A novel mechanism in recessive nephrogenic diabetes insipidus: wild-type aquaporin-2 rescues the apical membrane expression of intracellularly retained AQP2-P262L

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Vasopressin regulates water homeostasis through insertion of homotetrameric aquaporin-2 (AQP2) water channels in the apical plasma membrane of renal cells. AQP2 mutations cause recessive and dominant nephrogenic diabetes insipidus (NDI), a disease in which the kidney is unable to concentrate urine in response to vasopressin. Until now, all AQP2 mutants in recessive NDI were shown to be misfolded, retained in the endoplasmic reticulum (ER) and unable to interact with wild-type (wt)-AQP2, whereas AQP2 mutants in dominant NDI are properly folded and interact with wt-AQP2, but, due to the mutation, cause missorting of the wt-AQP2/mutant complex. Here, patients of two families with recessive NDI appeared compound heterozygotes for AQP2-A190T or AQP2-R187C mutants, together with AQP2-P262L. As mutations in the AQP2 C-tail, where P262 resides, usually cause dominant NDI, the underlying cell-biological mechanism was investigated. Upon expression in oocytes, AQP2-P262L was a properly folded and functional aquaporin in contrast to the classical mutants, AQP2-R187C and AQP2-A190T. Expressed in polarized cells, AQP2-P262L was retained in intracellular vesicles and did not localize to the ER. Upon co-expression, however, AQP2-P262L interacted with wt-AQP2, but not with AQP2-R187C, resulting in a rescued apical membrane expression of AQP2-P262L. In conclusion, our study reveals a novel cellular phenotype in recessive NDI in that AQP2-P262L acts as a mutant in dominant NDI, except for that its missorting is overruled by apical sorting of wt-AQP2. Also, it demonstrates for the first time that the recessive inheritance of a disease involving a channel can be due to two cell-biological mechanisms.

INTRODUCTION

In the renal proximal tubule epithelium and descending limbs of Henle, aquaporin-1 (AQP1) is expressed in both apical and basolateral membranes enabling an optimal water reabsorption due to a parallel sodium uptake across the cells. By this mechanism, ~90% of the water content from 180 l of pro-urine formed daily is constitutively reabsorbed. Water transport in collecting ducts, however, is tightly regulated by arginine vasopressin (AVP) (1,2), which binds to its G-protein coupled V2 receptor (V2R) in the basolateral membrane of its principal cells. This binding triggers a cyclic AMP (cAMP) signaling cascade which leads to protein kinase A activation and phosphorylation of aquaporin-2 (AQP2) (3).
Consequently, AQP2 is redistributed from intracellular vesicles to the apical membrane, initiating a transcellular influx of water that can exit the cells to the interstitium via AQP3 and AQP4, both present in the basolateral membrane. Conversely, withdrawal of AVP reverses this process, thereby terminating water reabsorption.

In congenital nephrogenic diabetes insipidus (NDI), a disease in which the kidney is unresponsive to AVP, this mechanism is impaired, resulting in a severe loss of water. NDI is caused by mutations in the gene encoding V2R, which comprises the X-linked form (4,5), or in AQP2 (OMIM *107777), which results in the autosomal recessive and dominant forms of NDI (6–8).

As found in several other recessive traits, AQP2 mutants encoded in recessive NDI can be functional or not, but are all retained in the endoplasmic reticulum (ER), which is due to misfolding and consequent retention by the ER quality control (9–11). Also, the resulting mutants do not interact with wt-AQP2, which is consistent with the recessive form of inheritance of NDI. Interestingly, all AQP2 mutations in recessive NDI are found in between the first and the last (sixth) transmembrane domain, which is thought to form the water pore (12,13).

In contrast to those mentioned earlier, AQP2 mutants in dominant NDI are functional water channels, but are sorted to other subcellular organelles than wild-type (wt)-AQP2 (8,14–16). As AQP2 is expressed as a homotetramer and these AQP2 mutants are properly folded in the ER, they form heterotetramers with wt-AQP2 and missort the wt/ mutant AQP2 complexes (17). The resulting lack of sufficient levels of wt-AQP2 in the apical membrane of collecting duct cells provides an explanation for dominant NDI. In contrast to mutants in recessive NDI, AQP2 mutations found in dominant NDI are all located in the C-terminal tail (Fig. 1C) and underscore the importance of this part of the protein in AQP2 sorting.

In this study, we present two families in which NDI was inherited in an autosomal recessive mode. Surprisingly, however, genomic analysis revealed that the patients were compound heterozygotes of AQP2 gene mutations of which one was located in the AQP2 core region, whereas the other was found in the AQP2 C-tail coding region. To unravel the cell-biological mechanism underlying the recessive nature of NDI in these families, we performed extensive functional and cell-biological analyses of the encoded AQP2 mutants in Xenopus oocytes and polarized epithelial cells.

