Regulation of the water channel aquaporin-2 by posttranslational modification

Hanne B. Moeller, Emma T. B. Olesen, and Robert A. Fenton

The Water and Salt Research Center, Department of Anatomy, Aarhus University, Aarhus, Denmark

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Moeller HB, Olesen ET, Fenton RA. Regulation of the water channel aquaporin-2 by posttranslational modification. Am J Physiol Renal Physiol 300: F1062–F1073, 2011. First published February 11, 2011; doi:10.1152/ajprenal.00721.2010—The cellular functions of many eukaryotic membrane proteins, including the vasopressin-regulated water channel aquaporin-2 (AQP2), are regulated by posttranslational modifications. In this article, we discuss the experimental discoveries that have advanced our understanding of how posttranslational modifications affect AQP2 function, especially as they relate to the role of AQP2 in the kidney. We review the most recent data demonstrating that glycosylation and, in particular, phosphorylation and ubiquitination are mechanisms that regulate AQP2 activity, subcellular sorting and distribution, degradation, and protein interactions. From a clinical perspective, posttranslational modification resulting in protein misroutting or degradation may explain certain forms of nephrogenic diabetes insipidus. In addition to providing major insight into the function and dynamics of renal AQP2 regulation, the analysis of AQP2 posttranslational modification may provide general clues as to the role of posttranslational modification for regulation of other membrane proteins.

POSTTRANSLATIONAL MODIFICATIONS (PTMs) are usually highly dynamic processes that alter the properties of the target protein. PTM of one or more amino acids within any given protein can occur by either 1) biochemical modification, e.g., addition of phosphate, lipids, carbohydrates; or 2) covalent attachment of short modifier proteins such as ubiquitin, small ubiquitin-like modifier (SUMO), or ubiquitin-like proteins (UBLs) (19). PTMs can modulate the activity of eukaryotic proteins and play a major role in intracellular signaling, protein maturation, and folding (57). PTMs can regulate proteins by altering function or activity, localization, stability, or interactions with other proteins (19, 57). Often, one particular type of PTM can influence the likelihood that neighboring amino acids undergo further PTM (27).

This review focuses on PTMs of the arginine vasopressin (AVP)-regulated water channel aquaporin-2 (AQP2). AQP2 is expressed in the principal cells of the kidney collecting duct (e.g., 5, 10, 72, 73, 98). In the absence of AVP, AQP2 is localized in a vesicle population at the subapical regions of the cell (72). In contrast, stimulation with AVP results in a predominant apical membrane localization of AQP2 (72). This renders the apical membrane highly permeable to water and is a key event in the formation of concentrated urine and thus in the regulation of body water balance. The antidiuretic actions of AVP occur predominantly via binding of this peptide hormone to the basolateral type 2 G-protein-coupled vasopressin receptor (V2R) (22, 80). In a simplified overview, AVP binding initiates a signaling cascade involving increased levels of intracellular cAMP (63–67), increased intracellular calcium (13, 20, 90), AQP2 phosphorylation (11), and subsequent AQP2 redistribution to the plasma membrane (72).

Although this review concentrates on the PTMs of AQP2 in the kidney, AQP2 is also expressed in other nonrenal epithelia: the colon (17, 26), the vas deferens of the male reproductive tract (71, 91), and the inner ear (48, 75, 88). In these tissues, regulation of AQP2 membrane targeting and/or abundance is different, implying that AQP2 can be regulated in different ways depending upon the cell type in which it is expressed. For example, in the inner ear AQP2 expression is regulated by AVP, whereas in the vas deferens AVP does not alter AQP2 abundance, and AQP2 is inserted into the apical plasma membrane via a nonregulated, constitutive pathway (91).

In the kidney, the trafficking of AQP2 (5, 10, 72, 73, 98) and the potential role of PTMs, e.g., phosphorylation, have been the focus of numerous studies. The precise mechanisms underlying this phenomenon are slowly being unraveled (50), but several fundamental questions remain unresolved. This review summarizes recent advances in the understanding of AVP-mediated regulation of AQP2 by PTM with major emphasis on phosphorylation, glycosylation, and ubiquitination.

Role of Protein Phosphorylation

Protein phosphorylation and dephosphorylation are common dynamic posttranslational processes often involved in regulation of protein function and cellular distribution (19). Of particular interest for this review, multisite phosphorylation forms a path for more than one phosphorylation event to take effect within a single protein. Multisite phosphorylation has been described to be “a key mechanism for achieving signal...
integration in a cell” and can play a major role in determining strength and duration of a response (16).

The role of phosphorylation in the regulation of AQP2 has been a major focus of several studies. It should be emphasized that although dephosphorylation is likely a key event in the modulation of AQP2, this process is less well described in the literature. However, increased AQP2 at the cell surface has been demonstrated in the presence of the phosphatase inhibitor okadaic acid (97), and the presence of endogenous phosphatase activity, which dephosphorylates AQP2, was observed in purified endosomes from the apical rat inner medullary collecting duct (IMCD) highly enriched in AQP2 (51). These events may be mediated by protein phosphatase 1c (PP1c), which has been shown to directly interact with the COOH-terminal tail of AQP2 (62, 106).

