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REVIEW

Localization and trafficking of aquaporin 2 in the kidney

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Abstract Aquaporins (AQPs) are membrane proteins serving in the transfer of water and small solutes across cellular membranes. AQPs play a variety of roles in the body such as urine formation, prevention from dehydration in covering epithelia, water handling in the blood–brain barrier, secretion, conditioning of the sensory system, cell motility and metastasis, formation of cell junctions, and fat metabolism. The kidney plays a central role in water homeostasis in the body. At least seven isoforms, namely AQP1, AQP2, AQP3, AQP4, AQP6, AQP7, and AQP11, are expressed. Among them, AQP2, the anti-diuretic hormone (ADH)-regulated water channel, plays a critical role in water reabsorption. AQP2 is expressed in principal cells of connecting tubules and collecting ducts, where it is stored in Rab11-positive storage vesicles in the basal state. Upon ADH stimulation, AQP2 is translocated to the apical plasma membrane, where it serves in the influx of water. The translocation process is regulated through the phosphorylation of AQP2 by protein kinase A. As soon as the stimulation is terminated, AQP2 is retrieved to early endosomes, and then transferred back to the Rab 11-positive storage compartment. Some AQP2 is secreted via multivesicular bodies into the urine as exosomes. Actin plays an important role in the intracellular trafficking of AQP2. Recent findings have shed light on the molecular basis that controls the trafficking of AQP2.

Keywords Water channel · AQP2 · Collecting duct · Vesicle · Trafficking

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Introduction

Aquaporins (AQPs) are membrane proteins serving in the transfer of water and small solutes across cellular membranes. A novel integral membrane protein of 28-kDa was identified from the human erythrocyte ghost during the isolation of the 32-kDa Rh polypeptides (Denker et al. 1988). Immunohistochemistry revealed that this 28-kDa protein was abundant in the kidney and localized in the proximal tubule cells. cDNA cloning identified a membrane protein with 6 membrane-spanning domains and intracellular N- and C-termini, and it was named channel-like integral

protein of 28 kDa (CHIP28) due to its structural similarity to membrane channel proteins (Preston and Agre 1991). When CHIP28 was expressed in *Xenopus* oocytes, a mercurial-sensitive increase in the water permeability of the plasma membrane was observed, demonstrating that CHIP28 was a long-sought water channel protein (Preston et al. 1992). Water channel proteins were later named aquaporins (AQPs) (Agre et al. 1993), and CHIP28 was classified as AQP1.

An AQP molecule is composed of approximately 270 amino acid residues. AQPs are usually glycosylated and form homotetramers in the membrane, with each having an independent channel pore for water in each monomer. Two asparagine-proline-alanine sequences, called NPA boxes, are conserved. The hourglass model of AQP predicted that two loops containing NPA boxes are folded into the center of the membrane, and form a critical portion of the pore in the channel where water molecules pass through. Crystallographic analyses using X-ray and electron beams have revealed the detailed molecular structure of AQPs (for review see Engel et al. 2008).

AQPs are widely distributed among bacteria, plants, and animals (Krane and Goldstein 2007; Rojek et al. 2008; Kaldenhoff et al. 2007). In mammalian cells, 13 isoforms, namely AQP0 through AQP12 have been identified to date (Agre et al. 2002; Matsuzaki et al. 2002; Nielsen et al. 2002; Takata et al. 2004b; Ishibashi 2006). AQPs are classified into three subfamilies: the aquaporin subfamily, aquaglyceroporin subfamily, and superaquaporin subfamily. The aquaporin subfamily is specific to water permeation, and is made up of AQP0, AQP1, AQP2, AQP4, AQP5, AQP6, and AQP8. Aquaglyceroporin serves in the transfer of water as well as small molecules such as glycerol and urea, and is made up of AQP3, AQP7, AQP9, and AQP10. The superaquaporin subfamily is composed of AQP11 and AQP12, which show a low homology (~20%) with other AQPs and have poorly conserved NPA boxes (Morishita et al. 2004; Ishibashi 2006).

Roles of AQPs in the body

AQPs are expressed in various organs and play important roles in homeostasis of the body (Takata et al. 2004b). Some of them are summarized in the following section. AQPs in the kidney will be described and discussed in the subsequent sections.

Prevention of dehydration

The aquaglyceroporin AQP3 is abundantly expressed in the transitional epithelia covering the urinary tract such as the renal pelvis, urinary bladder, and proximal part of the urethra (Matsuzaki et al. 1999a). AQP3 is also found in the epidermis of the skin, airway epithelia covering the respiratory tract, and stratified epithelia of the digestive tract. It is localized along the plasma membrane other than the apical membrane. In cultured cells, the expression of AQP3 is induced by hypertonic stimulation (Matsuzaki et al. 2001). In addition, AQP3 expression of the epidermis in the rat commenced late in fetal life just prior to birth (Matsuzaki et al. 1999a). These observations indicate that AQP3 may provide epithelial cells with water from the subepithelial side to protect them from dehydration (Matsuzaki et al. 1999a). In fact, AQP3-null mice showed impaired skin hydration (Ma et al. 2002), which was alleviated by the administration of glycerol (Hara and Verkman 2003). These results show that AQP3 plays an important role in preventing epithelial cells from dehydration by taking up water and glycerol via AQP3 at their plasma membrane. AQP3 also serves in the proliferation of epidermal cells by facilitating the uptake of glycerol, and thereby is involved in the development of skin cancer (Hara-Chikuma and Verkman 2008; Verkman et al. 2008).

Water handling in the blood-brain barrier

AQP4 is abundant in astrocytes of the brain, where it is concentrated at their endfeet. Freeze-fracture replica electron microscopic examination revealed arrays of orthogonally arranged intramembranous particles at the plasma membrane of astrocytes, and they were named orthogonal arrays (Landis and Reese 1974). Fracture label analysis using anti-AQP4 antibody revealed that the arrays are composed of AQP4 molecules (Rash et al. 1998). Endfeet together with endothelia sealed by tight junctions serve as the structural basis of the blood-brain barrier and AQP4 may serve in the transfer of water across the barrier. AQP4-null mice showed better survival rates compared to wild-type mice in the brain edema model caused by acute water intoxication, or focal ischemic stroke by cerebral artery occlusion (Manley et al. 2000). AQP4 may serve in water homeostasis in the central nervous system, and the control of water influx by modulating the water channel activity of AQP4 in astrocytes could have a therapeutic significance.

