Importance of the mercury-sensitive cysteine on function and routing of AQP1 and AQP2 in oocytes

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Mulders, Sabine M., Johan P. L. Rijss, Anita Hartog, Rene J. M. Bindels, Carel H. van Os, and Peter M. T. Deen. Importance of the mercury-sensitive cysteine on function and routing of AQP1 and AQP2 in oocytes. *Am. J. Physiol.* 273 (*Renal Physiol.* 42): F451–F456, 1997.—To discriminate between water transport of of aquaporin-2 (AQP2) mutants in nephrogenic diabetes insipidus and that of an AQP2 molecule used to drag them to the oolemma, we investigated the mercury sensitivity of wild-type and AQP2 C181S proteins in oocytes. Incubation with $HgCl_2$ inhibited the osmotic water permeability (P_f) of human (h) AQP2 by 40%, whereas inhibition of hAQP1 was 75%. Oocytes expressing hAQP1 C189S revealed a P_f comparable to wild-type hAQP1, but mercury sensitivity was lost. In contrast, no increase in P_f was obtained when hAQP2 C181S was expressed. Also, expression of rat AQP2 C181A and C181S mutants did not increase the P_f, which contrasts with published observations. Immunocytochemistry and immunoblotting revealed that only AQP1, AQP1 C189S, and AQP2 were targeted to the plasma membrane and that AQP2 mutant proteins are retarded in the endoplasmic reticulum. In conclusion, water transport through AQP2 is less sensitive to mercury inhibition than through AQP1. Furthermore, substitution of the mercury-sensitive cysteine for a serine results in an impaired routing of human and rat AQP2. Similar mutations have no effect on AQP1 function, which is indicative of structural differences between AQP1 and AQP2.

water channel; water transport; routing; endoplasmic reticulum

THE CLONING of the first discovered water channel, aquaporin-1 (AQP1), opened an exciting new field of research, and, at present, six different mammalian aquaporins are known (AQP0–AQP5) (5–7, 9, 10, 13, 16, 18). Aquaporins are members of the major intrinsic protein (MIP) family, traverse the membrane six times, and have intracellular amino and carboxyl termini (19). A characteristic amino acid stretch present in every member of the MIP family is the NPA box, found in the first intracellular loop (loop B) and in the third extracellular loop (loop E) (Fig. 1).

So far, the best-studied water channels are AQP1 and AQP2. AQP1 is constitutively expressed in erythrocytes, renal proximal tubules, descending limb of Henle, and in several other epithelia (15). AQP2 has been shown to be the vasopressin-regulated water channel that is exclusively expressed in renal collecting duct principal cells and inner medullary collecting duct cells (6).

Water permeation through AQP1 can be inhibited by binding of mercury to cysteine 189 (17, 22). When this cysteine is replaced by a serine, the water permeability (P_f) remains unaffected, but mercury sensitivity is lost. Recent reports show that mercury binding to cysteine 181 in AQP2 also results in inhibition of the P_f and substitution of cysteine 181 for serine or alanine results in loss of mercury sensitivity, together with a 20–50% reduction of P_f compared with wild-type (wt) AQP2 (1, 2).

Recently, we have reported mutations in the AQP2 gene, which are the cause of the autosomal recessive form of nephrogenic diabetes insipidus (NDI) (4, 20). All missense AQP2 proteins in NDI were found to be impaired in their routing to the oolemma (3). Jung et al. (11) reported that coexpression of AQP1 missense mutants and an AQP1 truncation mutant (D237Z) in oocytes overcomes the impaired routing to the plasma membrane (11). To apply a similar strategy as used by Jung et al. in the study of AQP2 missense mutants, we must be able to discriminate between water movement through the missense mutant and through a truncated AQP2 protein. Coexpression in oocytes of mercury-insensitive AQP2 missense proteins together with a truncated wild-type AQP2 should result in mercury-insensitive water flow when the missense mutant is still a functional water channel. Therefore, the usefulness of mercury (in)sensitivity of AQP2 proteins as a tool to discriminate between water permeation conferred by the truncated or the mutant AQP2 proteins was investigated.

