HUMAN MUTATION Mutation in Brief 30:E891–E903 (2009) Online

MUTATION IN BRIEF

HUMAN MUTATION

OFFICIAL JOURNAL
HGVS
HUMAN GENOME VARIATION SOCIETY
www.hgvs.org

p.R254Q Mutation in the Aquaporin-2 Water Channel Causing Dominant Nephrogenic Diabetes Insipidus Is Due to a Lack of Arginine Vasopressin-induced Phosphorylation

Paul JM Savelkoul[1#], Fabrizio De Mattia[1#], Yuedan Li[1], Erik-Jan Kamsteeg[1], Irene BM Konings[1], Peter van der Sluijs[2], and Peter MT Deen[1*]

[1]*Dept of Physiology, Radboud University Nijmegen Medical Center, Nijmegen, The Netherlands*
[2]*Dept of Cell Biology, UMC Utrecht, Utrecht, The Netherlands*

[#] Both authors contributed equally to this work

*Correspondence to Peter M.T. Deen, PhD, 286 Dept Physiology, Research Tower 7th floor, UMC St Radboud, PO Box 9101, 6500 HB Nijmegen. Phone: 31-243617347; Fax: 31-243616413. E-mail: p.deen@ncmls.ru.nl.

Grant Sponsor Information: See Acknowledgments

Communicated by Michel Goossens

ABSTRACT: Vasopressin regulates human water homeostasis by re-distributing homotetrameric aquaporin-2 (AQP2) water channels from intracellular vesicles to the apical membrane of renal principal cells, a process in which phosphorylation of AQP2 at S256 by cAMP-dependent protein kinase A (PKA) is thought to be essential. Dominant nephrogenic diabetes insipidus (NDI), a disease in which the kidney is unable to concentrate urine in response to vasopressin, is caused by AQP2 gene mutations. Here, we investigated a reported patient case of dominant NDI caused by a novel p.R254Q mutation. Expressed in oocytes, AQP2-p.R254Q appeared to be a functional water channel, but was impaired in its transport to the cell surface to the same degree as AQP2-p.S256A, which mimics non-phosphorylated AQP2. In polarized MDCK cells, AQP2-p.R254Q was retained and was distributed similarly to that of unstimulated wt-AQP2 or AQP2-p.S256A. Upon co-expression, AQP2-p.R254Q interacted with, and retained wt-AQP2 in intracellular vesicles. In contrast to wild-type AQP2, forskolin did not increase AQP2-p.R254Q phosphorylation at S256 or its translocation to the apical membrane. Mimicking constitutive phosphorylation in AQP2-p.R254Q with the p.S256D mutation, however, rescued its apical membrane expression. These date indicate that a lack of S256 phosphorylation is the sole cause of dominant NDI here, and thereby, p.R254Q is a loss of function instead of a gain of function mutation in dominant NDI.

KEY WORDS: water channel, AQP2, phosphorylation, PKA, membrane trafficking

INTRODUCTION

Water and salt balance in the body is maintained within strict boundaries. With hypernatremia or hypovolemia, vasopressin (AVP) is released from the pituitary gland and transported to the kidney where it binds to the

Received 16 April 2009; accepted revised manuscript 11 June 2009.

DOI: 10.1002/humu.21082

vasopressin-2 receptor (AVPR2) located in the basolateral membrane of collecting duct principal cells. This G-protein coupled receptor subsequently activates adenylyl cyclase to turn ATP into cAMP, which in turn increases protein kinase A (PKA) activity. PKA is then thought to phosphorylate several proteins, among which the aquaporin-2 (AQP2) water channel that resides in intracellular vesicles. Phosphorylation of at least three out of four monomers of an AQP2 tetramer at serine 256 (S256) is sufficient to redistributing AQP2 homotetramers from storage vesicles to the apical membrane of the principal cells, consequently rendering the cells permeable to water (Kamsteeg et al., 2000a; Van Balkom et al., 2002). Driven by the transcellular osmotic gradient, urinary water then enters the cells via AQP2 and leaves the cells via AQP3 and AQP4, which are constitutively expressed in the basolateral membrane. This solute-independent reabsorption of water from urine restores isotonicity and euvolemia, which leads to a diminished AVP release from the pituitary and re-distribution of AQP2 from the plasma membrane to intracellular vesicles (van Os and Deen, 1998; Nielsen et al., 1995).

Congenital nephrogenic diabetes insipidus (NDI), which is characterized by the inability of the kidney to concentrate urine in response to AVP, is caused by defects in the *AVPR2* (MIM# 300538) or the *AQP2* gene (MIM# 107777). Mutations in the *AVPR2* gene cause the X-linked recessive form of NDI, whereas autosomal recessive and dominant traits of NDI are caused by mutations in the *AQP2* gene (Rosenthal et al., 1992; Deen et al., 1994; Mulders et al., 1998; Robben et al., 2006; van den Ouweland et al., 1992). Expression studies revealed that nearly all missense AQP2 mutants encoded in recessive NDI are misfolded and retarded in the endoplasmic reticulum (ER). In contrast, AQP2 mutants identified in dominant NDI, including AQP2-c.761G>T (p.R254L), AQP2-c.772G>A (p.E258K), AQP2-c.721delG, AQP2-c.727delG, c.763_772del, c.812_818del, and AQP2-c.779_780insA, are properly-folded but missorted to different subcellular destinations depending on the mutation (Mulders et al., 1998; Kamsteeg et al., 2003; Kamsteeg et al., 1999; Kuwahara et al., 2001; Asai et al., 2003; Marr et al., 2002b; De Mattia et al., 2005). These mutants exert their dominant effect by inhibiting the proper sorting of wt-AQP2 to the apical membrane after the formation of heteroligomers (Kamsteeg et al., 1999; Asai et al., 2003).

A novel mutation in AQP2, p.R254Q, has been reported to result in dominant NDI in a 5 years old girl (pedigree reviewed but not shown; (Robertson and Kopp, 2002)). Sequencing revealed that this mutation was caused by a 761G>A substitution in one allele of the AQP2 gene, while no mutation was found in her *AVPR2* gene. This patient exhibited unusual thirst from birth, was noted to be polyuric at 18 months and was diagnosed DI at her 5 years age. There was no history of DI in her mother, father or only sibling (a 9 year old girl). Her physical examination, including height and weight were normal for her age (50th percentile). Her DI did not respond to standard therapeutic doses of desmopressin. On *ad libitum* intake of fluids, her basal plasma osmolality, sodium, potassium, urea and creatinine were within normal limits. However, her plasma vasopressin (Pvp) was slightly elevated (4.3 pg/ml), her urine osmolality (Uosmo) was very low (58 mOsmo/kgH_2O) and her 24 hour urine volume (Uvol) was high (280 ml/kg weight), indicating she had moderately severe NDI. When deprived of fluid for 5 hours, she developed mild hypertonic dehydration and raised her Pvp and Uosmo to 22.3 pg/ml and 220 mOsmo/kgH_2O. When rehydrated and given a supra-physiologic dose of desmopressin (6.3 µg intravenous in 20 minutes), her Uosmo rose to 258 mOsmo/kgH_2O after 2 hours, confirming a partial antidiuretic response to stimulation of the V2 receptors.

To identify whether AQP2-p.R254Q can be causal to NDI and to determine the underlying mechanism of this mutation in dominant NDI, we here expressed AQP2-p.R254Q in oocytes and polarized epithelial MDCK cells and analyzed these in detail.

