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Molecular cloning and expression of a member of the aquaporin family with permeability to glycerol and urea in addition to water expressed at the basolateral membrane of kidney collecting duct cells

(major intrinsic protein family/glycerol facilitator/urine concentration)

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ABSTRACT **Water transport in highly water-permeable membranes is conducted by water-selective pores—namely, water channels. The recent cloning of water channels revealed the water-selective characteristics of these proteins when expressed in *Xenopus* oocytes or reconstituted in liposomes. Currently, it is assumed that the function of water channels is to transport only water. We now report the cloning of a member of the water channel that also transports nonionic small molecules such as urea and glycerol. We named this channel aquaporin 3 (AQP3) for its predominant water permeability. AQP3 has amino acid sequence identity with major intrinsic protein (MIP) family proteins including AQP–channel-forming integral membrane protein, AQP–collecting duct, MIP, AQP–γ tonoplast intrinsic protein, nodulin 26, and glycerol facilitator (33–42%). Thus, AQP3 is an additional member of the MIP family. Osmotic water permeability of *Xenopus* oocytes measured by videomicroscopy was 10-fold higher in oocytes injected with AQP3 transcript than with water-injected oocytes. The increase in osmotic water permeability was inhibited by $HgCl_2$, and this effect was reversed by a reducing agent, 2-mercaptoethanol. Although to a smaller degree, AQP3 also facilitated the transport of nonionic small solutes such as urea and glycerol, while the previously cloned water channels are permeable only to water when expressed in *Xenopus* oocytes. AQP3 mRNA was expressed abundantly in kidney medulla and colon. In kidney, it was exclusively immunolocalized at the basolateral membrane of collecting duct cells. AQP3 may function as a water and urea exit mechanism in antidiuresis in collecting duct cells.**

Water channels have been postulated for the pathway of selective water permeation in highly water-permeable membranes. Recent molecular cloning of two types of water channels [aquaporin–channel-forming integral membrane protein (AQP-CHIP) (1) and AQP–collecting duct (CD) (2)] in mammals and one in plant [AQP–γ tonoplast intrinsic protein (γTIP) (3)] has given an insight into the molecular mechanism of water transport. AQP-CHIP was originally isolated from human erythrocytes (4) and expressed in a wide variety of tissues, including kidney, eye, spleen, intestine, lung, and choroid plexus (5–8). On the other hand, AQP-CD is expressed only in kidney (2). AQP-γTIP is present at the vacuolar membrane (tonoplast) of higher plant cells (9). All these proteins belong to an ancient channel family, the major intrinsic protein (MIP) family (10–12).

Water channels are highly selective to water permeation and exclude small solutes. The mechanism of this selectivity is postulated to be the small size of the pore. The pore diameter of water channels may be ≈0.3 nm, to exclude urea. As the pore size may not be the same in various water channels, some functional heterogeneity regarding the selectivity of water permeation may exist. So far, no water channel has been reported to permeate small solutes as well as water. Moreover, there has been controversy as to whether water and urea traverse the same pathway or separate pathways in biological membranes (13).

By using a PCR cloning strategy, we have cloned another member of the MIP family from rat kidney; aquaporin 3 (AQP3).** Functional expression in *Xenopus* oocytes confirms its water-channel function. Moreover, AQP3 also facilitates the transport of nonionic small solutes such as urea and glycerol, albeit to a smaller degree. Our results are provocative in suggesting that water channels can be functionally heterogeneous and possess water and solute permeation mechanisms.

MATERIALS AND METHODS

Reverse-Transcribed PCR. One microgram of rat kidney total RNA was reverse transcribed and used for PCR with a set of degenerative primers (5 μM) as reported (2). The PCR was conducted in the following profile: 94°C for 1 min, 50°C for 1 min, 72°C for 3 min for 30 cycles.