Secretion

AQP5 is widely expressed in exocrine cells in the body (Matsuzaki et al. 1999b, 2003). In the salivary glands, AQP5 is localized at the apical membrane of acinar cells, and sometimes in duct cells, and serves in the permeation of water across the apical membrane. AQP5 is found in the acinar cells in the digestive tract such as duodenal and esophageal glands, lacrimal gland, and acinar cells in the

respiratory tract (Matsuzaki et al. 2004; Ablimit et al. 2006, 2007).

AQP4 is expressed in the basolateral membrane of acid-secreting parietal cells in the stomach (Koyama et al. 1999; Fujita et al. 1999), and may be involved in acid secretion, but its detailed function has yet to be determined.

Conditioning of sensory systems

Maintaining the osmotic and ionic conditions seems to be critical in highly differentiated sensory systems such as the eye, ear, and olfactory and vomeronasal systems. In fact, a variety of AQPs are differentially expressed in the eye (Hamann et al. 1998) and the inner ear (Lopez et al. 2007). AQPs are also abundant in the sensory epithelia of the olfactory and vomeronasal mucosae for the reception of chemical stimuli (Ablimit et al. 2006, 2008). AQPs may play important roles in water homeostasis in these sensory organs.

Cell motility and metastasis

AQP1 is expressed in the kidney, erythrocytes, and endothelial cells. In AQP1-null mice, tumor growth was severely impaired because of the reduced tumor vascularity due to the abnormal vessel formation of endothelial cells (Saadoun et al. 2005). In migrating cells, AQP1 is concentrated in their leading edges and contributes to the rapid water influx for lamellipodia formation (Verkman et al. 2008).

In addition, AQP1 is strongly expressed in tumor cells and is involved in local tumor invasion, extravasation, and metastases. Lamellipodia formation by water influx via AQP1 may account for such cellular motility of cancer cells (Verkman et al. 2008).

Cell adhesion

AQP0 was originally identified as a membrane protein abundant in the lens fiber cells, and was called a major intrinsic protein (MIP). Later, the cloning and sequencing of MIP revealed a structural similarity to AQPs, and so it was named AQP0. In the lens fiber cells, AQP0 forms microdomains at the plasma membrane and was considered to form junctions between lens fibers (Zampighi et al. 2002). X-ray and electron crystallographic analyses of the double-layered two-dimensional crystals revealed the molecular structure of AQP0 and interaction between apposing AQP0 molecules. It has been proposed that AQP0 forms junctions between adjacent lens fiber membranes (Harries et al. 2004; Gonen et al. 2005; Engel et al. 2008). In addition to AQP0, AQP4 has been suggested to be involved in junctions between cells (Engel et al. 2008).

Fat metabolism

AQP7 and AQP9 serve as glycerol channels in adipocytes and hepatocytes, respectively, and are involved in glycerol and lipid metabolism in the body (Hibuse et al. 2006; Rodríguez et al. 2006). As was originally called AQP-adipose, AQP7 is expressed in adipocytes (Kuriyama et al. 1997). In the lipolytic condition, AQP7 serves as a channel that facilitates the exit of intracellular glycerol produced by the degradation of stored triglycerides. AQP7-null mice have a low plasma glycerol level and impaired glycerol release in response to beta3-adrenergic agonists, showing that AQP7 is important in the release of glycerol from adipocytes. In such cells, the accumulation of glycerol activates glycerol kinase, which accelerates triglyceride synthesis and finally leads to the development of obesity (Hibuse et al. 2005).

Recently, the expression of AQP7 in pancreatic beta cells was reported (Matsumura et al. 2007). AQP7 may play an important role in controlling the glycerol content and glycerol kinase activity in beta cells and affect their proliferation and insulin secretion.

Aquaporins in the kidney

The kidney is an organ specialized for water homeostasis. At least seven isoforms, namely AQP1, AQP2, AQP3, AQP4, AQP6, AQP7, and AQP11, are expressed (Nielsen et al. 2002, 2007; Takata et al. 2004b, 2005; Morishita et al. 2005). In addition, AQP8 was reportedly expressed in it (Elkjaer et al. 2001). As shown in Fig. 1, these AQP isoforms are differentially expressed along the tubules, ducts, and blood vessels of the kidney. AQP1 is expressed in the proximal tubules, descending limb of Henle's loop, and the vasa recta. AQP1 is localized at both the apical and basolateral membranes of these epithelial and endothelial cells. It serves in the reabsorption of water in the initial descending part of the nephron. AQP1-null mice became dehydrated after water deprivation due to impaired urine concentration. A microperfusion experiment showed decreased water permeability in this part of the kidney, demonstrating that water is reabsorbed transcellularly via AQP1 (Schnermann et al. 1998). However, the phenotype is not as severe as that in AQP2-null mice.

In addition to AQP1, the aquaglyceroporin AQP7 is expressed in the proximal straight tubules. It is localized along the apical membrane. A knockout experiment showed that AQP7 participates in the reabsorption of glycerol (Sohara et al. 2006).

AQP11 is expressed in the proximal tubules intracellularly (Morishita et al. 2005). The disruption of AQP11 produced vacuoles in the proximal tubules cells, and

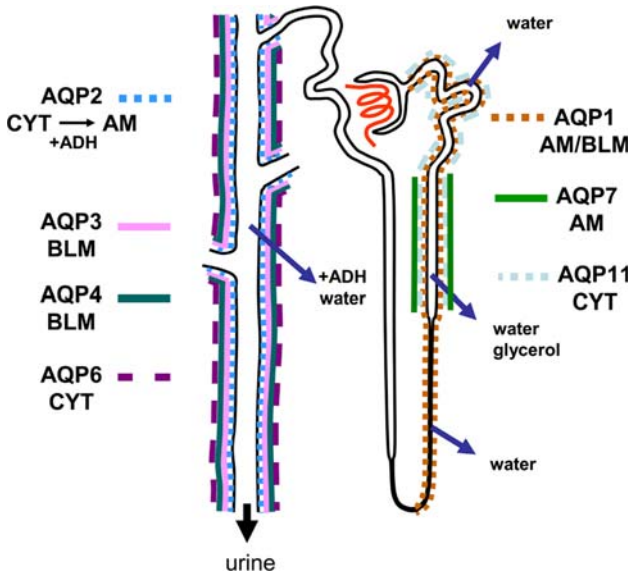

Fig. 1 A schema showing the expression of AQPs in the kidney. *AM* apical membrane, *ADH* anti-diuretic hormone, *BLM* basolateral membrane, *CYT* cytoplasmic compartment

finally, many cysts are formed (Morishita et al. 2005). The function of AQP11 remains to be clarified.