MATERIALS AND METHODS

Expression constructs. The human AQP2 C181S clone was obtained by introducing a C-to-G transition at position 634 and a G-to-C transition at position 636 in the human AQP2 cDNA using the Altered Sites II in vitro mutagenesis kit (Promega, Madison, WI). The clone that was identical to wt-AQP2, except for the above-mentioned transitions, was selected by sequence analysis (8). After digestion with *Bam*H I and *Kpn* I, a 282-bp fragment was isolated by gel electrophoresis and inserted into the corresponding sites of pT7TsAQP2 (4). In this vector, an *Xba* I-*Nde* I fragment had been removed from the polylinker to have a unique *Bam*H I site in the AQP2 cDNA. The rat AQP2 C181S and C181A cDNAs (2) in the pXBGev1 expression vector were kindly provided by Drs. K. Fushimi and S. Sasaki (Tokyo, Japan). These constructs were checked by restriction analysis. The human AQP1 and the AQP1 C189S cDNAs (17) in the pXBGev1 expression vector were kindly provided by Drs. G. M. Preston and P. Agre (Baltimore, MD).

Transcription. The constructs were linearized with *Sal* I (pT7Ts) or *Xba* I (pXBGevI) and in vitro transcribed using T7 RNA polymerase (pT7Ts) or T3 RNA polymerase (pXBGev1), according to Promega's (1991) Protocols and Principles Guide, except that nucleotide triphosphates and 7-methyl-diguanosine triphosphate were used at a final concentration of 1 mM. The cRNAs were purified and dissolved in diethyl pyrocarbonate-treated milliQ water. The integrity of the cRNA was

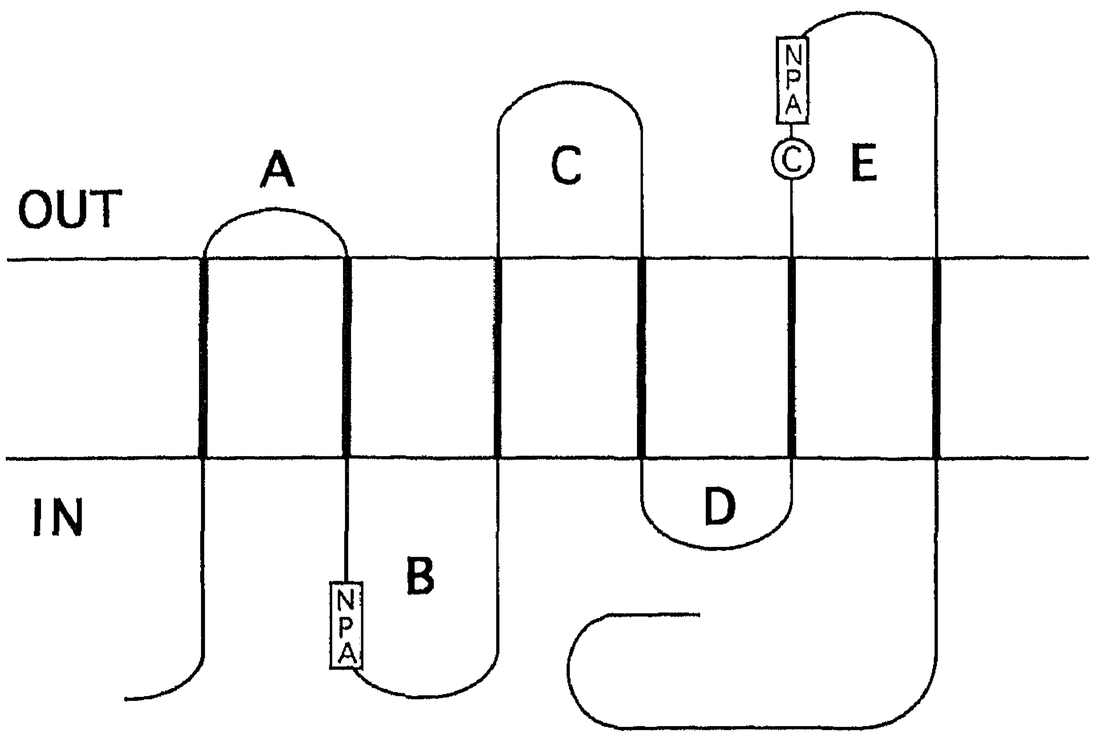

Fig. 1. Proposed topology model of aquaporin-1 and -2 (AQP1 and AQP2). Highly conserved NPA boxes in loops B and E and the mercury-sensitive cysteine (C189 in AQP1, C181 in AQP2) in loop E are indicated.

checked by agarose gel electrophoresis, and the concentration was determined spectrophotometrically.

