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An impaired routing of wild-type aquaporin-2 after tetramerization with an aquaporin-2 mutant explains dominant nephrogenic diabetes insipidus

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Autosomal recessive and dominant nephrogenic diabetes insipidus (NDI), a disease in which the kidney is unable to concentrate urine in response to vasopressin, are caused by mutations in the aquaporin-2 (*AQP2*) gene. Missense AQP2 proteins in recessive NDI have been shown to be retarded in the endoplasmic reticulum, whereas AQP2-E258K, an AQP2 mutant in dominant NDI, was retained in the Golgi complex. In this study, we identified the molecular mechanisms underlying recessive and dominant NDI. Sucrose gradient centrifugation of rat and human kidney proteins and subsequent immunoblotting revealed that AQP2 forms homotetramers. When expressed in oocytes, wild-type AQP2 and AQP2-E258K also formed homotetramers, whereas AQP2-R187C, a mutant in recessive NDI, was expressed as a monomer. Upon co-injection, AQP2-E258K, but not AQP2-R187C, was able to heterotetramerize with wild-type AQP2. Since an AQP monomer is the functional unit and AQP2-E258K is a functional but misrouted water channel, heterotetramerization of AQP2-E258K with wild-type AQP2 and inhibition of further routing of this complex to the plasma membrane is the cause of dominant NDI. This case of NDI is the first example of a dominant disease in which the 'loss-of-function' phenotype is caused by an impaired routing rather than impaired function of the wild-type protein.

Keywords: disease/oligomerization/oocytes/recessive

Introduction

To maintain water and osmolyte balance, the mammalian kidney forms 180 litres of pro-urine per day. Of this volume, nearly all water is reabsorbed, which occurs mainly through aquaporin-1 (AQP1) and aquaporin-2 (AQP2) water channels (Deen *et al*., 1994; Nielsen *et al*., 1998; Schnermann *et al.*, 1998). AQP1 is constitutively present in the apical and basolateral plasma membrane of proximal tubules and descending limbs of Henle, and is essential for this water reabsorption (King and Agre, 1996). Upon stimulation with the antidiuretic hormone arginine vasopressin (AVP), principal cells of the kidney collecting duct concentrate the pro-urine via AQP2 in the apical plasma membrane (Nielsen *et al.*, 1995) and via AQP3 and AQP4 in the basolateral plasma membrane (Ecelbarger *et al.*, 1995; Terris *et al.*, 1995). Binding of AVP to the vasopressin type-2 receptor (V2R) at the basolateral side of these cells increases intracellular cAMP levels, resulting in phosphorylation of, among other proteins, AQP2 by protein kinase A. Subsequently, intracellular vesicles containing AQP2 translocate to and fuse with the apical plasma membrane, rendering the cell water permeable. Upon removal of AVP, AQP2 is internalized by endocytosis and restores the water-impermeable state of the cell (Katsura *et al.*, 1995; Marples *et al.*, 1995; Nielsen *et al.*, 1995; Yamamoto *et al.*, 1995).

Nephrogenic diabetes insipidus (NDI) is a disease characterized by the inability of the kidney to concentrate urine upon stimulation by AVP and can be caused by mutations in either the *V2R* gene or the *AQP2* gene. Mutations in the *V2R* gene cause the X-linked form of inheritance of NDI (Ouweland *et al.*, 1992; Rosenthal *et al.*, 1992), whereas mutations in the *AQP2* gene are the cause of the autosomal form of inheritance of NDI (Deen *et al.*, 1994; Lieburg *et al.*, 1994; Mulders *et al.*, 1997). Upon expression in *Xenopus* oocytes, missense AQP2 mutants, encoded in the recessive form of NDI, showed an impaired routing to the plasma membrane due to retardation in the endoplasmic reticulum (ER) (Deen *et al.*, 1995; Mulders *et al.*, 1997). For some of these mutants, this has been confirmed in mammalian cells (Tamarappoo and Verkman, 1998). Recently, an autosomal dominant form of inheritance of NDI was described, caused by a mutation in the *AQP2* gene leading to a substitution of a lysine for a glutamic acid at position 258 (AQP2-E258K) (Mulders *et al.*, 1998). When expressed in oocytes, this mutant was not ER retarded, but localized mainly to the Golgi network by an as yet unknown mechanism. Co-expression of AQP2-E258K and wild-type AQP2 in oocytes confirmed the dominant-negative effect of AQP2-E258K on the function of wild-type AQP2.

A dominant inheritance of a 'loss-of-function' disease can only be explained when the mutant protein associates with the wild-type protein and inhibits the function or routing of this complex. Cryo-electron microscopy and freeze-fracture studies of AQP1 revealed that these molecules form homotetramers (Smith and Agre, 1991; Verbavatz *et al.*, 1993; Walz *et al.*, 1997), and it can be assumed that other aquaporins also form homotetramers. Radiation inactivation and oocyte expression studies, however, revealed that the individual monomers of AQP1 are functional water pores (Hoek *et al.*, 1991; Preston *et al.*, 1993). If AQP2 forms homotetramers, an impaired routing of wild-type AQP2 after oligomerization with AQP2-E258K would then explain the dominant inheritance of NDI. Consequently, in the recessive inheritance of NDI, oligomerization of the mutant AQP2 protein with the wild-type protein would not be expected.

