Aquaporin-2: COOH terminus is necessary but not sufficient for routing to the apical membrane

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The remaining 10% can be reabsorbed in collecting ducts and is under control of the antidiuretic hormone arginine vasopressin (AVP). In the principal cells of collecting ducts, binding of AVP to its V2-type receptor, which is located in the basolateral membrane, initiates a cAMP signaling cascade that results in the activation of protein kinase A (PKA). One of the physiologically important substrates for this kinase is the aquaporin-2 (AQP2) water channel protein. As a result, AQP2 is redistributed from intracellular vesicles to the apical membrane, thereby rendering this membrane water permeable. Removal of AVP initiates endocytosis of AQP2 and restores the water-impermeable state of the apical membrane (35, 44, 46).

AQP2 is essential in this process, because humans who lack functional AQP2 suffer from nephrogenic diabetes insipidus (NDI), a disease in which the kidneys are unable to concentrate urine in response to AVP, resulting in a daily water loss of 10–15 l (7). Expression studies (43) of AQP2 missense mutants identified in patients with recessive NDI revealed that all mutants were retained in the endoplasmic reticulum, presumably as a consequence of misfolding. When expressed in oocytes, the only AQP2 mutant associated with dominant NDI that has been studied to date was not misfolded but was retained in the Golgi complex region (31). Heterotetramerization with wild-type AQP2 (WT-AQP2), precluding its further transport to the membrane, provided an explanation for the dominant NDI phenotype (18).

As is the case in renal proximal tubules, AQP1 expressed in transfected Madin-Darby canine kidney (MDCK) cells is found in both the apical and basolateral membranes and confers a water permeability that is not influenced by activation of either PKA or protein kinase C (PKC) (5). Additionally, as in collecting duct cells, AQP2 heterologously expressed in MDCK cells is stored in intracellular vesicles without PKA stimulation.

THE KIDNEY IS THE MAIN ORGAN for the regulation of water homeostasis. Of the 180 liters of pro-urine produced daily, 90% of the water is constitutively reabsorbed in the renal proximal tubules and descending thin limbs of Henle. This massive reabsorption has been attributed to the aquaporin-1 (AQP1) water channel, which is expressed in the apical and basolateral membranes of the epithelial cells that line these nephron segments.

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tion, whereas on stimulation of the cAMP signaling cascade, AQP2 is redistributed to the apical membrane, which results in a three- to fourfold increase in transcellular osmotic water permeability (6). These differences in subcellular localization and regulation for AQP1 and AQP2 expressed in the same MDCK cell type indicate that the signals necessary for specifying the routing of the respective proteins must reside in their primary sequences.

The COOH-terminal tail of AQP1 and AQP2 might be responsible for the differential regulation of these proteins. Whereas human AQP1 and AQP2 have an overall identity of 48%, the COOH-terminal tails differ extensively. In addition, the COOH-terminal tail bears the PKA phosphorylation site of AQP2 (10), and two mutations of AQP2 that cause dominant NDI are located in this segment (25, 31). Prevention of phosphorylation of AQP2 at the PKA phosphorylation site (as has been shown for the AQP2-S256A construct in LLC-PK1 cells and oocytes) results in retention of this AQP2 protein in intracellular vesicles (9, 17, 20), whereas the AQP2-E258K construct in dominant NDI is retained in the Golgi complex region (31). Thus to identify signals potentially involved in the routing and regulation of AQP2, chimeras of the COOH-terminal tail of AQP2 coupled to AQP1 or aquaporin-unrelated reporter segments were generated, expressed in MDCK cells, and analyzed for routing and function.

MATERIALS AND METHODS

Expression constructs. pCB6-TMR-Plap consists of the mammalian expression vector pCB6 (which confers resistance to G418) and a cDNA that encodes the ectodomain of human placental alkaline phosphatase (Plap) linked to the transmembrane region (TMR) of the vesicular stomatitis virus G (VSV-G) protein (2). To generate pCB6-AQP2-Plap, the human AQP2 cDNA in pBluescript (pBS-AQP2) (7) was used as a template with the sense primer CGCAAGCTTTTTCGCCCAAGCAAGGCC and the antisense primer GCCTCTAGATCTAGGG (2). The AQP2 cDNA was amplified for 30 min in 3% paraformaldehyde in PBS-CM fixed for 30 min in 3% paraformaldehyde in PBS-CM and cloned into the corresponding sites of pCB6. For all of the final constructs, proper nucleotide sequences of the cDNA inserts were checked by double-stranded DNA sequence analysis.