Connecting tubules and collecting ducts are the most distal part of the tubule system in the kidney. Reabsorption from the glomerular filtrate and secretion in this part determine the final composition of the urine. The ducts are lined with two-types of epithelial cells: principal cells and intercalated cells. In the principal cells, AQP3 and AQP4 are localized along the basolateral plasma membrane (Fig. 2) (Ishibashi et al. 1994; Frigeri et al. 1995a, b). AQP2 is also expressed in the principal cells (Fig. 2a). In the basal state, AQP2 is mainly localized in the intracellular vesicles (Figs. 2b, 3a). Upon stimulation with anti-diuretic hormone (ADH), AQP2 is translocated from the intracellular compartment to the apical plasma membrane by exocytic fusion of AQP2-bearing vesicles (Figs. 2c, 3b). In this way, the water permeability of the apical membrane is regulated by the trafficking of AQP2 to the apical membrane. Once AQP2 appears at the apical membrane, water is easily reabsorbed by passing through the principal cell layer transcellularly, since AQP3 and AQP4 are constitutively present at the basolateral membrane. In the water reabsorption in the principal cells, the intracellular trafficking of AQP2 serves as an acute regulatory mechanism in urine concentration. Due to its expression in the furthest downstream part of urine formation and regulation by ADH, AQP2 is a critical water channel in the kidney. In fact, a defect in AQP2 results in diabetes insipidus, an inability to concentrate urine. (for review, see Nielsen et al. 2002; Brown 2003; Takata et al. 2004a, 2005; Noda and Sasaki 2006; Takata 2006).

AQP6 is expressed in the acid-secreting intercalated cells (Hazama et al. 2002). AQP6 is localized intracellularly and distributed throughout the cytoplasm (Yasui et al. 1999). It remains to be clarified whether AQP6 functions as a water channel intracellularly or AQP6 is translocated to the plasma membrane.

AQP2 in principal cells of the collecting ducts in the kidney

In the rat kidney principal cells, AQP2 is mainly localized in the supranuclear region (Fig. 2). In order to characterize the AQP2-storage compartment, double-labeling immunofluorescence microscopic examination was carried out using various organelle markers. It was shown that AQP2 is sometimes colocalized with the early endosome marker EEA1 (Tajika et al. 2002), suggesting that the endosomal system is involved in its trafficking. AQP2 is not localized at the endoplasmic reticulum, Golgi apparatus, trans-Golgi network, or lysosomes. These observations suggest that endosomal compartments play important roles in the storage and trafficking of AQP2.

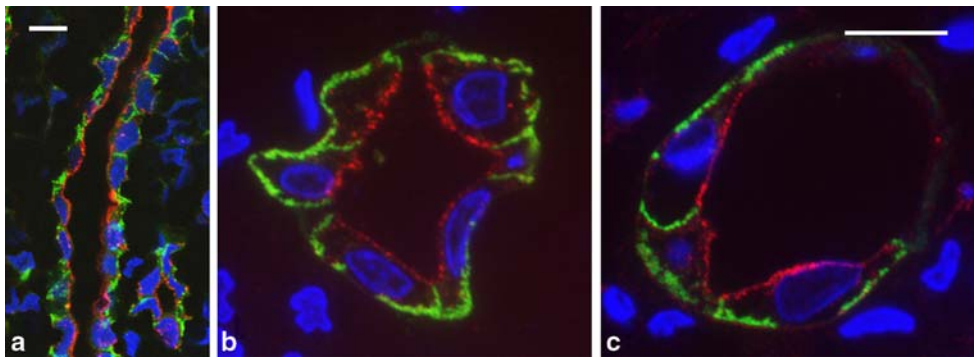

Fig. 2 AQP2 and AQP3 in the collecting ducts of the rat kidney. **a** AQP2 (*red*) and AQP3 (*green*) are expressed in the collecting ducts. **b**, **c** AQP2 (*red*) is localized in intracellular vesicles mainly in the subapical cytoplasm in the basal state (**b**). Administration of ADH results in the translocation of AQP2 (*red*) to the apical membrane (**c**). *Bars* 10 μm. Reproduced with permission from Takata et al. 2005

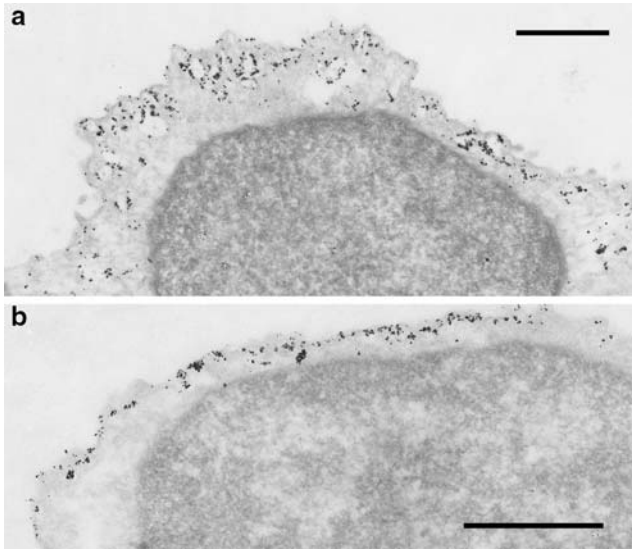

Fig. 3 AQP2 in principal cells of the rat kidney collecting duct. In the basal state, AQP2 is localized intracellularly in subapical vesicles (**a**). Translocation of AQP2 to the apical plasma membrane occurs by the administration of ADH (**b**). Cryostat sections were immunostained for AQP2 using Nanogold probes followed by silver enhancement and gold treatment and processed for electron microscopy. *Bars*: 1 μm