Protein Kinases

The complete set of protein kinases encoded by the genome of an organism is known as its kinome. For example, there are >500 different protein kinases in the human kinome, each with a specific substrate and cellular specificity (12, 30, 58). In eukaryotic cells, these various kinases can be grouped by their specific ability to catalyze phosphorylation on serine/threonine or tyrosine residues (82). AQP2 contains numerous putative phosphorylation sites for various protein kinases, e.g., PKA, PKG, PKC, and casein kinase II (10). Many of these sites are based upon bioinformatic analysis, which is useful for predicting consensus recognition sequences for various kinases. However, for a protein kinase to play a role in modulating channel function it must fulfill several criteria other than sequence recognition: 1) the kinase must be present in the same cell-type as the target protein; 2) the kinase must colocalize with the target protein; and 3) the kinase must be active under particular conditions, e.g., AVP stimulation. Phosphorylation of the serine at position 256 (S256) in the COOH-terminal tail of AQP2 was the first to be identified (see below) and, due to numerous studies, is the best characterized phosphorylation site of AQP2. In 2006, large-scale phospho-proteomic analysis demonstrated that this S256 phosphorylation site is part of a polyphosphorylated region in the COOH-terminal tail of AQP2 (see Fig. 1A) (36). In addition to S256, the polyphosphorylated region encompasses S261, S264, and S269.

S256-AQP2 is a target for PKA-induced phosphorylation, although other kinases may also target this residue (see below). Studies of LLC-PK1 cells treated with cpt-cAMP and metabolically labeled with phosphate demonstrated an increase in phosphorylation of AQP2, most likely at the S256 residue (25), which was supported by in vitro findings from other groups (44). Direct evidence of PKA-induced S256 phosphorylation was provided by in vitro phosphorylation assays of AQP2 COOH-terminal peptides (34). These assays also demonstrated that PKA alone cannot phosphorylate the downstream sites at S261, S264, and S269 (34).

Kinases other than PKA may be involved in AQP2 COOH-terminal phosphorylation. S256 is a substrate for Golgi casein kinase 2, and phosphorylation by this kinase is required for transition of AQP2 through the Golgi (84). PKG is another AGC kinase family member (like PKA) proposed to modulate AQP2 trafficking; however, its role remains controversial. The agonist of PKG, cGMP, has been shown to mediate translocation of AQP2 to the plasma membrane in AQP2-transfected LLC-PK1 cells and in isolated kidney slices (7). In the same study, PKG was able to phosphorylate the COOH-terminal tail of AQP2 in vitro, although the specific target site for phos-

For the remainder of the manuscript, the phosphorylation site is indicated by the single amino acid code and the amino acid number, e.g., S256, S261, etc.

Fig. 1. Illustration of the topology of aquaporin-2 (AQP2) and time course studies of AQP2 COOH-terminal phosphorylation. A: schematic illustration of the topology of AQP2 and the COOH-terminal phosphorylation sites of AQP2. Reproduced from Ref. 83. B: time course of changes in AQP2 phosphorylation at S256, S261, S264, and S269 in response to 1 nM dDAVP in rat inner medullary collecting duct (IMCD) tubule suspensions (modified figure). This research was originally published in Ref. 34. (J Biol Chem 283: 24617–24627, 2008. © The American Society for Biochemistry and Molecular Biology).
phosphorylation was not addressed. Activators of the cGMP pathway, such as atrial natriuretic peptide (ANP) or L-arginine, also induced AQP2 translocation to the plasma membrane in mouse cortical collecting duct (mpkCCD) cells (6). Combining these data with the finding that the cGMP phosphodiesterase type 5 (PDE5) inhibitors sildenafil citrate (Viagra) or 4-([3',4'-methylenedioxybenzyl]amino)-6-methoxyquinazoline elevates intracellular cGMP levels (8) resulting in plasma membrane accumulation of AQP2 suggests a positive role of PKG on AQP2 trafficking. However, in cultured IMCD cells, stimulating the cGMP/PKG pathway resulted in decreased S256-AQP2 phosphorylation, reduced AQP2 in the plasma membrane, and reduced IMCD cell swelling following hypotonic shock (46). Thus it appears that PKG may modulate AQP2 function differently in various regions of the kidney.

The protein kinases responsible for S261, S264, and S269 phosphorylation are currently debatable. In vitro phosphorylation of peptides suggests that S261 could be a target for JNK1, p38α, CDK1/cycB, and CDKS/p25NCK (36, 85). With respect to the specific kinase involved in S269 phosphorylation, it has been suggested that the last amino acids of the COOH-terminal tail are within a putative PKA site (36) or S269 could potentially be acted upon by PKG (45). Due to its involvement in AQP2 membrane accumulation (see below), identifying the kinases responsible for phosphorylation at S269 is likely to be an important step in future work.

