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The Role of Putative Phosphorylation Sites in the Targeting and Shuttling of the Aquaporin-2 Water Channel*

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In renal collecting ducts, a vasopressin-induced cAMP increase results in the phosphorylation of aquaporin-2 (AQP2) water channels at Ser-256 and its redistribution from intracellular vesicles to the apical membrane. Hormones that activate protein kinase C (PKC) proteins counteract this process. To determine the role of the putative kinase sites in the trafficking and hormonal regulation of human AQP2, three putative casein kinase II (Ser-148, Ser-229, Thr-244), one PKC (Ser-231), and one protein kinase A (Ser-256) site were altered to mimic a constitutively non-phosphorylated/phosphorylated state and were expressed in Madin-Darby canine kidney cells. Except for Ser-256 mutants, seven correctly folded AQP2 kinase mutants trafficked as wild-type AQP2 to the apical membrane via forskolin-sensitive intracellular vesicles. With or without forskolin, AQP2-Ser-256A was localized in intracellular vesicles, whereas AQP2-S256D was localized in the apical membrane. Phorbol 12-myristate 13-acetate-induced PKC activation following forskolin treatment resulted in vesicular distribution of all AQP2 kinase mutants, while all were still phosphorylated at Ser-256. Our data indicate that in collecting duct cells, AQP2 trafficking to vasopressin-sensitive vesicles is phosphorylation-independent, that phosphorylation of Ser-256 is necessary and sufficient for expression of AQP2 in the apical membrane, and that PMA-induced PKC-mediated endocytosis of AQP2 is independent of the AQP2 phosphorylation state.

In humans, the kidney is the prime organ for regulation of body fluid osmolarity, which is maintained within strict boundaries. To fine-tune this balance, principal cells of the renal collecting duct reabsorb water from pro-urine, which is under control of the anti-diuretic hormone arginine vasopressin (AVP).[1] Upon hypovolemia or hypernatremia, pituitary-derived AVP binds its V2 receptor in the basolateral membrane of these cells and initiates an intracellular cAMP signaling cascade that causes a transient increase in cytosolic calcium (1) and the activation of protein kinase A (PKA), which in turn phosphorylates homotetrameric aquaporin-2 (AQP2) water channels and possibly other proteins. Consequently, AQP2-containing vesicles fuse with the apical membrane, rendering the principal cells water-permeable (2, 3). Driven by an osmotic gradient, water will then enter these cells via AQP2 and will exit the cells via AQP3 and AQP4, located in the basolateral membrane, a process in which urine is concentrated.

By using antibodies that recognize Ser-256-phosphorylated AQP2 (p-AQP2), Nishimoto *et al.* (4) were able to show that *in vivo* AVP-induced redistribution of AQP2 from vesicles to the apical membrane coincides with phosphorylation of Ser-256. By using similar antibodies, Christensen *et al.* (5) demonstrated that p-AQP2 is, besides the apical membrane, also present in intracellular vesicles of principal cells and that the intracellular distribution of AQP2 is regulated via V2 receptors by altering the phosphorylation state of Ser-256 in AQP2. In a later study, water permeability analyses of *Xenopus* oocytes expressing different ratios of AQP2-S256A and AQP2-S256D (which mimic non-phosphorylated and phosphorylated AQP2, respectively) indicated that three or more monomers in an AQP2 tetramer need to be phosphorylated at Ser-256 for a steady state plasma membrane localization of AQP2 (6), which provided an explanation for the detection of p-AQP2 in intracellular vesicles. The retention of AQP2-S256A in intracellular vesicles of LLC-PK_1 cells upon treatment with forskolin, while wt-AQP2 in such cells migrated to the basolateral membrane, revealed that phosphorylation of Ser-256 in AQP2 is essential for re-distribution to the basolateral membrane (7, 8). At present, however, it is unclear whether phosphorylation of Ser-256 is essential and/or sufficient for AQP2 translocation to the apical membrane.

Retrieval of AQP2 from the apical membrane of principal cells, which results in a reduction in water reabsorption and urine concentrating ability, is mediated by removal of AVP and by several hormones that activate the protein kinase C (PKC) pathway. Some of these hormones (ATP/UTP, endothelin) are thought to activate PKCs that block the AVP-triggered increase in cAMP (9, 10). In contrast, other hormones, such as epidermal growth factor, prostaglandin E_2, and agonists of muscarinic receptors did not interfere with the AVP-mediated

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[1] The abbreviations used are: AVP, arginine vasopressin; AQP2, aquaporin-2; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; CKII, casein kinase II; MDCK, Madin-Darby canine kidney; DMEM, Dulbecco's modified Eagle's medium; BSA, bovine serum albumin; PBS, phosphate-buffered saline; ER, endoplasmic reticulum; CLSM, confocal laser scanning microscope; PKA, protein kinase A.

cAMP increase and, therefore, were suggested to act on the AQP2 shuttling process only (11–13). Because the inhibitory effect of these latter hormones was absent upon co-treatment with PKC inhibitors (10, 12) and phorbol 12-myristate 13-acetate (PMA), which is a specific activator of several PKCs (14), also inhibits AVP-induced water permeability (13), these hormones were suggested to activate PKC isotypes that interfere with shuttling of AQP2 to the apical membrane. For some proteins, it has been shown that PKCs exert their effect through activation of casein kinase II (CKII) proteins (15, 16). At present, however, it is unknown whether the AVP-counteracting PKCs mediate the re-distribution of AQP2 to intracellular vesicles via direct or indirect (*e.g.* through CKII) (de-)phosphorylation of AQP2 or whether it occurs independent of the AQP2 phosphorylation state.