Library Construction and Screening. An oligo(dT)-primed directional rat kidney cDNA library was constructed in the *Not* I/*Sal* I site of λgt22 (Superscript cDNA synthesis kit; BRL) to screen under high stringency (6× SSPE/5× Denhardt's solution/0.2% SDS/100 μg of salmon sperm DNA per ml/50% formamide at 42°C) with the 420-bp PCR clone labeled with [α-^{32}P]dCTP (3000 Ci/mmol; 1 Ci = 37 GBq; Amersham). A positive clone (AQP3) was isolated with a 1.8-kb insert and was subcloned into *Not* I- and *Sal* I-cut

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Abbreviations: AQP, aquaporin; CD, collecting duct; CHIP, channel-forming integral membrane protein; GI, gastrointestinal; GlpF, glycerol facilitator; IMCD, inner medullary CD; MIP, major intrinsic protein; TIP, tonoplast intrinsic protein; cRNA, complementary RNA.

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**The sequence reported in this paper has been deposited in the GenBank data base (accession no. D17695).

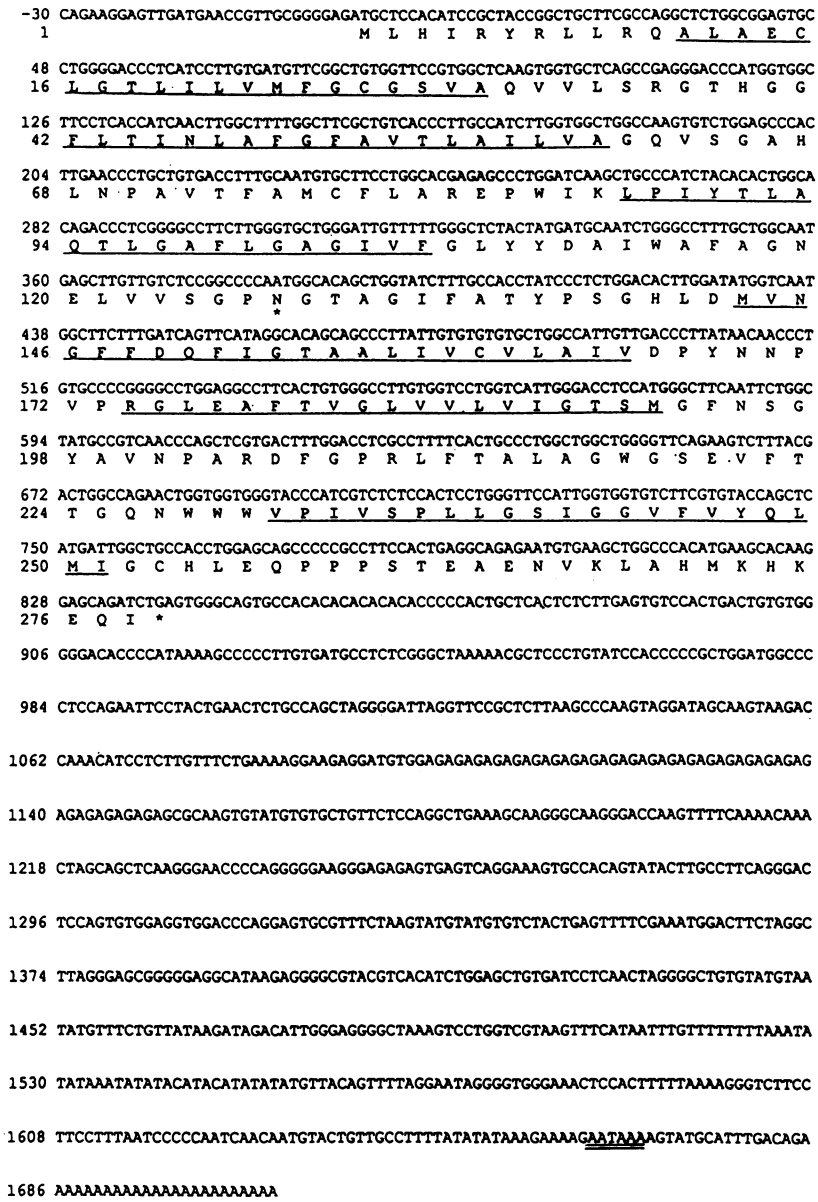

FIG. 1. Sequence and predicted structure of AQP3. Nucleotide sequence and deduced amino acid sequence. The 3′ noncoding sequence contains a consensus polyadenylylation signal (double underlined) with a poly(A) tail. One potential N-linked glycosylation site is indicated by an asterisk. Six potential transmembrane domains are underlined.

pSPORT and sequenced by the chain-termination method with Sequenase (United States Biochemical).