RESULTS
Phenotyping of the patients
Figure 1 shows partial pedigrees of the two NDI families. The proband of family A was an 11-year-old boy with a life-long history of polyuria and polydipsia. Detailed clinical evaluation revealed that he had severe NDI. By history, DI was not present in other members of his family. The proband of family B was a 6-year-old girl also referred for a documented life-long history of polyuria and polydipsia, and a 24-h urine output of 5.0 l (336 ml/kg of body weight). Her basal urine osmolality was <150 mOsm/kg and did not increase after administration of a therapeutic dose of 1-desamino-8-D-arginine (dDAVP). She is the only polyuric member of a three generation family.

Genotyping of the patients
Because mutations in the sex-linked V2R gene are the most common cause of congenital NDI and could, based on the pedigrees, not be excluded, its sequence was determined. However, no mutation in the V2R coding sequence was found in either family. Subsequent haplotype analysis and sequence analysis of the AQP2 gene revealed that the affected male in family A (Fig. 1) is a compound heterozygote for a 568G>A (AQP2 gene entry: NM 000486) nucleotide substitution in one allele and a 785C>T substitution in the other allele, encoding A190T and P262L amino acid changes, respectively. The affected female in family B was also a compound heterozygote, but now for 559C>T and 785C>T nucleotide substitutions, encoding R187C and P262L transitions, respectively. DNA analysis of several healthy relatives of the patients revealed that the parents and an uncle of patient A, and the mother and cousin of patient B encoded a normal wt-AQP2 and one of the two mutant AQP2 proteins, which proved the recessive inheritance of NDI in these families. The locations of the mutations in AQP2 are indicated in its topology model (Fig. 1C). The R187C and A190T mutations are located in the E-loop of AQP2, a region in which more mutations causing recessive NDI have been found. However, the P262L mutation was found in the C-terminal tail of AQP2 in which only mutations causing dominant NDI have been found.

Functional analysis in oocytes
Next, we wanted to determine whether the identified mutations could be the cause of NDI in these families or were polymorphisms. The R187C-encoding mutation present in the proband of family B has been found in other NDI patients and has been extensively studied (10,17). This mutant is a non-functional water channel that is retained in the ER. To investigate whether the A190T and P262L mutations were fundamental to NDI, these mutations were introduced into the AQP2 cDNA sequence. Subsequently, oocytes were injected with a concentration series of cRNA encoding wt-AQP2 (0.3, 1.0, 3.0 ng), AQP2-P262L (0.3, 1.0, 3.0 ng) or AQP2-A190T (1.0, 3.0 ng).

The water permeability (P) of oocytes expressing AQP2-A190T was not different from non-injected control oocytes (Fig. 2A). Immunoblotting of total membranes of these oocytes revealed 27, 29 and 32 kDa bands for AQP2-A190T (0.3, 1.0, 3.0 ng), AQP2-P262L (0.3, 1.0, 3.0 ng) or AQP2-A190T (1.0, 3.0 ng).

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indicated that AQP2-A190T is misfolded and retained in the ER, as found for other AQP2 mutants in recessive NDI. This was different, however, for AQP2-P262L. The $P_C$ of oocytes expressing AQP2-P262L was higher than that of control oocytes, which indicated that AQP2-P262L is a functional water channel (Fig. 2A). Interestingly, immunoblot analysis revealed that AQP2-P262L was, besides the normal unglycosylated 29 kDa form, expressed as a 30 kDa protein, which was clearly different from the 32 kDa high mannose glycosylated AQP2 form observed for AQP2-A190T (Fig. 2B).
Figure 2. Expression of AQP2-P262L and AQP2-A190T in oocytes. (A) Water permeability in oocytes. Three days after injection of the indicated amounts of wt-AQP2, AQP2-P262L or AQP2-A190T cRNAs, oocytes were subjected to a standard swelling assay. Non-injected oocytes were taken as a control (C). Mean water permeabilities ($P_l$) and SEM of 10 oocytes are shown. (B) Expression in oocytes. From 12 oocytes injected as described earlier, total membranes (TM) or plasma membranes (PM) were isolated. Subsequently, equivalents of one oocyte were immunoblotted for AQP2. The masses in kDa of unglycosylated AQP2 (29), high mannose glycosylated AQP2 (32) and a degradation product of AQP2 (27) are indicated. (C) Immunocytochemistry of oocytes. Non-injected oocytes (d), or those injected with 10 ng cRNA encoding AQP2-A190T (a), or 1 ng cRNA coding for AQP2-P262L (b) or wt-AQP2 (c), were fixed at 3 days after injection and embedded in paraffin. Sections were incubated with rabbit α-AQP2 antibodies followed by Alexa488-conjugated α-rabbit antibodies. AQP2 proteins were visualized using CLSM. The plasma membranes are marked with arrows.
Comparison of the AQP2 signals in plasma membranes versus total membranes revealed that the relative plasma membrane expression of AQP2-P262L (both bands) was not less than that of wt-AQP2, which indicated that the trafficking of AQP2-P262L to the plasma membrane was not impaired.