MATERIAL AND METHODS

Constructs

The constructs encoding pT7Ts-AQP2, pT7Ts-AQP2-p.R187C, pT7Ts-AQP2-p.S256A, pT7Ts-wt-AQP2-F, and G-AQP2 were as described (Deen et al., 1994; Mulders et al., 1998; Kamsteeg et al., 1999; Marr et al., 2002a). The cDNA coding for AQP2-p.R254Q or AQP2-p.R254Q-p.S256D were made by introducing the mutations into pT7Ts-AQP2 (GenBank reference sequence: NM_000486.5) with the Altered Sites mutagenesis kit (Promega, Madison, WI) using the primers 5'- CGAGGTGCGACGTCAGCAGTCGGTGG-3' and 5'-CGAGGTGCGACGTCAACAGGA CGTGGAGCTGC-3', respectively. Nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence, according to journal guidelines (www.hgvs.org/mutnomen). The initiation codon is codon 1. Introduction of only

the desired mutations was confirmed by DNA sequence analysis. To generate pCB6-AQP2-p.R254Q or pCB6-AQP2-p.R254Q-p.S256D, AQP2-p.R254Q and AQP2-p.R254Q-p.S256D were digested from pT7Ts-AQP2-p.R254Q or pT7Ts-AQP2-p.R254Q-p.S256D with *Bgl*II and *Spe*I and ligated into the *Bgl*II and *Xba*I sites of the eukaryotic expression vector pCB6.

To generate FLAG-tagged AQP2 expression constructs, pBluescript-FLAG-wtAQP2 (Kamsteeg et al., 1999) was cut with *Not*I, blunted and digested with *Hin*dIII, and the FLAG-tagged AQP2 (F-AQP2) cDNA fragment was isolated. In pCB7, which is similar to pCB6, but contains a hygromycin instead of neomycine resistance gene, the *Bam*HI site was cut, blunted and re-ligated. Next, the F-AQP2 fragment was ligated into the blunted *Kpn*I site and the *Hin*dIII of pCB7-Δ*Bam*HI to yield pCB7-Δ*Bam*HI-F-AQP2. To generate pCB7-F-AQP2-p.R254Q, pT7Ts-AQP2-p.R254Q plasmids were digested with *Bam*HI and *Kpn*I, a 300 bp fragment isolated and cloned into the corresponding sites of pCB7-Δ*Bam*HI-F-AQP2.

Oocytes

The obtained pT7Ts constructs were linearized with *Sal*I. Transcription of pT_7T_s-constructs and analysis of their integrity was done as described (Mulders et al., 1998). cRNA injection of oocytes and analysis of their water permeability in a standard swelling assay were done as reported (Deen et al., 1994). Statistical significance was determined using the Student's T-test and was considered significant when $p<0.05$. Total and plasma membranes were isolated as described (Kamsteeg and Deen, 2001).

MDCK cells

MDCK cells were cultured and stably transfected with expression constructs using the calcium-phosphate precipitation technique as described (Deen et al., 1997a). Selection of G418-resistant clones and immunocytochemistry of transfected MDCK cells was done as described (Deen et al., 2002). MDCK cell lines stably expressing wt-AQP2 (wt-10), AQP2-p.S256A or GFP-tagged AQP2 were as described (Deen et al., 1997b; Marr et al., 2002a; Van Balkom et al., 2002).

Monoclonal antibodies against FLAG (m2) and Golgi 58K were purchased from Sigma (St Louis, MO, USA) and against early endosome antigen-1 (EEA1) from BD Transduction Laboratories (Lexington, KY, USA). Polyclonal antibodies against the Golgi marker proteins GOS28 and Giantin were obtained from Dr. Y. Ikehara, Fukuoka University School of Medicine and against the ER marker protein PDI from Ineke Braakman, University of Utrecht (Benham et al., 2000). Monoclonal antibody (clone AC17) against the late endosomal/lysosomal marker Lamp2 (Marr et al., 2002b) were kindly provided by Dr. Le Bivic, Marseille, France. Anti-rabbit and anti-mouse secondary antibodies coupled to Alexa 488 or 594 were purchased from Molecular Probes (Eugene, OR, USA). Images were obtained with a Bio-Rad confocal laser-scanning microscope (CLSM) using a 60x oil-immersion objective and a 3-fold magnification.

Transient transfections of MDCK type I cells have been performed using LipofectAMINE^{TM} 2000 reagent (Invitrogen, CA, USA) according to the manufacturer's protocol.

Immunoblotting, immunoprecipitation and densitometrical analysis

Immunoblotting, immunoprecipitations and orthophosphate labelling of MDCK cells were done as described (Van Balkom et al., 2002; Kamsteeg et al., 1999). To semi-quantify AQP2 amounts or the level of phosphorylation of AQP2-p.R254Q relative to that of wt-AQP2, immunoblot and [^{32}P]-orthophosphate signals were semi-quantified by measuring the integrated optical densities (IOD) of the film signals using the Image-Pro Plus analysis software (Media Cybernetics, Silver Springs, CO). These signals were compared with those of two-fold dilution series of wt-AQP2, which were blotted in parallel or separated by SDS-PAGE and autoradiographed, respectively. Background IOD values were determined at unexposed areas of the film and subtracted from obtained IOD values for the different proteins. To determine the relative amounts of phosphorylated AQP2-p.R254Q protein, the level of phosphorylation was normalized for the amount of immunoprecipitated AQP2 and expressed as a percentile of phosphorylated wt-AQP2 protein under unstimulated condition, which was set at 100%.

RESULTS

Functional analysis of AQP2-p.R254Q in oocytes

Several expression studies have shown that phosphorylation of AQP2 at S256 is essential for its AVP-induced translocation from vesicles to the plasma membrane, because AQP2-p.S256A, which mimics non-phosphorylated AQP2, is retained in vesicles (Fushimi et al., 1997; Nishimoto et al., 1999; Kamsteeg et al., 2000a; Van Balkom et al., 2002). R254 in AQP2 is part of the PKA phosphorylation consensus site (R-R_{254}-X-S). Earlier, we showed that AQP2-p.R254L likely causes dominant NDI, because of its retention in intracellular vesicles due to an impaired phosphorylation at S256 (De Mattia et al., 2005). We hypothesized that the mechanism underlying NDI for the present mutation, p.R254Q, would be similar.

To determine whether the identified missense mutation could be the underlying cause for NDI in this patient, it was introduced into the AQP2 cDNA sequence and transcribed. Then, oocytes were injected with cRNA encoding AQP2-p.R254Q, wt-AQP2 or AQP2-p.S256A. Determination of the water permeability (Pf) revealed that the Pfs of oocytes expressing AQP2-p.R254Q (25.9±4.2; n=8) and AQP2-p.S256A (22.4±12; n=8) from 1 ng injections were significantly higher than those of controls ($p=4x10^{-3}$), which showed that both mutants are functional water channels (Fig. 1A). To relate the obtained Pfs to the amount of expressed AQP2, total membranes and plasma membranes were isolated from the same batches of oocytes. Subsequent immunoblotting for AQP2 and densitometric scanning of the signals revealed that the expression levels of AQP2-p.R254Q and AQP2-p.S256A with 1 ng injections were similar and in between that of wt-AQP2 expressing oocytes, injected with 1 and 3 ng cRNA (Fig. 1B, TM). In the plasma membranes of these oocytes, however, the expression levels of AQP2-p.R254Q and AQP2-p.S256A were about 6-fold less than that found for wt-AQP2 (Fig. 1B, PM). Consistently, the total expression of AQP2-p.R254Q from 1 ng injections was higher than that of oocytes injected with 0.3 ng wt-AQP2 cRNA, but their plasma membrane expression levels were less. As reported for AQP2-p.S256A (Kamsteeg et al., 2000a), these data indicated that AQP2-p.R254Q and AQP2-p.S256A were impaired in their trafficking to the plasma membrane. The similar level of retention of AQP2-p.S256A and AQP2-p.R254Q could indicate that the molecular cause of their retention in oocytes is identical.

