Expression and Immunolocalization of AQP6 in Intercalated Cells of the Rat Kidney Collecting Duct*

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Summary. The expression and localization of AQP6 were examined in rat kidneys. In the kidney compartments, the expression was more intense in the outer medulla than in the cortex or inner medulla, and was negative in the glomerulus. During development, the AQP6 mRNA expression in the kidney was not detected in the fetus, but was recognized at birth, increased gradually by 4 weeks of age, and was unchanged thereafter. In situ hybridization demonstrated significant signals for AQP6 mRNA along the outer and inner medullary collecting ducts. Since the localization of the AQP6 mRNA-expressing cells was comparable to that of immunoreactive H⁺-ATPase-bearing cells in the collecting duct, they were identified as intercalated cells. No AQP6 mRNA signals were recognizable in other cells in the kidneys, including glomerular cells. No glomerular expression of AQP6 mRNA was confirmed by RT-PCR using total RNA extracted from the glomeruli. Immunohistochemistry using an antibody raised against recombinant rat AQP6 protein could localize the immunoreactivity in a population of collecting duct cells. Serial section observations indicated that the AQP6-immunoreactive cells corresponded to H⁺-ATPase bearing intercalated cells.

The Aquaporins (AQPs) are water channels and are a family of major intrinsic proteins (MIP) (Görin et al., 1984). At present, ten AQP members (AQP0-9) have been found in various mammalian organs and tissues (Agre et al., 1995; Yamamoto and Sasaki, 1998; Nielsen et al., 1999). AQP1 was first identified as a channel-forming integral membrane protein with a molecular weight of 28 kDa (CHIP28) in human erythrocytes (Denker et al., 1988) and has also been found in the proximal tubules, descending thin limbs of Henle, and descending vasa recta of the rat kidney (Nielsen et al., 1995). AQP2 was demonstrated to occur in the collecting duct principal cells as a water channel member and to move from the cytoplasmic vesicles to the apical membrane when the cells were stimulated by vasopressin (Fushimi et al., 1993; Sabolic et al., 1995; Yamamoto et al., 1995b).

AQP6 genes have been isolated from rat and human kidneys with high homologies to AQP2 and MIP (AQP0) (Ma et al., 1993, 1996). The human AQP6 gene was mapped to chromosome locus 12q13, which is also shared by the AQP0, AQP2 and AQP5 genes (Sasaki et al., 1994; Saito et al., 1995; Lee et al., 1996). A previous study showed that AQP2, AQP5, and AQP6 genes are clustered in a 27-Kb segment of chromosome 12, suggesting a close evolutionary relationship among these genes (Ma et al., 1997). On the other hand, rat AQP6 was found to encode a 29 kDa protein with 39% amino acid identity to rat AQP1, and 50% to AQP2 (Ma et al., 1993). In addition, AQP6 mRNA was shown to be exclusively expressed in the kidney, as in the case of AQP2 mRNA which is up-regulated in a water-depleted condition (Ma et al., 1993). Recently, Yasui et al. (1999a) reported that AQP6 was immunolocalized in the glomerular podocytes and proximal tubules as well as intercalated cells in collecting ducts in rat kidneys. Another recent report recorded an exclusive localization of AQP6 in the intercalated cells of rat collecting ducts and showed upregulation of AQP6 mRNA expression in the kidneys of alkali-loaded or water-depleted rats (Promeneur et al., 2000).

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The present study aimed to settle this dispute over the localization of AQP6 by examining the mRNA expression and the localization of AQP6 immunoreactivity in the kidneys of rats. It further investigated changes in the expression of AQP6 in rats during their development and in an acidic or dehydrated condition. Consequently, it was found that AQP6 mRNA was only expressed in the kidney but not in the other representative organs examined. The expression was enhanced in rats acidified by drinking ammonium chloride for 9 days and was down-regulated in dehydrated rats. Both AQP6 mRNA and immunoreactive AQP6 were localized in the intercalated cells of collecting ducts but not in the glomerular cells or proximal tubules.