Besides modulating the steady state localization of a protein in response to hormonal stimulation, the transit of proteins from the endoplasmic reticulum (ER) to their final subcellular location can also be a phosphorylation-dependent process. For example, phosphorylation of the *N*-methyl-D-aspartate receptor NR1 and caveolin-1 enables these proteins to exit the ER (17, 18), whereas translocation of numerous proteins from the trans-Golgi network to intracellular compartments or the plasma membrane and vice versa is also regulated by phosphorylation events (19–20). Since such phosphorylation events can be transient, lasting only minutes (21, 22), the importance of a phosphorylation event can be easily missed, which will not occur when such a site is constitutively phosphorylated or non-phosphorylated. Several studies have shown that the negative charge introduced by phosphorylation can often be mimicked by changing a phosphorylation site Ser/Thr residue for a Glu or Asp (23, 24), whereas phosphorylation of such a site can be prevented by changing it to an Ala residue (25, 26).

Human AQP2 contains three phosphorylation consensus sequences for CKII (Ser-148, Ser-229, and Thr-244), one for PKC (Ser-231), and one for PKA (Ser-256), and in Madin-Darby canine kidney (MDCK) cells, the routing to intracellular storage vesicles and the AVP-regulated shuttling of heterologously expressed human AQP2 to and from the apical membrane is similar to those processes in principal renal cells (27). Therefore, to address the role of the putative phosphorylation sites in human AQP2 in these processes, MDCK cell lines that stably expressed AQP2 proteins, in which each putative kinase site was changed into an Ala or Glu/Asp residue, were generated and analyzed in detail.

EXPERIMENTAL PROCEDURES

Expression Constructs—For expression of AQP2-S256A and AQP2-S256D in MDCK cells, the encoding cDNA fragments were cut from pT7Ts-AQP2-S256A and pT7Ts-AQP2-S256D constructs (6, 28) with *Bgl*II and *Spe*I and cloned into the *Bgl*II and *Xba*I sites of the mammalian expression vector pCB6 (29).

To generate pCB6 constructs for expression of the other kinase site mutants, three point PCRs were performed. For this, sense primers for AQP2-S148A (5′-CATCTTCGCCGCCACCGATGA-3′), S148D (5′-GCATCTTCGCCGACACCGATGAGC-3′), S229A (5′-CCAGCCAAGGCCCTGTCGGA-3′), S229D (5′-CCGCCAGCCAAGGATCTGTCGGAG-3′), S231A (5′-AAGAGCCTGGCGGAGCGCCT-3′), S231D (5′-CAAGAGCCTGGATGAGCGCCTGG-3′), T244A (5′-GAGCCGGACGCCGATTGGGA-3′), and T244E (5′-GGAGCCGGACGAGGATTGGGAGG-3′) or their corresponding antisense primers were used in combination with a pT7Ts reverse primer (5′-GCTTAGAGACTCCATTCGGG-3′) or T7 primer, respectively, in a standard PCR with pT7Ts-AQP2 (28) as a template. The resulting fragments were isolated, and a second PCR, using both fragments as template, combined with the pT7Ts reverse and T7 primer was performed to generate the full-length mutated AQP2 fragment. Subsequently, these fragments were digested with *Bgl*II and *Spe*I and ligated into the *Bgl*II and *Xba*I sites of pCB6. Introduction of only the desired mutations was confirmed using DNA sequence analysis.

Cell Culturing and Transfection of MDCK Cells—MDCK type I cells (27) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% (v/v) fetal calf serum at 37 °C in 5% CO_2. For transfection of MDCK cells, 25 μg of purified circular DNA was transfected using the calcium-phosphate precipitation technique as described in detail previously (30, 31). Twenty four hours after transfection the cells were trypsinized, divided over 6 Petri dishes, and expanded in medium containing 800 μg/ml G418 (Invitrogen). Ten to fourteen days after transfection, individual clones were selected and grown on selection drug for 4 weeks.

Immunoblotting—Protein samples were denatured by incubation for 30 min at 37 °C in 1× Laemmli buffer, subjected to electrophoresis on a 13% SDS-polyacrylamide gel (Fluka Biochimica, Switzerland), and blotted onto polyvinylidene fluoride membranes (Millipore Corp., Bedford, MA) as described previously (32). Membranes were blocked for 1 h in 5% nonfat dried milk in TBS-T (20 mM Tris-HCl, 73 mM NaCl, 0.2% Tween 20, pH 7.6) and subsequently incubated with 1:3000 diluted affinity-purified rabbit AQP2 antibodies (raised against the 15 COOH-terminal amino acids of rat AQP2 (33)) or rabbit antibodies directed against Ser-256-phosphorylated AQP2 (AN83-2) ((4), diluted in TBS-T with 1% nonfat dried milk. As secondary antibodies, goat anti-rabbit antibodies coupled to horseradish peroxidase (1:5000 in TBS-T, Sigma) were used. Proteins were visualized using enhanced chemiluminescence (Pierce).

