosylation of these mutants when expressed in oocytes were not examined. An increase in the electrophoretic mobility of the S216P mutant in the immunoblot indicates alterations in the channel structure or glycosylation, but this mutation did not seem to significantly change the function of the aqueous pore. The findings that L22V and P262L had function nearly identical to WT-AQP2 was compatible with previous observations in oocyte experiments (8, 31), indicating that the first transmembrane segment and the amino- and carboxy-terminal domains are not important for the assembly of the aqueous pore. Rather, it has been suggested that mutations in the terminal domains may interfere with the interactions between the AQP2 protein and vesicular or cytoskeletal proteins that regulate the intracellular trafficking of AQP2 (6, 7, 35, 42). The finding that the regulatory exocytosis of AQP2 is controlled by the phosphorylation of serine-256 (17) suggests that mutations near this residue may cause defects in vesopressin-responsive channel translocation.

The channel water permeability of the AQP2 mutants in this yeast expression system strongly supports our model of AQP2 structure proposed on the basis of oocyte expression data which demonstrates that mutations in the third and fourth hydrophilic loops impair channel function (3). In this model, we have emphasized the critical roles of these domains in the formation of the aqueous pore and for the integrity of AQP2 function. Our data in this study further validate our AQP2 structure model, because analyses of new mutations also suggested the significance of the third and fourth hydrophilic loops, and because examinations completely eliminated the effects of mutation-related misrouting are fully compatible with findings in previous oocyte experiments. The lack of function of C181W and the partial effects of N68S and R187C mutations in this study partially support the hourglass model proposed from AQP1 mutation analyses (23). These are also in accord with our model, in which the importance of the NPA domains was proposed. Although differences in the mercury-sensitive site among members of the aquaporin family (26, 33) and differences in the sensitivity of osmotic water permeability to mercury between AQP1 and AQP2 have suggested that there are structural differences in the aqueous pore of aquaporins, our
studies have underscored the importance of the structure between the third and fifth hydrophilic loops at least for AQP2.

The present data have confirmed that the disruption of the intrinsic function of AQP2 by missense mutations may be a significant pathogenic cause of NDI. It has been suggested from experimental observations with oocytes that accelerated degradation and the inappropriate targeting of the AQP2 mutants are the major causes of NDI (13, 32, 41). The mechanisms by which gene mutations disrupt the function of the protein product include a decrease in protein synthesis, disorders in posttranslational modification including glycosylation and phosphorylation, accelerated protein degradation, mistargeting of the protein, and alterations in the intrinsic activity of the protein. For AQP2, mutation-related disruptions affecting regulatory redistribution may be potential causes of NDI. Previous studies using NDI-related AQP2 mutants expressed in Xenopus oocytes showed that AQP2 mutants other than L22V (8) and E258K (31) are retained in the endoplasmic reticulum and are less stable than WT-AQP2 (41). However, because the trafficking mechanisms of channel proteins in oocytes may be different from those in mammalian cells and there have been no studies using mammalian epithelial cells, it cannot be concluded that the misrouting of AQP2 mutants is mainly responsible for the pathogenesis of NDI.

In conclusion, we have directly evaluated channel water permeability of a series of AQP2 mutants that are related to NDI without observing the effects of intracellular misrouting. Mutations between the third and fifth hydrophilic loops are shown to impair water permeability, implicating the direct association of mutation-induced loss of channel water permeability and the pathogenesis of NDI. In addition, our observations have stressed the importance of the structure between the third and fifth hydrophilic loops for water permeability function, confirming our structure model for AQP2.

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