Culturing of MDCK cells and selection of transfected clones. Native MDCK-HRS (type I) cells, and MDCK cells expressing rat AQP1 [clone K (5)] or human AQP2 [WT10 (6)] were grown in DMEM supplemented with 5% (vol/vol) fetal calf serum at 37°C in 5% CO2. For transfection of MDCK cells, 25–30 μg of circular DNA (of expression constructs) purified over a Qiagen column were transfected using the calcium-phosphate precipitation technique as described previously (5). After 24 h, the cells were trypsinized, divided among six to eight petri dishes, and cultured in medium containing 800 μg/ml G418 ( Gibco BRL, The Netherlands). Within 10–14 days after transfection, individual colonies were selected (using cloning rings) and expanded. After approximately eight passages after selection of a clone, the selection drug was omitted from the medium.

Transcellular osmotic water permeability measurements. Cells derived from 0.33 cm² of confluent monolayers were seeded onto 0.33-cm² polycarbonate filters (Costar, Cambridge, MA). On the second day after seeding, the medium was aspirated and replaced by fresh medium in the presence of 5 × 10⁻⁶ M indomethacin to reduce basal intracellular cAMP levels (6). Osmotic water transport was assayed 3 days after seeding in the presence of indomethacin, with or without 5 × 10⁻⁵ M forskolin, by incubation of the apical compartment with 150 μl of 0.5× Krebs-Henseleit buffer (KHB; 1× KHB contains (in mM) 1.2 MgSO4, 128 NaCl, 5 KCl, 2 NaHPO4, 10 sodium acetate, 20 HEPES, 1 CaCl2, 1 l-alanine, and 4 l-lactate; pH 7.4) with 30 mg/l phenol red and the addition of 800 μl of KHB to the basal compartment. After incubation for 2 h at 37°C, the content of the apical compartment was mixed with a pipette, and two aliquots of 50 μl each were put into Eppendorf tubes and diluted to 600 μl with Tris-buffered saline (TBS; 20 mM Tris and 73 mM NaCl; pH 7.6) to which 1% (wt/vol) extrane was extrane (Merek, Darmstadt, Germany). Cells were mixed and centrifuged, and absorbance at 479 nm was measured. The facilitated osmotic water transport (Pf ± SE) was calculated from the acquired absorbances as described previously (5).

Fluid-phase endocytosis. Cells grown to confluence on 2-cm² filters and pretreated with indomethacin were subjected to a fluid-phase endocytosis assay essentially as described by Katsura (21). Briefly, cells were incubated for 30 min in medium with or without 1 × 10⁻⁵ M deamino-8-arginine vasopressin (dAVP), a chemical analog of AVP. The medium at the apical side of the cells was then replaced by a small volume of the same medium with drugs that also contained 10 mg/ml FITC-dextran (mol wt 9,400; Sigma, St Louis, MO) and incubated for 15 min at 37°C. The cells were washed twice with ice-cold PBS with 1 mM MgCl2 and 0.1 mM CaCl2 (PBS-CM), fixed in 3% paraformaldehyde in PBS, washed with PBS, mounted on slides in Vectashield (Vector Laboratories, Burlingame, CA), and analyzed by confocal laser scanning microscopy (CLSM) as described (see In vivo immunocytochemistry).

Immunocytochemistry. Cells grown on 2-cm² filters and incubated as described were rinsed with ice-cold PBS and fixed for 30 min in 3% (wt/vol) paraformaldehyde in PBS-CM.
at reverse transcription; this was followed by two washes with PBS-CM. Subsequently, the cells were permeabilized for 15 min in permeabilization buffer (0.3% Triton X-100 and 0.1% BSA in PBS), incubated for 15 min in 50 mM NH4Cl in PBS, washed with PBS, and washed with permeabilization buffer. Nonspecific binding was then blocked for 30 min in goat serum dilution buffer (GSDB: 16% goat serum, 0.3% PBS, washed with PBS, and washed with permeabilization buffer, and mounted on slides in Vectashield. As primary antibodies, a 1:100 dilution of affinity-purified rabbit or guinea pig anti-AQP2 antibodies raised against the AQP2 COOH terminus (4), a 1:100 dilution of affinity-purified rabbit anti-AQP1 antibodies, and/or a 1:100 dilution of rabbit antiserum against human Plap (Fitzgerald Industries, Concord, MA) were used. As secondary antibodies, a 1:100 dilution of affinity-purified goat anti-rabbit IgG coupled to Alexa 488 or goat anti-guinea pig IgG coupled to Alexa 594 (Molecular Probes, Leiden, The Netherlands) were employed.