Regulation of AQP2 COOH-Terminal Polyphosphorylated Region

The use of phospho-specific antibodies and mutational analysis of AQP2 in cell lines and oocytes has been a key component in examination of AQP2 phosphorylation (e.g., Ref. 11). In mutational studies, serine or threonine residues within AQP2 have been substituted using alanine (A), aspartic acid (D), or glutamic acid (E). The rationale for this analysis is based on 1) the assumption that replacement of serine or threonine residues with alanine prevents phosphorylation, and 2) the assumption that glutamic acid or aspartic acid mimics the structure and charge of a phosphorylated serine or threonine residue. In contrast to alanine substitution, glutamic acid or aspartic acid substitutions have the potential to directly assess the effects of modification at particular sites without the need for additional phosphorylation in vitro or in cells. However, in an analysis of results from mutational analysis or “phospho-mimics,” it is important to understand the limitations of the system, such as alterations in primary amino acid sequence and possible conformational changes.

In IMCD tubule suspensions, the use of phospho-specific antibodies demonstrated that the specific V2R agonist dDAVP (or addition of exogenous cAMP) increased phosphorylation of AQP2 at S256, S264, and S269 (see Fig. 1B) (34). Phosphorylation of S256 increases initially, which is followed by the downstream phosphorylation events. Maximal phosphorylation at S256 occurs within 60 s, whereas it takes longer for maximal phosphorylation to occur at the other sites. Interestingly, rather than being a transient event, phosphorylation at the S256, S264, and S269 sites remains high as long as the agonist is present (34). In 32P-labeled rat renal papilla, S256 phosphorylation also increased in response to dDAVP (74).

A method for estimating the abundance (relative quantification) of AQP2 phosphoforms at S256, S264, and S269 in the presence and absence of dDAVP in both rat IMCD and cultured mpkCCD cells was recently established (102). In these studies, the baseline level of phosphorylated S256 was constitutively high and did not significantly increase after dDAVP treatment. However, under the same conditions, large increases in pS269 abundance in response to dDAVP were observed (102). This data suggested that high levels of S256 phosphorylation do not only/necessarily depend on high levels of AVP (102). Furthermore, immunohistochemistry of kidney sections from AVP-deficient Brattleboro rats revealed that phosphorylated S269 was only found after dDAVP treatment, indicating that AVP is essential for the presence of this phosphoform (60). In contrast to the effects observed on S256, S264, and S269, dDAVP stimulation results in decreased phosphorylation at S261 (34).

The use of mutated AQP2 expressed in polarized kidney epithelial cell lines suggests that the complicated process of AQP2 COOH-terminal polyphosphorylation occurs as a hierarchical event, with S256 phosphorylation being required/strongly facilitating phosphorylation of S264 and S269 (34). This is based on the observation that the S264 and S269 phosphoforms of AQP2 are not observed in cells expressing the 256A mutated form of AQP2, even when means are taken to increase intracellular cAMP (34). In support of this, immunohistochemistry of kidney sections from a mouse model for congenital progressive hydronephrosis (CPH), with a mutation of S256 to leucine (L) preventing S256 phosphorylation, could not detect either the S264 or the S269 phosphoforms even after dDAVP treatment (Fig. 2) (34). These in vivo observations add to the evidence that S256 phosphorylation is a priming event for phosphorylation of S264 and S269 (34, 61).

Intracellular Localization of AQP2 Phosphoforms

Often, the specific intracellular distribution of a protein can provide clues to that particular protein’s overall function. Phosphorylated forms of a protein are likely to have an even narrower cellular distribution. This fundamental idea has led to the examination of the intracellular localization of AQP2 phosphoforms in vitro as well as in vivo using phospho-specific antibodies (see Fig. 3). In kidney sections from various animal models, application of these phospho-specific antibodies has revealed distinct subcellular pAQP2 distributions. Most notably, it was demonstrated by light microscopy, confocal microscopy, and immunogold electron microscopy that phosphorylated S269-AQP2 (pS269-AQP2) is not observed in any intracellular compartments but detected exclusively in the apical plasma membrane of the principal cell of the collecting duct (60).

pS256-AQP2 is detected in both intracellular vesicles and the apical plasma membrane in the collecting duct, where its abundance is increased in response to AVP treatment (15). It must be emphasized that although regulated by AVP, AQP2 phosphorylated at S256 is detected in nonstimulated Brattleboro rats, indicating that even in low levels of circulating AVP, S256 is highly phosphorylated (15).

pS261-AQP2 is predominantly localized within the cell in compartments different from the endoplasmic reticulum, Golgi, and lysosomes (35). The majority of pS264-AQP2
resides in plasma membrane-associated compartments and early endocytic pathways. In addition, in rats pS264-AQP2 increases in abundance in both the apical and basolateral plasma membrane of principal cells following acute dDAVP treatment (23). Whether this is due to increased pS264-AQP2 trafficking to the basolateral membrane or increased phosphorylation of AQP2 at this residue within the membrane remains undetermined.