**MATERIALS AND METHODS**

**PCR cloning of AQP6 cDNA**

Rat kidney cDNA was synthesized from RNA samples of the organ and glomeruli using oligo dT primers and reverse transcriptase. Then the coding sequence of AQP6 gene was amplified by PCR using 5'-AACGGTAATGACGGTCACATCC-3' (sense) and 5'-GCTTGCTCCATCGTCCTGAG-3' (antisense) primers. The PCR products were subcloned into plasmid vectors (pGEM-T, Promega, Madison, WI). The cloned cDNA was sequenced by a DNA sequencer (PRIZM, Applied Biosystems Japan, Urayasu) and was digested with EcoI to linearize the circular plasmid containing 355-bp AQP6 cDNA fragment. A 114-bp fragment of the gene encoding rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and linearized with BamHI was used for detection of a housekeeping gene, GAPDH mRNA.

The linearized plasmids were used as templates to synthesize 32P-labeled antisense cRNA probes by in vitro transcription with either SP6 (for AQP6 probe) or T7 (for GAPDH probe) RNA polymerase. Total cellular RNA was extracted from 17 organs (cerebrum, cerebellum, salivary gland, heart, lung, liver, kidney, adrenal gland, colon, ileum, pancreas, spleen, thymus, testis, ovary, eye, striated muscle) of Wistar-Kyoto (WKY) rats (Charles River Japan, Atsugi). Four kidney compartments (cortex, outer medulla, inner medulla, and glomerulus) were separated as described previously (Yamamoto et al., 1995b). Fetal and postnatal rat kidneys were also obtained at 18 days of gestation, 1 day, 1, 2, and 4 weeks and 2, 6, and 14 months of age. Ribonuclease protection assay was employed to detect mRNA expression in the RNA samples as reported previously (Goto et al., 1995). Ten micrograms of each RNA sample were hybridized with a mixture of the 32P-labeled AQP6 and GAPDH antisense cRNA probes (1×106 cpm each) overnight at 45°C.

To examine the effects of chronic dehydration or metabolic acidosis on the AQP6 mRNA expression in the kidneys, male WKY rats (200–300 g, three animals each) were either dehydrated by being denied drinking water for 3 days or were given 0.5 N ammonium chloride as drinking water for 9 days. Control animals were allowed free access to water during these experiments. After sacrifice, total cellular RNA samples were extracted from the whole kidneys, cortex, and medulla compartments. For quantification of the mRNA expression, the autoradiography bands for the AQP6 and GAPDH mRNA were analyzed by computer-based densitometry using an NIH Image software and the ratio (AQP6/GAPDH mRNA) was calculated as reported previously (Yamamoto et al., 1997).

**Antibody preparation**

PCR using the primers; 5'-CCGCTACGCCATCC-TTGTGGGTACCAC-3' (sense) and 5'-CGGACGTGTACCGCTCATTCTGTCGTC-3' (antisense), allowed the rat AQP6 gene encoding the C-terminus to be amplified from rat kidney cDNA and cloned into an expression vector (pET-40b+, Novagen, Madison, WI) at KpnI-SacI sites. E. coli (BL21) transfected with the vector was cultured in Luria-Bertani (LB) medium supplemented with kanamycin and was stimulated with 0.4 mM isopropyl-1-thio-D-galactoside (IPTG) for 3-4 h to induce recombinant protein synthesis. After harvesting, the cells were disrupted by ultrasonication, and the target recombinant protein with a molecular mass of 36.5 kDa was purified by a mini whole gel elutor (Bio-Rad Lab., Japan) after electrophoresis on a SDS-polyacrylamide gel (2 mm thickness).

The purified recombinant protein was emulsified with complete Freund's adjuvant and injected subcutaneously into each New Zealand White rabbit twice at 2-week intervals (1 mg each). The blood was collected both before the immunization to obtain pre-immune serum and then one week after the second immunization.