In vivo immunocytochemistry. To detect only the population of chimeric Plap proteins expressed in the plasma membrane, the selected AQP2-Plap and TMR-Plap clones grown and incubated as described were washed in ice-cold PBS-CM and incubated for 3 h with rabbit anti-Plap antibodies in 1% BSA in PBS-CM at 4°C. After three washes with ice-cold PBS-CM, the cells were fixed in 3% paraformaldehyde in PBS and incubated for 15 min in permeabilization medium. Subsequent wash steps, incubation with secondary antibodies, and mounting were as described. Horizontal extended-focus images and vertical images were obtained with a Bio-Rad MRC-1000 laser scanning confocal imaging system using a ×60 oil-immersion objective, a 32 Kalman collection filter, an aperture diaphragm of 2.5, and an axial resolution of 0.14 μm/μs. The images were contrast-stretched, and a threshold value with pseudocolor was applied. As controls, incubation of native MDCK cells or omission of primary or secondary antibodies revealed no labeling.

Sucrose-gradient centrifugation. Stably transfected MDCK clones grown to confluence in 75-cm² flasks were scraped from the flasks in 10 ml of PBS, spun down, and homogenized in 5 ml of homogenization buffer A [HbA; 20 mM Tris (pH 7.4), 5 mM MgCl2, 5 mM NaH2PO4, 1 mM EDTA, 80 mM sucrose, 1 mM PMSF, and 5 μg/ml leupeptin and pepstatin]. After centrifugation at 1,000 g for 10 min and 4°C to remove cellular debris and nuclei, the supernatant was spun for 30 min at 200,000 g at 4°C to recover the microsomal membranes. These membranes were dissolved in 300 μl of solubilization buffer [4% sodium deoxycholate, 20 mM Tris (pH 8.0), 5 mM EDTA, 10% glycerol, 1 mM polymethylsulfonyl fluoride (PMSF), and 5 μg/ml leupeptin and pepstatin] for 1 h at 37°C and centrifuged at 100,000 g for 1 h at 4°C to remove undissolved membranes essentially according to Neely and Agre (34). Briefly, 5–17.5% sucrose-step gradients were prepared with 533 μl of 5, 7.5, 10, 12.5, 15, and 17.5% sucrose each in medium containing 20 mM Tris (pH 8.0), 5 mM EDTA, 0.1% Triton X-100, 1 mM PMSF, and 5 μg/ml of leupeptin and pepstatin. Samples (300 μl) of membrane proteins in sodium deoxycholate were loaded and subjected to 150,000 g centrifugation for 16 h at 8°C. Fractions (200 μl) were taken off carefully, designated a through s, and analyzed by immunoblotting. As sedimentation markers, a mixture of BSA (67 kDa), phosphorylase B (97 kDa), yeast alcohol dehydrogenase (150 kDa), and catalase (232 kDa) was used. All markers were from Sigma.

Side-specific biotinylation. Selected clones were seeded and grown on 10-cm² semipermeable filters as described. After incubation for 2 h with or without forskolin, the cells were washed in ice-cold PBS-CM and incubated twice for 20 min at 4°C with 500 μl of 1.5 mg/ml Sulfo-SS-Biotin (Pierce, Rockford, IL) in biotinylation buffer [that contained (in mM) 10 triethanolamine, 2 CaCl2, and 125 NaCl; pH 8.9] applied to the apical surface of the cells. After quenching the biotin with a 5-min incubation in 50 mM NaH2PO4 in PBS-CM, the filters were washed twice with PBS-CM and excised from the support. The cells were then lysed for 30 min at 37°C in 1 ml of lysis buffer (that contained 150 mM NaCl, 5 mM EDTA, 50 mM Tris, and 1% Triton X-100; pH 7.5) to which protease inhibitors had been added (1 μg/ml leupeptin and pepstatin and 1 mM PMSF). Next the cells were scraped from the filters and spun down for 5 min to remove cellular debris. The supernatant of each sample was added to 30 μl of washed Immunopure streptavidin beads (Pierce) and rotated for at least 2 h at 4°C. The beads were washed twice with high-salt buffer (that contained 500 mM NaCl, 5 mM EDTA, 50 mM Tris, and 0.1% Triton X-100; pH 7.5), twice with lysis buffer, and once with 10 mM Tris (pH 7.5). After removal of all liquid using a 30-g needle, the proteins bound to the beads were solubilized in 30 μl of 1.5% Laemmli buffer, denatured for 30 min at 37°C, and subjected to immunoblot analysis.