Immunocytochemistry—Cells seeded at 1.5×10^{-5} cells/cm^2 were grown on 1.13-cm^2 polycarbonate filters (Corning Costar Europe, Badhoevedorp, The Netherlands) for 2 days. After subsequent overnight treatment with 5×10^{-5} M indomethacin, cells were incubated for 45 min with DMEM with or without 5×10^{-5} M forskolin. To activate PKCs following forskolin treatment, filters were subsequently incubated in DMEM with forskolin and 10^{-7} M PMA (Sigma) for 45 min. In these cases, cells incubated for 90 min with indomethacin with or without forskolin were taken as controls. After these incubations cells were washed twice with PBS-CM (PBS with 0.1 mM $CaCl_2$, 1 mM $MgCl_2$) and fixed in 3% paraformaldehyde for 30 min. Following quenching of aldehyde groups with 50 mM NH_4Cl in PBS for 15 min, cells were permeabilized with 0.2% SDS in PBS for 5 min, incubated with goat serum dilution buffer (GSDB; 16% goat serum, 0.3% Triton X-100, 0.3 M NaCl in PBS) for 30 min to block nonspecific antibody binding, and incubated overnight with a mixture of a 1:100 dilution of affinity-purified rabbit anti-AQP2 antibody (33) in GSDB. After washing twice with permeabilization buffer (0.3% Triton X-100, 0.1% BSA in PBS), filters were incubated with 1:100 diluted goat anti-rabbit antibodies coupled to Alexa 594 (Molecular Probes, Eugene, OR) in GSDB for 45 min. Next, filters were rinsed twice with permeabilization buffer and mounted on glass slides with Vectashield (Vector Labs, Burlingame, CA). Images were obtained with a Bio-Rad confocal laser scanning microscope (CLSM) using a 60× oil-immersion objective.

Side-specific Biotinylation—MDCK cells were seeded at 1.5×10^5 cells/cm^2 on 9.6-cm^2 polycarbonate filters (Corning Costar Europe, Badhoevedorp, The Netherlands), grown, and treated as described above. Next, the cells were washed twice with ice-cold PBS-CM and incubated twice for 20 min at 4 °C with 500 μl of 1.5 mg/ml Sulfo-NHS-SS-Biotin (Pierce) in biotinylation buffer (10 mM triethanolamine, 2 mM $CaCl_2$, and 125 mM NaCl, pH 8.9) applied to the apical surface of the cells. Subsequently, the filters were incubated for 5 min with quenching solution (50 mM NH_4Cl in PBS-CM) at 4 °C and rinsed twice with cold PBS-CM. After the filters were cut from their plastic support, 1 ml of lysis buffer (150 mM NaCl, 20 mM Tris-HCl, pH 8.0, 5 mM EDTA, 1% Triton X-100, 0.2% BSA, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, and 5 μg/ml pepstatin) was added and incubated for 30 min at 37 °C. Subsequently, the cells were scraped and transferred to Eppendorf tubes. After centrifugation for 5 min, the supernatant was added to streptavidin beads (30 μl/sample), which had been pre-washed twice with high salt buffer (500 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl, pH 7.5, 0.1% Triton X-100) and twice with lysis buffer. After incubation for 16 h at 4 °C, the beads were centrifuged for 5 min and washed twice with high salt buffer, twice with lysis buffer, and once with 10 mM Tris-HCl, pH 7.5. Finally, the beads were sucked dry with a 30-gauge needle, resuspended in 30 μl of 1× Laemmli buffer, and denatured for 30 min at 37 °C.

Orthophosphate Labeling—Transfected MDCK cells were seeded as described for side-specific biotinylation. Two days after seeding, cells were treated overnight with medium containing indomethacin (Sigma). Next, filters were cut from their support and washed once with serum/phosphate-free DMEM (ICN Biomedicals, The Netherlands, Europe). Subsequently, the medium was replaced by serum/phosphate-free DMEM (1 ml/filter) containing indomethacin and 20 μCi/ml

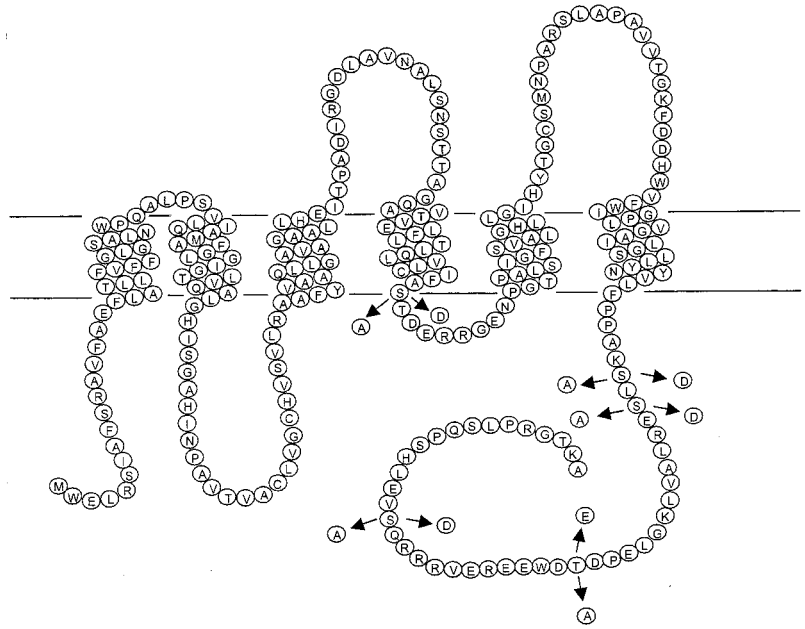

FIG. 1. **Putative phosphorylation sites in AQP2.** AQP2 consist of six transmembrane domains and has its NH_2 and COOH termini intracellularly. The putative phosphorylation sites and the Ala or Glu/Asp residues in which they were changed to mimic constitutively non-phosphorylated or phosphorylated AQP2 forms, respectively, are indicated by *arrows*.