Intracellular storage compartment and trafficking of AQP2

For characterization of the AQP2 compartment, a proteomics approach was performed by analyzing the immunoisolated AQP2 vesicles from the rat kidney medullary collecting ducts (Barile et al. 2005). A long list of proteins was generated comprising proteins of the endosomes, trans-Golgi network, and rough endoplasmic reticulum. Among them, Rab proteins, namely, Rab4, Rab5, Rab18 and Rab21 of early endosomes, Rab7 of late endosomes, and Rab11 and Rab25 of recycling endosomes, were identified, suggesting a close relationship between AQP2 and the endosomal system. SNARE proteins and motor proteins such as non-muscle myosins were identified. Immunogold electron microscopy showed that Rab5, Rab7 and Rab11 were present in AQP2-immunoisolated vesicles (Barile et al. 2005). Among them, Rab11 seems to be associated with the AQP2-storage compartment. In fact, immunoblot analysis showed that Rab11 is present in these AQP2-bearing vesicles (Barile et al. 2005; Nedvetsky et al. 2007).

For the detailed analyses of the intracellular AQP2-storage compartment and trafficking, cultured cell systems have been widely used. MDCK cells expressing human AQP2 were analyzed by a double-labeling immunofluorescence study with various organelle markers. AQP2 is stored in vesicles in the supranuclear region, and is distinct from the endoplasmic reticulum, Golgi apparatus, trans-Golgi network, and lysosomes in the basal state, as is observed in the principal cells of the kidney collecting duct (Tajika et al. 2004). Stored AQP2 is mostly colocalized with Rab11 (Fig. 4) (Tajika et al. 2004, 2005; Nedvetsky et al. 2007; Vossenkämper et al. 2007), a marker of apical recycling endosomes. The colocalization of AQP2 and Rab11 was also observed in the primary culture of rat kidney inner medullary collecting duct cells (Vossenkämper et al. 2007). In addition to Rab11, AQP2 is occasionally colocalized with the early endosome marker EEA1, but not with basal endosomes marked with endocytosed transferrin from the basal side. These results indicate that AQP2 is stored in a Rab11-positive compartment in the apical cytoplasm, and early endosomes may play a part in its trafficking.

By elevating the cAMP level with forskolin, AQP2 is translocated to the apical plasma membrane, as is seen in the kidney after ADH stimulation. The disappearance of AQP2 from the Rab11-positive compartment coincides with the increase of surface AQP2, showing the cAMP-mediated translocation of AQP2 to the plasma membrane from the AQP2/Rab11 vesicles. Washout of forskolin terminates the stimulation and triggers the synchronized retrieval of surface AQP2 by endocytosis. Time-course observation showed that endocytosed AQP2 is not directly routed to the Rab11-positive compartment, but first delivered to EEA1-positive early endosomes en route to Rab11-positive vesicles (Figs. 4, 5) (Tajika et al. 2005). When Rab11 was depleted with RNA interference, the retention of AQP2 at the subapical storage compartment was impaired (Tajika et al. 2005).

Transfer from early endosomes to the Rab11-storage compartment is sensitive to wortmannin and LY294002, showing that this process is phosphatidylinositol 3-kinase-dependent. The actin filament is involved in this process, since the disruption of actin filaments with cytochalasin D or latrunculin B resulted in the accumulation of AQP2 in the EEA1-positive early endosomes (Tajika et al. 2005). AQP2 in early endosomes is insensitive to ADH stimulation since AQP2 accumulated in EEA1-positive vesicles by the disruption of actin filaments with cytochalasin D or latrunculin B failed to respond to ADH stimulation (Tajika et al. 2005). On the other hand, the disruption of microtubules with nocodazol or colcemid did not affect this transfer process and AQP2 in Rab11-positive vesicles retained their reactivity to ADH stimulation, although the intracellular distribution of AQP2 was perturbed (Tajika et al. 2005; Vossenkämper et al. 2007). These observations suggest that AQP2 vesicles with Rab11comprise a ‘ready-to-go’ compartment on ADH stimulation.

Rab11 associates with the Rab11 family interacting protein 2 (Rab11-FIP2), through which it binds to myosin Vb (Hales et al. 2002). The disruption of recycling by expressing the dominant-negative myosin Vb tail and Rab11-FIP2