Immunoblotting. Cells were seeded and grown as described. The medium was aspirated and the cells were lysed in 1× Laemmli sample buffer containing 100 mM dithiothreitol, and subsequently denatured for 30 min at 37°C. Sometimes cells were homogenized in HbA and subjected to endoglycosidase F digestion according to the protocol provided by the manufacturer (New England Biolabs, Hertfordshire, U.K.) to remove the sugar moieties. After SDS-PAGE on a 13% acrylamide gel, the proteins were immunoblotted onto Immobilon polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA). The efficiency of protein transfer was checked by staining the membranes with Ponseau red. Subsequently the blots were blocked for 1 h with 5% nonfat dried milk (NFDM) in TBS with Tween (TBS-T; 20 mM Tris and 73 mM NaCl (pH 7.6), supplemented with 0.2% (vol/vol) Tween) and incubated with a 1:3,000 dilution of affinity-purified rabbit anti-AQP2 antibodies, a 1:1,000 dilution of rabbit anti-human Plap antiserum, or a 1:50 dilution of a mouse monoclonal antibody raised against rabbit AQP1 (14) in TBS with 1% NFDM. As secondary antibodies, a 1:5,000 dilution of affinity-purified goat anti-rabbit antibodies or a 1:2,000 dilution of affinity-purified sheep anti-mouse antibodies, all coupled to horseradish peroxidase (Sigma), were used. Sites of antigen-antibody reactions were visualized with enhanced chemiluminescence according to the protocol provided by the manufacturer (ECL, Boehringer Mannheim, Mannheim, Germany). To compare relative aquaporin-expression levels, concentration series of AQP2 and AQP2-Plap were immunoblotted (data not shown).

RESULTS

Subcellular localization of AQP2 chimeric proteins. In MDCK cells, AQP1 and AQP2 are routed and regulated as in epithelial cells of the renal proximal tubules and collecting ducts, respectively. Therefore, to identify trafficking signals contained in the COOH tail of AQP2, a mammalian AQP1 expression construct was made that encodes a protein in which the COOH terminus of AQP1 was exchanged with that of AQP2.

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[starting at Asn220 (AQP1/2-N220)] and transfected to MDCK cells. In this chimera, the site of exchange was at the point of loss of amino acid identity between AQP1 and AQP2, which is located in the latter part of the sixth TMR of AQP1. To test whether the AQP2 tail itself would be sufficient for trafficking to (and shuttling from) the apical membrane, a construct was made that encodes the ectodomain of Plap coupled to the VSV-G protein TMR and the COOH terminus of AQP2 starting at F224 (AQP2-Plap). As a negative control, the construct coding for the Plap ectodomain coupled to the VSV-G protein TMR was used (TMR-Plap), because in MDCK type II cells, this protein targeted to the basolateral membrane (Muth TR and Caplan MJ, unpublished observations). Figure 1 shows diagrams of the proteins encoded by the different constructs. All constructs were transfected into MDCK cells, and G418-resistant clonal cell lines were selected. With immunoblotting of endoglycosidase F-treated cell homogenates, clones were selected in which the expression levels of the chimeric proteins were closest to that of WT-AQP2 as found in the established WT-AQP2-expressing MDCK cell line, WT10 ([6]; see Fig. 2). From this and other blots, it appeared that the amounts of AQP2 (29 kDa) and AQP1/2-N220 (28 kDa) were similar, whereas those of AQP2-Plap (63 kDa) and TMR-Plap (58 kDa) were threefold less (note that antibodies against AQP2 and Plap were used). The amount of AQP1 could not be related to that of the others, because AQP1 was detected with different antibodies.

To determine the subcellular localization of the different chimeras in the absence or presence of forskolin, the selected clones were grown to confluence on semipermeable inserts and subjected to immunocytochemistry. Subsequent CLSM revealed that with or without forskolin stimulation, AQP1/2-N220 was mainly localized in the apical membrane, whereas some staining was found in the basolateral

Fig. 1. Schematic representation of the studied proteins. Aquaporin (AQP)1, AQP2, and the AQP1-AQP2 chimeric protein AQP1/2-N220 traverse the membrane six times with both termini located inside the cell. Characteristic NPA boxes are indicated. In the dimeric AQP2 tail coupled to placental alkaline phosphatase (AQP2-Plap) and the same protein without the AQP2 tail (TMR-Plap), the ectodomain of Plap and the vesicular stomatitis virus G (VSV-G) protein transmembrane region (TMR) are shown in light and dark gray, respectively.