**Western blotting**

Reactivity of the antisera to the recombinant AQP6 protein was examined by Western blot analysis using whole lysate of E. coli transfected with the AQP6 cDNA-inserted vector. A portion of the lysate was treated with human thrombin overnight at 20°C to liberate the recombinant AQP6 C-terminus peptide.
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According to the manufacture's protocol (Novagen), the reaction mixture was fractionated to separate molecules into groups of above or below 10 kDa using a microconcentrator (Centricon-10, Amicon Inc., Beverly, MA). The samples were then electrophoresed on a SDS-polyacrylamide gel (4–20% gradient gel) and protein bands were electroblotted to polyvinylidene difluoride membrane. The membrane was preincubated with a blocking buffer (10% nonfat milk-PBST; 0.05% Tween 20 and 0.5% NaN₃ in PBS) for 2 h at room temperature and incubated with the anti-AQP6 antibody (diluted 1:1,000 in blocking buffer) overnight at 4°C. After washing in PBST three times, the membrane was incubated with goat anti-rabbit immunoglobulins conjugated to peroxidase-labeled dextran polymer (DAKO, Carpinteria, CA) containing 2% normal rat serum for 1 h, and the peroxidase reaction products were colored with 0.5 mg/ml 3’-diaminobenzidine tetrahydrochloride and 0.01% hydrogen peroxide.

**Immunohistochemistry**

The blocks of WKY rat kidneys were immersed in 4% paraformaldehyde overnight, dehydrated in ethanol, and embedded in paraffin. They were sectioned at a thickness of 4 μm. The sections were incubated with the anti-rat AQP6 antibody (1:1000 dilution) for several hours at room temperature, rinsed with PBS, and then with goat anti-rabbit immunoglobulins conjugated to peroxidase-labeled dextran polymer for 1 h.

To verify the specificity of the immunoreactivity, the anti-AQP6 antibody was preabsorbed with 10 times excess (molar ratio) the recombinant AQP6 protein and used for immunohistochemistry. After washing in PBS, the AQP6 antibody binding sites were visualized by using 0.5 mg/ml 3’-diaminobenzidine tetrahydrochloride and 0.01% hydrogen peroxide. Serial sections were stained with either the AQP6 antibody or anti-56-kDa subunit of the medullary H⁺ ATPase antibody (B56-CT), kindly provided by Dr. Stephen GLUCK (Washington University School of Medicine, St. Louis, Mo) and shown to react to rat H⁺ ATPase in the intercalated cells (BROWN et al., 1987).

**In situ hybridization**

In situ hybridization was performed by Wilcox's method with a minor modification (WILCOX, 1993), using a digoxigenin-labeled cRNA antisense probe for AQP6 mRNA. The kidney blocks were fixed in 4% paraformaldehyde for 5 h at room temperature and immersed in 30% sucrose overnight at 4°C. They were frozen in hexane chilled to −80°C and sectioned

![Image](image-url)
Fig. 2. AQP6 mRNA expression in the kidneys of dehydrated rats. a. The expression in the medulla is downregulated in rats after dehydration for three days (D) compared with that in the hydrated rats (H). The downregulation is not observed in the whole kidneys or cortex. b. Quantitative analysis shows a significant downregulation of AQP6 mRNA expression in the medulla of dehydrated rat kidneys compared with that in hydrated rat kidneys. *p<0.05.

Fig. 3. Effects of chronic metabolic acidosis on the expression of AQP6 mRNA in the rat kidney. a. Upregulation of AQP6 mRNA expression is evident in the cortex and slightly detectable in the outer medulla of acidotic rats (A) compared with normal rats (N). b. A significant upregulation of AQP6 mRNA expression in the cortex of acidified rat kidneys is verified by quantitative analysis compared with that of control rat kidneys. *p<0.05.

in a cryostat at a 7 μm thickness. Then they were post-fixed in 4% paraformaldehyde in PBS, treated with 5 mg/ml proteinase K (Promega) for 2 min at room temperature, and hybridized with the probe in a hybridization buffer (10 mM Tris-HCl, pH 7.6, 50% formamide, 10% dextran sulfate, 1×Denhart’s solution, 0.25 mg/ml tRNA, 600 mM NaCl, 0.25% SDS and 1 mM EDTA) overnight at 55°C. After washing in 2×SSC and 50% formamide twice for 20 min at 55°C, the sections were treated with 20 μg/ml ribonuclease A for 30 min at room temperature, and washed sequentially in 2× and 0.2×SSC for 20 min at 55°C. They were subsequently blocked with a 1.5% blocking reagent for 60 min and incubated with 1:500 diluted anti-digoxigenin antibody conjugated with alkaline phosphatase for 30 min at room temperature. Alkaline phosphatase was visualized by an overnight reaction with 2% nitroblue tetrazolium and 5-bromo-4-chloro-3-indoly1 phosphate (Boehringer Mannheim, Indianapolis, IN) in the dark at room temperature.