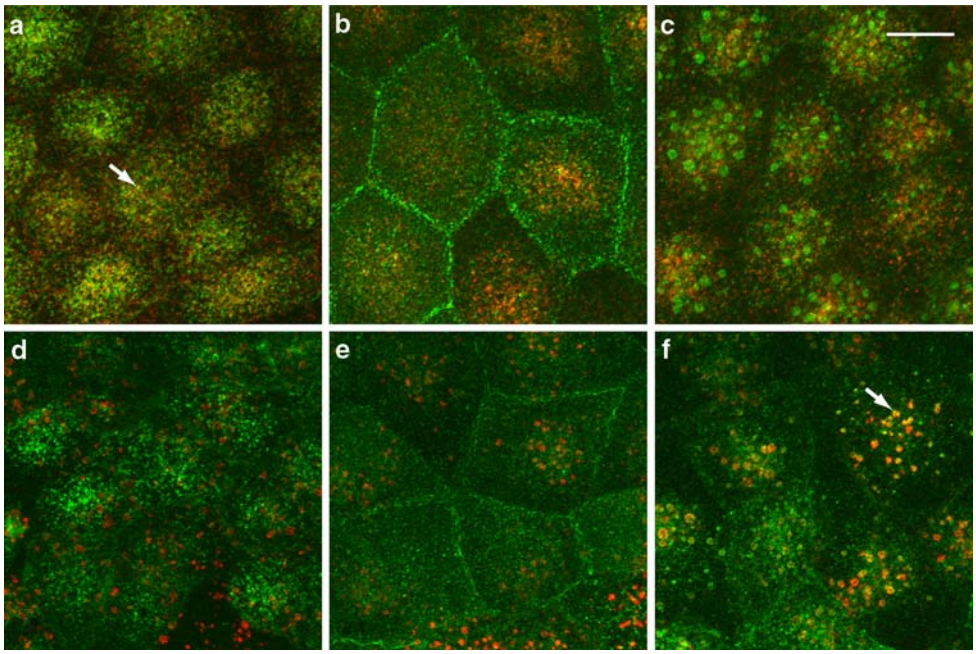

Fig. 4 Localization and trafficking of AQP2 in MDCK cells. MDCK cells transfected with AQP2 were double-labeled for either AQP2 (*green*) and Rab11 (*red*) (**a**–**c**), or AQP2 (*green*) and EEA1 (*red*) (**d**–**f**). **a**, **d** In the basal state, AQP2 resides in Rab11 compartment (*arrow* in **a**). **b**, **e** Forskolin stimulates the translocation of AQP2 to the cell surface. **c**, **f** By washout of forskolin and chasing in forskolin-free medium for 30 min, AQP2 is retrieved to EEA1-positive early endosomes (*arrow* in **f**). *Bar* 10 μm

lacking the C2 domain in CD8 cells abolished AQP2 recycling and resulted in the accumulation of AQP2 in Rab11-positive vesicles (Nedvetsky et al. 2007).

The involvement of myosin and myosin light chain kinase in AQP2 translocation by ADH was reported. In addition to the cAMP-protein kinase A (PKA)-mediated phosphorylation of AQP2, ADH also triggers intracellular calcium mobilization and the subsequent activation of calmodulin (Chou et al. 2000, 2004; Balasubramanian et al. 2008). It is considered that the activation of calmodulin activates myosin light chain kinase and results in the phosphorylation of myosin light chain. In fact, the association of myosin and myosin light chain kinase in AQP2-bearing vesicles was shown by the identification of AQP2-binding proteins and proteomics analyses (Noda et al. 2004b; Noda and Sasaki 2006; Barile et al. 2005). These evidences suggest that myosin might be involved in the trafficking of AQP2 vesicles through Rab11 and Rab11-FIP2, but the detailed molecular mechanism remains to be clarified.

Endocytosis of surface AQP2

AQP2 at the cell surface is retrieved to the intracellular vesicles by endocytosis. Immunogold electron microscopic examination revealed that AQP2 is concentrated in the clathrin-coated pits at the plasma membrane of collecting duct principal cells from ADH-treated rats (Sun et al. 2002). Concentration of AQP2 was also confirmed in LLC-PK1 cells expressing AQP2 by the fracture label method. The overexpression of dominant-negative dynamin 1 or dynamin 2 inhibited the detachment of clathrin-coated pits and induced the accumulation of clathrin and AQP2 at the cell surface (Sun et al. 2002). These results show that AQP2 at the cell surface is retrieved to the intracellular compartment via the clathrin-coated pit in a dynamin-dependent manner (Fig. 5).

Endocytosis and the intracellular fate seem to be regulated by the ubiquitination of AQP2. A portion of AQP2 is modified with two to three ubiquitin moieties at lysine 270 (Kamsteeg et al. 2006). In MDCK cells expressing AQP2, the ubiquitination of AQP2 occurs preferentially when present at the apical membrane, and the endocytosis of AQP2 proceeds. Ubiquitination enhances the endocytosis of AQP2, and it is delivered to internal vesicles of multivesicular bodies. Also, the lysosomal degradation was extensive in ubiqitinated AQP2 compared to non-ubiquitinated one. These results indicate that the short-chain ubiquitination of AQP2 regulates its endocytosis and subsequent sorting to multivesicular bodies and lysosomes (Fig. 5) (Kamsteeg et al. 2006).

Regulation by phosphorylation of AQP2

ADH controls the translocation of AQP2 from the intracellular compartment to the apical plasma membrane by its phosphorylation (Fig. 5). ADH binds to the vasopressin V2 receptor at the basolateral membrane, and activates PKA by elevating the cAMP level. Serine 256, located at the cytoplasmic C-tail of the molecule, is phosphorylated by PKA, which is critical in the ADH-elicited trafficking of AQP2, since mutation of this site S256A resulted in the failure of translocation to the plasma membrane (Katsura et al. 1997).