[^{32}P]orthophosphate (Amersham Biosciences). After 3 h of incubation at 37 °C the medium was replaced by medium containing indomethacin with or without forskolin and 20 μCi/ml [^{32}P]orthophosphate for 1 h. Subsequently, for PKC induction, cells treated with forskolin were incubated with medium containing indomethacin, forskolin, PMA, and 20 μCi/ml [^{32}P]orthophosphate for an additional hour. Next, the cells were washed twice with ice-cold wash buffer (PBS + 2 mM EDTA) containing 10 mM NaF and 0.5 mM Na_3VO_4 to inhibit dephosphorylation. Subsequently, cells were scraped and homogenized in 750 μl of ice-cold lysis buffer (100 mM Tris-HCl, 150 mM NaCl, 2 mM EDTA, 0.5% sodium deoxycholate, 0.1% SDS, 0.5% Nonidet P40) containing 10 mM NaF and 0.5 mM Na_3VO_4. The lysates were transferred to Eppendorf tubes and centrifuged for 10 min at 4 °C. The cleared lysate was subjected to immunoprecipitation.

Immunoprecipitation—10 μl of protein A-agarose beads (Kem-En-Tec A/S, Copenhagen, Denmark) per sample were washed twice in lysis buffer + 1% BSA. Per sample, 4 μl of rabbit 7 anti-AQP2 antibodies was added to 400 μl of lysis buffer and rotated overnight at 4 °C. Before use, the antibody-coupled protein A beads were washed twice in ice-cold lysis buffer. The washed antibody-bound beads were incubated with cleared lysate for 16 h, washed four times with lysis buffer containing phosphatase inhibitors, sucked dry with a 30-gauge needle, and resuspended in 30 μl of 1× Laemmli buffer. [^{32}P]Orthophosphate-labeled samples were split into two equal portions of which one was immunoblotted for AQP2, and the second was subjected to SDS-PAGE. The gels were dried and exposed to film for ~3 days using two amplifying screens at −80 °C. Relative quantification of the signals was performed with a PhosphorImager.

RESULTS

Trafficking of AQP2 to Intracellular Vesicles Is Phosphorylation-independent—To determine the role of phosphorylation of Ser-148, Ser-229, Ser-231, Thr-244, and Ser-256 in the targeting and regulation of shuttling of AQP2, all these sites were independently mutated into alanines to mimic a non-phosphorylated state or into glutamic (Thr-244) or aspartic acids (others) to mimic a phosphorylated state (Fig. 1). Eucaryotic expression constructs coding for these proteins were stably transfected into MDCK cells. Immunoblot analysis of the selected clones revealed that, except for AQP2-S148D, all AQP2 mutants were mainly expressed as unglycosylated 29-kDa proteins, which is also the most prominent band for wt-AQP2 in MDCK cells (Fig. 4). AQP2-S148D, however, was mainly expressed as 29- and 32-kDa AQP2 proteins (Fig. 2), which has been shown to be indicative for AQP2 proteins retained in the ER (34). Therefore, this mutant was left out of further analyses.

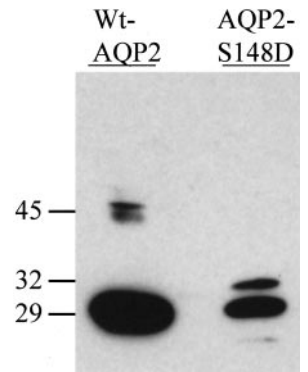

FIG. 2. **Immunoblot analysis of AQP2-S148D.** Lysates of representative cell lines expressing wt-AQP2 or AQP2-S148D were immunoblotted for AQP2. For wt-AQP2, the unglycosylated 29-kDa and complex-glycosylated 40–45-kDa bands were obtained. Besides the 29-kDa band, a 32-kDa high mannose band is detected for AQP2-S148D, which indicates that this mutant is retained in the ER.

To determine the effects of the introduced mutations on the targeting of AQP2 from the organelle of synthesis (ER) to intracellular vesicles, the cell lines were treated with indomethacin to lower endogenous cAMP levels and subjected to immunocytochemistry. With the exception of AQP2-S148D and AQP2-S256D, CLSM analysis revealed a vesicular localization for all AQP2 mutants that was similar to that of wt-AQP2 (Fig. 3, *left column*). AQP2-S148D revealed a dispersed intracellular staining, which did not change with forskolin or forskolin/PMA treatments and is typical for ER-retained proteins. Strikingly, AQP2-S256D was, in contrast to wt-AQP2 and all other AQP2 mutants, expressed in the apical membrane.