The expression of AQP6 mRNA in the glomeruli was further examined by RT (reverse transcriptase)-PCR using RNA samples (10 μg each) purified from isolated glomeruli and the whole kidney. Equal qualities and quantities of these RNA samples were analyzed by RT-PCR for GAPDH.

RESULTS
Expression of AQP6 mRNA
The expression of AQP6 mRNA in 17 organs and kidney compartments was determined by ribonuclease protection assay (Fig. 1a). AQP6 mRNA was exclusively expressed in the kidney among the organs and tissues tested. In the kidney, AQP6 mRNA was more abundant in the medulla, especially in the outer medulla, than in the cortex. The expres-
sion was negative in the glomerulus (Fig. 1b).

AQP6 mRNA was negligible in the 18d fetal kidney but was evident at birth and increased gradually until 4 weeks of age (Fig. 1c). Thereafter, the AQP6 mRNA expression continued at the same level to the end of the experimental period (14 months of age).

Effects of dehydration of the animals on the AQP6 expression in the kidney were examined. Obvious differences were not detected in the whole kidney or the cortex after dehydration, whereas the AQP6 expression in the medulla was apparently suppressed (Fig. 2a). Quantitative analysis showed that the medullary AQP6 mRNA expression level was significantly lower in the dehydrated rats than in the hydrated control rats (AQP6/GAPDH mRNA ratio: 0.76 ± 0.08 vs 1.12 ± 0.10, mean ± SD, p < 0.05) (Fig. 2b).

The kidney AQP6 mRNA expression in a chronic metabolic acidosis condition was also investigated. No changes in the AQP6 mRNA expression were recognized in the outer medulla of the acidosis rat kidneys, whereas the cortical expression was apparently enhanced (Fig. 3a). A computerized densitometry analysis (0.41 ± 0.09 and 0.20 ± 0.06, mean ± SD, p < 0.05) (Fig. 3b) showed the difference in enhancement between the acidic and normal conditions to be approximately twofold.

**Localization of immunoreactive AQP6**

Specificity and reactivity of the anti-AQP6 antibody prepared by immunizing the recombinant C-terminus AQP6 protein was examined by Western blot analysis (Fig. 4). The antibody reacted to a major band of 36.5 kDa, which corresponded to the speculated size of the recombinant protein expressed in the vector. After digestion with thrombin, two bands of 7.3 kDa (AQP6 C-terminus protein) and 29 kDa (vector-derived fusion protein, DsbC) were immunoblotted. The results indicated that the antibody reacted to the C-terminus portion of AQP6 protein.

Immunohistochemistry using the anti-AQP6 antibody revealed that immunoreactive AQP6 was exclusively localized in a population of epithelial cells forming the collecting ducts, and not in any other nephron segments or glomeruli in the kidney (Fig. 5a). The immunoreactive cells were dispersed separately and seldom coupled or grouped. The immunoreaction was found in their cytoplasm in a fine-vesicular pattern (Fig. 5b). Approximately 25% to 50% of the cortical and outer medullary collecting duct cells were immunolabeled with the anti-AQP6 antibody. The staining intensity was the highest in the outer medullary collecting ducts.

Examination using serial sections revealed that the immunoreactive AQP6-bearing cells precisely corresponded to the cells labeled with the anti-56-kDa H+ ATPase (Fig. 5c, d). This observation suggested that the immunoreactive AQP6-positive cells were the intercalated cells. The AQP6 immunostaining was abolished when the anti-AQP6 antibody preabsorbed with the thrombin-digested recombinant AQP6 protein was used.

**In situ hybridization**

The localization of AQP6 mRNA-expressing cells in the kidney was examined by in situ hybridization. Distinct signals for AQP6 mRNA were recognized along the collecting ducts in the cortex, outer, and inner medulla (Fig. 6a). The signal was found in a population of the collecting duct cells (Fig. 6b, c, d), and its distribution pattern well coincided with that of the AQP6 immunoreactive cells. It was thus most reasonable to say that the AQP6 mRNA was expressed in the intercalated cells. No signals for AQP6 mRNA were detected in the glomerulus or other nephron segments.