AQP2-p.R254Q heteroligomerizes with wt-AQP2

To determine whether AQP2-p.R254Q would form heteroligomers with wt-AQP2 and thereby exert a dominant negative effect as other AQP2 mutants in dominant NDI do (Kamsteeg et al., 1999; Kuwahara et al., 2001; Marr et al., 2002b; Kamsteeg et al., 2003), it was co-expressed with flag-tagged wt-AQP2 (F-AQP2) in oocytes and subjected to immunoprecipitation using Flag antibodies. Previously, we have shown that this N-terminal FLAG tag does not interfere with proper plasma membrane expression of wt-AQP2 (Kamsteeg et al., 1999). As a negative control, AQP2-p.R187C was taken along, which is an ER-retained AQP2 mutant in recessive NDI and does not heteroligomerize with wt-AQP2 (Kamsteeg et al., 1999). Two days after cRNA injections, membranes of oocytes expressing combinations of F-AQP2 with AQP2-p.R254Q or AQP2-p.R187C were solubilized and subjected to immunoprecipitation. Immunoblotting of total membranes using anti-AQP2 antibodies revealed clear expression of the 31 kDa band of F-AQP2, the 29 kDa bands of AQP2-p.R254Q and AQP2-p.R187C, and the 32 kDa high-mannose glycosylated band of AQP2-p.R187C, which migrates slightly slower than F-AQP2 (Fig. 1C, TM). Immunoblot analysis of the FLAG immunoprecipitates showed that AQP2-p.R254Q, but not AQP2-p.R187C, co-precipitated with F-AQP2 (Fig. 1C, IP). Also, no AQP2 was detected in the immunoprecipitates from oocytes expressing untagged AQP2 only, which showed that the immunoprecipitations were specific for Flag-tagged proteins (not shown). Therefore, from these results it could be concluded that AQP2-p.R254Q forms heteroligomers with wt-AQP2.

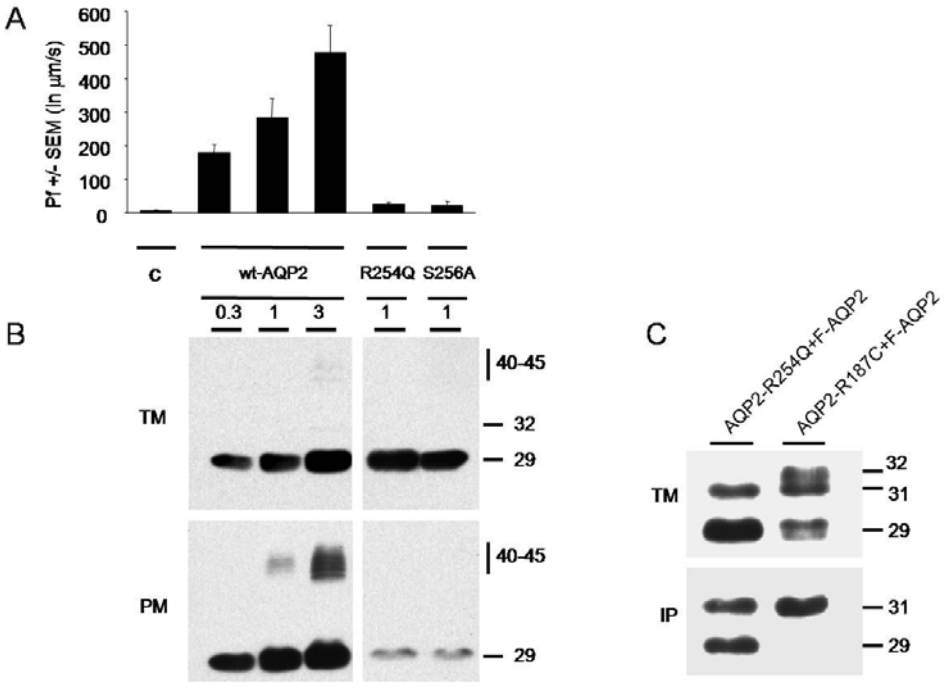

Figure 1. Expression of AQP2-p.R254Q in oocytes. **A)** Water permeability. Three days after injection of the indicated amounts of wt-AQP2, AQP2-p.R254Q or AQP2-p.S256A cRNA, oocytes were subjected to a standard swelling assay. Non-injected oocytes were taken as a control (C). Mean water permeabilities (P_f) and SEM of 8 oocytes are shown. **B)** AQP2 expression. From 12 oocytes injected as described above, total membranes (TM) or plasma membranes (PM) were isolated. Subsequently, equivalents of one (TM) or four (PM) oocytes were immunoblotted for AQP2. **C)** Heteroligomerization of AQP2-p.R254Q with wt-AQP2. Of 30 oocytes expressing combinations of F-AQP2 with AQP2-p.R254Q or with AQP2-p.R187C, total membranes (TM) were isolated. Equivalent fractions were solubilized in deoxycholate and subjected to immunoprecipitation (IP) using FLAG antibodies. Subsequently, total membranes and IPs were immunoblotted for AQP2. Molecular masses (in kDa) are indicated on the right.

Localization of AQP2-p.R254Q in MDCK cells

Though oocytes are a good cell system to establish functionality and interacting ability of AQP2 mutants in NDI, they are not polarized as with collecting duct cells. Also, we were unable to study the level of phosphorylation of AQP2-p.R254Q in these cells. Madin-Darby Canine kidney (MDCK) cells are polarized, and heterologously-expressed AQP2 in these cells is redistributed from intracellular vesicles to the apical membrane upon forskolin treatment (Deen et al., 1997b). Therefore, to study AQP2-p.R254Q further, a eukaryotic expression construct encoding AQP2-p.R254Q was generated and transfected into MDCK cells. Single clones were grown to confluence, incubated with or without forskolin, and subjected to immunocytochemistry using α-AQP2 antibodies. MDCK-wt-AQP2 cells were taken along as controls. CLSM analysis revealed that AQP2-p.R254Q localized to intracellular vesicles and that this distribution was not changed upon incubation with forskolin (Fig. 2A, upper panel). In contrast and as shown before (Deen et al., 1997b), wt-AQP2 was translocated from intracellular vesicles to the apical membrane (Fig. 2A, lower panel). The AQP2-positive vesicles in unstimulated MDCK-AQP2 and MDCK-AQP2-p.R254Q cells, however, were of similar size and were mainly located on the apical side of the cell, which is also similar to that found for AQP2-p.S256A (Van Balkom et al., 2002). To further identify the subcellular organelles in which AQP2-p.R254Q resides, representative cell lines were tested for co-localization with various marker proteins for subcellular organelles. MDCK-AQP2 and MDCK-AQP2-p.S256A cell lines were taken along as controls. None of the studied AQP2 proteins appeared to co-localize with marker proteins for the

ER (PDI), cis-Golgi (GOS28), median-trans-Golgi (Giantin) or late endosomes/lysosomes (Lamp2; not shown). However, AQP2-p.R254Q, wt-AQP2 and AQP2-p.S256A appeared to partially co-localize with the early endosomal marker protein EEA1 (Fig. 2B, arrows). Semi-quantification of the signals revealed an overlap of 9, 15 and 12% for AQP2-p.R254Q, wt-AQP2 and AQP2-p.S256A respectively, which indicated that they co-localized to a similar level with EEA1.