Phosphorylation of Ser-256 Is Necessary for Expression of AQP2 in the Apical Membrane—To determine whether the introduced kinase site mutations affected the cAMP-induced re-distribution of AQP2 from intracellular vesicles to the apical membrane, all cell lines were treated with forskolin and subjected to immunocytochemistry. CLSM analysis revealed that all AQP2 mutants were mainly expressed in the apical plasma membrane as has been found previously for wt-AQP2, except for AQP2-S256A (Fig. 3, *middle column*). Of this latter mutant, the vesicular localization did not change upon forskolin treatment, which indicated that phosphorylation of Ser-256 is es-

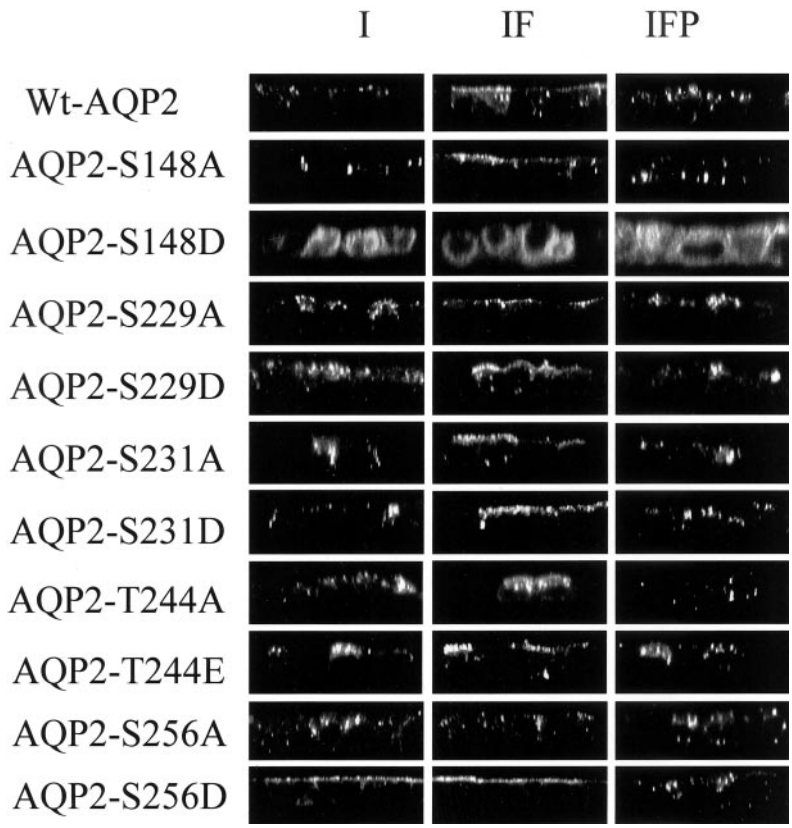

FIG. 3. **Immunocytochemical analysis of MDCK cells expressing the different phosphorylation site mutants.** X-Z confocal images of MDCK cells expressing wt-AQP2 or the different phosphorylation site mutants are indicated. These cells were grown to confluence and incubated overnight with indomethacin (*I*) to reduce basal cAMP levels. Cells were then incubated with forskolin (*IF*) for 45 min or with forskolin for 45 min followed by PMA and forskolin for 45 min (*IFP*), both in the presence of indomethacin. After fixation, the cells were subjected to immunocytochemistry using anti-AQP2 antibodies. AQP2-S148D shows a dispersed ER-like pattern. All mutants, except AQP2-S256A/S256D, were sorted and redistributed as observed for wt-AQP2. With or without forskolin, AQP2-S256A was retained in vesicles, whereas AQP2-S256D was localized in the apical membrane. Upon the combined forskolin/PMA treatment, all AQP2 proteins were internalized, except AQP2-S256A.

sential for re-distribution of AQP2 from vesicles to the apical membrane.

PMA-induced Endocytosis of AQP2 from the Apical Plasma Membrane Is Independent of the Phosphorylation State of AQP2—In several *in vivo* studies, it has been shown that hormones and PMA that activate the PKC pathway, counteract the AVP-induced re-distribution of AQP2 from vesicles to the apical membrane in collecting duct cells and, therefore, the concentration of urine (13, 35). To test whether this process could be simulated in MDCK cells and whether the kinase site mutants act differently upon activation of the PKC pathway, all cell lines were pre-treated with forskolin and subsequently treated with forskolin and PMA followed by immunocytochemistry. CLSM analysis revealed that wt-AQP2 as well as all kinase site mutants were re-distributed from the apical membrane to intracellular vesicles (Fig. 3, *right column*). The vesicular localization of AQP2-S256A was not changed by the combined forskolin-PMA treatment. These results indicated that neither phosphorylation of the PKC consensus site nor modification of any other putative kinase site was needed for PMA-induced translocation of AQP2 from the apical membrane to intracellular vesicles. Because AQP2-S256D is also internalized with PMA, these data also suggested that the PKC-induced redistribution of AQP2 to intracellular vesicles is an event that is independent of de-phosphorylation of the PKA phosphorylation site.

To biochemically establish expression of the AQP2 proteins in the apical membrane, all cell lines were treated as above and subjected to an apical cell surface biotinylation assay. Immunoblotting revealed that forskolin treatment strongly increased the apical membrane expression of all AQP2 proteins (only shown for wt-AQP2, AQP2-S229A/S229D, and AQP2-S231A/S231D), except for AQP2-S256A and AQP2-S256D (Fig. 4). AQP2-S256A was not detected in the apical membrane, whereas the apical membrane expression of AQP2-S256D in unstimulated cells was not further increased with forskolin.

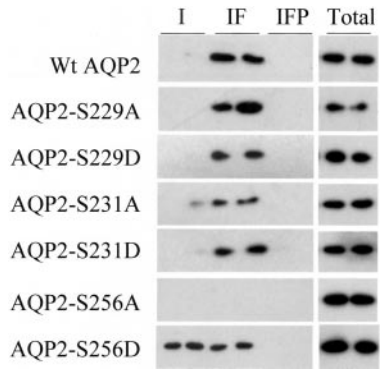

FIG. 4. **Apical cell surface expression of the kinase site mutants of AQP2.** Cells expressing wt-AQP2 or the AQP2 kinase site mutants (indicated) were grown and treated with indomethacin (*I*), indomethacin/forskolin (*IF*), or indomethacin/forskolin/PMA (*IFP*) as described in the legend of Fig. 3 and subjected to a cell surface biotinylation assay. Biotinylated proteins were precipitated with streptavidin-agarose beads and immunoblotted for AQP2. A sample of the lysed cells was immunoblotted in parallel to visualize the amount of mutant proteins expressed. The data confirmed the results obtained by immunocytochemistry.