Immunocytochemistry of oocytes

To determine the localization of AQP2-A190T and AQP2-P262L, oocytes of the same batch were subjected to immunocytochemistry. Confocal laser scanning microscopy (CLSM) revealed a dispersed staining for AQP2-A190T (Fig. 2Ca). AQP2-P262L and wt-AQP2, however, were only detected in the plasma membrane (Fig. 2Cb and c), while non-injected controls did not show any staining (Fig. 2Cd). The expression pattern of AQP2-A190T is similar to the pattern obtained for other AQP2 mutants in recessive NDI (9,11), which further indicated that AQP2-A190T is a typical ER-retained AQP2 mutant in recessive NDI. Since AQP2-R187C and AQP2-A190T are both typical AQP2 mutants in recessive NDI, AQP2-R187C was taken as a representative in following experiments.

AQP2-P262L forms heteroligomers with wt-AQP2

The ability of AQP2 mutants in dominant NDI, but not those in recessive NDI, to form heteroligomers with wt-AQP2 is fundamental to the mode of inheritance of NDI (17). Indeed, it has been shown that AQP2-R187C cannot form heteroligomers with AQP2 (17). To assess whether AQP2-P262L forms heteroligomers with AQP2 or AQP2-R187C, AQP2-P262L was co-expressed with FLAG-tagged AQP2 (F-AQP2) or AQP2-R187C (F-R187C) in oocytes. As a positive control, AQP2 was co-expressed with F-AQP2. The FLAG-tag allows specific immunoprecipitation of F-AQP2 and F-R187C and, owing to the 2 kDa mass of the FLAG-tag, discrimination of both F-AQP2 and F-R187C from AQP2-P262L on immunoblots (17). Also, the N-terminal FLAG tag does not interfere with oligomerization and plasma membrane expression of wt-AQP2 in oocytes. Two days after cRNA injections, total membranes of oocytes were isolated. After solubilization, one fraction was directly immunoblotted for AQP2 (Fig. 3, TM), while the other fraction was first subjected to anti-FLAG immunoprecipitation and then immunoblotted (Fig. 3, IP). Immunoblotting of the total membrane fractions for AQP2 revealed expression of all proteins [untagged AQP2 and AQP2-P262L of 29 kDa, F-AQP2 of 31 kDa and F-R187C of 31 (non-glycosylated) and 33 kDa (high mannose glycosylated)]. Immunoblotting of the FLAG-tag immunoprecipitates showed that AQP2-P262L and AQP2 co-precipitated with F-AQP2, but that AQP2-P262L did not co-precipitate with F-R187C (Fig. 3, IP). These data revealed that AQP2-P262L is able to form heteroligomers with wt-AQP2, but not with AQP2-R187C. Altogether, the oocyte experiments showed that AQP2-P262L is a functional water channel of 29 and 30 kDa, is expressed in the plasma membrane, heteroligermizes with AQP2, and is not retained in the ER (based on the absence of the 32 kDa high mannose glycosylated band). Thus, if AQP2-P262L would contribute to recessive NDI in our families, it clearly showed a different cellular behavior in oocytes than other mutants in recessive NDI.

Localization of AQP2-P262L in polarized cells

Heterologously expressed in polarized MDCK cells, wt-AQP2 is redistributed from vesicles to the apical membrane upon stimulation with AVP or forskolin (18) and, therefore, AQP2-P262L was studied further in these cells. Following stable transfection, several MDCK cell clones expressing AQP2-P262L were grown to confluence, incubated with indomethacin (to reduce intracellular cAMP levels), treated with or without forskolin (to activate adenylate cyclase) and subjected to immunocytochemistry. CLSM analysis revealed that in unstimulated cells, AQP2-P262L mainly resides in intracellular vesicles (Fig. 4A, upper panel). Upon forskolin treatment, most AQP2-P262L remained localized in intracellular vesicles, whereas a small fraction translocated to the basolateral membrane, as shown by its partial co-localization with E-Cadherin (Fig. 4A, lower panel). wt-AQP2, which was taken along as a control, was translocated from intracellular vesicles to the apical membrane with forskolin (Fig. 4B). The vesicular and basolateral expressions of AQP2-P262L were also clearly different from the reticular pattern observed for the ER marker protein, protein disulfide isomerase (PDI) (Fig. 4C; note the complete absence of PDI from the plasma membrane). This again indicated that AQP2-P262L does not localize to the ER. AQP2-P262L also did not co-localize with the lysosome associated membrane protein 2 or the early endosome antigen 1 (data not shown), which indicated that the steady state localization of AQP2-P262L is not early or late endosomes, or lysosomes.