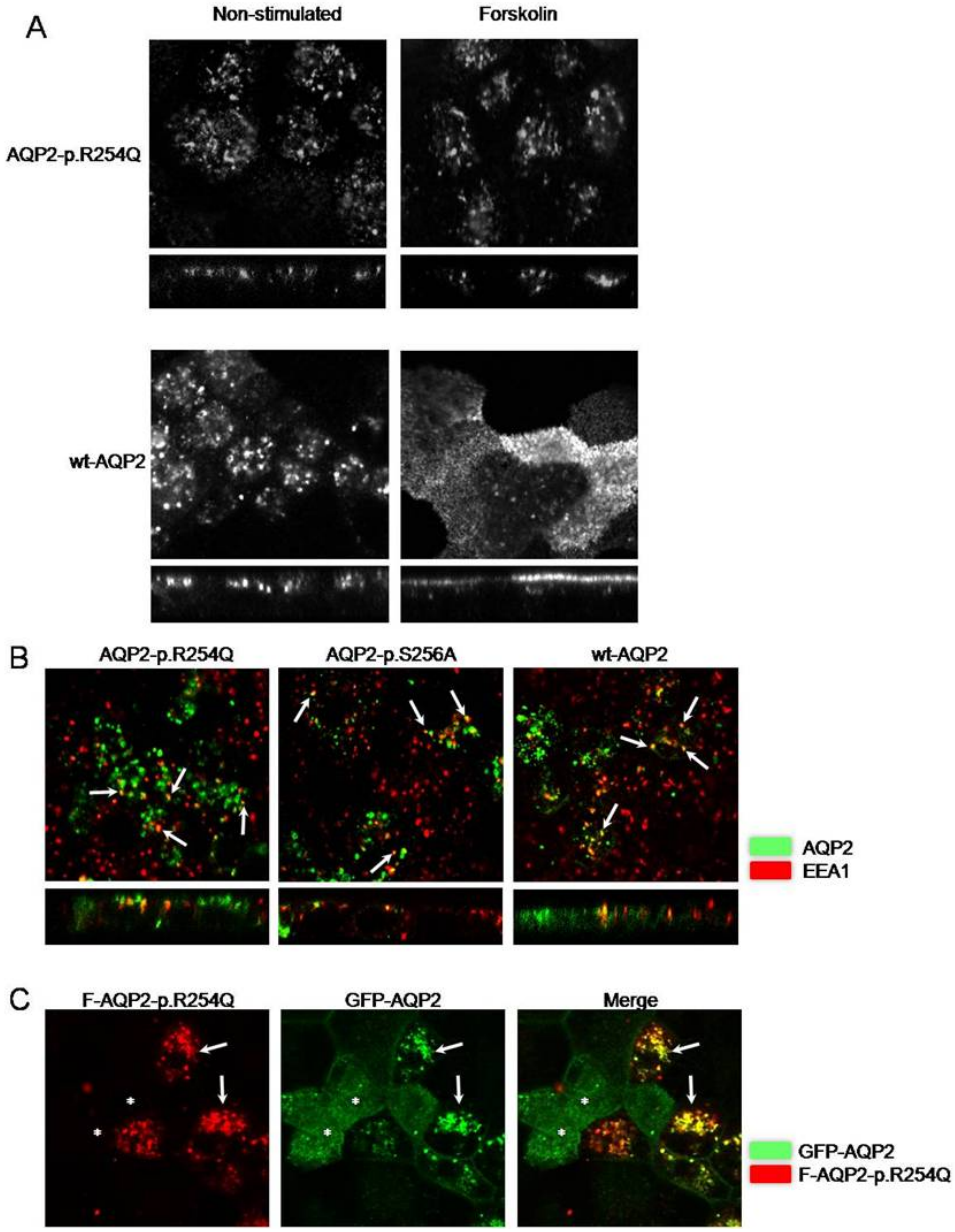

Figure 2. Localization of wt-AQP2 and AQP2-p.R254Q in MDCK cells. **A** and B) MDCK cell lines stably-expressing or wt-AQP2, AQP2-p.R254Q or AQP2-p.S256A (indicated) were grown to confluence on semi-permeable filters and incubated O/N with indomethacin to reduce basal cAMP levels (A, non-stimulated; B). Subsequently, cells were treated for 45 minutes with forskolin in the presence of indomethacin (A, stimulated). After fixation and permeabilization, the filters were incubated with rabbit anti-AQP2 antibodies and monoclonal antibodies recognizing the early endosomal marker protein EEA1, followed by Alexa 488 conjugated anti-rabbit and Alexa 594 conjugated anti-mouse antibodies. XY and XZ images were obtained using confocal laser scanning microscopy. Co-localization of the AQP2 proteins (green) and EEA1 (red) is indicated by arrows. C) Dominant effect of AQP2-p.R254Q on GFP-AQP2. MDCK cells expressing GFP-tagged wt-AQP2 (GFP-AQP2) and (transiently) FLAG-tagged AQP2-p.R254Q (F-AQP2-p.R254Q) were grown to confluence, stimulated with forskolin as described above, fixed and permeabized. The filters were incubated with mouse FLAG antibodies and subsequently Alexa 594 conjugated anti-mouse antibodies. Upon co-expression, GFP-AQP2 and F-AQP2-p.R254Q co-localize (asterisks) in intracellular vesicles, while GFP-AQP2 expressed alone is found in the apical membrane (indicated by arrows).

AQP2-p.R254Q impairs trafficking of wt-AQP2 to the apical membrane

Since AQP2-p.R254Q was retained in intracellular vesicles in MDCK cells, we next wanted to investigate whether AQP2-p.R254Q exhibit a dominant negative effect on the trafficking of wt-AQP2. For this, MDCK cells stably-expressing GFP-tagged wt-AQP2 (GFP-AQP2) was transiently transfected with FLAG-tagged AQP2-p.R254Q (F-AQP2-p.R254Q), which resulted in cells expressing GFP-AQP2 alone as well as together with F-AQP2-p.R254Q. Immunocytochemistry and CLSM analysis of cell monolayers revealed that in cells expressing GFP-AQP2 and AQP2-p.R254Q, both proteins co-localized in intracellular vesicles (Fig. 2C; arrows). In contrast and as reported before (Marr et al., 2002a), cells only expressing GFP-AQP2 showed a dispersed expression pattern, which is typical for apical membrane expression (Fig. 2C; asterisks; see also forskolin-treated wt-AQP2 in Fig. 2A). These data revealed that AQP2-p.R254Q exert a dominant-negative effect on wt-AQP2 in that it interferes with its trafficking to the apical membrane.