Fig. 2. Immunoblot analysis of Madin-Darby canine kidney (MDCK) cell lines expressing AQP2-tail chimeric proteins. Cells of MDCK clonal lines stably expressing AQP1 (K), AQP2 (WT10), AQP1/2-N220 (Ch. N220), AQP2-Plap, or TMR-Plap were homogenized. Protein equivalents were directly lysed in Laemmli buffer (−) or after treatment (+) with endoglycosidase F (endo-F) and subjected to immunoblotting using rabbit AQP1 antibodies (K) or a combination of rabbit antibodies directed against AQP2 and Plap. Molecular masses of size markers are indicated in kilodaltons.
membrane (Fig. 3A, top). In contrast, AQP2-Plap was predominantly localized in an intracellular compartment, which was not changed with forskolin treatment (Fig. 3A, middle). TMR-Plap, however, was only found in the apical membrane independent of the presence of forskolin (Fig. 3A, bottom). As reported (5, 6), AQP1 was steadily expressed in the apical and basolateral membranes (Fig. 3B, top), whereas

Fig. 3. Immunocytochemical analysis of MDCK cells expressing AQP2-tail chimeric proteins. Cells of selected MDCK clones expressing AQP1/2-N220 (Ch. N220), AQP2-Plap, and TMR-Plap (A), and wild-type AQP2 (WT-AQP2) and WT-AQP1 (B) were grown to confluence on semipermeable filters, incubated with or without forskolin for 2 h, fixed, and subjected to immunocytochemistry. Confocal images were taken in the xy-(top) and xz-axes (bottom) for each cell type.
AQP2 was redistributed from intracellular vesicles to the apical membrane with forskolin (Fig. 3B, bottom).

If the Plap chimeric proteins are (partly) expressed in the plasma membranes, they should be well detectable by in vivo immunocytochemistry using the Plap antibody, because the Plap protein would be on the extracellular surface of the cell. Using this technique, CLSM revealed that some AQP2-Plap was present in the apical and basolateral membranes which was not redistributed upon forskolin treatment (Fig. 4, top). Furthermore, in vivo labeling confirmed the forskolin-independent apical localization of TMR-Plap (Fig. 4, bottom).

Expression of AQP2 chimeric proteins in apical membrane and conferred transcellular water permeabilities. To determine the effect of forskolin on the apical expression of the AQP2 chimeric proteins, the selected clones were subjected to side-specific biotinylation with subsequent immunoblotting for AQP2 (Fig. 5) and densitometric analysis of the signals. In the MDCK clone that expresses AQP1/2-N220, forskolin increased its apical expression 1.4-fold. Interestingly, apical expression of AQP1 was increased 1.5-fold with forskolin. In contrast, forskolin increased the apical expression of WT-AQP2 by 4.5-fold, whereas the drug had no effect on the level of apical expression of AQP2-Plap nor on that of TMR-Plap.

To test the effect of forskolin on the conferred water permeability (measured as $P_f$), the selected clones were subjected to a standard transcellular osmotic water-transport assay (Fig. 6). In AQP1/2-N220-expressing cells, the $P_f$ value of $42.9 \pm 2$ was not changed by forskolin ($42.4 \pm 3$). Also, in clone K cells, forskolin did not change the $P_f$ value (from $41.6 \pm 2$ to $44.4 \pm 2$). In contrast, forskolin increased the $P_f$ value of WT-AQP2-expressing cells 2.8-fold (from $13.4 \pm 1$ to $37.9 \pm 2$). As expected, the $P_f$ values for cells that expressed AQP2-Plap ($7.3 \pm 1$ and $11.7 \pm 1$) or TMR-Plap ($5.8 \pm 1$ and $7.7 \pm 2$) were not different from that for nontransfected MDCK cells ($6.2 \pm 0$ and $9 \pm 1$).