Incubation with forskolin-PMA following forskolin treatment again decreased the apical membrane expression of all kinase site mutants to undetectable levels. Immunoblotting of equivalents of the biotinylated and lysed cells revealed that all cell lines expressed well detectable levels of wt-AQP2 or of the AQP2 mutants (Fig. 4, *Total*).

Forskolin Specifically Enhances Phosphorylation of Ser-256—To determine whether the forskolin and forskolin-PMA-induced redistribution of AQP2 coincided with a changed phosphorylation of the AQP2 protein, wt10 cells and AQP2-S256A-expressing cells were not stimulated or treated with forskolin or forskolin-PMA in the presence of radioactive orthophosphate. After this treatment, AQP2 proteins were im-

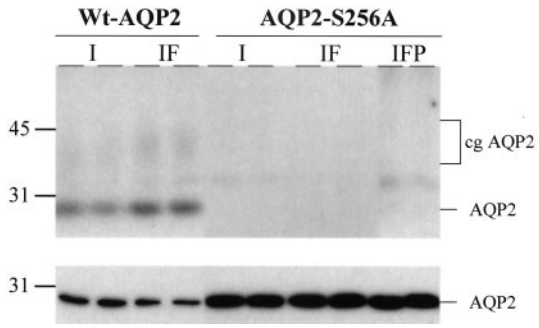

FIG. 5. **Phosphorylation of wt-AQP2 and AQP2-S256A in MDCK cells.** Cells expressing wt-AQP2 or AQP2-S256A were grown and treated with indomethacin (*I*), indomethacin/forskolin (*IF*), or indomethacin/forskolin/PMA (*IFP*) as described in the legend of Fig. 3 and subjected to [^{32}P]orthophosphate labeling. After lysis, the AQP2 proteins were immunoprecipitated, split in two portions of which one was separated on SDS-PAGE and autoradiographed (*upper panel*). The second portion was immunoblotted for AQP2 (*lower panel*). Unglycosylated (*AQP2*) and complex-glycosylated AQP2 (*cg-AQP2*) are indicated, and the mass of marker proteins in kDa is given on the *left*. Wt-AQP2 shows a clear increase in phosphorylation with forskolin, whereas AQP2-S256A is not labeled under any condition.

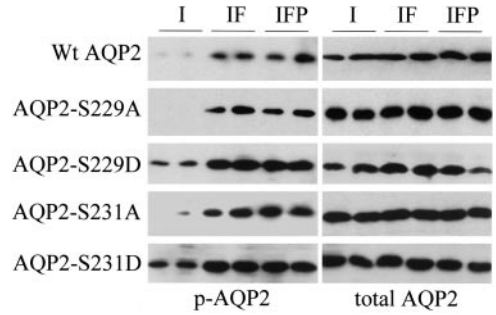

FIG. 6. **Phosphorylation of wt-AQP2 and the kinase site mutants at Ser-256.** Cells expressing wt-AQP2 or the AQP2 kinase site mutants (only Ser-229 and Ser-231 mutants are shown) were grown and treated with indomethacin (*I*), indomethacin/forskolin (*IF*), or indomethacin/forskolin/PMA (*IFP*) as described in the legend of Fig. 3 and immunoblotted using an antibody specifically recognizing PKA-phosphorylated AQP2 (*p-AQP2*) or recognizing all AQP2 forms (total AQP2). Equal amounts of AQP2 proteins were loaded (*right panel*). With all AQP2 kinase site mutants, phosphorylation at Ser-256 was increased with forskolin, which did not decrease upon subsequent incubation with forskolin and PMA.

munoprecipitated from lysed cells, split into two equal portions, and immunoblotted for AQP2 or loaded on a gel and exposed to film. Immunoblotting revealed that per cell line equal amounts of AQP2 were loaded for each condition tested (Fig. 5, *lower panel*). Determination of the level of phosphorylation of wt-AQP2 in wt10 cells revealed that wt-AQP2 was already phosphorylated without stimulation, which was, following normalization for the amounts of AQP2 loaded, increased 2-fold upon stimulation with forskolin.

In contrast to wt-AQP2, AQP2-S256A was not labeled in unstimulated, forskolin-stimulated or forskolin-PMA-treated cells, whereas the AQP2-S256A expression was higher than wt-AQP2 (Fig. 5). These results showed that under steady state conditions only Ser-256 in AQP2 is detected as being phosphorylated and that its level of phosphorylation is increased with forskolin.