Phylogenetic Analysis of MIP Family Proteins. We estimated the numbers of amino acid substitutions per site between two amino acid sequences by use of Kimura's equation (14), $d_a = -\ln(1 - p - 0.2p^2)$, where p is the proportion of different amino acid substitutions per site between them. With use of the values of d_a, the phylogenetic tree was constructed by the neighbor-joining method (15).

Expression of AQP3 in *Xenopus* Oocytes. The *Sal* I/*Eco*RI fragment of AQP3 cDNA (containing an open reading frame) was blunt-ligated into the *Bgl* II site of a pSP64T-derived BlueScript vector containing 5′ and 3′ untranslated sequences of the β-globin gene of *Xenopus* [pXβG-ev1; a generous gift from P. Agre (1)]. Capped complementary RNA (cRNA) was synthesized by using T7 RNA polymerase. Oocytes were injected with 20 ng of AQP3 cRNA (1 μg/μl) and incubated at 18°C.

Osmotic Water Permeability of Oocytes. After 48–62 h of incubation, osmotic water permeability (P_f) was measured as described (2, 16). Oocytes were transferred from 200 mosM to 70 mosM modified Barth's buffer and osmotic volume increase was observed at 24°C by videomicroscopy.

Substrate Uptake into Oocytes. After 48–62 h of incubation, the uptakes of radioisotope-labeled substrates were measured. Oocytes were incubated at room temperature in Barth's solution with each isotope: [^{14}C]urea (specific activity, 2.02 GBq/mmol; Amersham), [U-^{14}C]glycerol (specific activity, 5.88 GBq/mmol; Amersham), α-[1-^{14}C]methylaminoisobutyric acid (specific activity, 1.8 GBq/mmol; Amersham), or methyl (α-D-[U-^{14}C]gluco)pyranoside (specific activity, 5.33 GBq/mmol; Amersham). At 0, 15, and 30 min after incubation, oocytes were rapidly rinsed five times in ice-cold Barth's solution. The individual oocytes were lysed in 200 μl of 10% SDS overnight, and the radioactivity was measured by liquid scintillation counting.

Voltage Clamp of Oocytes. Two-electrode voltage clamp experiments were performed at room temperature. Voltage pulse protocols and data acquisition were performed by the pCLAMP program.

Northern Blots. RNA was extracted from various rat tissues. Poly(A)$^+$ RNA (6 μg each; except urinary bladder, 2

μg) was electrophoresed in agarose gel containing formamide. After transfer to nylon membranes, blots were hybridized under high stringency with AQP3 cDNA labeled with [α-^{32}P]dCTP.

Immunostaining of AQP3. Rabbit antisera were raised against the synthetic peptide (Peptide Institute, Osaka) corresponding to the 15 C-terminal amino acids of AQP3 (EAENVKLAHMKHKEQ) as described (2). Sections (4 μm) of rat kidney fixed with paraformaldehyde were mounted on slides. The slides were preincubated with nonimmune goat serum and then incubated with dilute rabbit serum (1:500) for 60 min at 37°C. After rinsing, the slides were incubated with fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin (1:100) for 60 min at 37°C. The antigen (peptide) absorption by dilute rabbit serum (1:500) was conducted at 4°C overnight with the molar ratio more than 100 times in excess of peptide to test the specificity of rabbit serum staining.

Statistical Analysis. All data were expressed as the means ± SD. A P value of <0.05 was assumed to indicate a statistically significant difference (Student's t test). The number of oocytes is shown in parentheses.

RESULTS

Cloning of cDNA and Analysis of Amino Acid Sequence of AQP3. We screened a rat kidney cDNA library for clones with a PCR product whose sequence was similar to that of the MIP family. A cDNA clone of 1.8 kb, AQP3 (Fig. 1), was isolated and found to encode a 279-amino acid protein (M_r, 29,848) with six transmembrane regions by hydropathy analysis. There are two possible start codons in-frame. The second ATG is assigned as +1 because the position −3 is a purine, consistent with a consensus 5′ noncoding sequence (17) and the conservation of only the second ATG in the human homologue (K.I., unpublished observation). The 3′ noncoding sequence contains a consensus polyadenylylation signal with a poly(A) tail. One potential N-linked glycosylation site is present in the third loop. Six potential transmembrane domains predict that both N and C termini are located in the cytosol. Searching the protein data base revealed the highest amino acid sequence identity with *Escherichia coli* glycerol facilatator (GlpF) (42%; refs. 18 and 19) and lesser identity with the other MIP family proteins including AQP-CHIP (33%; ref. 4), AQP-CD (34%; ref. 2), MIP (35%; ref. 20), AQP-γTIP (35%; ref. 9), and nodulin 26 (33%; ref. 21). A phylogenetic comparison between AQP3 and eight MIP family proteins revealed that AQP3 is most related to GlpF and developed in a different branch from other water channels (Fig. 2).