AQP2-mediated increase in AVP-induced endocytosis. With the use of extracellularly supplied FITC-dextran as a fluid-phase marker for endocytosis, it has
been shown that in LLC-PK1 cells, heterologous expression of AQP2 but not AQP1 significantly increases the AVP-induced exo- and endocytosis of vesicles (21). We wished to test whether a similar phenomenon occurs for MDCK cells expressing AQP1 or AQP2. Toward this end, native MDCK, clone K (AQP1), and WT10 (AQP2) cells were subjected to the fluid-phase endocytosis assay with or without dDAVP. With native and AQP1- or AQP2-transfected MDCK cells, hardly any intracellular staining was observed without dDAVP, whereas substantial intracellular staining was obtained with AVP treatment; this clearly indicates that AVP induces an increase in exocytosis and, consequently, endocytosis also in MDCK cells (Fig. 7; only WT10 cells). However, at 15 min of treatment with AVP, no difference in the level of endocytosis was observed between native, AQP1-, or AQP2-expressing MDCK cells (only shown for native and WT10 cells), which illustrates that in MDCK cells, neither AQP2 nor AQP1 has an additive effect on AVP-induced endocytosis.

Are AQP2-Plap and WT-AQP2 localized in the same intracellular compartment? Without forskolin stimulation, AQP2-Plap and WT-AQP2 are located in intracellular compartments (see Fig. 3). It might be that AQP2-Plap is stored in the same intracellular compartment as WT-AQP2 without stimulation but that it is not redistributed to the apical membrane with forskolin. To investigate this, WT10 cells were transfected with the AQP2-Plap expression construct, and G418-resistant clones were selected. A representative clone was subjected to immunocytochemical analysis using antibodies directed against Plap and AQP2. Because the Plap antibodies only recognize AQP2-Plap whereas the AQP2 antibodies will reveal AQP2-Plap and WT-AQP2, colocalization would be indicated when all sites stained by AQP2 antibodies were also stained with Plap antibodies. CLSM analysis of the cell clone expressing WT-AQP2 and AQP2-Plap, however, revealed many AQP2-stained sites that did not stain for Plap (Fig. 8), which indicates that AQP2-Plap and WT-AQP2 are predominantly located in different vesicles. To determine the localization of AQP2-Plap, MDCK cells expressing this chimera were subjected to immunocytochemistry with antibodies directed against Plap and the endoplasmic reticulum marker protein, protein disulphide isomerase (PDI), the Golgi marker proteins giantin, 58K, or mannosidase II, or the late endosomes/lysosomal marker protein LAMP-1. CLSM analysis, however, revealed that AQP2-Plap did not colocalize with any of the marker proteins (data not shown).

Oligomerization state of AQP2-Plap and WT-AQP2. Using sucrose-gradient centrifugation, we have previously shown that renal AQP2 and WT-AQP2, heterologously expressed in *Xenopus* oocytes, occur as homotetramers (18). To test whether in MDCK cells a difference in the oligomerization state might explain the different subcellular localization of WT-AQP2 and AQP2-Plap, membrane proteins of the WT10 cell line expressing AQP2-Plap were subjected to a sucrose-gradient sedimentation centrifugation. Immunoblotting of fractions of this gradient for AQP2 revealed that AQP2-Plap peaked in fraction $H$, whereas WT-AQP2 peaked in fraction $I$ (Fig. 9). By comparison with parallel sedimented marker proteins, these fractions correspond with a molecular mass for AQP2-Plap and WT-AQP2 between 97 and 150 kDa.

**DISCUSSION**

In general, conditions of hypovolemia or hypernatremia stimulate the pituitary to release AVP, which induces the reabsorption of water in renal collecting duct cells in the short term by initiating the redistribution of AQP2 from intracellular vesicles to the apical membrane and in the long term by increasing the
expression of AQP2. This process is reversed with the removal of AVP. However, in some conditions this process is not so straightforward. For example, in lithium-treated rats that developed NDI, the expression of AQP2 was reduced and AQP2 was located in intracellular vesicles (29). Treatment of these rats with dDAVP for 7 days redistributed AQP2 to the apical membrane but did not increase the AQP2 expression levels. In contrast, dehydration of such rats for 48 h increased AQP2 expression but did not result in a redistribution of AQP2 to the apical membrane. In addition, in rats that are given a water load after being infused with dDAVP for many days, AQP2 remained localized in the apical membrane of collecting duct cells, but the AQP2 expression level was instantaneously decreased. This process is known as the “vasopressin escape response” (8).