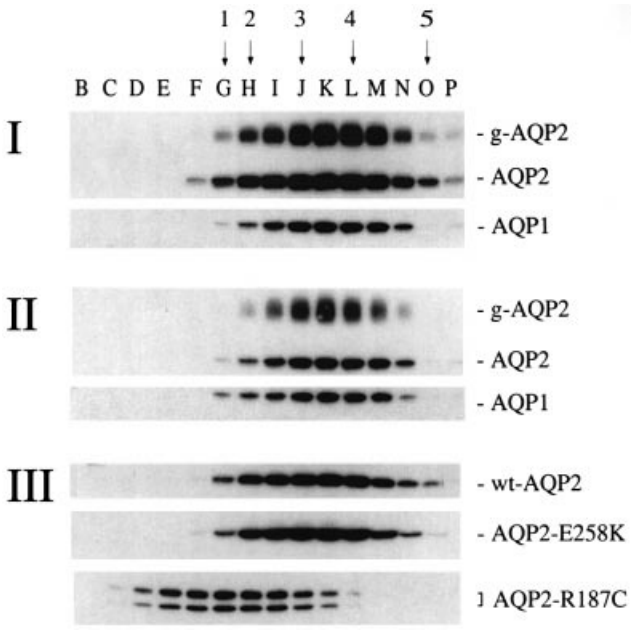

Fig. 1. Immunoblot analyses of the oligomerization state of AQP1 and AQP2. Membranes from rat (I) and human (II) kidney sections or oocytes expressing AQP2 proteins (III) were solubilized in 4% deoxycholate and subjected to sucrose gradient centrifugation. Fractions were collected and immunoblotted for AQP1 and AQP2. In kidney material, native AQP1, AQP2 and complex-glycosylated AQP2 (g-AQP2) are indicated. Oocytes were injected with 0.3 ng of cRNA encoding wild-type (wt)-AQP2, the AQP2 mutant in dominant NDI (AQP2-E258K) or a mutant in recessive NDI (AQP2-R187C). The fractions with peak intensities of the marker proteins ovalbumin (43 kDa), BSA (67 kDa), phosphorylase B (97 kDa), yeast alcohol dehydrogenase (150 kDa) and catalase (232 kDa) are indicated by the numbers 1–5, respectively.

To explain dominant and recessive NDI, we determined the oligomerization state of AQP2 and delineated the molecular mechanisms of both forms of inheritance of this disease.

Results

Wild-type AQP2 is present as a homotetramer in membranes of native tissue

To reveal the oligomerization state of AQP2 in native tissue, microsomal membranes were isolated from human and rat kidney sections, solubilized and subjected to sucrose gradient sedimentation centrifugation. Immunoblotting of fractions taken from the gradient revealed that the peak intensity of AQP2 from rat and human kidney was found in fraction K (Figure 1, I and II). Re-probing of the same immunoblots for AQP1 revealed that this protein also peaked in fraction K (Figure 1). The sedimentation marker proteins ovalbumin (43 kDa), bovine serum albumin (BSA) (67 kDa), phosphorylase B (97 kDa), yeast alcohol dehydrogenase (150 kDa) and catalase (232 kDa), which were loaded on a parallel sucrose gradient, peaked in fractions G, H, J, L and O, respectively (see arrows in Figure 1). A plot of the fraction with peak intensity versus the molecular mass of the marker proteins (not shown) revealed that in renal tissue, AQP2 occurs as a complex with a molecular mass of 120–150 kDa. Since renal AQP1, which is known to form homotetramers (Verbavatz *et al.*, 1993; Walz *et al.*, 1997), peaked in the same fraction as AQP2, and since the molecular masses of AQP1 (28 kDa) and AQP2 (29 kDa) are similar, our results strongly indicated that in human and rat kidney, AQP2 also forms homotetramers.

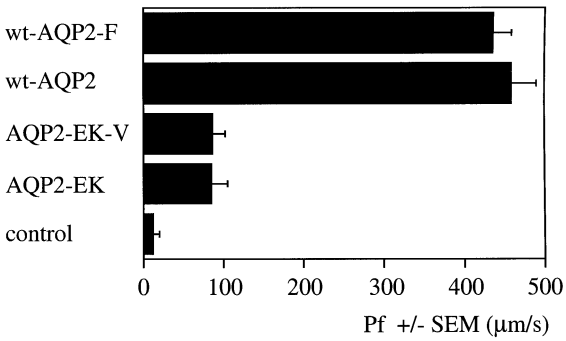

Fig. 2. Osmotic water permeability (P_f) of oocytes expressing wild-type or mutant AQP2 proteins. Oocytes were not injected (control) or injected with 10 ng of cRNA encoding wild-type (wt) AQP2 or AQP2-E258K (AQP2-EK). These proteins were untagged or tagged with a FLAG tag (-F extension) or a vesicular stomatitis virus glycoprotein tag (-V extension). Two days after injection, the P_fs were determined in a standard swelling assay. The means ± SE of at least 12 oocytes are shown.