Phosphorylation of AQP2-p.R254Q in MDCK cells

Since the p.R254Q mutation destroys the phosphorylation consensus sequence of PKA, we next tested whether AQP2-p.R254Q could be phosphorylated at S256. For this, MDCK cells expressing wt-AQP2, AQP2-p.R254Q or AQP2-p.S256A were subjected to orthophosphate labeling in the presence or absence of forskolin. Subsequently, AQP2 proteins were immunoprecipitated and equivalents were immunoblotted for AQP2 (Fig. 3A, IB) or loaded on a gel and exposed to a film for 3 days (Fig. 3A, ^{32}P). Analysis of the autoradiogram revealed that wt-AQP2 and AQP2-p.R254Q were already phosphorylated in the absence of forskolin stimulation. In contrast, AQP2-p.S256A

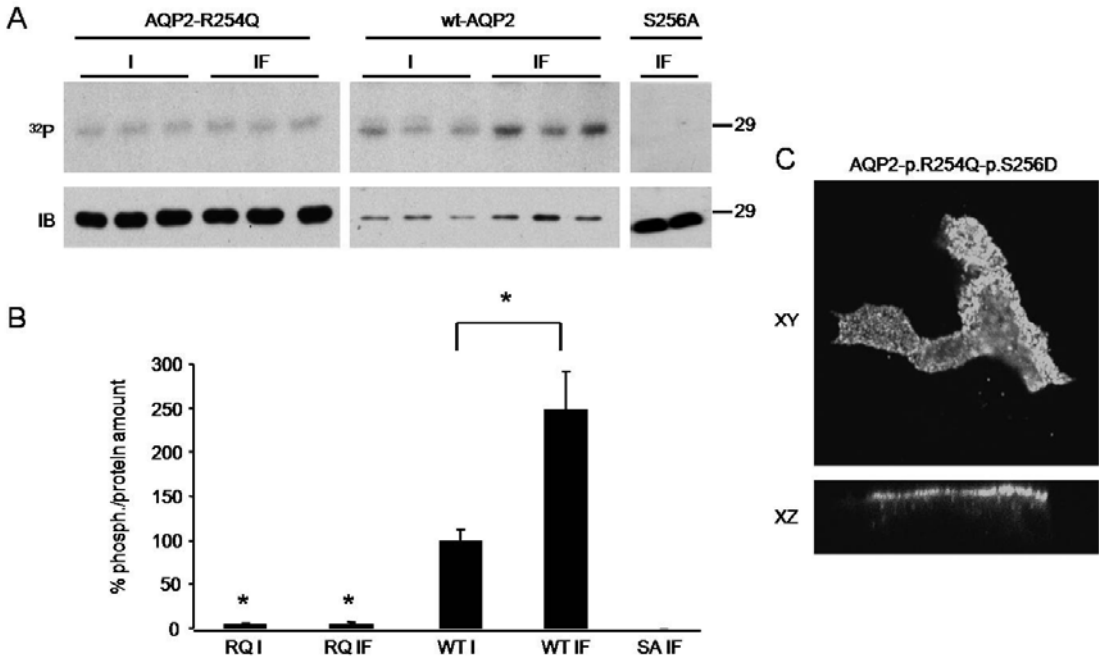

Figure 3. Phosphorylation of AQP2-p.R254Q in MDCK cells. **A)** MDCK cells expressing wt-AQP2, AQP2-p.R254Q or AQP2-p.S256A were grown and independent triplicates from the cell lines were treated with indomethacin (I), or indomethacin/forskolin (IF), as described in the legend of Fig. 2 and subjected to [^{32}P]orthophosphate labelling. After cell lysis, the AQP2 proteins were immunoprecipitated, split into two portions of which one was separated on SDS-PAGE and autoradiographed (upper panel), while the second portion was immunoblotted for AQP2 (lower panel). Wt-AQP2 shows a clear increase in phosphorylation with forskolin, while AQP2-p.R254Q is phosphorylated to low extent, with or without forskolin treatment. AQP2-p.S256A is not labelled with forskolin. **B)** Relative phosphorylation levels. The [^{32}P] signals of the blots under A were densitometrically scanned and normalization for the particular AQP2 protein expression levels. Bars represent the ratio of phosphorylated AQP2 versus the total amount of AQP2 protein. **C)** Apical localization of AQP2-p.R254Q-p.S256D in MDCK cells. MDCK cells transiently expressing AQP2-p.R254Q-p.S256D mutant were incubated without forskolin, fixed, and immunostained for AQP2 proteins using rabbit anti-AQP2 and Alexa-488 conjugated anti–rabbit antibodies. XY and XZ images were made with a confocal laser-scanning microscope.

was not phosphorylated, although the cells were stimulated with forskolin and AQP2-p.S256A was expressed at high levels (Fig 3A, IB). This indicated that the basal phosphorylation of wt-AQP2 and AQP2-p.R254Q was at S256. Semi-quantification of the [^{32}P] signals and normalization for the expression levels, however, revealed that the basal level of phosphorylation of AQP2-p.R254Q was only about 5% of that of unstimulated wt-AQP2 (Fig. 3B). Upon treatment with forskolin, phosphorylation of wt-AQP2 was about 2.5-fold increased, whereas the level of AQP2-p.R254Q phosphorylation was not increased. With forskolin stimulation, the level of AQP2-p.R254Q phosphorylation was thus only 2% of that of wt-AQP2. These data revealed that AQP2-p.R254Q was impaired in its forskolin-induced phosphorylation at S256, in contrast to wt-AQP2.

To test whether the lack of phosphorylation is the only determinant for the retention of AQP2-p.R254Q, we introduced the p.S256D mutation into AQP2-p.R254Q, generating AQP2-p.R254Q-p.S256D. Previously, we have shown that the p.S256D transition in wt-AQP2 mimics constitutively-phosphorylated AQP2 in that this AQP2-p.S256D is located in the apical membrane of MDCK cells, independent of stimulation by forskolin (Van Balkom et al., 2002). Following transient transfection of MDCK cells, growth to confluent layers and immunocytochemistry, CLSM analysis indeed revealed that AQP2-p.R254Q-p.S256D was localized in the apical membrane, independent of forskolin treatment (Fig. 3C), which indicated that the retention of AQP2-p.R254Q in intracellular vesicles is solely due to the lack of phosphorylation of S256.

DISCUSSION

AQP2-p.R254Q is a typical mutant in dominant NDI and lack of S256 phosphorylation is likely the molecular cause

As shown previously, AQP2 mutants in dominant NDI are 1) functional water channels of 29 and 40-45 kDa, 2) missorted to other subcellular organelles than wt-AQP2, and 3) able to form heteroligomers with wt-AQP2. Because of these characteristics, they cause missorting of wt-AQP2 (Mulders et al., 1998; Kamsteeg et al., 1999; Kuwahara et al., 2001; Marr et al., 2002b; Kamsteeg et al., 2003). In contrast, AQP2 mutants in recessive NDI are misfolded, retained in the ER and unable to interact with wt-AQP2 (Marr et al., 2002a; Deen et al., 1994; Kamsteeg et al., 2000b). Indeed, like other mutations in dominant NDI (Mulders et al., 1998; Kuwahara et al., 2001; Marr et al., 2002b; Kamsteeg et al., 2003), upon expression in oocytes and MDCK cells, AQP2-p.R254Q was observed to be a functional water channel of 29 and 40-45 kDa and retained in the cell, but not in the ER, as revealed by the absence of 32 kDa high-mannose bands (Fig. 1). Also, AQP2-p.R254Q formed heteroligomers with wt-AQP2 and impaired the further trafficking of wt-AQP2 to the plasma membrane (Figs. 1C and 2C). Therefore, similar to other mutants in dominant NDI, AQP2-p.R254Q is likely to cause dominant NDI in this particular NDI patient by inhibiting the trafficking of enough wt-AQP2 to the apical membrane of the principal cells. And due to this dominant negative feature of p.R254Q mutation, presence of wt-AQP2 in the heterozygous patient would not rescue the phenotype by providing simply 50% of functional channels.

In oocytes, wt-AQP2 is always maximally phosphorylated. Similar to polarized cells, however, phosphorylation of AQP2 at S256 is also essential in these cells, because AQP2-p.S256A is retained in vesicles, whereas AQP2-p.S256D, which mimics continuously phosphorylated AQP2, is completely localized in the plasma membrane (Kamsteeg et al., 2000a). Consistent with an impaired phosphorylation of S256 in AQP2-p.R254Q, this protein was retained to a similar extent in oocytes as AQP2-p.S256A (Fig. 1). Also, immunoblotting of membranes of injected oocytes with antibodies recognizing S256-phosphorylated AQP2 (Christensen et al., 2000) did not reveal any signal for AQP2-p.R254Q, whereas wt-AQP2 was well detected (not shown).