Functional Characterization of AQP3. We examined the function of AQP3 when expressed in *Xenopus* oocytes. To determine the P_f of oocytes injected with AQP3 transcript, the cell volume increase induced by hypotonic shock was measured by videomicroscopy. The P_f of AQP3 cRNA-injected oocytes was 10 times higher than the P_f of water-injected oocytes as summarized in Fig. 3*A*. The induction was comparable to the levels observed in the other water channels. This increase in P_f was reduced to 35% by incubation in 0.3 mM $HgCl_2$, which was reversed by a reducing agent, 2-mercaptoethanol (Fig. 3*A*). The result suggested the involvement of SH residues in water permeation. The deduced amino acid sequence of AQP3 contains 5 cysteine residues. To estimate the activation energy (E_a) from the Arrhenius plot of P_f, we measured P_f at 10, 20, and 30°C. E_a was 3.7 kcal/mol (1 cal = 4.184 J), a value in the range expected for a water channel (24). The results suggest that the expressed AQP3 protein is a water channel.

The water specificity of AQP3 channel function was examined by measuring the uptake of radiolabeled solutes. Oocytes were incubated in the presence of 6.4 μM [^{14}C]-glycerol and the intracellular radioactivity was measured after 30 min. AQP3 cRNA injection stimulated [^{14}C]glycerol uptake 4-fold (Fig. 3*B*). Glycerol uptake was completely blocked by 1 mM $HgCl_2$ (Fig. 3*B*). The calculated glycerol permeability of AQP3-injected oocytes was 23×10^{-6} cm/sec. The results were comparable with the previous report on glycerol uptake by GlpF expressed in *Xenopus* oocytes where glycerol uptake was increased 5-fold (3). The results were reproduced four times in different sets of oocytes. To estimate E_a from the Arrhenius equation, glycerol uptake was measured at 4 and 24°C. The mean was 6.0 kcal/mol, a value larger than free molecule movement but smaller than movement through lipid bilayers (10–20 kcal/mol). The reported E_a value of GlpF in *E. coli* is 5 kcal/mol (25).

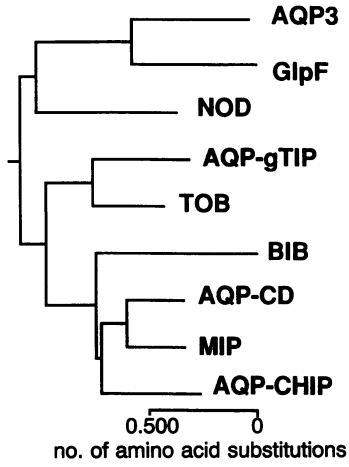

FIG. 2. Phylogenetic tree for AQP3 and related proteins based on amino acid sequences. NOD, soybean nodulin 26 (21); TOB, TobRB7 of tobacco root (22); BIB, big brain of *Drosophila* (23); MIP, bovine MIP (20).

Next we examined urea transport as urea is as small as glycerol and GlpF slightly permeates urea (26). AQP3 cRNA injection stimulated the uptake of [^{14}C]urea (90.6 μM) into oocytes 2-fold (Fig. 3*C*). The calculated urea permeability of AQP3-injected oocytes was 2.3×10^{-6} cm/sec, which was 1 order of magnitude lower than that of the recently cloned vasopressin-regulated urea transporter (27) (45×10^{-6} cm/sec). The results were reproduced four times in different sets of oocytes. The effect of mercuric ion on AQP3-induced urea uptake was complicated by stimulation of urea transport in the oocytes by mercuric ion (data not shown). We used a classic inhibitor of urea transport, phloretin. The urea uptake was completely blocked by 0.35 mM phloretin. Previous studies in erythrocytes (13) and inner medullary CD (IMCD) (28) showed that water permeability was not inhibited by phloretin and the results were cited as the basis for the separate pathways for water and urea. We then examined the effect of phloretin on P_f of oocytes expressing AQP3. Surprisingly, 0.35 mM phloretin inhibited P_f by 58% [177 ± 31.2 (n = 27 oocytes) vs. 75 ± 20 (n = 11 oocytes)]. The toxic or nonspecific effects of phloretin to oocytes are negligible as P_f of AQP-CD cRNA-injected oocytes was not inhibited by phloretin [218.6 ± 59.7 (n = 26 oocytes) vs. 204.9 ± 53.0 (n = 26 oocytes)].