Studies of various AQP2 mutants in cell lines have demonstrated that the phosphorylation-deficient form, S256A-AQP2, is predominantly located within the cell even in the presence of forskolin (44, 84, 99). Contrastingly, the phospho-mimicking S256D-AQP2 form is predominantly located in the plasma membrane (70, 99) in the absence of stimulation. These data suggest that S256 phosphorylation is essential for accumulation of AQP2 in the plasma membrane. Similar to the S256D

Fig. 2. Immunohistochemistry of AQP2 in a mouse model of hydronephrosis on the basis of a mutation of S256 to leucin (L) in the gene for AQP2 demonstrates that phosphorylation of AQP2 at S264 and S269 depends on phosphorylation at S256 in vivo. Immunohistochemistry of inner medulla from wild-type control and S256L-AQP2 cph mice using total AQP2 antibody (top row), and phospho-specific antibodies against pS261-AQP2 (second row), pS264-AQP2 (third row), and pS269-AQP2 (bottom row). pS264- and pS269-AQP2 are not observed, even after dDAVP administration, in kidney sections from mice with an AQP2-S256L mutation. Collecting duct lumen in nonlabeled tubules is indicated by an asterisk.
mutation, S269D-AQP2 is predominantly localized to the plasma membrane in basal, unstimulated conditions. However, in contrast to S256A-AQP2, S269A-AQP2 retained the ability to accumulate in the plasma membrane in response to forskolin treatment (34). Taken together, these observations strongly suggest a role for both S256 and S269 in membrane accumulation of AQP2. With respect to other phosphorylation sites, S261A-, S261D-, S264A-, and S264D-AQP2 all translocate from an intracellular localization to a predominant localization in the plasma membrane in response to forskolin treatment in Madin-Darby canine kidney (MDCK) (62) or LLC-PK1 cells (56), suggesting that these sites play minimal roles in AQP2 plasma membrane targeting.

**Roles of AQP2 Phosphorylation**

The COOH-terminal phosphorylation sites in AQP2 are highly conserved between species (Fig. 4), suggesting essential functions. In theory, phosphorylation can regulate AQP2 in numerous ways. Below, we discuss the proposed roles of AQP2 phosphorylation for channel gating and membrane accumulation (endocytosis and exocytosis). We also briefly address other cellular processes suggested to involve AQP2 phosphorylation.

**Channel gating:** phosphorylation-dependent regulation of AQP2 water permeability. In some cases, permeability properties of AQPs have been reported to be regulated by gating. In plant aquaporins, gating by conformational changes have been described, e.g., phosphorylation of SoPIP2;1, an aquaporin present in the spinach plasma membrane, regulates the permeability properties of the channel, likely due to channel gating (38, 94). In mammalian cells, AQP0 is gated by phosphorylation (32), and phosphorylation-mediated gating of AQP4 has been suggested (29, 105). Thus it is plausible that phosphorylation of the COOH-terminal tail of AQP2 could be involved in gating of the channel, leading to direct changes in water transport function; however, alternative studies on this using different systems, or similar systems from different research groups, have failed to unanimously agree. In particular, the effect of PKA-induced phosphorylation of S256 on gating has been examined. It has been reported that PKA phosphorylation at S256 of AQP2 reconstituted in proteoliposomes enhances the water permeability ($P_f$) of the channel compared with WT-AQP2 (21). A similar $P_f$ was observed for a S256D-AQP2 mutant, whereas WT-, S256A-, S261A- and S261D-AQP2 $P_f$ did not differ (21). An earlier study in oocytes also suggested that AQP2-mediated $P_f$ could be directly regulated by cAMP.
Although direct measurements of plasma membrane AQP2 abundance compared with $P_f$ was not assessed. In contrast, earlier studies of apical endosomes from rat IMCD cells containing AQP2 did not indicate a role for PKA-mediated phosphorylation of AQP2 in $P_f$ (51). Studies of oocytes injected with S256D-AQP2 indicated similar $P_f$ and membrane abundance compared with WT-AQP2 (40). In a recent, comprehensive analysis of relative unit $P_f$ of AQP2 in oocytes, there was no evidence that S256A-, S261A-, S264A/D-, and S269A/D-AQP2 directly alter the water transport function of AQP2 (61). Taken together, the role of phosphorylation-mediated AQP2 gating remains debatable. In our opinion, the use of a “nonphysiological” proteoliposome system that is free of interacting proteins for studying AQP2, which interacts with numerous other proteins depending on its phosphorylation status (see below), is unreliable. In the renal principal cell, no protein ever exists alone and its physiological function is influenced by several other proteins. The controversy regarding the gating of AQP2 is likely only to be resolved with direct measurements of AQP2 membrane abundance and $P_f$ in mammalian cells. Alternatively, a crystal structure of AQP2 in various phosphorylated states would be a way, at the atomic level, to determine whether COOH-terminal phosphorylation is involved in gating of AQP2.

**Exocytosis.** Phosphorylation of S256 is considered a critical event in AVP-induced AQP2 trafficking to the plasma membrane and membrane accumulation of AQP2 (25, 40, 44). Trafficking of AQP2 can also occur as a constitutive event in the absence of AVP, or when S256 is replaced by an alanine residue that prevents phosphorylation at this position (25, 54, 81, 87, 92). Thus AQP2 membrane targeting can occur independently of S256 phosphorylation. Nunes et al. (81) proposed that AQP2 can reach the apical membrane as a result of two different mechanisms/pathways: 1) AVP-induced accumulation of AQP2 at the cell surface facilitated by S256 phosphorylation and 2) insertion of AQP2 in the membrane as a result of constitutive recycling.