By RT-PCR, expression of AQP6 mRNA was detected in the whole kidney RNA sample but not in the glomerular RNA sample (Fig. 7). The quality of the RNA samples was verified by a similar amplification of GAPDH cDNA by RT-PCR using these samples. The results indicated AQP6 mRNA expression was negative in the glomeruli.
Fig. 5. Immunohistochemistry for AQP6. a. Immunoreactive AQP6 exclusively present in a population of cells (C) lining the collecting duct (arrowheads). Note that the glomerulus (G) and proximal tubules (P) are free of the immunoreaction. b. Closer view of the outer medulary portion immunostained in a fine granular pattern with the anti-AQP6 antibody. Specific domed cells lining the collecting duct (C) are immunostained, while no other cells such as the thick ascending limb of Henle (TA) are stained. c and d. Sections adjacent to a and b, respectively, immunostained for H⁺ ATPase. The same cells as those bearing the immunoreactive AQP6 (arrowheads) are immunolabeled. a, c: ×360, b, d: ×1,800
Fig. 6. Localization of AQP6 mRNA-expressing cells by in situ hybridization. a. An overview micrograph showing a population of the collecting ducts intensely expressing AQP6 mRNA. C cortex, OM outer medulla, IM inner medulla. b. A minor population of crossly cut inner medullary collecting duct (★) cells expressing AQP6 mRNA. c. AQP6 mRNA expression is intense in a population of outer medullary collecting ducts. d. AQP6 mRNA is weakly expressed in the cortical collecting ducts (CD). No significant signals are observed in the glomerulus (G). a: ×50, b, c, d: ×800
DISCUSSION

The present study confirms the report by MA et al. (1993) who recognized the expression of AQP6 mRNA in the kidney among several rat organs. We were further able to demonstrate that AQP6 mRNA was intensely expressed in the outer medulla among several kidney compartments. This expression profile suggests that AQP6 is localized in a special cell population in the kidney.

By immunohistochemistry and in situ hybridization technique, immunoreactive AQP6 and its mRNA expression were coincidentally demonstrated along the collecting duct epithelia. The co-localization of immunoreactive AQP6 and H⁺ ATPase in the collecting ducts indicated that AQP6 was present in the intercalated cells. It is worth noting that AQP6 was immunolocalized in the cytoplasm and not distinctly on the plasma membranes of the intercalated cells, because all other AQP members, except AQP2, previously shown in the kidney have been primarily localized on the plasma membranes of various cell types (YAMAMOTO and SASAKI, 1998). The cytoplasmic localization of AQP6 suggests that this channel might be associated with cytoplasmic vesicles, as we have already demonstrated the localization of AQP2 on the vesicular membrane in principal collecting duct cells by immunoelectron microscopy (YAMAMOTO et al. 1995a). The AQP2-bearing vesicles were demonstrated to translocate to the apical plasma membrane of collecting duct principal cells in response to stimulation with vasopressin (NIELSEN et al., 1994, YAMAMOTO et al., 1995b). To identify the subcellular localization of this water channel, immunoelectron microscopy would seem suitable. Our preliminarily attempt along this line, however, failed to immunolocalize AQP6 in renal tissues fixed in 2% paraformaldehyde or periodate-lysine-paraformaldehyde.

The intercalated cells are characterized by a rich inclusion of mitochondria in their cytoplasm and are known to be differentiated from the neighboring principal cells (VENKATACHALAM and KRIZ, 1998). These cells have been shown to have H⁺ ATPase and Cl⁻/HCO₃⁻ exchanger and to transport proton and bicarbonate, respectively. These molecules function to balance the body's acid/alkaline nature (VENKATACHALAM and KRIZ, 1998). Several studies have demonstrated that the H⁺ ATPase is shuttled from the cytoplasmic vacuoles to the apical plasma membrane in the intercalated cells in response to acid-base changes (MAIDEN and TISHER, 1984; VERLANDER et al., 1987; BROWN, 1989).