Next we tested whether AQP3 permeates larger molecules (amino acids and sugars). The uptake of [^{14}C]methylaminoisobutyric acid (10.5 μM) was not stimulated by AQP3 expression at 30 min [437 ± 77 (n = 3) vs. 574 ± 106 (n = 5) fmol per hr per oocyte]. The uptake of methyl α-[^{14}C]glucopyranoside (14 μM) was not stimulated by AQP3 expression at 30 min [512 ± 96 (n = 3) vs. 587 ± 93 (n = 5) fmol per hr per oocyte]. Therefore, AQP3 may have a narrow pore that passes water and small solutes such as glycerol and urea but excludes larger molecules such as amino acids and sugars.

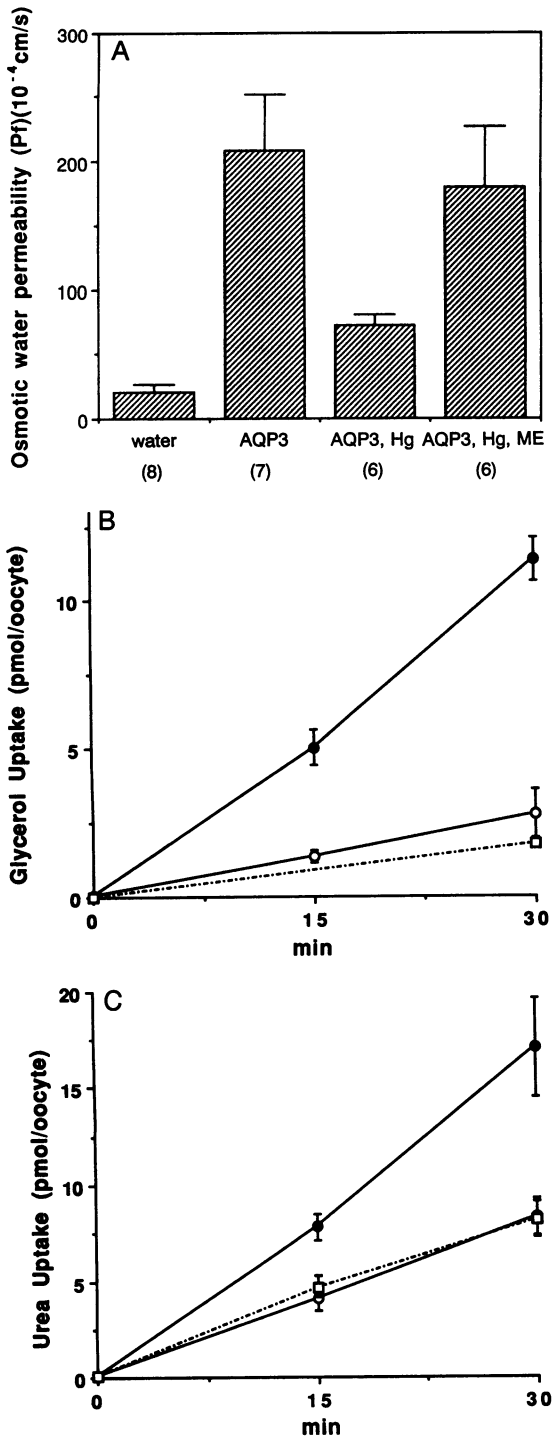

FIG. 3. Functional expression of AQP3 in *Xenopus* oocytes. (*A*) P_f values in cRNA or water-injected oocytes. When indicated, the assay was performed in the presence of $HgCl_2$ (Hg; 0.3 mM; 5-min preincubation) or 2-mercaptoethanol (10 mM) after mercuric ion pretreatment (Hg, ME; 5-min preincubation with 0.3 mM $HgCl_2$ followed by 15-min preincubation and the assay with 10 mM 2-mercaptoethanol). (*B*) Time course of [^{14}C]glycerol uptake into oocytes injected with water (○) or AQP3 cRNA (●) and 1 mM $HgCl_2$-treated oocytes with AQP3 cRNA (□). Values are averaged from measurements of five oocytes at each point. (*C*) Time course of [^{14}C]urea uptake into oocytes injected with water (○) or AQP3 cRNA (●) and 0.35 mM phloretin-treated oocytes with AQP3 cRNA (□). Values are averaged from measurements of five oocytes at each point.

This property is distinct from the other water channels: AQP-CHIP excludes urea (1) and glycerol (3); AQP-γTIP excludes glycerol (3); AQP-CD excludes urea (K.F. and S.S., unpublished observation). Therefore, AQP3 is a unique water channel permeating not only water but also small solutes.

To determine whether AQP3 could transport ions, we examined the electrical conductance of the oocyte plasma membrane expressing AQP3. No newly expressed membrane currents were detected in the oocytes when voltage was changed over the range −130 to +30 mV (data not shown). Although the passage of nonabundant ions cannot be ruled out, AQP3 does not appear to function as an ion channel.