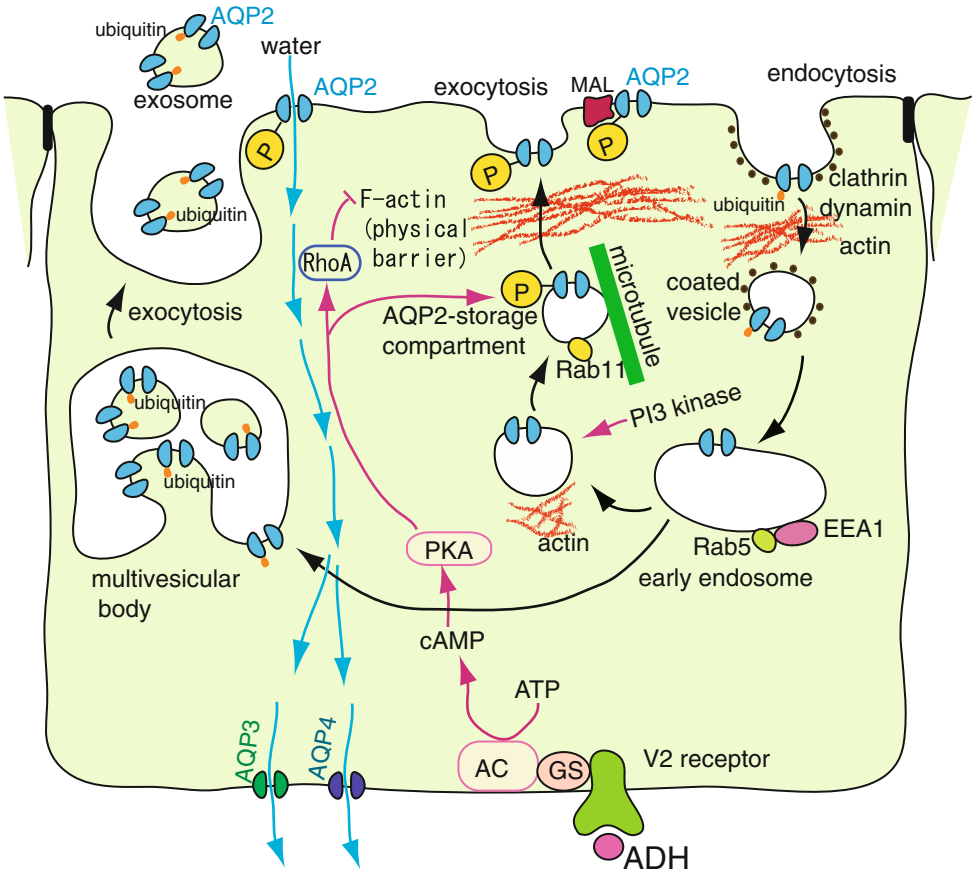

Fig. 5 A schema showing the signal transduction of ADH, trafficking of AQP2, and transcellular water transfer. *AC* adenylate cyclase, *ADH* anti-diuretic hormone, *MAL* myelin and lymphocyte-associated protein, *PKA* protein kinase A. AQP2 is stored in Rab11 vesicles. Binding of ADH to V2 receptor activates AC, and elevation of the cAMP level activates PKA. Phosphorylation of AQP2 in the storage compartment by PKA triggers its translocation to the apical plasma membrane. AQP2-associated proteins (not illustrated in detail in this schema) may play important roles in penetrating the cortical F-actin cytoskeleton that forms a physical barrier. Such a protein complex may serve as a force generator complex in the trafficking of vesicles (Noda et al. 2005, 2006). Once AQP2 reaches the apical plasma membrane, water is reabsorbed transcellularly via AQP2 at the apical side, and AQP3/AQP4 at the basolateral side. Phosphorylated AQP2 interacts with MAL and is preferentially retained to the apical membrane. As soon as ADH-mediated stimulation is terminated, surface AQP2 is endocytosed via the clathrin-coated pit and delivered to the early endosome. PI3 kinase plays an important role in the transfer of AQP2 from the early endosome back to the AQP2-storage compartment. Some of the AQP2 is routed to the multivesicular body. Ubiquitinated AQP2 (ubiquitination is shown as an *orange dot*) is preferentially sorted to the multivesicular body. The internal vesicles are formed by the budding of the limiting membrane. Exocytosis of the multivesicular body results in the release of exosomes containing AQP2 into the urine

On the other hand, S256D was constitutively localized at the apical membrane, suggesting that phosphorylation at serine 256 is necessary for the localization of AQP2 at the apical membrane (van Balkom et al. 2002). The activation of protein kinase C with PMA counteracts the surface expression of AQP2 by inducing the endocytosis of AQP2 independent of the AQP2 phosphorylation state (van Balkom et al. 2002). The internalization of AQP2 by prostaglandin E2 and dopamine is also independent of AQP2 dephosphorylation (Nejsum et al. 2005). Taken together, phosphorylation at serine 256 is necessary for the cell surface translocation of AQP2, but other mechanisms independent of phosphorylation also regulate the surface localization of AQP2.

The phosphorylation of AQP2 at serine 256 is critical in its targeting to the apical cell surface by ADH stimulation. However, the localization of AQP2 does not seem to be simply determined by the phosphorylation of serine 256 alone. The direct visualization of the phosphorylation of serine 256 of AQP2 was performed with antibodies specific to this molecule (Christensen et al. 2000). By immunohistochemical analysis with this antibody, phosphorylated AQP2 was shown to be localized at the apical membrane as well as in the intracellular vesicles. Other possible sites of phosphorylation such as serine 261, 264, and 269 may affect the trafficking of AQP2. Recent proteome analyses have shed light on the roles of serine 261 and 264.

Phosphoproteomics analysis of rat inner medullary collecting duct cells revealed AQP2 phosphorylation at serine 261. ADH induced an increase in monophosphorylation at serine 256 and diphosphorylation at serines 256 and 261,

whereas monophosphorylation at serine 261 decreased, suggesting that phosphorylation of both sites is involved in AQP2 trafficking (Hoffert et al. 2006). Also ADH stimulation resulted in a reciprocal change of phosphorylation: the decrease of phosphorylation at serine 261 and increase at serine 256. Immunofluorescence microscopy showed that AQP2 phosphorylated at serine 261 was mainly localized intracellularly, whereas AQP2 phosphorylated at serine 256 was localized at the apical membrane (Hoffert et al. 2007). The intracellular localization of AQP2 phosphorylated at serine 261 was distinct from the endoplasmic reticulum, Golgi apparatus, and lysosomes. Recent findings regarding point mutation analysis of serine 261 and serine 256 indicate that the phosphorylation state of AQP2 at serine 261 does not detectably affect the regulated or constitutive trafficking of AQP2 (Lu et al. 2008). The precise role of phosphorylation at serine 261 remains to be clarified.

In addition, the phosphorylation at serine 264, and its possible role in the regulation of the trafficking of AQP2 was reported (Fenton et al. 2008). The mechanism of cell surface delivery and retention of phosphorylated AQP2 is poorly understood. Recently, the interaction of phosphorylated AQP2 with an apical membrane protein was revealed. Myelin and lymphocyte-associated protein (MAL), also known as vesicle integral protein of 17 kDa (VIP17), is localized at the apical membrane in principal cells of the kidney collecting ducts (Kamsteeg et al. 2007). MAL preferentially interacts with phosphorylated AQP2 (Kamsteeg et al. 2007). AQP2 phosphorylated at serine 256 appears to interact more extensively with MAL compared with non-phosphorylated AQP2. MAL increases the surface expression of AQP2 by attenuating the internalization of AQP2 from the cell surface.