The H⁺ ATPase on the plasma membrane secretes proton from the cells by using ATP supplied by the numerous mitochondria. Since the enhanced expression of H⁺ ATPase mRNA have been reported in acidic conditions rather than in alkaline conditions (FEJES-TOOTH and NARAY-FEJES-TOOTH, 1995), we presumed that the AQP6 mRNA expression might also be altered in acidic or alkaline changes in the body. Thus, the present study demonstrated that in the chronic acidosis rats, AQP6 mRNA expression in the cortex was upregulated compared with that in the control rats. This observation suggests a role of AQP6 in the regulation of acid and alkaline balance or secretion of proton. AQP6 was presumed to act as an anion channel in addition to a water channel under low pH values (YASUI et al., 1999b). Alternatively, AQP6 may transport water to facilitate proton secretion from the intercalated cells as AQP5 transports water to increase the output of saliva containing amylase and other proteins from the salivary gland (MA et al., 1999).

Collecting ducts are the sites determining the final concentration of urine. In the rat, a marked increase in the concentration of urine has been observed to reach the maximal state by the first 3-4 weeks of life (FALK, 1955). There are several factors involved in completing this maturation of the urine concentrating capacity and regulation of the AQP family expression may be one of them. Among AQP2, AQP3 and AQP4 expressed in the collecting ducts, AQP2 was demonstrated to be expressed in a close relation with the urine concentrating ability (YAMAMOTO et al., 1997). The expression profile of AQP6 in rat kidneys during development and maturation was
similar to that of AQP2, suggesting that AQP6 also might act as a factor in the maturation of the urine concentrating activity in the rat collecting ducts.

A recent immunohistochemical study by Yasui et al. (1999a) reported that AQP6 was localized in glomerular podocytes and proximal tubules as well as in the intercalated cells. In a clear discrepancy from that result, neither immunoreactive AQP6 nor AQP6 mRNA signals could be detected in the podocytes and proximal tubules in the current study. No AQP6 mRNA expression in the glomeruli was confirmed by RT-PCR.

Several members of the AQP family have been identified in the kidney: AQP1 in the proximal tubules, descending thin loop of Henle, descending vasa recta, AQP2, AQP3, and AQP4 in the principal cells of collecting ducts, and AQP7 in a portion of the proximal tubules. Among these AQP members, the expression of AQP2, AQP3 and AQP4 has been shown to be upregulated in dehydrated animals (Yamamoto et al., 1995a; Umenishi et al., 1996), while AQP1 expression was unchanged (Yamamoto et al., 1995a; Umenishi et al., 1996). Interestingly, AQP6 mRNA expression in the medulla was downregulated by the dehydration of animals in contrast to the upregulation of other AQP members. The difference in the expression between AQP6 and other AQP members in a water-depleted condition may be due to differences in their localization and functions. Enhancement of AQP2, AQP3 and AQP4 expression is suitable to retain water in the body in a dehydrated condition since they are primary water channels located in the principal cells and reabsorb water from urine. On the other hand, the intercalated cells expressing AQP6 are not primarily responsible for water intake but for acid-base and potassium transport (Verlander et al. 1987). AQP6 may facilitate the excretion of proton by increasing water transport through the apical plasma membrane of intercalated cells. Therefore, it may be suitable to suppress water movement from the intercalated cells into urine through AQP6 in a dehydrated condition.

An increase in AQP6 mRNA and protein expression has been recently reported in alkali-loaded rats, while no changes have been observed in acid-loaded animals (Promeneur et al., 2000). Since the upregulation of AQP6 in alkali-loaded animals was associated with increased water intake, water intake but not acid loading per se was considered to influence the AQP6 expression. This speculation may be in agreement with our observation showing that AQP6 expression was downregulated in dehydrated rats. We were unable to detect an increase in the AQP6 expression in metabolic alkalosis rats induced by ingesting 0.28 N sodium bicarbonate for 6 days (data not shown). The lack of any change of water intake in our experimental animals might be the reason. On the other hand, we also found a significant increase in AQP6 mRNA expression in the cortex of chronic acidosis rats. Intercalated cells in the cortical collecting ducts but not in the medullary ones may respond mainly to acidosis by increasing AQP6 expression for proton secretion into urine.

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