PMA-induced Endocytosis of AQP2 Is Independent of Ser-256 De-phosphorylation—Data obtained for AQP2-S256A and AQP2-S256D described above demonstrated the importance of phosphorylation of Ser-256 to re-distribute AQP2 from intracellular vesicles to the apical membrane. We also wanted to determine whether forskolin-induced translocation to the plasma membrane and subsequent re-location to intracellular vesicles with forskolin-PMA treatment coincides with an increased, respectively decreased, phosphorylation of AQP2 at Ser-256. Therefore, the different cell lines were treated as described above and lysed. Immunoblotting of the obtained samples, using antibodies specifically recognizing Ser-256-phosphorylated AQP2 (4), revealed that forskolin strongly increased the level of Ser-256 phosphorylation of all AQP2 kinase site mutants that were re-distributed from vesicles to the apical membrane (Fig. 6; shown for wt-AQP2, AQP2-S229A/S229D and AQP2-S231A/S231D), with the exception of AQP2-S256A/S256D. Neither AQP2-S256A nor AQP2-S256D could be detected with these antibodies, presumably because these mutations disrupt the epitope recognized by the antibody (not shown).

Surprisingly, treatment with forskolin-PMA following forskolin treatment did not appear to reduce the level of phosphorylation of Ser-256 (Fig. 6). These results demonstrated that the PMA-induced re-distribution of AQP2 from the apical membrane to intracellular vesicles occurred independent of de-phosphorylation of Ser-256.

DISCUSSION

AQP2 Routing to cAMP-sensitive Storage Vesicles Is a Phosphorylation-independent Process—In this study, putative kinase sites were changed into Ala or Glu/Asp residues to investigate the involvement of putative phosphorylation sites in AQP2 routing and regulation of its shuttling in MDCK cells.

Immunocytochemical analysis revealed that, except for AQP2-S148D, all AQP2 kinase site mutants were routed to intracellular vesicles, as shown by the observed spot-like structures. The dispersed staining of AQP2-S148D (Fig. 3) is typical for an ER-retained protein. This was confirmed by the appearance of a 32-kDa band on immunoblot (Fig. 2), which has been shown to represent a high mannose glycosylated form of AQP2 (32). One could argue that the ER retention of AQP2-S148D indicates that AQP2 is phosphorylated at Ser-148 while residing in the ER and needs to be de-phosphorylated to continue its route to storage vesicles. Inconsistent with this, however, is that AQP2-S148A, which cannot be phosphorylated at Ser-148, is not impaired in its routing to intracellular vesicles (Fig. 3). Also Ser-148 is located close to or is part of transmembrane domain four (Fig. 1), and transmembrane domains are considered to be highly sensitive to amino acid changes, resulting in improperly folded proteins. Indeed, many of the misfolded AQP2 proteins, encoded in patients suffering from nephrogenic diabetes insipidus, are caused by mutations in transmembrane domains (36). Most likely, therefore, AQP2-S148D is a misfolded protein. Because all other AQP2 kinase site mutants, except AQP2-S256A (see below), are, as wt-AQP2, translocated from vesicles to the apical membrane upon forskolin treatment (Figs. 3 and 4), our data indicate that the routing of AQP2 to cAMP-sensitive storage vesicles is (de-)phosphorylation-independent.

Phosphorylation of Ser-256 Is Necessary and Sufficient for Localization of AQP2 in the Apical Plasma Membrane—By using LLC-PK$_1$ cells expressing AQP2-S256A, it has been shown that phosphorylation of Ser-256 is needed for AQP2 translocation from vesicles to the basolateral membrane (7, 8). The vesicular localization of AQP2-S256A in oocytes indicated that also in these cells phosphorylation of Ser-256 was essential for plasma membrane expression (6). In contrast, the exclusive plasma membrane expression of AQP2-S256D in oocytes indicated that this protein mimics constitutively phosphorylated AQP2, because this localization is identical to that of wt-AQP2, which is in these cells under basal conditions phosphorylated at Ser-256 to a high level (6). Because the level

of Ser-256 phosphorylation could not be modulated in oocytes, its role in the regulation of AQP2 shuttling could not be studied further in these cells.

In MDCK cells, however, induction of the cAMP pathway by AVP, cAMP, or forskolin is needed to induce the re-distribution of wt-AQP2 from storage vesicles to the apical membrane (37), which coincides with an increased level of Ser-256 phosphorylation (Figs. 5 and 6). In these cells, forskolin treatment did not change the vesicular localization of AQP2-S256A (Fig. 3), which was underscored by the lack of detection of AQP-S256A in the apical membrane with the biotinylation assay (Fig. 4). This clearly showed that phosphorylation of Ser-256 in wt-AQP2 is also needed for AQP2 translocation to the apical plasma membrane. Recently, it was speculated that Ser-256 needs to be phosphorylated by the Golgi CKII and subsequently de-phosphorylated before its itinerary to storage vesicles could be continued (38). Co-localization studies using antibodies that recognize the Golgi marker proteins 58K and giantin, however, did not reveal any co-localization with AQP2-S256A nor AQP2-S256D (not shown), which showed that in MDCK cells (de-)phosphorylation of Ser-256 is not needed to exit the Golgi complex.

In contrast to AQP2-S256A, AQP2-S256D was already expressed in the apical plasma membrane without stimulation of the cAMP cascade with forskolin (Figs. 3 and 4). This revealed that phosphorylation of just Ser-256 in AQP2 is sufficient for AQP2 localization in the apical membrane and suggests that other vasopressin-induced intracellular changes are not needed for translocation of AQP2. This seems inconsistent with the existing literature, because vasopressin-induced tethering of PKA to AQP2-containing vesicles and depolymerization of the actin cytoskeleton via inhibition of the Rho GTPase were shown to be essential for AQP2 translocation to the plasma membrane (39–41).