Water permeability. Stage V and VI oocytes of *Xenopus laevis* were isolated and injected with water or 10 ng of cRNA. After incubation for 3 days in modified Barth solution (MBS) at 18°C, oocytes were analyzed in a swelling assay as described previously (4). Oocyte swelling was performed at 22°C following transfer from 200 to 70 mosmol. For the mercury inhibition studies, oocytes that exhibited a high P_f were selected and incubated for 5 min in MBS containing 1 or 3 mM $HgCl_2$. During the swelling assay, the same concentration of $HgCl_2$ was present in the diluted buffer. After this assay, the same oocytes were incubated for 15 min in buffer containing 5 mM β-mercaptoethanol and assayed again in diluted buffer containing 5 mM β-mercaptoethanol.

Oocyte lysate and membrane isolation. To determine the stability and size of the AQP1 and AQP2 proteins, eight oocytes were homogenized in 160 μl homogenization buffer A [(in mM): 20 tris(hydroxymethyl)aminomethane (Tris, pH 7.4), 5 $MgCl_2$, 5 NaH_2PO_4, 80 sucrose, 1 M EDTA, 1 dithiothreitol DTT, 1 phenylmethylsulfonyl fluoride, and 5 μg/ml leupeptin and pepstatin] at 4 °C at 1, 2 and 3 days after injection. Subsequently, the lysates were centrifuged twice for 10 min at 125 *g* to remove yolk proteins. On the 3rd day after injection, plasma membranes were isolated from 25 oocytes according to Wall and Patel (21). Oocyte lysates of an equivalent of one oocyte were digested with recombinant endoglycosidase H (endo H) (New England Biolabs, Beverly, MA) according to the manufacturer, except that protein samples were digested for 18 h after denaturation for 30 min at 37°C.

Immunoblotting. Lysates or plasma membranes equivalent to 0.1 oocyte or 8 oocytes, respectively, were denatured for 30 min at 37°C in sample buffer [2% (wt/vol) sodium dodecyl sulfate (SDS), 50 mM Tris (pH 6.8), 12% (vol/vol) glycerol, 0.01% (wt/vol) Coomassie brilliant blue, 100 mM DTT], electrophoresed through a 13% SDS-polyacrylamide gel (12), and transferred to a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany) as described previously (3). Efficiency of protein transfer was checked by reversible staining of the membrane with Ponceau red. For immunodetection, the membrane was incubated with a 1:10,000 dilution of affinity-purified rabbit polyclonal antibodies directed against the 15 COOH-terminal amino acids of rat AQP2 (3) or a 1:200 dilution of a mouse monoclonal antibody directed against dog AQP1 (gift from M. L. Jennings, Galveston, TX). As a secondary antibody, a 1:5,000 dilution of affinity-purified anti-rabbit or anti-mouse immunoglobulin G (IgG) conjugated to horseradish peroxidase (Sigma, St. Louis, MO) was used. Proteins were visualized using enhanced chemiluminescence (Boehringer, Mannheim, Germany).

Immunocytochemistry. Three days after injection, oocytes were stripped from remaining vitelline membranes and were incubated for 1 h in 1% wt/vol paraformaldehyde fixative (14), dehydrated, and embedded in paraffin. After blocking with 10% (vol/vol) goat serum in Tris-buffered saline (TBS), sections of oocytes expressing AQP2 proteins were incubated overnight at 4°C with affinity-purified polyclonal AQP2 antibodies diluted 1:500 in 10% (vol/vol) goat serum in TBS. The sections of oocytes expressing AQP1 proteins were incubated with a 1:100 dilution of a rabbit polyclonal AQP1 antibody. This antibody was prepared by immunization of rabbits with a synthetic peptide representing the last 15 COOH-terminal amino acids of rat AQP1 coupled to rabbit serum albumin. After three washes for 10 min in TBS, the sections were incubated for 1 h in a 1:100 dilution of anti-rabbit IgG coupled to fluorescein isothiocyanate (FITC) (Sigma). The sections were again washed three times for 10 min, dehydrated by washing in 70–100% (vol/vol) ethanol, and mounted in mowiol 4–88, 2.5% (wt/vol) NaN_3. Photographs were taken with a Zeiss Axioskop microscope equipped with epifluorescent illumination, and Kodak EPH P1600X films were used.