Wild-type AQP2 and AQP2-E258K, but not AQP2-R187C, are expressed as homotetramers in oocytes

To assess whether in oocytes wild-type AQP2 and mutant AQP2 proteins in recessive and dominant NDI are also expressed as homotetramers, membranes of oocytes expressing wild-type AQP2, AQP2-E258K or AQP2-R187C were solubilized and subjected to sucrose gradient centrifugation. Immunoblotting of the obtained fractions showed that the peak intensities for wild-type AQP2 and AQP2-E258K were also found in fraction K (Figure 1, III). However, AQP2-R187C peaked in fraction G, which corresponds to a molecular mass of 30–60 kDa. These results show that in oocytes, wild-type AQP2 and AQP2-E258K, a mutant causing dominant NDI, are capable of forming homotetramers, whereas AQP2-R187C, a mutant found in recessive NDI, is unable to form homotetramers.

Wild-type AQP2 and AQP2-E258K form heterotetramers

Since wild-type AQP2 and AQP2-E258K form homotetramers in oocytes, co-expression of these proteins in oocytes could lead to formation of either homotetramers or heterotetramers of wild-type AQP2 and AQP2-E258K. To be able to discriminate between wild-type and mutant AQP2 proteins in putative AQP2 tetramers, wild-type AQP2 was N-terminally tagged with a FLAG epitope (wt-AQP2-F), whereas AQP2-R187C and AQP2-E258K were N-terminally tagged with a vesicular stomatitis virus glycoprotein (VSV-G) epitope (AQP2-R187C-V and AQP2-E258K-V, respectively). The water permeabilities of oocytes injected with 10 ng of wt-AQP2-F cRNA or AQP2-E258K-V cRNA were not different from oocytes injected with 10 ng of wild-type AQP2 cRNA or AQP2-E258K cRNA, respectively (Figure 2), which indicated that the tags did not disturb AQP2 function or routing. Subsequently, oocytes were co-injected with cRNAs encoding wt-AQP2-F and AQP2-R187C-V or wt-AQP2-F

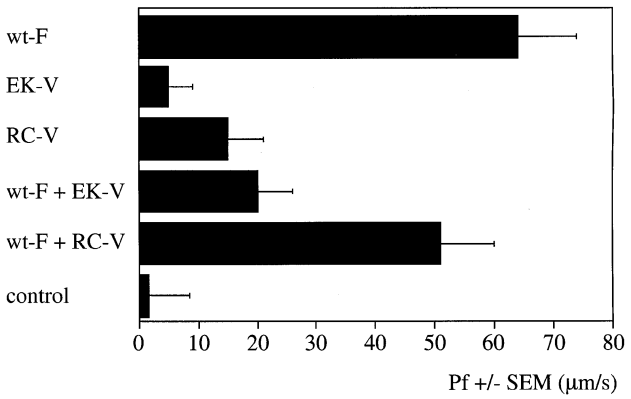

Fig. 3. Osmotic water permeability (P_f) of oocytes co-expressing wild-type and mutant AQP2 proteins. Oocytes were not injected (control) or injected with 0.3 ng quantities of cRNAs coding for FLAG-tagged wild-type AQP2 (wt-F), VSV-G-tagged AQP2-E258K (EK-V) or VSV-G-tagged AQP2-R187C (RC-V) alone or in combination (wt-F + EK-V and wt- F + RC-V). Two days after injection, the P_fs were determined in a standard swelling assay. The means ± SE of at least 12 oocytes are shown.

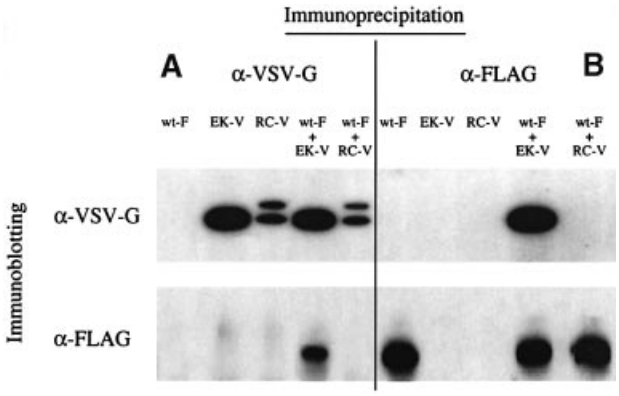

Fig. 4. Co-immunoprecipitation of tagged AQP2 proteins. Membranes of oocytes expressing FLAG-tagged wild-type AQP2 (wt-F), VSV-G-tagged AQP2-E258K (EK-V) or VSV-G-tagged AQP2-R187C (RC-V) alone or in combination (wt-F + EK-V and wt-F + RC-V) were solubilized and subjected to centrifugation on a sucrose gradient. Fractions containing the expressed proteins were pooled, divided into two equal portions and immunoprecipitated with either VSV-G antibodies (**A**) or FLAG antibodies (**B**). All precipitates were divided into two equal portions and immunoblotted with either VSV-G antibodies (α-VSV-G) or FLAG antibodies (α-FLAG).