AQP2 phosphorylation can directly influence membrane targeting of AQP2 (77). In an elegant model proposed by Noda et al. (77), AQP2 itself is able to critically regulate local actin depolymerization and subsequent exocytosis of AQP2. In this model, the direct binding of AQP2 to G-actin (77, 79) is reduced via PKA-induced phosphorylation of AQP2 at S256. Following phosphorylation, the affinity of AQP2 to tropomyosin 5b (TM5b) (78) is increased. This phosphorylation-dependent interaction results in a reduction in the quantity of TM5b bound to F-actin, an event resulting in F-actin destabilization and allowing translocation of AQP2 vesicles to the plasma membrane (see Fig. 5) (77).

**Endocytosis.** It is likely that AQP2 internalization also occurs as both a constitutive and a regulated process, although the molecular basis for regulated internalization is poorly understood. Although phosphorylation at S256 is important for AVP-stimulated accumulation of AQP2 in the apical plasma membrane, under specific conditions, internalization can possibly occur even when this position is phosphorylated (70, 104). This process may be dependent upon PKC activation (99).

In cell lines, the subcellular localization of S256D- and S269D-AQP2 under control conditions, in combination with the exclusive apical plasma membrane localization of pS269-AQP2 in collecting duct cells, suggested a role for these phosphorylation sites in the regulation of AQP2 membrane abundance. Recently, a biotin-based internalization assay of multiple mutated AQP2-expressing cell lines demonstrated that mimicking phosphorylation of S256 and S269 significantly slows down the internalization rate of AQP2 compared with wild-type AQP2 (62). In contrast, internalization rates of all

Fig. 5. Proposed model of AQP2 trafficking to the membrane resulting from direct interaction with G-actin. A: in basal conditions, AQP2 interacts with G-actin. B: upon vasopressin-stimulated cAMP signaling, PKA is activated and phosphorylates AQP2. AQP2 phosphorylation dissociates G-actin from AQP2. C: this promotes AQP2 association with TM5b. In this way, TM5b is sequestered from F-actin by phosphorylated AQP. This induces destabilization of the F-actin network, allowing efficient transport of AQP2 toward the apical membrane. This figure, originally published in Ref. 77, is used by permission of Rockefeller University Press.
other mutated forms of AQP2 did not differ significantly from wild-type AQP2.

Thus it appears that AVP stimulation increases AQP2 membrane accumulation by both 1) increasing insertion of AQP2 in the membrane, a process facilitated/depending on S256 phosphorylation; and 2) decreasing endocytosis of AQP2, resulting from S256 and/or S269 phosphorylation of AQP2 inserted in the membrane due to either regulated or constitutive trafficking. As AQP2 recycling occurs at high rates (54), the contribution of decreased internalization may contribute significantly to the total AQP2 membrane abundance. In line with this, it was proposed by Nunes et al. (81) that S256 plays a more pronounced role in decreasing AQP2 internalization rates than AQP2 exocytic events.

Other cellular processes regulated by AQP2 phosphorylation. Within the plasma membrane, phosphorylation may be involved in determining the “active structure” of AQP2. Aquaporins can oligomerize and are usually present as homotetramers in the plasma membrane (89, 100). Using AQP1 as a model, each monomer within a tetramer constitutes an independent water channel (68). Some aquaporins are present in higher-order oligomers in the membrane, e.g., certain isoforms of AQP4 form square arrays that have been suggested to affect their P$_i$ properties (24). Based on analysis of oocytes coinjected with various ratios of S256A- and S256D-AQP2, it has been proposed that three of four monomers within an AQP2 tetramer are required to be phosphorylated at S256 for its plasma membrane localization (40). It must be emphasized that this conclusion is based on the assumption that random assembly of the tetramers occurs and results in expression of three S256Ds to one S256A, independently of phosphorylation. Whether this is truly the case is not known. In MDCK cells, it was demonstrated that AQP2 tetramers are likely formed in the endoplasmic reticulum or early after protein synthesis (33). The functional role of tetramerization or possible higher order oligomerization of AQP2 in the membrane is unknown.

Phosphorylation may also play a role in AQP2 degradation. Using cyclohexamide to block protein synthesis, the rate of AQP2 degradation in various AQP2 mutants has been examined (62). In these studies, 256A-AQP2 had the lowest protein half-life compared with wild-type AQP2, whereas both the mutants 256D- and 269D-AQP2 had increased half-life. Thus phosphorylation of these sites decreases AQP2 degradation, either directly, or more likely, due to the decreased rate of internalization observed for these mutants.

In addition to the importance of AQP2 in the regulation of body water homeostasis by participation in concentrating the urine, it has also been suggested that AQP2 and phosphorylation of the protein play a role in the regulation of cell volume recovery after tonicity-induced cell shrinkage (53). Furthermore, AQP2 has been shown to be involved in cell motility and cell-cell adhesion via its interactions in the second external loop with integrins (103). Whether regulated phosphorylation plays a role in this process is not currently known.