To test further whether the introduced leucine or the deleted proline causes AQP2-P262L retention, MDCK cells stably expressing AQP2-P262A were generated. CLSM analysis of several single clones revealed that without stimulation, AQP2-P262A is located in intracellular vesicles, while...
forskolin stimulation induced its translocation to the apical membrane (Fig. 4D). As a similar redistribution was found for wt-AQP2 (Fig. 4B), these data indicated that the retention of AQP2-P262L is due to the introduction of a leucine at position 262.

Heteroligomerization with wt-AQP2 overrules the missorting of AQP2-P262L

Missorting of wt-AQP2 upon co-expression and heteroligomerization with missorted AQP2 mutants underlies dominant NDI (14–16). As AQP2-P262L is retained in intracellular vesicles (Fig. 4) and interacts with wt-AQP2 in oocytes (Fig. 3), we wanted to determine whether the recessive trait of NDI in these families would become apparent when AQP2-P262L would be co-expressed with wt-AQP2 in MDCK cells. To discriminate AQP2-P262L and wt-AQP2, MDCK cells stably-expressing GFP-tagged wt-AQP2 (G-AQP2) were stably transfected for FLAG-tagged AQP2-P262L (F-AQP2-P262L). As we did not succeed to generate polarized cell lines stably expressing G-AQP2-R187C and F-AQP2-P262L, polarized MDCK cells stably expressing G-AQP2-R187C were transiently transfected for F-AQP2-P262L. MDCK cells stably expressing each AQP2 protein alone were taken along as references.

Immunoblot analysis of cells expressing F-AQP2-P262L alone revealed bands of 31 and 32 kDa (Fig. 5A), which is
consistent with the mass of the 2 kDa FLAG-tag added to untagged AQP2-P262L of 29 and 30 kDa, respectively. G-AQP2 was expressed as 40 and 60 kDa proteins (second and third lanes), while G-AQP2-R187C (Fig. 5A, right panel) was expressed as 40 and (about) 43–46 kDa proteins. On the basis of the masses observed for the untagged forms, the 40, 43–46 and 60 kDa proteins likely represent unglycosylated, high mannose and complex glycosylated G-AQP2 proteins, respectively. Interestingly, in cell lines expressing both G-AQP2 and F-AQP2-P262L, the 32 kDa band of F-AQP2-P262L was not observed, in contrast to its 31 kDa form, whereas both F-AQP2-P262L bands were observed upon co-expression with G-AQP2-R187C.

To analyze whether F-AQP2-P262L interacts with G-AQP2 and/or G-AQP2-R187C in MDCK cells, cells co-expressing these proteins were subjected to immunoprecipitations using FLAG antibodies. Immunoblots of the lysates of these cells revealed that all three proteins were well detectable (Fig. 5B, lysate). However, immunoblot analysis of the precipitates revealed strong signals for F-AQP2-P262L and G-AQP2, but G-AQP2-R187C could not be detected. This indicated that G-AQP2, but not G-AQP2-R187C, interacts with F-AQP2-P262L in MDCK cells.

To analyze the subcellular localization of AQP2-P262L, the cells were pre-incubated with indomethacin, treated with or without forskolin and subjected to immunocytochemistry. CLSM analysis demonstrated that, independent of forskolin administration, the subcellular localization of F-AQP2-P262L was not different from that of untagged AQP2-P262L and, as reported (9), G-AQP2 was expressed in the...
apical membrane (data not shown). In cells co-expressing G-AQP2 and F-AQP2-P262L, however, both proteins seemed to co-localize in the apical membrane, whereas some co-staining was found in the basolateral membrane (Fig. 5C, upper panel). In contrast, in cells co-expressing F-AQP2-R187C and F-AQP2-P262L, both proteins showed a similar distribution when expressed alone (Fig. 4) and did not co-localize (Fig. 5C, lower panel). These localizations were independent of stimulation with forskolin (data not shown). As we were not able to generate MDCK cells stably expressing wt-AQP2 and F-AQP2-P262L, we transiently co-transfected both expression constructs into MDCK cells and stimulated them with forskolin. Subsequent confoocal analysis revealed that in the few cells that co-expressed both proteins, they co-localized mainly in the apical membrane (Fig. 5D).