and AQP2-E258K-V. As shown for untagged AQP2 proteins (Mulders *et al*., 1998), the water permeabilities of oocytes injected with wt-AQP2-F cRNA alone or in combination with AQP2-R187C-V cRNA were not different (Figure 3). Also, the water permeability of oocytes co-injected with wt-AQP2-F cRNA and AQP2-E258K-V cRNA was significantly reduced compared with wt-AQP2-F (Figure 3), confirming the dominant-negative effect of AQP2-E258K on the function of wild-type AQP2. The oocyte membranes were isolated, solubilized and subjected to sucrose gradient centrifugation. Sedimentation fractions containing the AQP2 proteins were pooled, divided into two equal portions and subjected to immunoprecipitation with FLAG antibodies or VSV-G antibodies. Precipitates were divided into two equal portions, loaded on parallel gels and blotted. Immunodetection with FLAG antibodies of proteins immunoprecipitated with VSV-G antibodies showed that wt-AQP2-F co-precipitated with AQP2-E258K-V, but not with AQP2-R187C-V (Figure 4A). In addition, immunodetection with VSV-G antibodies of proteins immunoprecipitated with FLAG antibodies revealed that AQP2-E258K-V, but not AQP2-R187C-V, co-precipitated with wt-AQP2-F (Figure 4B). The absence of AQP2-R187C-V in the immunoprecipitate, produced using FLAG antibodies, from solubilized membranes of oocytes co-expressing wt-AQP2-F and AQP2-R187C-V (Figure 4B) was not a consequence of low expression or inefficient immunoprecipitation of AQP2-R187C-V, because immunoprecipitation of the same fractions with the VSV-G antibodies and subsequent immunoblotting for VSV-G-tagged proteins clearly showed AQP2-R187C-V (Figure 4A). Therefore, these results clearly revealed that, in oocytes, wild-type AQP2 oligomerizes with AQP2-E258K, but not with AQP2-R187C.

AQP2-R187C is present as a monomer in oocyte membranes

When expressed in oocytes, AQP2-R187C is present in a 29 and a 32 kDa form. In the sucrose gradient sedimentation, however, AQP2-R187C peaked in the same fraction as the marker protein ovalbumin (43 kDa), which suggested that AQP2-R187C could be present in either a monomeric or a dimeric form. To reveal the state of oligomerization of AQP2-R187C, this mutant was tagged with a FLAG (AQP2-R187C-F) or a VSV-G (AQP2-R187C-V) epitope at its N-terminus. Equal amounts of the encoding cRNAs were co-injected into oocytes, and 2 days later solubilized membranes of these oocytes were subjected to sucrose gradient centrifugation. Pooled sedimentation fractions containing both tagged AQP2-R187C forms were immunoprecipitated with FLAG antibodies. Subsequent immunoblotting for the presence of VSV-G-tagged proteins revealed that AQP2-R187C-V did not co-precipitate with AQP2-R187C-F (Figure 5B). In addition, immunoprecipitation of the same fractions with VSV-G antibodies followed by immunoblotting for FLAG-

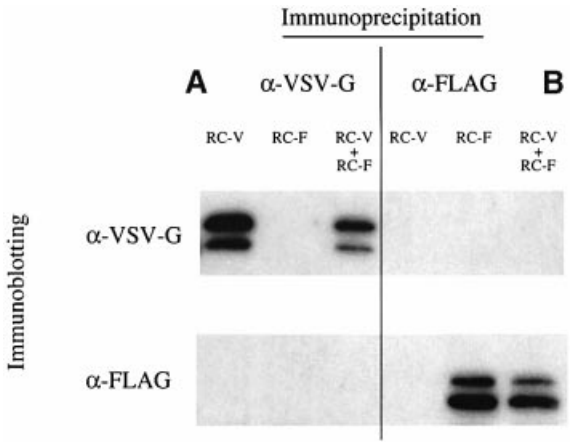

Fig. 5. Co-immunoprecipitation of tagged AQP2-R187C proteins. Membranes of oocytes expressing VSV-G-tagged AQP2-R187C (RC-V) or FLAG-tagged AQP2-R187C (RC-F) alone or in combination (RC-V + RC-F) were solubilized and subjected to centrifugation on a sucrose gradient. Fractions containing the expressed proteins were pooled, divided into two equal portions and immunoprecipitated with either VSV-G antibodies (**A**) or FLAG antibodies (**B**). All precipitates were divided into two equal portions and immunoblotted with either VSV-G antibodies (α-VSV-G) or FLAG antibodies (α-FLAG).

tagged proteins also did not show co-precipitation of AQP2-R187C-F with AQP2-R187C-V (Figure 5A). Therefore, it can be concluded that in oocytes AQP2-R187C is present in a monomeric form.

Discussion

With the identification in a family with dominant NDI of the G866A transition in the *AQP2* gene, resulting in the E258K substitution, it became apparent that mutations in the *AQP2* gene could cause recessive as well as dominant NDI. Upon expression in oocytes and other cells, missense AQP2 proteins in recessive NDI were all shown to be impaired in their export from the ER, whereas AQP2-E258K was shown to be retained in the Golgi apparatus (Deen *et al.*, 1995; Mulders *et al.*, 1997, 1998; Tamarappoo and Verkman, 1998).