Ubiquitination of AQP2

Compared with phosphorylation, ubiquitination is a more complex PTM whereby small proteins (ubiquitins), expressed in virtually all types of cells, are covalently attached to a target protein by a process involving a hierarchical set of three enzymes (27). A ubiquitin-activating enzyme, E1, activates ubiquitin. Next, the activated ubiquitin is transferred to a ubiquitin-conjugating enzyme, E2. Finally, a ubiquitin ligase E3 catalyzes the transfer of ubiquitin from E2 to the target protein. Attachment of ubiquitin can affect proteins in numerous ways, e.g., promote endocytosis, degradation, and at the molecular level affect binding of interaction partners. Similar to phosphorylation, ubiquitination can be induced by various stimuli and is also a reversible process involving deubiquitination enzymes (DUBs) (101). Adding to the complexity, ubiquitin can be attached to the target protein as monomers or as polyubiquitin chains, which likely generate the diversity in ubiquitin signaling (101). Interestingly, it has been described that ubiquitination often requires protein phosphorylation that allows recognition of the substrate by the ubiquitinating enzymes (27).

Involvement in AQP2 internalization. It has recently been reported that AQP2 undergoes short-chain ubiquitination with two to three moieties on the COOH-terminal lysine residue K270. This ubiquitination is suggested to mediate endocytosis (41). An obvious link between phosphorylation and ubiquitination to consider is that inhibition of ubiquitination may occur due to phosphorylation of S256-AQP2 and/or S269-AQP2, thus slowing down endocytosis of the water channel (62).

Kamsteeg and colleagues (41) demonstrated that ubiquitination of S256A-AQP2 was similar, or slightly lower, than unstimulated WT-AQP2. In contrast, ubiquitination of the S256D-AQP2 mutant, which is predominantly associated with the plasma membrane, was greater. These data suggest that ubiquitination takes place preferentially at the apical plasma membrane; although the greater levels of S256D-AQP2 ubiquitination seem to contradict the decreased internalization rate for this mutant. Two possible explanations for this observation exist. 1) It is possible that endocytic pathways alternative to ubiquitination are slowed down in S256D-AQP2. This possibility is supported by evidence from Kamsteeg et al. that ubiquitination is not an absolute requirement for AQP2 internalization. Thus it remains feasible that physiological, transient ubiquitination of AQP2 is a facilitator of AQP2 internalization, but additional downstream events are required to exert ubiquitination’s full effect. 2) If ubiquitination is considered a constitutive process taking place at the membrane at all times, then only membrane-bound proteins can be ubiquitinated. In S256D-AQP2 cells, the pool of AQP2 readily available for ubiquitination is much greater than in wild-type AQP2 or S256A-AQP2 cells. Therefore, it is plausible that phosphorylated S256-AQP2 actually inhibits ubiquitination, even though total levels of AQP2 ubiquitination are increased per se.

The results from that study (41) open many interesting questions of how internalization of AQP2 may be mediated and also raises many questions of how ubiquitination may occur in a regulated fashion is renal principal cells. Future studies, focusing on whether ubiquitination is an inducible process mediated by various hormones or rather is to be considered a constitutive process that can be inhibited will be informative. Additionally, identifying the possible DUBs involved in removing AQP2 ubiquitin moieties will also be a focus of future studies. For example, Staub and colleagues (9) identified an AVP-sensitive deubiquitinating enzyme (Usp10), which is in-
Involvement in regulation of protein abundance/degradation. The generation of a cell line expressing constitutively ubiquitinated AQP2 (AQP2-Ub) suggested that AQP2, when ubiquitinated, is automatically directed toward degradation pathways, thus indicating a role for ubiquitin in regulation of AQP2 degradation (41). Additionally, when the dominant nephrogenic diabetes insipidus (NDI)-causing mutant E258K-AQP2 was expressed in cells (42), AQP2 was misrouted to multivesicular bodies and lysosomes. Compared with wild-type AQP2, the E258K-AQP2 mutant was determined to be monoubiquitinated at K228, providing a possible explanation for this missorting of the protein. This study (42) suggests that the amino acid mutations observed in other forms of NDI, resulting in protein missorting or degradation, may be explained by alterations in ubiquitination.

Recently, a role of phosphorylation via p38-MAPK and polyubiquitination in the control of AQP2 protein degradation has been suggested (69). Although highly contrasting with several previous observations (e.g., Refs. 23 and 60), the authors found that short-term AVP or forskolin treatment is sufficient to increase AQP2 abundance in cultured IMCD cells due to decreased AQP2 degradation. AQP2 S261 phosphorylation was increased by PKA activation, via decreased phosphorylation of p38-MAPK, an effect mimicked by a p38-MAPK inhibitor. Furthermore, the inhibition of p38-MAPK resulted in decreased polyubiquitination of AQP2 and subsequent proteasomal degradation. One problem with the interpretation of the data in this study is that polyubiquitination and degradation of AQP2 are described as parallel events without using a model where polyubiquitination is inhibited. Thus it cannot be concluded directly that polyubiquitination is necessarily involved in controlling AQP2 levels. As hypertonicity-induced membrane targeting of AQP2 via p38-MAPK has also been shown (31), the role of S261 in the effects of hypertonicity on AQP2 distribution could be an interesting future objective.