To discriminate expression in the apical membrane or sub-apical regions of the cell, MDCK cells expressing wt-AQP2, G-AQP2 or F-AQP2-P262L only, or co-expressing G-AQP2 and F-AQP2-P262L, were stimulated with forskolin and subjected to apical cell surface biotinylation assays (Fig. 5E). Following immunoblotting, semi-quantification of the signals revealed ~5-fold less AQP2-P262L expression in the apical membrane than that of wt-AQP2 [1.3 versus 6.3 arbitrary units (AU), upper panel]. Upon co-expression with G-AQP2, however, ~10-fold more F-AQP2-P262L was expressed in the apical membrane than when expressed alone (16.6 versus 1.3 AU). For G-AQP2, co-expression with AQP2-P262L led to a slight reduction in apical membrane expression (10.9–8.0 AU). The absence of actin in the biotinylation samples (middle panel) and its presence in the corresponding cell lysates (lower panel) reveal the specificity of the biotinylation assays for proteins present in the apical membrane. These blotting data confirmed the increased expression of AQP2-P262L in the apical membrane upon co-expression with G-AQP2 and indicated that AQP2-P262L is sorted to the apical membrane upon heterotetramerization with wt-AQP2.

**DISCUSSION**

**Analysis of the patients**

In this study, haplotype and sequence analysis of the V2R and AQP2 genes revealed novel mutations in two separate families with recessive NDI. The affected male in family A (Fig. 1) appeared to be a compound heterozygote for 568G>A and 785C>T substitution in the AQP2 gene, encoding A190T and P262L mutations, respectively. The female proband of family B (Fig. 1) appeared to be a compound heterozygote for 559C>T and 785C>T AQP2 gene mutation, coding for R187C and P262L mutations, respectively. Haplotype analysis of both families revealed that the P262L mutation co-segregated with identical lengths for the simple sequence tags D12S131 and AFMb007yg5, whereas the length of the AFM259v9-fag differed. If a recombination event occurred between this common haplotype and the more distant locus AFM259v9, the P262L mutation could have the same ancestry. However, we have been unable to reconstruct the family trees to that degree to demonstrate this.

**AQP2-A190T and AQP2-R187C are classical AQP2 mutants in recessive NDI**

Until now, all patients with recessive NDI due to AQP2 gene mutations encode mutations found in between the first and last transmembrane domain. Although some showed a residual water transport capacity, most are non-functional (9–11) and all were retained in the ER, as concluded from their 29 (unglycosylated) and 32 kDa (high mannose glycosylated) expression forms, their dispersed ‘reticular’ expression pattern and their reduced stability compared with wt-AQP2 (10,19,20). As found for AQP2 mutants and many other proteins in diseases, this is likely due to misfolding, ER retention by quality control molecular chaperones and subsequent degradation via the proteasomal pathway (21–23). Presumably due to their misfolding, these AQP2 mutants are expressed as monomers and are not able to heteroligomerize and interfere with the further maturation and routing of wt-AQP2 in its pathway to the apical membrane, which is in line with the recessive nature of inheritance (17). As reported (10,16) and as shown in this study (Figs 2, 3 and 5), AQP2-R187C exerts all these characteristics. In line with a study from Kuwahara et al. (24), AQP2-A190T also showed the typical 29 and 32 kDa bands, was severely impaired in its trafficking to the plasma membrane and showed a dispersed staining pattern (Fig. 2). Therefore, both AQP2-R187C and AQP2-A190T can be classified as typical AQP2 mutants in recessive NDI.

**The cellular phenotype of AQP2-P262L is similar to AQP2 mutants in dominant NDI**

Our expression studies, however, showed a completely different cellular phenotype for AQP2-P262L when compared with classical mutants in recessive NDI. At first, the absence of the 32 kDa high mannose form (Fig. 2B), the plasma membrane expression in oocytes (Fig. 2B and C) and the lack of co-localization with an ER marker protein in MDCK cells (Fig. 4C) indicated that AQP2-P262L escaped the ER quality control and was, as such, not considered to be misfolded by the cell. Secondly, AQP2-P262L was able to assemble into heteroligomers with AQP2 (Figs 3 and 5B), which is never observed for classical AQP2 mutants in recessive NDI (17). Thirdly, in contrast to most other mutants in recessive NDI, AQP2-P262L is a functional water channel (Fig. 2). Although not corroborated by a study from Kuwahara et al. (24), this is in line with water permeability measurements on AQP2-P262L in yeast (25).

As such, AQP2-P262L showed a cellular phenotype clearly different from other AQP2 mutants in recessive NDI. Instead, and consistent with the molecular location of the mutation, it exerted all the characteristics that have been found for AQP2 mutants in dominant NDI (8,14–16).