Mutations causing a dominant form of inheritance of a disease lead to either a 'gain-of-function' or a 'loss-of-function' phenotype, while mutations causing a recessive inheritance of a disease lead to a 'loss-of-function' phenotype. A dominant inheritance of a disease implies that the protein involved is part of a multimeric complex, whereas in recessively inherited diseases this is not necessarily the case. Recent investigations on mutant proteins, causal for recessive and dominant 'loss-of-function' diseases, have shown or indicated that in the dominant forms of the disease the function of the complex is impaired by the interacting mutant protein, whereas the recessive form of inheritance can be brought about by different mechanisms. For example, in dominantly inherited Crigler–Najjar type II (CNII), a syndrome characterized by hyperbilirubinaemia, the mutant bilirubin UDP-glucuronosyltransferase subunit appeared to retain its ability to tetramerize with the wild-type protein, thereby impairing its functioning (Koiwai *et al.*, 1996). In recessive CNII, the mutant protein did not interact with the wild-type protein and had a low expression level due to impaired interaction with the ER translocation machinery and the subsequent degradation of the mutant protein (Seppen *et al.*, 1996). In recessive and dominant osteochondrodysplasias, a disorder affecting skeletal morphogenesis in mice and humans, the recessive form is explained differently (Vikkula *et al.*, 1995). In healthy subjects, collagen XI α1, α2 and α3 chains trimerize to form fibrillar collagen XI in cartilage. In the dominant form of the disease, a mutant α2 chain, which lacks 18 amino acids, is speculated to associate with the other chains, but to interfere with normal triple helix formation. In recessive osteochondrodysplasias, a mutant α2 chain was speculated also to be part of collagen XI fibres, but affecting its stability (Li *et al.*, 1995). Its participation in oligomerization was suggested on the basis of the fact that complete absence of the collagen XI α1 chain in mice resulted in perinatal lethal osteochondrodysplasias.

A dominant form of NDI indicated that AQP2 was also part of a complex. AQP0 and AQP1 have been shown to form homotetramers (Konig *et al.*, 1997; Walz *et al.*, 1997). Since it has also been shown that an AQP1 monomer is the functional unit (Hoek *et al.*, 1991; Preston *et al.*, 1993), we speculated that (i) AQP2 would also form homotetramers and (ii) the AQP2 mutant in dominant NDI would exert its effect by impairing the routing of wild-type AQP2 after oligomerization. In contrast, a mutant in recessive NDI was expected not to be able to oligomerize and therefore not to inhibit the routing of wild-type AQP2.

In this study, sedimentation analysis on human and rat renal tissue revealed that AQP2 sedimented as a complex of 120–150 kDa that peaked in the same fraction as AQP1 (Figure 1). When expressed in oocytes, the sedimentation profile of AQP2 was identical (Figure 1), which makes it very unlikely that in renal tissue proteins other than AQP2 are part of the AQP2 complex, and indicate a homotetrameric structure. Since AQP1 is expressed as a homotetramer and AQP1 (28 kDa) and AQP2 (29 kDa) are of a similar molecular mass, it can be concluded that AQP2 is also expressed as a homotetramer.

Expression in oocytes revealed that the mutant in dominant NDI (AQP2-E258K) also formed homotetramers, whereas a mutant in recessive NDI (AQP2-R187C) sedimented as a 30–60 kDa protein (Figure 1, III). Although this latter size is in between a monomeric (29 kDa) and a dimeric (58 kDa) form of AQP2, immunoprecipitation of differently tagged AQP2-R187C proteins co-expressed in oocytes revealed that AQP2-R187C is expressed as a monomer (Figure 5). It is possible that the high sedimentation value obtained for AQP2-R187C might have been caused by a different sedimentation of membrane proteins compared with the water-soluble marker proteins. A similar finding, resulting in overestimation of the sedimentation value of membrane proteins, was obtained in gel filtration analysis (Le Maire *et al.*, 1986).

The homotetrameric structure of AQP2-E258K in oocytes indicated that, in contrast to AQP2-R187C, AQP2-E258K might be able to associate with wild-type AQP2 when co-expressed. To be able specifically to immunoprecipitate wild-type AQP2 or the AQP2 mutants, they were tagged differently. Analyses revealed that the water permeabilities of oocytes expressing similar levels of tagged or non-tagged wild-type AQP2 or AQP2-E258K were identical, indicating that the tags did not change the normal behaviour of the respective AQP2 proteins in oocytes (Figure 2). Therefore, cRNAs coding for tagged AQP2 proteins were injected in the remaining experiments. After confirmation of the dominant effect of AQP2-E258K-V on wt-AQP2-F functioning (Figure 3), oocytes injected with cRNA encoding wt-AQP2-F, AQP2-R187C-V or AQP2-E258K-V alone, or injected with wt-AQP2-F cRNA together with AQP2-E258K-V cRNA or AQP2-R187C-V cRNA, were subjected to the co-precipitation assay. Immunoblotting of immunoprecipitates of AQP2-E258K-V or wt-AQP2-F, co-expressed in oocytes, indeed revealed that AQP2-E258K-V co-precipitated with wt-AQP2-F, and vice versa (Figure 4). However, co-precipitation of AQP2-R187C-V with wt-AQP2-F and vice versa was not observed (Figure 5). These results clearly showed that AQP2-E258K is able to hetero-tetramerize with wild-type AQP2, whereas AQP2-R187C is not.