Several studies have reported on the mechanism and proteins involved in AQP2 shuttling. It is believed that the docking and fusion of AQP2-containing vesicles with the apical membrane occurs via specialized integral membrane proteins that are similar to proteins found in neuronal synaptic vesicles (the SNARE hypothesis; see Refs. 39, 40), because vesicle-associated membrane protein 2 (VAMP-2) has been found to colocalize with AQP2 in vesicles of collecting duct cells (15, 26, 36), and the vesicle-targeting receptor syntaxin 4, to which VAMP-2 might bind, has been identified in the apical membrane of the same cells (27). The essential role of an AVP-induced cAMP cascade is exemplified by the findings that in cultured cells, this hormone induces an A-kinase anchoring-protein-dependent tethering of PKA to AQP2-containing vesicles and a RhoA-mediated rearrangement of the cytoskeleton, both of which appear to be essential for AQP2 translocation to the plasma membrane (22, 23). Recent studies in oocytes and collecting duct cells furthermore suggest that AVP might induce the translocation of AQP2 to the apical plasma membrane of inner medullary collecting duct cells by increasing the number of PKA-phosphorylated monomers in AQP2 tetramers, although this might be overruled by alternative regulatory mechanisms (3, 17, 41, 48).

Despite this knowledge on the regulation of the AQP2 shuttling mechanism, hardly any information exists on the parts of AQP2 that are important in these processes or on the proteins that AQP2 interacts with. In transfected MDCK cells, AQP1 is routed as in renal proximal tubules in that the random (i.e., apical and basolateral) localization of AQP1 is not influenced by activation of PKA through forskolin (5). In contrast, forskolin redistributes AQP2 from intracellular vesicles to the apical membrane in transfected MDCK cells (6). These differences in routing and regulation of AQP1 and AQP2 in the same cell indicate that the determinants for this behavior must reside in the protein itself. The aquaporin isoforms differ from one another mainly in the termini. Interestingly, many...
routing determinants of proteins are found in the COOH termini (e.g., see Refs. 16, 45). Furthermore, the COOH tail of AQP2 contains the PKA phosphorylation site (10), which has been shown to be essential for redistribution of AQP2 to the plasma membrane (9, 20). The present studies were undertaken, therefore, to determine whether the COOH tail of AQP2 was necessary and sufficient for routing of AQP2 to the apical membrane of MDCK cells.

**COOH terminus of AQP2 is necessary for routing to apical membrane.** To study the role of the AQP2 tail in targeting AQP2 to the apical membrane, we decided to exchange it with the tail of AQP1 for three reasons. First, all aquaporins analyzed are expressed as tetramers (18, 24, 32, 34), a structure that is believed to be essential for stability. This hypothesis is underscored by the steady-state localization and oligomeric states of AQP1/2-N220, AQP2-Plap, and TMR-Plap (see Figs. 3 and 9). Second, in most cells, AQP1 is expressed in vivo in the basolateral and apical membranes in contrast to other AQPs, which are expressed in the basolateral membrane [AQP3 and AQP4 (12)], apical membrane [AQP5 (12)], or vesicles [AQP6 (47)]. Because basolateral and possibly vesicular targeting sequences are thought to be dominant over apical targeting signals, whereas proteins without targeting sequences (and thus delivered by bulk-flow transport) are mostly found in both plasma membranes (i.e., as with AQP1), AQP1 seemed a more sensitive protein to use to determine whether the AQP2 tail contains apical targeting signals. Third, the steady-state localization of expression of AQP1 is similar in proximal tubules and MDCK type I cells and has been well documented in these latter cells (5).

Immunocytochemical (see Fig. 3), biotinylation (see Fig. 5), and water transport (see Fig. 6) analyses of MDCK cells expressing AQP1/2-N220, in which the COOH-terminal tail of AQP1 has been totally exchanged for that of AQP2, revealed that, like AQP2, this chimera was predominantly routed to the apical membrane. However, its regulation was more similar for that of AQP2, revealed that, like AQP2, the COOH tail of AQP2 is necessary for its redistribution from vesicles to the plasma membrane (9, 20). In this respect, the predominant apical localization in nonstimulated cells of AQP1/2-N220, which contains the intact PKA phosphorylation consensus site of AQP2, is striking. Because AQP2 is thought to be continuously shuttled, irrespective of whether its steady-state localization is at the apical membrane or in intracellular vesicles (17, 19), a possible explanation for the apical localization is that AQP1/2-N220 lacks a portion of AQP2 that is essential for its targeting to storage vesicles. Alternatively, retrieval to the storage vesicles might be mediated by the COOH tail of AQP2, which could be blocked by a segment of the AQP1 protein in the AQP1/2-N220 chimeric protein. This latter explanation is corroborated by the constitutive plasma membrane localization of AQP2 coupled to green fluorescent protein (GFP) at its COOH terminus in transfected LLC-PK1 cells (11).