Additional evidence was obtained from transfected MDCK cells. First, the distribution and size of AQP2-p.R254Q-containing vesicles was alike those of wt-AQP2 in unstimulated cells and of AQP2-p.S256A (Fig. 2B; (Van Balkom et al., 2002)). Moreover, neither one of these proteins co-localized with marker proteins of the ER, cis-median Golgi complex, TGN, late endosomes/lysosomes or basolateral membrane (not shown), but showed a similar level of co-localization with the endosomal marker protein EEA1 (Fig. 2B). Most convincingly, however, forskolin treatment did not increase phosphorylation of AQP2-p.R254Q or AQP2-p.S256A, in contrast to wt-AQP2 (Fig. 3A), and introduction of the ‘constitutive phosphorylation’ mutation p.S256D, which resulted in the steady state apical membrane localization when introduced in wt-AQP2 (Van Balkom et al., 2002), also rescued the apical membrane expression of AQP2-p.R254Q (Fig. 3C).

It has been well-accepted that phosphorylation of S256 is essential for AQP2 trafficking. Non-phosphorylatable AQP2-p.S256A mutant showed impaired trafficking to the plasma membrane in several cell culture studies (Fushimi et al., 1997; Katsura et al., 1997; Kamsteeg et al., 2000a; Van Balkom et al., 2002). Using antibodies recognizing phosphorylated AQP2, it has been also been shown that AQP2 is phosphorylated at S256 *in vivo* (Nishimoto et al., 1999; Christensen et al., 2000). Same as AQP2-p.R254L, AQP2-p.R254Q is only impaired in its phosphorylation of S256 and causes dominant NDI, suggesting the fundamental importance of AQP2 phosphorylation at S256 *in vivo*.

The p.R254Q mutation is a dominant NDI due to a loss of function mutation

Different AQP2 mutants in dominant NDI are missorted to different locations within the cell. Except AQP2-p.R254L, all six other AQP2 mutants described are thought to be missorted due to a signal *introduced* with the mutation. In oocytes, AQP2-p.E258K is retained in the Golgi complex region. Deletion of the region surrounding E258, however, greatly restored its plasma membrane expression, which indicated that the introduction of a Lys instead of the loss of E258 caused the dominant feature (Mulders et al., 1998). AQP2-c.779_780insA, which leads to an +2 reading frame shift of the AQP2 C-tail was targeted to the basolateral membrane, because of two introduced basolateral sorting signals in its mutant C-terminal tail (Kamsteeg et al., 2003). Although starting at different positions, the other four described mutations (c.721delG, c.727delG, c.763_772del and c.812_818del) introduce a +1 reading frame shift, which results in a similar extended C-terminal tail. Studies in polarized cells, revealed that AQP2-727delG accumulates in late endosomes/lysosomes and, to some extent, in the basolateral plasma membrane of MDCK cells (Marr et al., 2002b), whereas the other three mutants have been reported to localize to the basolateral membrane. Since the extended tail in AQP2-812_818del starts only at the stop codon of wt-AQP2 and the extended tails contain a di-leucine motif, which is a known basolateral membrane targeting motif (Heilker et al., 1999), the dominancy of these mutants in NDI is suggested to be due to this introduced basolateral sorting signal.

In contrast to the above mutants which introduce a missorting signal, AQP2-p.R254Q caused dominant NDI, same as AQP2-p.R254L, is due to the *loss of function* of wt-AQP2, namely the phosphorylation at S256. Consistent with this, AQP2-p.R254Q seems to accumulate in the vesicles in which unstimulated wt-AQP2 and AQP2-p.S256A also reside, and upon stimulation impairs the trafficking of wt-AQP2 to the similar level as AQP2-p.S256A (Fig. 2).

However, many, if not all, membrane proteins are continuously transported between several organelles along microtubular and actin cytoskeletal systems (Sonnichsen et al., 2000; Rohn et al., 2000; Ghosh et al., 1998). The predominant subcellular localization of the protein in such a dynamic equilibrium is then determined by several factors, including the activity of protein kinases and phosphatases (Molloy et al., 1999; Bao et al., 2000). In parallel, it has been shown that in renal collecting duct and/or transfected cells, AQP2 is continuously shuttled between intracellular vesicles and the apical plasma membrane (Strange et al., 1988; Brown et al., 1988) and that the predominant localization of AQP2 (intracellular vesicles or plasma membrane) in a steady state (without or with AVP stimulation, respectively) is determined by whether AQP2 is phosphorylated or not (Wade et al., 1981; Katsura et al., 1997; Nielsen et al., 1995). Indeed, AQP2-p.S256A, which mainly resided in intracellular vesicles of LLC-PK1 cells, appeared to continuously cycle between these vesicles and the plasma membrane, as blocking endocytosis resulted in its accumulation at the plasma membrane (Sun et al., 2002; Lu et al., 2003). AQP2-p.R254Q, which seemed to accumulate in storage vesicles that also harbour non-phosphorylated wt-AQP2 or AQP2-p.S256A, is therefore also likely to cycle to the plasma membrane. This is partly underscored by the partial co-localization of wt-AQP2, AQP2-p.S256A and AQP2-p.R254Q with EEA1 (Fig. 2), which is known to regulate the endosome docking and fusion with the plasma membrane in the exocytotic process (Christoforidis et al., 1999).

Fluid deprivation test and dDAVP infusion have been reported to increase renal AQP2 expression (Nielsen et al., 1993; van Os et al., 1994). Although this will presumably not result in increased phosphorylation of AQP2, and thus not in a changed distribution of AQP2-p.R254Q/wt-AQP2 complexes in the renal cells, the increased wt-AQP2 and AQP2-p.R254Q expression levels will result in higher total amounts of wt-AQP2/AQP2-p.R254Q complexes in the apical membrane. This might provide an explanation for the weak ability of the AQP2-p.R254Q patient to concentrate her urine, from 58 to 220 and 258 mOsmo/ kgH_2O, upon the fluid deprivation test and dDAVP infusion, respectively (Fig. 4).

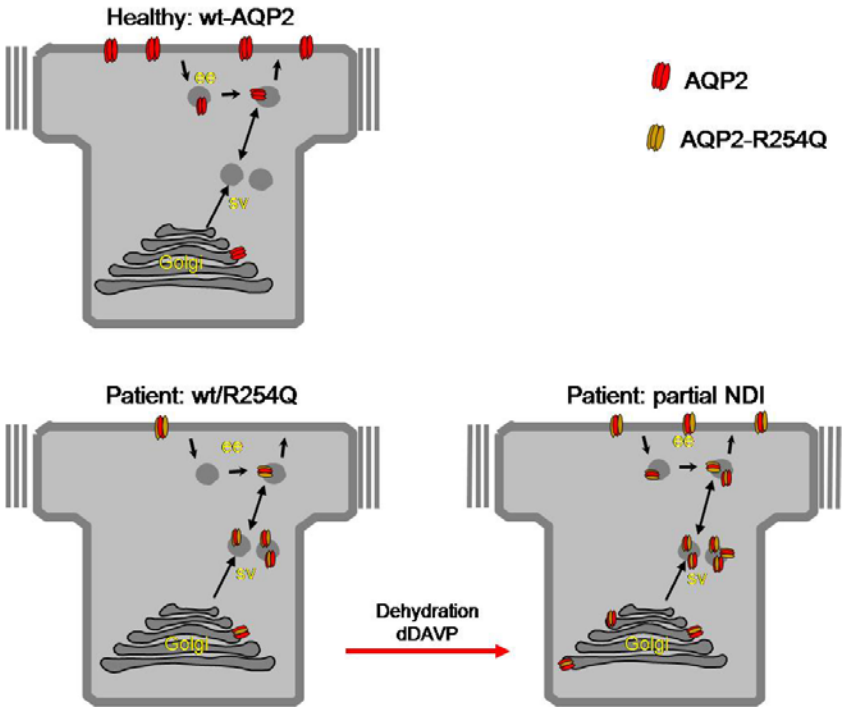

Figure 4. Hypothesis to explain the increased urine osmolality in the dominant NDI patient. Besides continuous cycling, AQP2 translocates from intracellular vesicles to the apical membrane under antidiuretic conditionin in healthy individuals (upper panel). In the AQP2-p.R254Q dominant NDI patient, translocation of AQP2 to the apical membrane is impaired (lower left panel), which can be rescued upon dehydration, or administration of dDAVP (lower right panel).