On the basis of the present study, we can conclude that the subcellular localization of the respective AQP2 mutants provides the explanation for the mechanisms involved in recessive and dominant NDI (Figure 6): all studied AQP2 mutants in recessive NDI have been shown to be ER retarded. This ER retardation, which has been found for

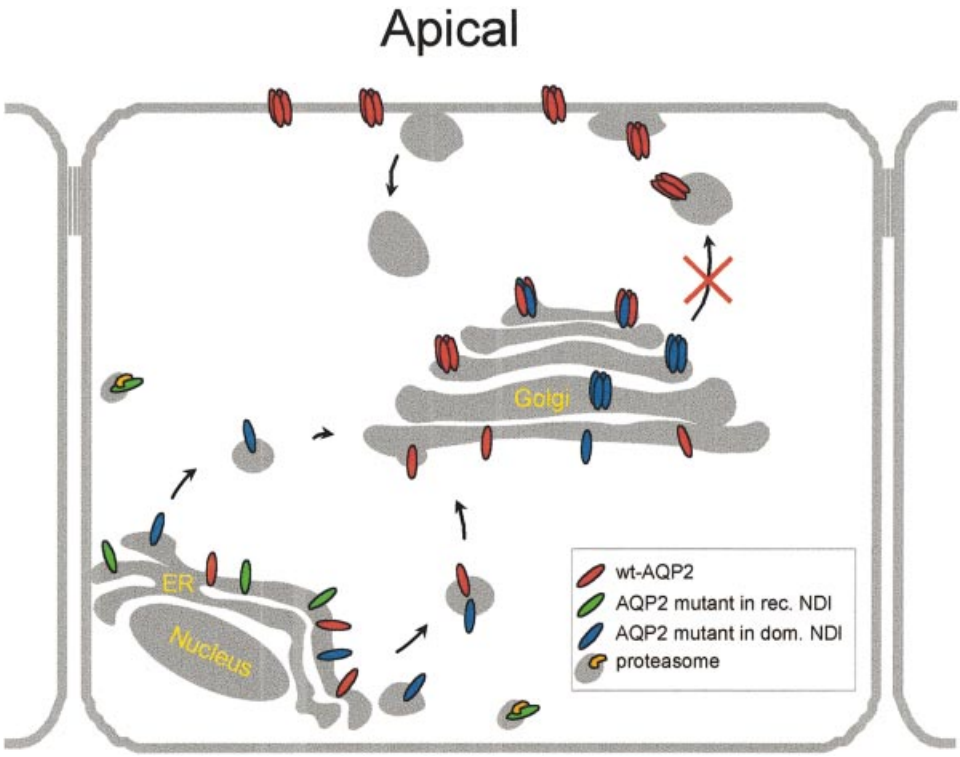

Fig. 6. Schematic representation of the postulated molecular mechanisms underlying recessive and dominant NDI caused by AQP2 mutants. See text for details.

many other proteins in recessive diseases, is a result of the quality control mechanism in the ER for misfolded proteins and is usually followed by degradation of the proteins by proteasomes (Kopito, 1997). The mutant in dominant NDI (AQP2-E258K) is not recognized as misfolded by the ER quality control, is as stable as wild-type AQP2, but is retained in the Golgi complex (Mulders *et al.*, 1998). The absence of AQP2 mutants in the urine of patients with recessive NDI, while AQP2 can be detected in the urine of patients with dominant NDI, might reflect the relative stability of the mutant proteins *in vivo* (Deen *et al.*, 1996; Mulders *et al.*, 1998). In recessive NDI, the AQP2 mutants are trapped in the ER and are not able to oligomerize with wild-type AQP2. Consequently, in the healthy parents of patients with a recessive form of NDI, whose genome encodes mutant and wild-type AQP2 proteins, wild-type AQP2 proteins are transported from the ER, can form homotetramers and fulfil their role in the concentration of urine. The genome of patients with dominant NDI also encodes a mutant (AQP2-E258K) and a wild-type AQP2 protein. However, the AQP2-E258K mutant exits the ER, after which it can heterotetramerize with wild-type AQP2. Since this mutant is retained in the Golgi, the heterotetramer is entrapped in this cell organelle. This precludes routing of enough wild-type AQP2 proteins to the apical membrane of collecting duct cells, resulting in NDI.

Conceptually, subunits of plasma membrane protein complexes undergo specific multisubunit assembly in the ER before they can be transported through the Golgi complex to the plasma membrane (Rose and Doms, 1988; Hurtley and Helenius, 1989). However, connexin 43 forms oligomers after exit from the ER (Musil and Goodenough, 1993) and influenza hemagglutinin trimerizes in the ER and the intermediate compartment (Tatu *et al.*, 1995). Although compartmentalization in the ER of wild-type AQP2 and AQP2 mutants in recessive NDI cannot be excluded, this study suggests that AQP2 assembly into tetramers occurs after exit from the ER.