**Dominancy of apical sorting signals in wt-AQP2 over the retention signals of AQP2-P262L explains recessive NDI**

A cell-biological explanation for its involvement in recessive instead of dominant NDI became apparent when AQP2-P262L was co-expressed with GFP-tagged or untagged AQP2 in MDCK cells (Fig. 5). Expressed on its own, most of AQP2-P262L resided in intracellular vesicles of unknown
identity, of which only a small fraction located in the basolateral (Figs 4 and 5D) and apical membrane (Fig. 5E) upon stimulation with forskolin. With forskolin stimulation, the level of apical membrane localization of AQP2-P262L was strongly reduced compared with that of wt-AQP2 (Figs 4 and 5E). Upon co-expression with G-AQP2 or untagged AQP2, however, intracellular retention of AQP2-P262L was greatly reduced (Fig. 5C–E). Instead and independent of forskolin stimulation, both proteins mainly localized in the apical membrane, whereas some were detected in the basolateral membrane (Fig. 5C and D). In contrast and in line with the recessive nature of NDI in these families, co-expression with G-AQP2-R187C did not result in apical membrane expression of F-AQP2-P262L (Fig. 5C).

Several data indicate that the localization of F-AQP2-P262L in the apical membrane is a consequence of its heterologomerization with AQP2. At first, in oocytes and MDCK cells AQP2-P262L interacted with wt-AQP2, but not with AQP2-R187C (Figs 3 and 5B). This is in line with our findings that AQP2 assembly into tetramers occurs in the ER and that AQP2 mutants that pass the ER are multimers (14–17,26). Secondly, the apical membrane localization of F-AQP2-P262L in MDCK cells was strongly increased upon co-expression with untagged or tagged AQP2 (Fig. 5C–E), but did not with G-AQP2-R187C (Fig. 5C). As reported, G-AQP2 mainly resides in the apical membrane, independent of forskolin stimulation (9), but the rescue of apical membrane expression of F-AQP2-P262L upon co-expression with G-AQP2 or untagged AQP2 shows that the rescue is not due to the GFP-tag. In addition, co-expression of G-AQP2 with two AQP2 mutants in dominant NDI resulted in the retention of the formed G-AQP2/mutant complexes in intracellular structures (unpublished data). The slightly reduced apical membrane expression of G-AQP2 upon co-expression with F-AQP2-P262L (Fig. 5E) and appearance of G-AQP2 in the basolateral membrane (Fig. 5B) might be due to its interaction with F-AQP2-P262L, because with forskolin some AQP2-P262L was targeted to this domain. Thirdly, the 32 kDa band of F-AQP2-P262L, which seems indicative for its missorting, is absent upon co-expressing with G-AQP2, but not with G-AQP2-R187C (Fig. 5A).

Extrapolated to the NDI patients, the recessive inheritance of NDI in the studied families can be explained as follows: In the two patients, AQP2-R187C and AQP2-A190T mutants are retained in the ER, and do not interact with AQP2-P262L. AQP2-P262L is properly folded and assembles into homotetramers, but will be mainly retained in intracellular vesicles. The consequent lack of sufficient AQP2 proteins in the apical membrane of their collecting duct cells then explains their NDI phenotype. In the parents encoding wt-AQP2 and AQP2-R187C/A190T, wt-AQP2 will not interact with either mutant, but will form homotetrameric complexes, of which the insertion into the apical membrane in collecting duct cells will be properly regulated by vasopressin. Although the AQP2 expression levels in these humans might be reduced, because all AQP2 is derived from one allele, the apparent absence of an NDI phenotype indicates that AQP2 derived from one allele suffices for a healthy phenotype. In the proband’s healthy relatives encoding wt-AQP2 and AQP2-P262L, both proteins are likely to assemble into heterotetramers.

The dominancy of wt-AQP2 on the localization of AQP2-P262L will then result in a proper AVP-regulated trafficking of the wt-AQP2/AQP2-P262L complexes to the apical membrane of their collecting duct cells.

**Molecular cause of the intracellular retention of AQP2-P262L**

It is at present unclear which molecular mechanism is fundamental to the retention of AQP2-P262L. AQP2-P262A is, similar to wt-AQP2, re-distributed from intracellular vesicles to the apical membrane with forskolin (Fig. 4), which revealed that the introduced Leu instead of the lost Pro residue causes the missorting of AQP2-P262L.

Although the AQP2-P262L band with a 1 kDa increased mass could not always be discriminated from the smaller band because of the small difference in size, it seems indicative for its missorting. As AQP2-P262L is also expressed as a band with a size similar to that of unglycosylated wt-AQP2, the increased mass AQP2-P262L band might be due to a post-translational modification or a conformational instability induced by the P262L mutation. However, PNGaseF treatment of lysates of AQP2-P262L-expressing oocytes did not result in its disappearance, which indicated that its higher mass was not due to a modification at an N-glycosylation site (data not shown), and bio-informatical analysis revealed that the P262L mutation did not introduce or remove putative post-translational modification sites.