**COOH terminus of AQP2 is not sufficient for routing to apical membrane.** Expressed in Heidelberg MDCK type II cells, the TMR-Plap protein is targeted to the basolateral membrane (Muth TR and Caplan MJ, unpublished observations). Because this protein does not contain any intracellular amino acids, a priori it seemed a good reporter protein to investigate whether the AQP2 COOH tail would be sufficient for apical targeting. In MDCK type I cells, however, it appeared to localize to the apical membrane (see Fig. 3A). Such a difference in targeting between different MDCK cell lines has also been encountered for the Na-K-ATPase (30) and underscores the dependence of the cell type on the routing of a particular protein, which is also encountered in vivo (28, 37). Because AQP2-Plap was impaired in its routing to the apical membrane, however, in retrospect this reporter protein appeared to be highly informative. Although some AQP2-Plap was localized in the plasma membrane (see Fig. 4), the majority was retained inside the cell (see Fig. 3B), which indicates that the AQP2 COOH terminus itself is not sufficient for routing to the apical membrane. This chimeric protein was not localized in the endoplasmic reticulum, Golgi complex, late endosomes, or lysosomes and appeared to be mainly localized in vesicles that were different from the WT-AQP2 storage vesicles (see Fig. 8). The apical localization of TMR-Plap, which differs from AQP2-Plap in that it lacks the AQP2 tail, clearly indicates that the AQP2 tail induces the intracellular retention of AQP2-Plap. This retention might be caused by the lack of tetramerization of AQP2-Plap. All aquaporin proteins are expressed as homotetramers, but because the monomer is the functional unit (42), it is unclear why aquaporins exist in a tetrameric complex. From studies on AQP2 mutants encoded in recessive and dominant NDI, it became clear that AQP2 mutants in recessive NDI (which are retained in the endoplasmic reticulum) are not able to oligomerize with WT-AQP2, whereas an AQP2 mutant in dominant NDI (AQP2-E258K, which was retained in the Golgi complex region) was able to form heterologomers with WT-AQP2 (18). Possibly, WT-AQP2 tetramerizes in the endoplasmic reticulum as do many other proteins (13,
38), and endoplasmic reticulum-retained mutants are precluded from heteroligomerizing with WT-AQP2 because of compartmentalization. Alternatively, and as found for connexin 43, AQP2 homotetramerization may occur in the Golgi complex and might be essential for further routing of AQP2 to the plasma membrane and/or to intracellular vesicles. As shown before for AQP2 expressed in renal tissue and Xenopus oocytes (18), sucrose-gradient centrifugation revealed that WT-AQP2 expressed in MDCK cells sedimented as a homotetramer (see Fig. 9). AQP2-Plap, however, which has a molecular mass of 63 kDa, sedimented as a dimeric complex (see Fig. 9). This sedimentation as a dimer is presumably caused by the Plap ectodomain, because it has been shown that the Plap protein is able to form dimers (1). The retention of the dimeric AQP2-Plap protein in an organelle different from endoplasmic reticulum, Golgi complex, late endosomes, or lysosomes, while tetrameric AQP2 and TMR Plap are routed to the apical membrane, indicates that proper packing of an AQP2 tetramer is essential for its further routing to the apical membrane and/or to intracellular vesicles. Analysis of a chimera in which the AQP2 tail is combined with an aquaporin-unrelated protein that also forms homotetramers might elucidate whether a homotetrameric AQP2 tail would be sufficient for further routing.

In conclusion, analysis of different chimeric AQP2 proteins revealed that the AQP2 tail is essential but not sufficient for routing of AQP2 to the apical membrane. Further studies on AQP1 and AQP2 chimeras in which a shorter part of the AQP1 COOH-terminal tail is exchanged for that of AQP2 might indicate a critical segment determining the apical localization of AQP2. In addition, the apical localization of AQP1/2-N220 in unstimulated MDCK cells indicated that another intracellular part of AQP2, possibly the NH2 terminus, might be necessary for endocytosis and subsequent routing of AQP2 to intracellular storage vesicles. Because trafficking of proteins occurs through interaction with other proteins, elucidation of the AQP2 routing signals will provide tools for identifying AQP2-interacting proteins, which eventually will lead to a better understanding of apical routing and the regulation of shuttling of AQP2 in health and disease.

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