Until now, the 'loss-of-function' in dominant diseases has always been explained by an impaired function of wild-type proteins (Vikkula *et al.*, 1995; Koiwai *et al.*, 1996; Cosma *et al.*, 1998). However, AQP2-E258K is a functional water channel (Figure 2) and an AQP monomer is the functional unit (Hoek *et al.*, 1991; Preston *et al.*, 1993). Therefore, dominant NDI in the family encoding AQP2-E258K is the first example of a dominant disease in which the 'loss-of-function' phenotype is caused by an impaired routing, and not function, of the wild-type protein. Other plasma membrane channels involved in recessive forms of diseases have also been shown to be expressed as homotetramers [e.g. ROMK1, KIR6.2 and MIP26 (Glowatzki *et al.*, 1995; Shiels and Bassnett, 1996; Tinker *et al.*, 1996; Verkarre *et al.*, 1998)]. It is likely that dominant forms of inheritance for these diseases will also be identified, in which the mutant protein impairs the routing of the wild-type protein.

Materials and methods

Epitope-tagged constructs

To be able to discriminate different AQP2 proteins co-expressed in oocytes, DNAs encoding FLAG (IBI, Kodak, New Haven, CT) or VSV-G (Kreis, 1986) epitope tags were cloned at the 5′ ends of mutant and wild-type AQP2 cDNAs. The 5′-FLAG-DNA was constructed by annealing and extension of the forward primer 5′-GCGGCCGCCA-CC**ATG**GACTACAAGGATGAC-3′ and the reverse primer 5′-GCCCG-

GGTCTTGTCATCGTCATCCTT-3′. VSV-G DNA was amplified with cloned *Pfu* polymerase (Stratagene, La Jolla, CA) using the forward primer 5′-GCGGCCGCCACC**ATG**GAGATTTATACAGACATA-3′ and the reverse primer 5′- GCCCGGGTCTTTCCAAGTCGGTTCAT-3′ in a PCR using a VSV-G tag-containing construct as a template. The obtained PCR fragments were digested with *Not*I and *Sma*I (underlined in the sequences) and cloned into pBlueScript II KS^+ (Stratagene, La Jolla, CA). Then a 423 bp blunted *Pst*I fragment of human wild-type AQP2 cDNA was isolated and ligated into the *Sma*I site of the tag-encoding vectors. Clones in which the AQP2 cDNA fragment was cloned in-frame with a proper FLAG or VSV-G tag were selected by DNA sequence analysis. To restore the entire wild-type AQP2 cDNA, a 635 bp *Apa*I–*Eco*RI fragment, coding for the C-terminal portion of AQP2, was ligated into the corresponding sites of the FLAG-tagged construct. To express FLAG-tagged AQP2 faithfully in oocytes, this construct was treated with *Not*I, Klenow and *Bam*HI, and the 650 bp fragment encoding the N-terminal portion of FLAG-tagged AQP2 was isolated and ligated into the blunted *Bgl*II site and the *Bam*HI site of pT_7T_S AQP2 (Deen *et al.*, 1994) and pT_7 T_SAQP2R187C (Deen *et al.*, 1994). The cloning of the VSV-G-tagged AQP2 fragment into pT_7T_SAQP2-E258K (Mulders *et al.*, 1998) and pT_7 T_SAQP2-R187C was carried out in an identical manner. With these cloning steps, constructs were obtained which code for AQP2 proteins with an N-terminal FLAG epitope (DYKDDDDK) or a VSV-G epitope (EIYTDIEMNRLGK), connected via a threonine residue.

Transcription of constructs

The obtained pT_7T_S constructs were linearized with *Sal*I and g-capped cRNA transcripts were synthesized *in vitro* using T_7 RNA polymerase according to Promega's Protocols and Principles guide (1991), except that 1 mM final concentrations of nucleotide triphosphates and 7-methyl-di-GTP were used. The cRNAs were purified and dissolved in diethylpyrocarbonate (DEPC)-treated water. The integrity of the cRNAs was checked by agarose gel electrophoresis and their concentrations were determined with a spectrophotometer.

Isolation, injection and water permeability (Pf) measurements of Xenopus oocytes

Oocytes were isolated from *Xenopus laevis* and defolliculated by digestion at room temperature for 2 h with 2 mg/ml collagenase A (Boehringer Mannheim, Mannheim, Germany). Stage V and VI oocytes were selected and stored at 18°C in modified Barth's solution [88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 10 mM HEPES (pH 7.5), 0.82 mM $MgSO_4$, 0.33 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$], supplemented with 25 μg/ml neomycin. Oocytes were (co-)injected with 0.3 or 10 ng of wild-type or mutant AQP2 cRNA and, 2 days after injection, were analysed in a standard swelling assay (Deen *et al.*, 1994).