In summary, we believe that it is difficult to draw conclusions with regard to the effect of Ps261 on polyubiquitination (69), and as yet no direct link has been established between phosphorylation and ubiquitination. For example, higher basal levels of polyubiquitination in S261D-AQP2 and the lower levels in S261A-AQP2 could possibly be explained by AQP2 accumulation in compartments where the protein has a higher vs. lower chance of polyubiquitination, an effect due to compartmentalization and not directly due to phosphorylation.

Glycosylation

The process of attaching both O- and N-glycans to extracellular domains of membrane proteins can be important for the apical location of these proteins (95). Some glycoproteins that are transported to the plasma membrane move though the Golgi and post-Golgi without further processing of high-mannose N-glycans. However, the majority of N-glycans are altered in the Golgi to become complex glycosylated proteins. N-glycosylation is the process of trimming, as well as elongation, of N-linked oligosaccharides and takes place in the Golgi apparatus. These oligosaccharides have various functions, including regulation of cell surface protein activity (86). AQP2 glycosylation. AQP2 undergoes complex N-glycosylation. However, examination of glycosylation in mammalian cell cultures is likely to be complicated by various factors, including the cell type used and cell culture conditions (37). As demonstrated for other membrane proteins, e.g., NHE3, glycosylation is highly species specific (4). The extent of AQP2 glycosylation varies considerably between the different cell culture systems and animal models examined. In our analysis of immunoblotting data presented for mouse (62) and human AQP2 (18) in MDCK cell culture studies, or rat AQP2 expressed in LLC-PK1 cells (porcine cells) (25), glycosylation was low or absent. However, our interpretation of this and other data is complicated because not all publications show full-length immunoblots. Contrastingly, in mpkCCD cells (derived from mouse collecting duct) (47, 52) or in a human cell line transfected with rat-AQP2 (96), both the nonglycosylated and the glycosylated forms of AQP2 are observed. Taken together, the variations in glycosylation levels suggest that this process not only varies between species, maybe as a result of differences in the composition or expression of glycosylating enzymes, but is potentially also a result of other variations, e.g., culture conditions.

In oocytes, unglycosylated (29 kDa) and high-mannose glycosylated (32 kDa) AQP2 mutants (representative of AQP2 mutations in recessive NDI) are often observed (59). These mutants are often retained in the endoplasmic reticulum (59) due to a defect in maturation or folding of the protein.

Role of AQP2 glycosylation. The functional role of AQP2 N-glycosylation is poorly understood. It is clear from studies of P1 in oocytes that glycosylation does not affect the biophysical function of AQP2. In two different studies, oocytes injected with cRNA encoding a constitutively nonglycosylated mutant of the water channel had comparable P1 to oocytes injected with wild-type AQP2 (1, 33). Thus, in oocytes, glycosylation is apparently not necessary for protein folding and water transport per se. Another study showed that treatment with tunicamycin, an inhibitor of N-glycosylation, prevented the stimulatory effect of dDAVP on AQP2 in MDCK cells (2). The authors point out, however, that this general inhibition of N-glycosylation could affect the dDAVP-induced intracellular signaling pathways at several different sites. In contrast studies with direct cAMP stimulation, tunicamycin did not affect P1 or apical cell surface expression of AQP2. It was therefore concluded that glycosylation did not affect membrane targeting and function of AQP2 (3).

Concerns regarding the unpredictability of tunicamycin treatment, i.e., direct vs. nondirect effects on AQP2, caused the issue of AQP2 glycosylation to be revisited using a different approach of transfecting MDCK cells with an AQP2 mutant, N123Q-AQP2, which cannot be glycosylated (33). AQP2 was not detected in biotinylated protein samples from these cells, and immunocytochemistry showed a clear perinuclear localization of the protein. Colocalization studies and immunogold electron microscopy were performed and revealed localization within the Golgi complex. This study (33) concludes, therefore, that glycosylation is necessary for exit of the water channel from the Golgi complex and for membrane targeting of the protein. However conclusive this seems, some questions remain as to the requirement for glycosylation in the trafficking of AQP2. One study showed that the glycosylated form was not observed in LLC-PK1 cells
transiently transfected with AQP2, but these cells were able to respond to cAMP with increased osmotic P1 and membrane targeting of AQP2 (25). Therefore, it appears that in certain cell types, AQP2 traffics to the plasma membrane without the requirement for glycosylation.

PTMs: Regulating Interaction with Other Proteins

PTMs can directly alter the interaction of a protein with other proteins in its immediate vicinity. For example, since phosphorylation can change hydrophobic regions of a protein into polar (negatively charged) or hydrophilic, one possible hypothetical mechanism of action of phosphorylation is by alteration of “recognition domains.” Phosphorylation could generate an overall change in charge state or induce structural changes in a protein. These changes may serve to mask or demask such recognition domains for interaction. Structural changes could also in themselves result in changed functionality. The idea that a change in charge of the COOH-terminal tail of AQP2 can critically affect AQP2 sorting was recently supported by the identification of a COOH-terminal motif (RRxxxxKx35L) known to cause NDI (43). This mutation results in misrouting of AQP2, possibly due to increased endocytosis or lysosomal targeting.