Regulation of AQP2 trafficking by actin

Cytoskeletons play important roles in vesicular trafficking. In the amphibian bladder and collecting ducts of the mammalian kidney, ADH promotes the depolymerization of actin filaments and the fusion of vesicles containing water channels with the apical plasma membrane occurs (Hays et al. 1993). The confocal visualization of the actin cytoskeleton with fluorescently labeled phalloidin revealed that AQP2-transfected CD8 cells showed a well-organized meshwork of actin filaments in the basal state (Valenti et al. 2000). On the other hand, very few organized actin filaments were seen in forskolin-treated cells. The protein phosphatase inhibitor okadaic acid also had a similar effect: it induced the disorganization of actin filaments and AQP2 translocation to the cell surface (Valenti et al. 2000). These results suggest a close relationship between the disassembly of actin filaments and translocation of AQP2 to the plasma membrane. In fact, when CD8 cells were treated with the PKA inhibitor H89 and stimulated with forskolin, neither the disassembly of actin filaments nor translocation of AQP2 to the plasma membrane occurred (Valenti et al. 2000). On the other hand, when H89-treated cells were incubated with okadaic acid, disorganization of actin filaments and concomitant decrease of AQP2 were observed.

In AQP2-transfected CD8 cells, the inhibition of Rho GTPase with *Clostridium difficile* toxin B or *C. limosum* C3 fusion toxin, as well as incubation with the Rho kinase inhibitor Y-27632, caused actin depolymerization and the translocation of AQP2 from the intracellular pool to the cell surface in the absence of forskolin (Tamma et al. 2001). The expression of constitutively active RhoA induced actin polymerization and abolished AQP2 translocation in the presence of forskolin. When actin filaments were depolymerized by cytochalasin D or latruncurin B, AQP2 was translocated to the plasma membrane (Tamma et al. 2001; Klussmann et al. 2001; Tajika et al. 2005). These results suggest that the disassembly of actin filaments induces the translocation of AQP2 to the plasma membrane. In other words, actin filament meshworks may serve in preventing the uncontrolled exocytosis and retain AQP2-bearing vesicles in the cytoplasm in resting cells (Fig. 5).

When AQP2-transfected CD8 cells were stimulated with forskolin, active RhoA decreased (Tamma et al. 2003), with a concomitant decrease in the Rho GDP dissociation inhibitor (Rho-GDI) in the membrane fraction. Co-immunoprecipitation experiments revealed that the level of association of Rho-GDI with RhoA increased by forskolin stimulation. Under this condition, RhoA is phosphorylated on a serine residue, which stabilizes the inactive form of RhoA and increases its interaction with Rho-GDI. Taken together, the phosphorylation of RhoA and its association with Rho-GDI control the polymerization of actin filaments, which regulates the exocytosis of AQP2-bearing vesicles.

Careful examination of the effect of cytochalasin D and latrunculin B on the localization of AQP2 in MDCK cells revealed that the disruption of actin filaments results in the translocation of AQP2 from Rab11-positive storage vesicles to the plasma membrane, but AQP2 does not remain at the cell surface and is endocytosed to the EEA1-positive early endosomal compartment and is accumulated there (Tajika et al. 2005). This observation suggests that actin filaments are also important in retaining AQP2 in the plasma membrane (Fig. 5). In addition, the failure to transfer endocytosed AQP2 from early endosomes to the Rab11-positive storage compartment in cytochalasin D- or latrunculin B-treated cells indicates that actin filaments play a critical role in this transfer (Tajika et al. 2005).

The involvement of ERM (ezrin, radixin, moesin) proteins that cross-link actin filaments with the plasma

membrane was reported (Tamma et al. 2005). Forskolin stimulation induced the redistribution of moesin from intracellular sites to the cell cortex in CD8 cells expressing AQP2. A short peptide containing the F-actin binding site of moesin mimicked the effect of forskolin including the disassembly of actin filaments and translocation of AQP2 from the intracellular vesicles to the plasma membrane. Forskolin stimulation reduced the level of moesin phosphorylation. Phosphorylation stabilizes moesin in its active state, which modulates actin depolymerization and reorganizes the F-actin-containing cytoskeletal meshwork in the cellular cortex in favor of the exocytotic translocation of AQP2 to the plasma membrane (Tamma et al. 2005).

In search for AQP2 binding proteins that may control its trafficking, a PDZ-domain containing protein SPA-1 (signal-induced proliferation-associated gene-1) was identified (Noda et al. 2004a). SPA-1 is a GTPase-activating protein (GAP) for Rap1 and is colocalized with AQP2 in the rat kidney collecting duct cells. Translocation of AQP2 to the apical membrane was inhibited by the SPA-1 mutant lacking Rap1-GAP activity and by the constitutive active mutant of Rap1 (Noda et al. 2004a). Moreover, AQP2 trafficking was impaired in SPA-1-deficient mice. SPA-1 may regulate the meshwork of actin filaments by Rap1 and possibly of Rho through its GAP activity, and affect the trafficking of AQP2 vesicles.

Anti-AQP2 affinity column chromatography of the rat kidney extract and subsequent analysis of bound proteins by two-dimensional gel electrophoresis and mass spectrometry generated a list of AQP2 binding proteins (Noda et al. 2004b; Noda and Sasaki 2006). Actin was identified as one of these proteins. By the surface plasmon resonance analyses using a C-terminal fragment of AQP2, high affinity binding of actin was observed, showing that actin itself is one of the AQP2 binding proteins (Noda et al. 2004b).

In addition to actin, a list of related proteins has been obtained by this method. It includes ionized calcium binding adaptor molecule 2, myosin regulatory light chain smooth muscle isoforms 2-A and 2-B, alpha-tropomyosin 5b, annexin A2 and A6, scinderin, gelsolin, alpha-actinin 4, alpha-II spectrin, and myosin heavy chain nonmuscle type A, most of which could be involved in the motility function of actin (Noda et al. 2005). It has been proposed that AQP2 and the above binding proteins could form a multi-protein "force generator complex" and serve in the translocation of AQP2 (Noda et al. 2005; Noda and Sasaki 2006).

The above observations suggest that actin plays multiple roles in the regulation of the intracellular trafficking of AQP2 (Fig. 5). Firstly, cortical actin filaments in the subapical region of the cell may serve as a mechanical obstacle in the movement of AQP2-bearing vesicles toward the apical plasma membrane. RhoA seems to play a regulatory role in the assembly of the cortical actin meshworks. Secondly, actin filaments associated with the plasma membrane may serve in retaining AQP2 molecules on the cell surface. Thirdly, actin filaments serve in the transfer of AQP2 from the early endosomes to the Rab11-positive storage compartment. In this part, actin filaments may serve as possible motor machinery. Fourthly, actin that binds to AQP2 in the storage vesicle may constitute a part of a multi-protein "force generator complex" that provide driving force in the translocation of AQP2 vesicles.