At present, it is also unclear whether the modification of AQP2-P262L is causing its missorting or is the result of its missorting. Interestingly, the ratio of 30 versus 29 kDa AQP2-P262L in plasma membranes of oocytes is inversely related to its expression levels. Similarly, with expression of different amounts of AQP2-R187C in oocytes, the ratio of high mannose glycosylated versus non-glycosylated forms was inversely related to its expression levels, which is thought to be a reflection of the level of retention of AQP2-R187C in the ER (27). The solution to these issues has to await detailed analysis of the location and identity of the modification.

In conclusion, we identified two families in which the recessive inheritance of NDI coincides with a P262L mutation that was located in a part of AQP2 that would have been anticipated to lead to dominant NDI. Detailed cell-biological analysis revealed that it indeed exerted most of the features of an AQP2 mutant in dominant NDI, but that, in contrast to mutants in dominant NDI, the apical sorting information in wt-AQP2 was dominant over the retention information in AQP2-P262L. As such, the AQP2-P262L mutant provides a unique and alternative cell-biological explanation for the occurrence of recessive NDI. Although it has been shown that recessive inheritance of diseases involving G-protein coupled receptors can have different cell-biological causes (e.g. misfolding, lack of agonist or G-protein binding), this has, to our recollection, never been reported for channels. Besides AQP2, it has been shown that misfolding of homotetrameric plasma membrane channels (e.g. ROMK1, KIR6.2, MIP26) underlies the recessive inheritance of other diseases (28,29). Possibly, missorting of the mutant and the rescue thereof by the wild-type protein also underlie the recessive nature of these diseases in some families.
MATERIALS AND METHODS

Analysis of patients
The proband in family A was evaluated clinically as previously described (30). Infusion of dDAVP was performed as described (0.3 μg/kg of body weight infused in 30 min) (31). The V2R genes of the patients and their relatives were sequenced as described (32). The AQP2 gene of the patients and their relatives was sequenced from genomic DNA as described (33). Genotyping of three loci (AFM259vf9, D12S131, AFMbo07yg5) that flank the AQP2 gene in chromosome region 1q13 was done to follow the segregation of the NDI allele in each family (34). AFM259vf9, D12S131 and AFMbo07yg5 are dinucleotide repeats. A haplotype, which is the specific combination of alleles at closely linked loci, was assigned for these three loci. For our interpretation of haplotype data provided support that the pedigree was consistent with stated biologic parentage.

Constructs
The oocyte expression constructs pT7Ts-AQP2 and pT7Ts-AQP2-R187C, AQP2-F and AQP2-R187C-F (here annotated as F-AQP2 and F-AQP2-R187C, because the tag was N-terminally fused) were as described (6,9,10,17). To facilitate cloning of mutant AQP2 cDNAs into the eukaryotic N-terminally fused) were as described (6,9,10,17). To facilitate cloning of mutant AQP2 cDNAs into the eukaryotic

Immunoprecipitation and immunoblotting
An aliquot of 15 μl of protein-G agarose beads per sample of solubilized total membranes of 25 oocytes was washed three times with 500 μl IPP500 [500 mM NaCl, 10 mM Tris (pH = 8.0), 0.1% NP-40, 0.1% Tween-20] plus protease inhibitors [1 mM PMSF, 5 μg/ml leupeptin and pepstatin A]. Subsequently, 3 μl of the FLAG-antibody (Sigma-Aldrich, St Louis, MO, USA) or 1:5000 diluted rabbit anti-GFP antibody (257–271, 1:20 000 diluted mouse anti-β-actin (AC15; Sigma, St Louis, MO, USA) or 1:5000 diluted mouse anti-β-actin (AC15; Sigma, St Louis, MO, USA), and rabbit anti-GFP antibodies (10,36). As secondary antibodies, a 1:50 000 diluted mouse anti-β-actin (AC15; Sigma, St Louis, MO, USA), and rabbit anti-GFP antibodies (10,36). As secondary antibodies, a 1:50 000 diluted mouse anti-β-actin (AC15; Sigma, St Louis, MO, USA), and rabbit anti-GFP antibodies (10,36). As secondary antibodies, a 1:5000 diluted goat anti-rabbit IgGs (Sigma) coupled to horseradish peroxidase was used. For semi-quantification purposes, 2-fold dilution series of wt-AQP2 were blotted in parallel.