Isolation of oocyte membranes and microsomal membranes from human and rat kidney

Forty to sixty injected or non-injected oocytes were homogenized in 1 ml of homogenization buffer [HbA; 20 mM Tris (pH 7.4), 5 mM $MgCl_2$, 5 mM NaH_2PO_4, 1 mM EDTA, 80 mM sucrose, 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 μg/ml leupeptin and pepstatin] and centrifuged twice for 5 min at 200 *g* at 4°C to remove yolk proteins. Next, membranes were isolated by 20 min centrifugation at 4°C at 14 000 *g*. The renal medulla was dissected from a rat deprived of water for 24 h to increase AQP2 expression. A section of a human renal cortex was obtained from a normal part of a kidney which had been removed because of a renal cell carcinoma. Both tissues were homogenized in 5 ml of HbA and centrifuged for 10 min at 1000 *g* at 4°C to remove nuclei and unbroken cells. Subsequently, the supernatant was spun for 30 min at 200 000 *g* at 4°C. The pellet contained the microsomal membranes.

Sedimentation by gradient centrifugation

Oocyte and kidney microsomal membranes were dissolved in solubilization buffer (4% Na-deoxycholate, 20 mM Tris pH 8.0, 5 mM EDTA, 10% glycerol, 1 mM PMSF, 5 μg/ml leupeptin and pepstatin) for 1 h at 37°C and centrifuged at 100 000 *g* for 1 h at 4°C to remove undissolved membranes, essentially according to Neely and Agre (1997). Sedimentation by gradient centrifugation was done essentially as described by Jung *et al.* (1994). Sucrose gradients (5–17.5%) were prepared of 533 μl of 5, 7.5, 10, 12.5, 15 and 17.5% sucrose each in 20 mM Tris (pH 8.0), 5 mM EDTA, 0.1% Triton X-100, 1 mM PMSF and 5 μg/ml of leupeptin and pepstatin. Samples (300 μl) of membrane proteins in Na-deoxycholate were loaded and subjected to 150 000 *g* centrifugation for 16 h at 8°C. Then 200 μl fractions were taken off carefully, designated A–S and analysed by immunoblotting with or without previous immunoprecipitation. As sedimentation markers, a mixture of ovalbumin (43 kDa), BSA (67 kDa), phosphorylase B (97 kDa), yeast alcohol dehydrogenase (150 kDa) and catalase (232 kDa) was used. All markers were from Sigma, St Louis, MO.

Immunoprecipitation

Fifteen microlitres equivalents of protein G–agarose beads (Pharmacia, Uppsala, Sweden) were pre-incubated for 12 h at 4°C with 1 μl of monoclonal FLAG antibody (m2; Sigma, St Louis, MO) or 1 μl of monoclonal VSV-G antibody (P5D4-ascitus; Kreis, 1986) in IPP500 (500 mM NaCl, 10 mM Tris pH 8.0, 0.1% NP-40, 0.1% Tween-20, 1 mM PMSF and 5 μg/ml of leupeptin and pepstatin) and 0.1% BSA. Fractions from the sucrose gradient that contained AQP2 proteins (G–L) were pooled and NaCl was added to a final concentration of 100 mM. Subsequently, the pools were divided into two equal fractions (600 μl each) and incubated for 4 h at 4°C with washed antibody-bound protein G–beads, washed three times with IPP100 (100 mM NaCl, 10 mM Tris pH 8.0, 0.1% NP-40, 0.1% Tween-20, 1 mM PMSF and 5 μg/ml of leupeptin and pepstatin) and dissolved in 30 μl of Laemmli buffer [2% SDS, 50 mM Tris (pH 6.8), 12% glycerol, 0.01% Coomassie Brilliant Blue, 25 mM dithiothreitol (DTT)]. On parallel gels, equal portions were loaded, blotted and subjected to immunodetection with either VSV-G antibodies or FLAG antibodies.

Biotinylation of antibodies

Purified IgGs from P5D4 were biotinylated with Sulfo-NHS biotin (Pierce, Rockford, IL) according to the manufacturer.

Immunoblotting

Protein samples were denatured by incubation for 30 min at 37°C in Laemmli buffer, subjected to electrophoresis on a 12% SDS–polyacrylamide gel and immunoblotted onto PVDF membranes (Millipore Corporation, Bedford, MA) by standard procedures. Marker proteins from sedimentation gradients were subjected to PAGE and stained with Coomassie Brilliant Blue. Blots with samples from the sucrose gradient were incubated with 1:3000 diluted affinity-purified rabbit AQP2:257–271 antibodies, raised against the 15 C-terminal amino acids of rat AQP2 (Deen *et al.*, 1996), or 1:100 diluted mouse monoclonal AQP1 antibodies (Jennings, 1992). Blots with samples from immunoprecipitations were incubated with 1:1000 diluted chicken α-FLAG (Aves Lab, Tigard, OR) or biotinylated α-VSV-G, both in a buffer supplemented with 1% non-fat dried milk. Blots subsequently were incubated with 1:5000 diluted goat α-rabbit IgG (Sigma, St Louis, MO), 1:2000 diluted sheep α-mouse IgG (Sigma), 1:3000 diluted goat α-chicken IgY (Aves Lab) or 1:10 000 streptavidin (Jacksons, West Grove, PN), which were all coupled to horseradish peroxidase. The latter two were subjected to the blots in a buffer supplemented with 1% non-fat dried milk. Finally, AQP2 proteins were visualized using enhanced chemiluminescence (Pierce, Rockford, IL).

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