Excretion of AQP2

AQP2 is excreted in human urine and serves as a good index of ADH action in the kidney (Kanno et al. 1995). AQP2 was recovered in small vesicles from the urine, and immunogold electron microscopy revealed that the membrane of vesicles is oriented cytoplasmic-side inward. Proteomics analyses revealed the presence of proteins of multivesicular bodies and the endosomal pathway (Pisitkun et al. 2004). It was hypothesized that AQP2 vesicles in the urine may be exosomes (Février and Raposo 2004) released by the fusion of multivesicular bodies to the apical membrane (Fig. 5) (Pisitkun et al. 2004). As noted in a preceding section, the short-chain ubiquitination of AQP2 at the apical membrane seems to affect endocytosis and the sorting of AQP2 to internal vesicles of multivesicular bodies and regulate the recycling of AQP2 (Kamsteeg et al. 2006).

Comparison of AQP2 and GLUT4

Water permeability of the apical plasma membrane of the principal cells of the kidney collecting ducts is regulated through ADH by the exocytic and endocytic trafficking of AQP2-bearing vesicles. Hormonal regulation of the plasma membrane activity by vesicular trafficking is also typically seen in the insulin-regulated glucose transport activity of the plasma membrane in adipocytes and muscle cells mediated by the glucose transporter GLUT4 (Watson et al. 2004). Both AQP2 and GLUT4 are stored in the intracellular vesicles, and are translocated to the plasma membrane upon ADH and insulin stimulation, respectively. Storage compartments of AQP2 and GLUT4 were compared in MDCK cells expressing both of them (Hasegawa et al. 2007). Triple-labeling with AQP2, GLUT4, and various organelle markers revealed that AQP2 and GLUT4 are differentially localized inside the cell in the basal condition. During the translocation and retrieval of AQP2, they share common EEA1-positive early endosomes, suggesting that they share a common compartment after retrieval from the plasma membrane, but their storage compartments are distinct from each other.

When AQP2 was expressed in 3T3-L1 adipocytes, the cAMP-responsive AQP2 compartment was distinct from the insulin-responsive GLUT4 compartment (Procino et al. 2006). The translocation of GLUT4 upon insulin stimulation did not affect the localization of AQP2, and forskolin-induced translocation of AQP2 did not affect the localization of GLUT4. These results show that AQP2 and GLU4 have distinct intracellular storage compartments and their translocation to the plasma membrane is differentially regulated, while they seem to share a common system of retrieval from the cell surface.

Basolateral localization of AQP2

Intracellular AQP2 is, in most cases, routed to the apical membrane upon ADH stimulation. In principal cells in the collecting ducts and connecting tubules, the basolateral localization of AQP2 is seen (Nielsen et al. 1993; Christensen et al. 2003). A detailed immunohistochemical examination including gold particle counting on immunogold electron microscopy revealed the axial heterogeneity in the basolateral localization of AQP2 along the connecting tubules and collecting ducts in normal and vasopressin-deficient Brattleboro rats (Christensen et al. 2003; van Balkom et al. 2003). The basolateral localization of AQP2 is seen in the connecting tubules and collecting ducts in the inner medulla. Little basolateral localization occurs in principal cells in the cortex and outer medulla. ADH treatment did not affect the basolateral localization in any segments. AQP2 and AQP3, or AQP2 and AQP4, do not form heterotetramers in vivo or in vitro, indicating that basolateral localization is not due to the heterotetramer formation of AQP2 with other basolateral AQPs, and subsequent missorting (van Balkom et al. 2003). Basolateral localization is not due to the high-level expression either, since basolateral localization was not seen in MDCK cells over-expressing AQP2. Rather, basolateral localization may be caused by the hypertonic environment of the inner medulla (van Balkom et al. 2003).

Mutation of AQP2 and nephrogenic diabetes insipidus

Nephrogenic diabetes insipidus is a disorder characterized by the inability to concentrate urine in response to ADH in the kidney collecting ducts. Vasopressin V2 receptor (V2R) and AQP2 are critical in this disorder.

AQP2 plays an important role in the onset of diabetes insipidus since it is located in the furthest downstream of the urine concentration cascade and is the only ADH-responsive water channel of the apical membrane. AQP2 is expressed in both the connecting tubules and collecting ducts. Mice that lack AQP2 in both of them die shortly after birth. Mice that express AQP2 in the connecting tubule but lack AQP2 in the collecting ducts exhibited severe urinary concentration defects, demonstrating that AQP2 in the collecting ducts is critical in water homeostasis (Rojek et al. 2006).

A decrease of the AQP2 expression level is often caused by lithium therapy and results in the onset of nephrogenic diabetes insipidus (Marples et al. 1995). In addition, the mutation of AQP2 is responsible for congenital nephrogenic diabetes insipidus. Two types of mutation of AQP2 are known: autosomal recessive and autosomal dominant types. In the recessive type, the mutated AQP2 molecules lose their water channel function, or are misrouted to the endoplasmic reticulum. In the dominant type, mutated AQP2 is localized in aberrant intracellular compartments such as the Golgi apparatus, late endosomes, lysosomes, or the basolateral membrane. Details of the molecular and cellular mechanisms of pathogenesis of such mutation of AQP2 can be found elsewhere (for review see Fujiwara and Bichet 2005; Robben et al. 2006).

Concluding remarks

Water is the most fundamental molecule of life, and therefore AQP, which mediates the influx and efflux of water, is also one of the most fundamental molecules of cells. Recent progress in AQP research, especially analyses of knockout animals, revealed that AQPs are involved in a wide range of phenomena (Verkman 2005). AQP2 in the kidney plays a critical role in maintaining water balance in the body, and the regulation of AQP2 trafficking in kidney collecting ducts is the key issue regarding water handling in the kidney. Proteomics analyses have identified a catalogue of proteins associated with AQP function. Figure 5 illustrates the putative mechanism of the regulation of AQP2 trafficking. The unveiling of this process will provide insight into the water homeostasis of the body.

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