Furthermore, McDill *et al.* reported that due to the lack of S256 phosphorylation by PKA, a p.S256L mutation in AQP2 caused congenital NDI in mice (McDill et al., 2006). Interestingly, although both result in the loss of phosphorylation at S256, the p.S256L mutation causes recessive NDI in mice, whereas the R254 mutations cause dominant NDI in humans. In contrast to our *in vitro* experiments, a considerable portion of wt-AQP2 (and possibly AQP2-S256L) was found in the apical membrane of the AQP2-p.S256L heterozygote. This clearly indicates that with AQP2 mutants interacting with wt-AQP2, the relative strength of the apical sorting signal in wt-AQP2 *vs.* the missorting signal in the AQP2 mutant determines recessive or dominant NDI, and that the mutation and its location within the AQP2 C-tail determines the strength of the apical sorting signal.

In conclusion, we identified that the mutation AQP2-p.R254Q causes dominant NDI, due to a lack of AVP-induced phosphorylation of S256 in the mutant protein and translocation to the plasma membrane. These data also illuminate that AQP2 phosphorylation at S256 is important for its translocation to the apical membrane *in vivo* and reveal that in this reported patient the dominant NDI is caused by a loss of function mutation instead of a gain of *mis*function. As a dominant negative mutant, AQP2-p.R254Q likely retains complex wt-AQP2 in storage vesicles from which they likely recycle to a low extent to the apical membrane. Therefore, the slightly-improved urine concentrating ability and reduced polyuria with the dehydration and dDAVP treatment may be explained by increased wt-AQP2/AQP2-p.R254Q expression levels.

ACKNOWLEDGMENTS

We thank Dr. Gary Robertson and Peter Kopp from Feinberg School of Medicine of Northwestern University, Chicago, IL, USA for their medical input and contribution of clinical information. We gratefully acknowledge Drs S. Nielsen from Aarhus, Denmark, Y. Ikehara from Fukuoka, Japan. I. Braakman from Utrecht, The Netherlands and Le Bivic, Marseille, France for their kind gifts of antibodies against p-AQP2, GOS28 and Giantin, Protein disulphide Isomerase and Lamp2, respectively. PMTD is a recipient of a VICI grant (865.07.002) of the Netherlands Organization for Scientific research (NWO).

Contract grant sponsor: The Netherlands Organization for Scientific Research (NWO) to PMTD; Contract grant number: VICI 865.07.002. Contract grant sponsor: NWO to PMTD and PVDS; Contract grant number: 902.18.292. Contract grant sponsor: NWO to EJK; Contract grant number: 916.36.122. Contract grant sponsor: The European Union to PMTD; Contract grant number: QLRT-2000-00778 and QLK3-CT-2001-00987. Contract grant sponsor: Coordination Theme 1 of the European Community's 7th Framework Program to PMTD; Contract grant number: HEALTH-F2-2007-201590 (entitled EUNEFRON), and RTN aquaglyceroporins 035995-2. Contract grant sponsor: The Dutch Kidney Foundation to PMTD; Contract grant number: PC159.

REFERENCES

Asai T, Kuwahara M, Kurihara H, Sakai T, Terada Y, Marumo F, Sasaki S. 2003. Pathogenesis of nephrogenic diabetes insipidus by aquaporin-2 C-terminus mutations. Kidney Int 64:2-10.

Bao J, Alroy I, Waterman H, Schejter ED, Brodie C, Yarden Y. 2000. Threonine phosphorylation diverts internalized EGF-receptors from a degradative pathway to the recycling endosome. J Biol Chem.

Benham AM, Cabibbo A, Fassio A, Bulleid N, Sitia R, Braakman I. 2000. The CXXCXXC motif determines the folding, structure and stability of human Ero1-Lalpha. EMBO J 19:4493-4502.

Brown D, Weyer P, Orci L. 1988. Vasopressin stimulates endocytosis in kidney collecting duct principal cells. Eur J Cell Biol 46:336-341.

Christensen BM, Zelenina M, Aperia A, Nielsen S. 2000. Localization and regulation of PKA-phosphorylated AQP2 in response to V(2)-receptor agonist/antagonist treatment. Am J Physiol Renal Physiol 278:F29-F42.

Christoforidis S, McBride HM, Burgoyne RD, Zerial M. 1999. The Rab5 effector EEA1 is a core component of endosome docking. Nature 397:621-625.

De Mattia F, Savelkoul PJ, Kamsteeg EJ, Konings IB, van der SP, Mallmann R, Oksche A, Deen PM. 2005. Lack of Arginine Vasopressin-Induced Phosphorylation of Aquaporin-2 Mutant AQP2-R254L Explains Dominant Nephrogenic Diabetes Insipidus. J Am Soc Nephrol 16:2872-2880.

Deen PMT, Nielsen S, Bindels RJM, van Os CH. 1997a. Apical and basolateral expression of Aquaporin-1 in transfected MDCK and LLC-PK cells and functional evaluation of their transcellular osmotic water permeabilities. Pflugers Arch 433:780-787.

Deen PMT, Rijss JPL, Mulders SM, Errington RJ, van Baal J, van Os CH. 1997b. Aquaporin-2 transfection of Madin-Darby canine kidney cells reconstitutes vasopressin-regulated transcellular osmotic water transport. Journal of the American Society of Nephrology 8:1493-1501.

Deen PMT, Van Balkom BWM, Savelkoul PJ, Kamsteeg EJ, Van Raak M, Jennings ML, Muth TR, Rajendran V, Caplan MJ. 2002. Aquaporin-2: COOH terminus is necessary but not sufficient for routing to the apical membrane. Am J Physiol Renal Physiol 282:F330-F340.

Deen PMT, Verdijk MAJ, Knoers NVAM, Wieringa B, Monnens LAH, van Os CH, van Oost BA. 1994. Requirement of human renal water channel aquaporin-2 for vasopressin-dependent concentration of urine. Science 264:92-95.

Fushimi K, Sasaki S, Marumo F. 1997. Phosphorylation of serine 256 is required for cAMP- dependent regulatory exocytosis of the aquaporin-2 water channel. J Biol Chem 272:14800-14804.

Ghosh RN, Mallet WG, Soe TT, McGraw TE, Maxfield FR. 1998. An endocytosed TGN38 chimeric protein is delivered to the TGN after trafficking through the endocytic recycling compartment in CHO cells. J Cell Biol 142:923-936.

Heilker R, Spiess M, Crottet P. 1999. Recognition of sorting signals by clathrin adaptors. Bioessays 21:558-567.