One concept behind the reduced AQP2 endocytosis is that phosphorylation alters the binding affinity and interaction of AQP2 with other proteins that subsequently are involved in sorting or trafficking of AQP2 (34). A number of phosphorylation-dependent AQP2 interactions have been recently reported, including hsp70 and annexin-2 (55, 106). Cell studies of S256A-AQP2 revealed increased binding to annexin-2 (62), and accordingly this protein was also found to preferentially bind to a nonphosphorylated AQP2 COOH-terminal peptide (106). Annexin-2 is a Ca2+-dependent protein involved in organizing membrane domains (28). Annexin also plays a role in exocytosis, and it has been demonstrated that inhibition of annexin function impairs the water permeability of cultured cells (93). Another example is myelin- and lymphocyte-associated protein (MAL), which is localized to the apical membrane of the principal cells. MAL preferentially interacts with S256 phosphorylated AQP2 (39). By attenuation of internalization, MAL was shown to increase the surface expression of AQP2 (39).

Using coimmunoprecipitation of various mutated AQP2 forms expressed in MDCK cells, various proteins involved in endocytic processes were significantly less abundant in pull-downs from S256D- and S269D-AQP2 expressing cells (62). Clathrin heavy chain and dynamin, both involved in clathrin-mediated endocytosis, interacted less with S256D- and S269D-AQP2 mutants, indicating that those forms are less abundant in clathrin-coated pits/vesicles. There is strong evidence that AQP2 endocytosis begins in clathrin-coated pits and blocking of clathrin-mediated endocytosis results in membrane accumulation of AQP2 (54, 92). Hsc70 is involved in uncoating of clathrin-coated vesicles and was previously shown to interact directly with the AQP2 COOH-terminal tail (55). In this study, the authors proposed that hsc70 binding potentiates AQP2 endocytosis and that S256 phosphorylation inhibits this process. In line with this, significantly reduced interaction of hsc70 and hsp70 with S256D and S269D-AQP2 was observed in MDCK cell lines (62). Thus decreased interaction with proteins of the endocytic machinery provides a possible explanation for the decreased endocytosis of S256D- and S269D-AQP2. It still remains to be addressed whether S269 phosphorylation alone can modulate AQP2 endocytosis.

Other mechanisms by which S269 phosphorylation (or any other phosphorylation site) could directly influence internalization is via modulation of AQP2 ubiquitination. Alternatively, phosphorylation could modulate the PDZ ligand in the final amino acids of AQP2 (GSKA) through which AQP2 is likely to associate with PDZ domain proteins. Sasaki and coworkers (76) suggested that trafficking of AQP2 depends on recognition of a COOH-terminal PDZ ligand by PDZ domain proteins. SPA-1 could be such an interactor (76).

Concluding Remarks and Perspectives

In this review, we have aimed to address the role of PTMs in regulation of AQP2 with a focus on phosphorylation, ubiquitination, and glycosylation. It is clear that AQP2 is subjected to diverse PTMs that may act synergistically or in competition in intracellular signaling processes to regulate the subcellular localization, trafficking, and function of AQP2.

Protein phosphorylation has been extensively studied. Data from many studies imply that phosphorylation is essential for plasma membrane accumulation of AQP2. However, many questions remain to be solved: 1) what kinases are responsible for S261, S264, and S269 phosphorylation?; 2) is the order of AQP2 phosphorylation upstream of S256 sequential?; 3) is there interaction between the individual phosphorylation sites?; 4) which phosphatases are involved in dephosphorylating AQP2 at specific residues?; and 5) can phosphorylation alter the structure of the AQP2 COOH-terminal tail? Importantly, the vast literature that has been accrued on the role of phosphorylation in regulation of AQP2 is likely applicable to various other membrane proteins.

Compared with phosphorylation, understanding the role of ubiquitination in AQP2 regulation is still in its infancy. Although initial studies suggest a role in AQP2 endocytosis and degradation, more studies are required to gain profound insight into the involvement of ubiquitination in AQP2 regulation: 1) which ubiquitin ligases are involved in AQP2 regulation?; 2) which DUBs are involved?; and 3) how is the ubiquitin/deubiquitin machinery regulated in the collecting duct principal cell?

The role of AQP2 glycosylation remains unclear, although it is likely that glycosylation plays a role in protein folding and maturation under normal conditions. Glycosylation of AQP2 is associated to the second extracellular loop of AQP2, in a similar region to where AQP2 interacts with integrin (103). Thus could glycosylated AQP2 be involved in cell-cell interactions or cell-extracellular interactions? Although speculative, it is possible that AQP2 in the apical plasma membrane could be regulated from the luminal side by, e.g., proteases (as is the case with ENaC), and that glycosylation plays a role in such a potential interaction.

Although not currently described, AQP2 may be a target for further PTMs (e.g., SUMOylation, acetylation) that may modulate AQP2 function. In the future, it is likely that high-throughput quantitative proteomic approaches will be utilized to further elucidate this. For example, proteomic approaches
could be used to examine whether AQP2 is acetylated (14), SUMOylated (57), or further ubiquitinated (57).

Finally, the true indication of the importance of any particular PTM for AQP2 is in its final biological/physiological adaptation of the role of AQP2 in urine concentration. Thus generation of knockout/knockin mice with point mutations for specific target sites causing lack of specific PTMs are essential to clarify this.

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