RESULTS

To determine the water permeability of wt-human (h)AQP2 and hAQP2 C181S, in comparison with wt-hAQP1 and hAQP1 C189S, cRNAs encoding these proteins were injected into *Xenopus* oocytes. P_f measurements revealed that the water transport mediated by hAQP2 and hAQP1 was comparable (Fig. 2). After incubation of the same oocytes in 1 mM $HgCl_2$, the percentage inhibition of water transport was 40 ± 8% (mean ± SE) for AQP2, whereas the inhibition of water transport was 75 ± 5% for AQP1. Incubation of the same oocytes in 5 mM β-mercaptoethanol fully restored the P_f of hAQP2 and hAQP1. Stronger inhibition was

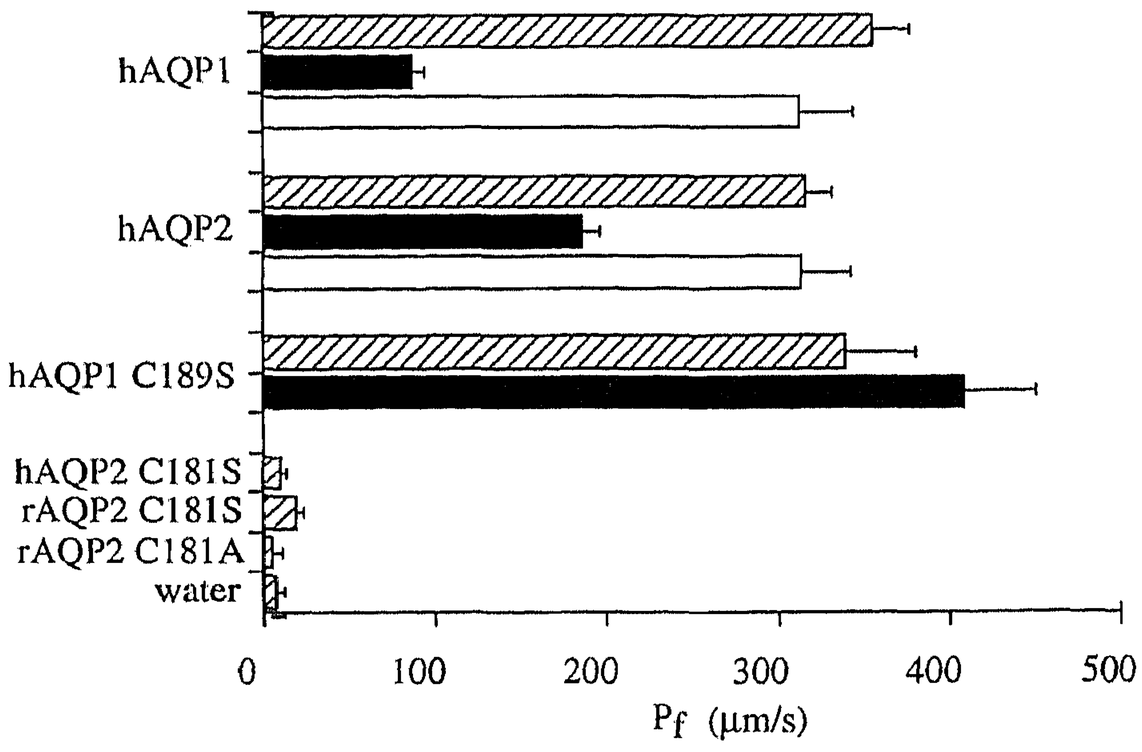

Fig. 2. Osmotic water permeability (P_f) of oocytes 3 days after injection of water or 10 ng of the following cRNAs: hAQP1, hAQP2, hAQP1 C189S, hAQP2 C181S, rAQP2 C181S, or rAQP2 C181A. Identical oocytes were subjected to the standard osmotic swelling assay (hatched bars), after incubation with 1 mM $HgCl_2$ (closed bars), and after subsequent incubation with 5 mM β-mercaptoethanol (open bars). Values are means ± SE of 15–40 oocytes.