Kamsteeg EJ, Bichet DG, Konings IB, Nivet H, Lonergan M, Arthus MF, van Os CH, Deen PMT. 2003. Reversed polarized delivery of an aquaporin-2 mutant causes dominant nephrogenic diabetes insipidus. J Cell Biol 163:1099-1109.

Kamsteeg EJ, Deen PMT. 2001. Detection of aquaporin-2 in the plasma membranes of oocytes: a novel isolation method with improved yield and purity. Biochem Biophys Res Commun 282:683-690.

Kamsteeg EJ, Heijnen I, van Os CH, Deen PMT. 2000a. The Subcellular Localization of an Aquaporin-2 Tetramer Depends on the Stoichiometry of Phosphorylated and Nonphosphorylated Monomers. J Cell Biol 151:919-930.

Kamsteeg EJ, Mulders SM, Bichet DG, Deen PMT, van Os C. 2000b. Consequences of aquaporin 2 tetramerization for genetics and routing. Nephrol Dial Transplant 15 Suppl 6:26-28.

Kamsteeg EJ, Wormhoudt TA, Rijss JPL, van Os CH, Deen PMT. 1999. An impaired routing of wild-type aquaporin-2 after tetramerization with an aquaporin-2 mutant explains dominant nephrogenic diabetes insipidus. EMBO J 18:2394-2400.

Katsura T, Gustafson CE, Ausiello DA, Brown D. 1997. Protein kinase A phosphorylation is involved in regulated exocytosis of aquaporin-2 in transfected LLC-PK1 cells. Am J Physiol 41:F816-F822.

Kuwahara M, Iwai K, Ooeda T, Igarashi T, Ogawa E, Katsushima Y, Shinbo I, Uchida S, Terada Y, Arthus MF, Lonergan M, Fujiwara TM, Bichet DG, Marumo F, Sasaki S. 2001. Three families with autosomal dominant nephrogenic diabetes insipidus caused by aquaporin-2 mutations in the C-terminus. Am J Hum Genet 69:738-748.

Lu H, Sun TX, Bouley R, Blackburn K, McLaughlin M, Brown D. 2003. Inhibition of endocytosis causes phosphorylation (S256)-independent plasma membrane accumulation of AQP-2. Am J Physiol Renal Physiol.

Marr N, Bichet DG, Hoefs S, Savelkoul PJ, Konings IB, De Mattia F, Graat MP, Arthus MF, Lonergan M, Fujiwara TM, Knoers NVAM, Landau D, Balfe WJ, Oksche A, Rosenthal W, Muller D, van Os CH, Deen PMT. 2002a. Cell-Biologic and Functional Analyses of Five New Aquaporin-2 Missense Mutations that Cause Recessive Nephrogenic Diabetes Insipidus. J Am Soc Nephrol 13:2267-2277.

Marr N, Bichet DG, Lonergan M, Arthus MF, Jeck N, Seyberth HW, Rosenthal W, van Os CH, Oksche A, Deen PMT. 2002b. Heteroligomerization of an Aquaporin-2 mutant with wild-type Aquaporin- 2 and their misrouting to late endosomes/lysosomes explains dominant nephrogenic diabetes insipidus. Hum Mol Genet 11:779-789.

McDill BW, Li SZ, Kovach PA, Ding L, Chen F. 2006. Congenital progressive hydronephrosis (cph) is caused by an S256L mutation in aquaporin-2 that affects its phosphorylation and apical membrane accumulation. Proc Natl Acad Sci U S A 103:6952-6957.

Molloy SS, Anderson ED, Jean F, Thomas G. 1999. Bi-cycling the furin pathway: from TGN localization to pathogen activation and embryogenesis. Trends Cell Biol 9:28-35.

Mulders SM, Bichet DG, Rijss JPL, Kamsteeg EJ, Arthus MF, Lonergan M, Fujiwara M, Morgan K, Leijendekker R, van der Sluijs P, van Os CH, Deen PMT. 1998. An aquaporin-2 water channel mutant which causes autosomal dominant nephrogenic diabetes insipidus is retained in the Golgi complex. J Clin Invest 102:57-66.

Nielsen S, Chou CL, Marples D, Christensen EI, Kishore BK, Knepper MA. 1995. Vasopressin increases water permeability of kidney collecting duct by inducing translocation of aquaporin-CD water channels to plasma membrane. Proc Natl Acad Sci U S A 92:1013-1017.

Nielsen S, Digiovanni SR, Christensen EI, Knepper MA, Harris HW. 1993. Cellular and subcellular immunolocalization of vasopressin- regulated water channel in rat kidney. Proc Natl Acad Sci U S A 90:11663-11667.

Nishimoto G, Zelenina M, Li D, Yasui M, Aperia A, Nielsen S, Nairn AC. 1999. Arginine vasopressin stimulates phosphorylation of aquaporin-2 in rat renal tissue. Am J Physiol 276:F254-F259.

Robben JH, Knoers NV, Deen PM. 2006. Cell biological aspects of the vasopressin type-2 receptor and aquaporin 2 water channel in nephrogenic diabetes insipidus. Am J Physiol Renal Physiol 291:F257-F270.

Robertson GL, Kopp P. 2002. A novel dominant mutation of the aquaporin-2 gene resulting in partial nephrogenic diabetes insipidus.

Rohn WM, Rouille Y, Waguri S, Hoflack B. 2000. Bi-directional trafficking between the trans-Golgi network and the endosomal/lysosomal system. J Cell Sci 113 (Pt 12):2093-2101.

Rosenthal W, Seibold A, Antaramian A, Lonergan M, Arthus M-F, Hendy GN, Birnbaumer M, Bichet DG. 1992. Molecular identification of the gene responsible for congenital nephrogenic diabetes insipidus. Nature 359:233-235.

Sonnichsen B, De Renzis S, Nielsen E, Rietdorf J, Zerial M. 2000. Distinct membrane domains on endosomes in the recycling pathway visualized by multicolor imaging of Rab4, Rab5, and Rab11. J Cell Biol 149:901-914.

Strange K, Willingham MC, Handler JS, Harris HW, Jr. 1988. Apical membrane endocytosis via coated pits is stimulated by removal of antidiuretic hormone from isolated, perfused rabbit cortical collecting tubule. J Membr Biol 103:17-28.

Sun TX, van Hoek A, Huang Y, Bouley R, McLaughlin M, Brown D. 2002. Aquaporin-2 localization in clathrin-coated pits: inhibition of endocytosis by dominant-negative dynamin. Am J Physiol Renal Physiol 282:F998-1011.

Van Balkom BWM, Savelkoul PJ, markovich D, Hofman E, Nielsen S, van der Sluijs P, Deen PMT. 2002. The role of putative phosphorylation sites in the targeting and shuttling of the aquaporin-2 water channel. J Biol Chem 277:41473-41479.

van den Ouweland AM, Dreesen JC, Verdijk MAJ, Knoers NVAM. 1992. Mutations in the vasopressin type 2 receptor gene (AVPR2) associated with Nephrogenic Diabetes Insipidus. Nat Genet 2:99-102.

van Os CH, Deen PMT. 1998. Aquaporin-2 water channel mutations causing nephrogenic diabetes insipidus. Proc Assoc Am Physicians 110:395-400.

van Os CH, Deen PMT, Dempster JA. 1994. Aquaporins: water selective channels in biological membranes. Molecular structure and tissue distribution. Biochim Biophys Acta 1197:291-309.

Wade JB, Stetson DL, Lewis SA. 1981. ADH action: evidence for a membrane shuttle mechanism. Ann N Y Acad Sci 372:106-117.