Culturing, transfection and immunocytochemistry of MDCK cells
MDCK cells stably expressing wt-AQP2 or GFP-tagged AQP2 (G-AQP2) have been described (9,18). MDCK cell culturing, stable cell transfection and selection of clones were done as described (37). Transient transfection was done with Lipofectamine 2000 reagent (Invitrogen, Breda, The Netherlands) according to manufacturer’s protocol.
Immunocytochemistry and side-specific biotinylation

Two days after injection of cRNAs, vitelline membranes were removed from oocytes, after which they were fixed, paraffin embedded, sectioned and used for immunocytochemistry as described (17). Treatment with indomethacin and forskolin, and immunocytochemistry of polarized MDCK cells were done as described (37), except that blocking occurred for 20 min in phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin, cells were permeabilized with 0.05% saponin in PBS and that antibody incubations were done for 30 min. As reported, indomethacin is an inhibitor of prostaglandin synthesis and reduces basal CAMP levels in MDCK-AQP2 cells (18). How prostaglandins increase the cAMP levels is unclear, but is thought to be due to paracrine activation of adenylyl cyclase-stimulating E-prostanoid (EP)2 or EP4 receptors. As primary antibodies, 1:100 dilutions of rat anti-E-Cadherin (DECMA, Sigma-Aldrich), rabbit anti-AQP2 antibodies, mouse anti-DDI (38) (kindly provided by D. Vaux, Oxford, UK) or FLAG antibodies (m2, Sigma-Aldrich) were used. As secondary antibodies, 1:100 diluted anti-rat, anti-rabbit or anti-mouse antibodies, conjugated to Alexa-488, Alexa-594 or Cy5 were used (Molecular Probes, Oxford, UK) or FLAG antibodies (m2, Sigma-Aldrich) were used. As secondary antibodies, 1:100 diluted anti-rat, anti-rabbit or anti-mouse antibodies, conjugated to Alexa-488, Alexa-594 or Cy5 were used (Molecular Probes, Oxford, UK) or FLAG antibodies (m2, Sigma-Aldrich) were used. As secondary antibodies, 1:100 diluted anti-rat, anti-rabbit or anti-mouse antibodies, conjugated to Alexa-488, Alexa-594 or Cy5 were used (Molecular Probes, Oxford, UK) or FLAG antibodies (m2, Sigma-Aldrich) were used. As secondary antibodies, 1:100 diluted anti-rat, anti-rabbit or anti-mouse antibodies, conjugated to Alexa-488, Alexa-594 or Cy5 were used (Molecular Probes, Oxford, UK) or FLAG antibodies (m2, Sigma-Aldrich) were used. As secondary antibodies, 1:100 diluted anti-rat, anti-rabbit or anti-mouse antibodies, conjugated to Alexa-488, Alexa-594 or Cy5 were used (Molecular Probes, Oxford, UK) or FLAG antibodies (m2, Sigma-Aldrich) were used. As secondary antibodies, 1:100 diluted anti-rat, anti-rabbit or anti-mouse antibodies, conjugated to Alexa-488, Alexa-594 or Cy5 were used (Molecular Probes, Oxford, UK) or FLAG antibodies (m2, Sigma-Aldrich) were used. As secondary antibodies, 1:100 diluted anti-rat, anti-rabbit or anti-mouse antibodies, conjugated to Alexa-488, Alexa-594 or Cy5 were used (Molecular Probes, Oxford, UK) or FLAG antibodies (m2, Sigma-Aldrich) were used. As secondary antibodies, 1:100 diluted anti-rat, anti-rabbit or anti-mouse antibodies, conjugated to Alexa-488, Alexa-594 or Cy5 were used (Molecular Probes, Oxford, UK) or FLAG antibodies (m2, Sigma-Aldrich) were used. As secondary antibodies, 1:100 diluted anti-rat, anti-rabbit or anti-mouse antibodies, conjugated to Alexa-488, Alexa-594 or Cy5 were used (Molecular Probes, Oxford, UK) or FLAG antibodies (m2, Sigma-Aldrich) were used. As secondary antibodies, 1:100 diluted anti-rat, anti-rabbit or anti-mouse antibodies, conjugated to Alexa-488, Alexa-594 or Cy5 were used (Molecular Probes, Oxford, UK) or FLAG antibodies (m2, Sigma-Aldrich) were used.

Densitometrical analysis

To relate the plasma membrane expression of AQP2-P262L and wt-AQP2 in oocytes to their total expression, immunoblot signals were semi-quantified by measuring the integrated optical densities (IOD) on the films using the Image-Pro Plus analysis software (Media Cybernetics, Silver Springs, MD, USA). The subcellular localization of the different proteins was analyzed using a Bio-Rad CLSM with a 60× oil-immersion objective, a 32 Kalmann collection filter, an aperture diaphragm of 2.8 and an axial resolution of 0.4 μm per pixel. Side-specific biotinylation of MDCK cells was done as described (37).

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