AQP3 mRNA Expression and Tissue Distribution. Northern blot analysis revealed that AQP3 mRNA (1.8 kb) was expressed most abundantly in colon and kidney medulla (Fig. 4). Fainter bands were detected in kidney cortex, small intestine, stomach, and spleen, and a much fainter band was detected in urinary bladder. After a longer exposure, a faint band was detected in lung, but no band was seen in brain, liver, pancreas, and heart. In kidney, it was expressed more in the medulla than in the cortex.

Immunohistochemical Localization of AQP3 in the Kidney. A polyclonal antibody was made against a synthetic peptide corresponding to the C-terminal amino acids of AQP3 and fixed rat kidney slices were stained. Immunofluorescence staining was observed only in CDs and not in other nephron segments (Fig. 5). The basolateral membranes were predominantly stained in CD cells, which was in contrast with the apical staining of AQP-CD (2). A minority of the cortical CD cells, which were stained negatively, may be intercalated cells.

DISCUSSION

We have cloned an additional type of water channel (AQP3) that transports small nonionic molecules such as urea and glycerol as well as water. Because of its predominant water permeation, we placed it as a member of water channels and named it aquaporin 3 (29). Previous functional expression studies in *Xenopus* oocytes have shown that glucose transporters (30, 31) and cystic fibrosis transmembrane conductance regulator (CFTR) (32) stimulated osmotic water permeability, suggesting the formation of aqueous pores by these transporters. However, the degree of stimulation was very small compared with water channels. The glucose transporters increased P_f by 9.3–30 × 10^{-4} cm/sec and CFTR increased P_f by 5 × 10^{-4} cm/sec, while AQP3 increased P_f by

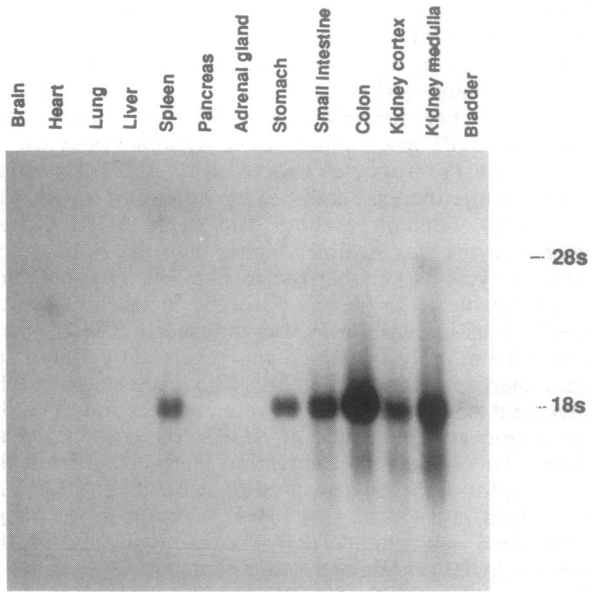

FIG. 4. Northern blot analysis of AQP3 expression in different rat tissues. AQP3 mRNA (1.8 kb) was detected in spleen, stomach, small intestine, colon, kidney, and urinary bladder.