The following model might explain these possible contradictions. AQP2 is thought to reside in two vesicle pools: a recycling pool, which continuously shuttles AQP2 to and from the apical membrane but rapidly changes the balance of AQP2 expression to the apical membrane upon hormonal stimulation, and a storage pool, which is transported along microtubules to the apical pole and delivers its cargo to the recycling pool upon stimulation (42–45). This hypothesis is consistent with the biphasic increase in vasopressin-induced AQP2-mediated water permeability in collecting ducts (46) and MDCK cells,[2] because the latter process is much slower than the former. The study presented here is consistent with this hypothesis of two vesicle pools. The storage vesicles are detected as clear intracellular spots, as observed for AQP2-S256A, and wt-AQP2 and other AQP2 kinase mutants in unstimulated cells, whereas AQP2 in the recycling pool is detected as being localized in the apical membrane, as found for AQP2-S256D with or without stimulation or for wt-AQP2 in stimulated cells. The assumption that the latter protein cycles to and from the apical membrane is corroborated by the data that treatment of stimulated wt-AQP2 or AQP2-S256D cells with cytochalasin D, which affects the actin cytoskeleton, resulted in numerous vesicles distributed throughout the cytoplasm (not shown). The constitutively phosphorylated state of Ser-256, mimicked in AQP2-S256D, thus conveys the protein a strong tendency to localize in recycling vesicles instead of storage vesicles.

Of further interest is that apical cell surface biotinylation experiments revealed that, although AQP1 and AQP2-Asn-220, which is an AQP1 protein in which the C-tail is exchanged for that of AQP2, are already expressed in the apical membrane of MDCK cells without stimulation, forskolin still caused a nearly 2-fold increase in their apical membrane expression (47). In contrast, the reporter protein TMR-Plap (47) and AQP2-S256D (Fig. 4) did not show any increase in apical expression with forskolin. These data suggest that, within the continuous shuttling process between recycling vesicles and the plasma membrane, AQP2-S256D (and TMR-Plap) in unstimulated cells is preferentially localized in the apical membrane, in contrast to AQP1 and AQP2-Asn-220. Because the tendency of AQP2-S256D to localize in the apical membrane is continuously present, a slow uni-directional transport of AQP2-S256D from storage vesicles to the apical membrane and/or a strongly reduced endocytosis of AQP2-S256D from the plasma membrane following the 3 days of culturing, provides an explanation for the apical expression of AQP2-S256D in unstimulated cells, for which PKA tethering and cytoskeletal rearrangements might not be needed. Therefore, we believe that the apical membrane localization of AQP2-S256D is not dependent on the translocation machinery needed to shuttle wt-AQP2 from storage vesicles to the apical membrane but does reveal that, on a long term, phosphorylation of AQP2 is sufficient for apical membrane localization.

PMA-induced Endocytosis of AQP2 Occurs Independently of the Phosphorylation State of AQP2—In terminal IMCD segments, activation of the PKC pathway has been shown to counteract the AVP-induced AQP2-mediated water permeability by increasing the vesicular *versus* apical membrane localization of AQP2. In MDCK cells, this process could be mimicked by PMA treatment, because, following forskolin stimulation, this drug triggered the re-distribution of AQP2 proteins from the apical membrane to intracellular vesicles.

Three different types of experiments provided information on the role of phosphorylation of the putative AQP2 kinase sites in the PMA-induced PKC-mediated endocytosis of AQP2. First, wt-AQP2 and all kinase site mutants, with the exception of AQP2-S256A/S256D mutants, were targeted to intracellular vesicles without stimulation and were re-distributed to the apical membrane upon forskolin treatment (Figs. 3 and 4). This indicated that neither the constitutively phosphorylated state of the putative PKC site, nor the constitutively (de-)phosphorylated state of any CKII site, was sufficient to maintain a steady state vesicular localization upon treatment with forskolin. Second, PMA treatment did not result in the phosphorylation of any putative phosphorylation site, as shown by the lack of phosphate labeling of AQP2-S256A (Fig. 5). Third, PMA-forskolin treatment following forskolin stimulation re-distributed all kinase site mutants from the apical membrane to vesicles throughout the cell, which indicated the PMA-induced endocytosis overruled the apical membrane targeting triggered by Ser-256 phosphorylation. This was underscored by the finding that all kinase site mutants were still phosphorylated at Ser-256 to a high extent although they were located in vesicles (Fig. 6).

Our data, therefore, strongly suggest that a PKC-induced retrieval of AQP2 from the apical membrane of collecting duct cells is a process that occurs independently of the phosphorylation state of AQP2. For Ser-256 in AQP2, this hypothesis is in line with data from Zelenina *et al.* (35), who showed that in isolated rat inner medulla prostaglandin E_2 induces internalization of AQP2 without decreasing the amount of PKA-phosphorylated AQP2. In collecting ducts, vasopressin is known to induce a de- and re-polymerization of the actin cytoskeleton (48), and cytochalasins, which disrupt actin filaments, markedly inhibit the vasopressin response in target epithelia (49). In addition, it has been shown that Rho, which belongs to a family of proteins involved in the regulation of F-actin polymerization, inhibits the translocation of AQP2 to the plasma membrane in

[2] P. M. T. Deen, unpublished data.

cultured cells (40, 41). Because PKC activation has been shown to disintegrate the actin cytoskeleton in confluent MDCK monolayers (14, 50), it is therefore most likely that the inhibitory effect of PMA-activated PKC pathways on vasopressin-induced AQP2 translocation to the apical membrane is conveyed through cytoskeletal rearrangements.

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The Role of Putative Phosphorylation Sites in the Targeting and Shuttling of the Aquaporin-2 Water Channel

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