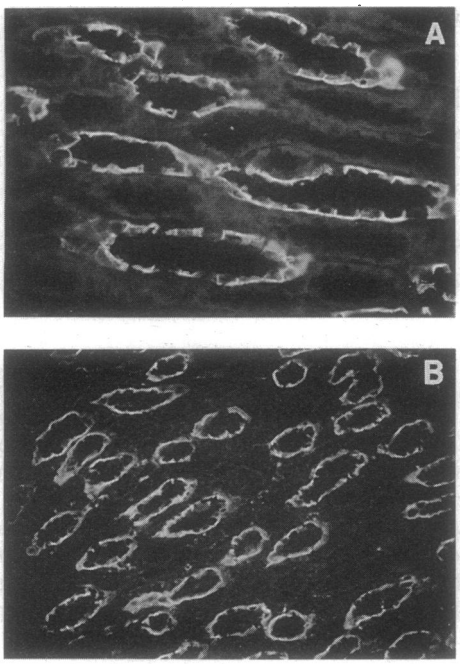

FIG. 5. Immunofluorescence staining of rat kidney cortex (*A*) and outer medulla (*B*) with polyclonal antibody against C-terminal peptide of AQP3 (1:500 dilution). AQP3 was detected at the basolateral membrane of CD cells. Immunostaining was specific since it was abolished when the antibody was pretreated with excess antigen (data not shown).

$150–200 \times 10^{-4}$ cm/sec. As the transport of water and solutes by AQP3 was inhibited by mercuric ion and phloretin, water and solutes most likely go through the same pathway. The lower E_a of water and glycerol transport suggests the channel-like character of the transport. AQP3 may have a pore with a diameter of ≈0.4 nm to permit glycerol to pass through. The phylogenetic analysis revealed the separate clustering of AQP3 and GlpF from other water channels (Fig. 2). AQP3 is more homologous to GlpF than to other water channels. However, GlpF failed to permeate water when expressed in oocytes (3). Thus, GlpF may have hydrophobic sites to exclude water, and AQP3 may have lost these sites. Alternatively, AQP3 may have acquired water permeating segments that are absent in GlpF.

The inhibition of water permeability with phloretin is an unreported property of water channels, suggesting the functional heterogeneity of water channels. A previous study with isolated terminal IMCD has shown the absence of effect of phloretin on P_f (28). The reason for the discrepancy between their study and the present oocyte expression study may be the difference of the tissues. Alternatively, apical water transport may be the rate-limiting step for IMCD water transport so that partial inhibition of basolateral water transport by phloretin may not affect transtubular water transport appreciably. Or there may be another water channel that is phloretin insensitive in terminal IMCD.

AQP3 mRNA was mainly expressed in kidney and the gastrointestinal (GI) tract. In kidney, it was expressed exclusively at the basolateral membrane of CD cells. As antidiuretic hormone (ADH) stimulates water and urea transport at the apical membrane, AQP3 may serve as a pathway for both water and urea exit in antidiuresis. A previous study of cortical CDs on water permeability has shown that the basolateral membrane possesses a water permeability as high as the ADH-treated apical membrane (33).

Although massive water secretion and absorption are conducted in the GI tract, the mechanism of water transport is poorly understood. The expression of AQP3 mRNA in colon and small intestine suggests that AQP3 may play an important role in GI water transport. AQP3 may open the way to study the mechanism of fluid transport in the GI tract. The presence of AQP3 in spleen suggests that AQP3 may function beyond epithelial tissues as has been shown with AQP-CHIP (7). Moreover, the role of AQP3 in nonionic small molecule transport is unclear at the moment. AQP3 may play some role in glycerol metabolism.

In summary, we have cloned a member of the water channel (AQP3) that is expressed abundantly in kidney medulla and colon. AQP3 is localized at the basolateral membrane of kidney collecting duct cells. AQP3 transports nonionic small molecules such as urea and glycerol in addition to water when expressed in *Xenopus* oocytes. AQP3 may help to solve a long-standing controversy on the independence of water and urea transport in biological membranes.

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