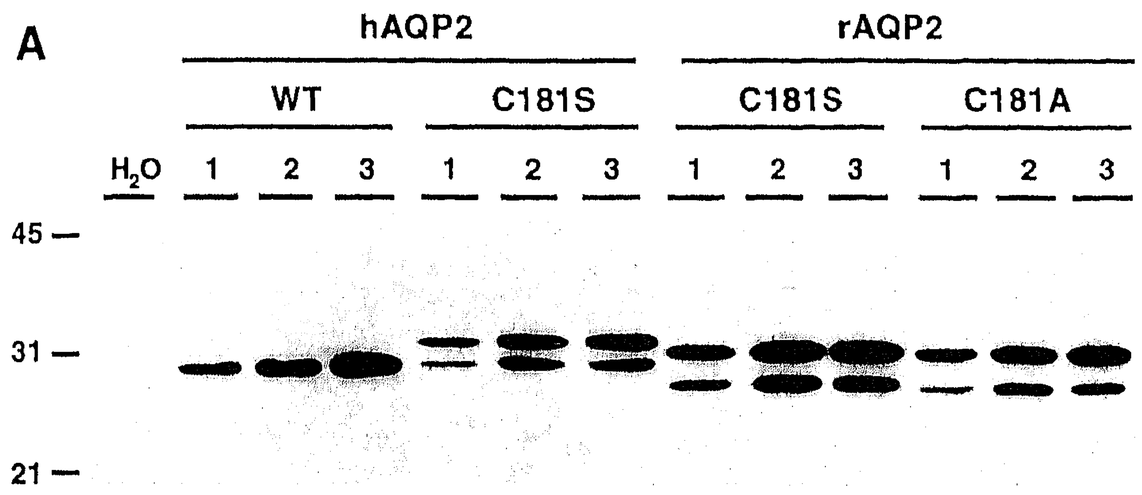

Fig. 3. Immunoblot analysis of oocyte lysates. At 1, 2, and 3 days after injection, lysates were prepared from 8 oocytes injected with water or 10 ng of cRNAs encoding hAQP2, hAQP2 C181S, rAQP2 C181S, rAQP2 C181A, hAQP1, or hAQP1 C189S. Equivalents of 0.1 oocyte were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by chemiluminescence. *A*: AQP2 proteins were visualized using affinity-purified rabbit polyclonal AQP2 antibodies and anti-rabbit immunoglobulin G (IgG) coupled to peroxidase. *B*: AQP1 proteins were visualized using monoclonal AQP1 antibodies and anti-mouse IgG coupled to peroxidase. WT, wild type.

observed after incubation for 5 min in 3 mM $HgCl_2$, with the same relative difference in P_f between AQP2 and AQP1 (data not shown). Expression of hAQP1 C189S resulted in a high P_f, which was unchanged in the presence of 1 mM $HgCl_2$. In contrast, oocytes injected with cRNA coding for hAQP2 C181S revealed a P_f that was not different from water-injected control oocytes. Because the latter result is totally in contrast to similar studies with rat AQP2 (1, 2), cRNAs encoding rat AQP2 C181S or AQP2 C181A were also injected into oocytes. Swelling tests on these oocytes also revealed P_f values that were not different from water-injected control oocytes (Fig. 2).

The absence of P_f in oocytes injected with cRNA encoding the AQP2 C181 mutants could be caused by the absense of the protein or a disturbed trafficking to the plasma membrane. To confirm the presence of AQP2 and to determine the stability, oocyte lysates were prepared at 1, 2, and 3 days after injection and subjected to immunoblotting. Reversible Ponceau red staining showed that comparable amounts of oocyte lysates were loaded (data not shown). Chemiluminescence revealed a band of ~29 kDa in all lanes of oocytes injected with cRNA encoding AQP2, representing the native, unglycosylated form of AQP2 (Fig. 3*A*). The hAQP2 C181S mutant protein showed, besides the native 29-kDa band, a strong additional band of ~32 kDa. Oocytes expressing rAQP2 C181S or rAQP2 C181A mutants also showed unglycosylated AQP2 and a larger AQP2-specific band, but these bands migrated somewhat faster than the human AQP2 protein (~27 and ~31 kDa) (Fig. 3*A*).

Immunoblots of oocytes expressing hAQP1 revealed one band of 28 kDa representing the native unglycosylated form of AQP1. Oocytes expressing hAQP1 C189S showed the same native band and a minor additional

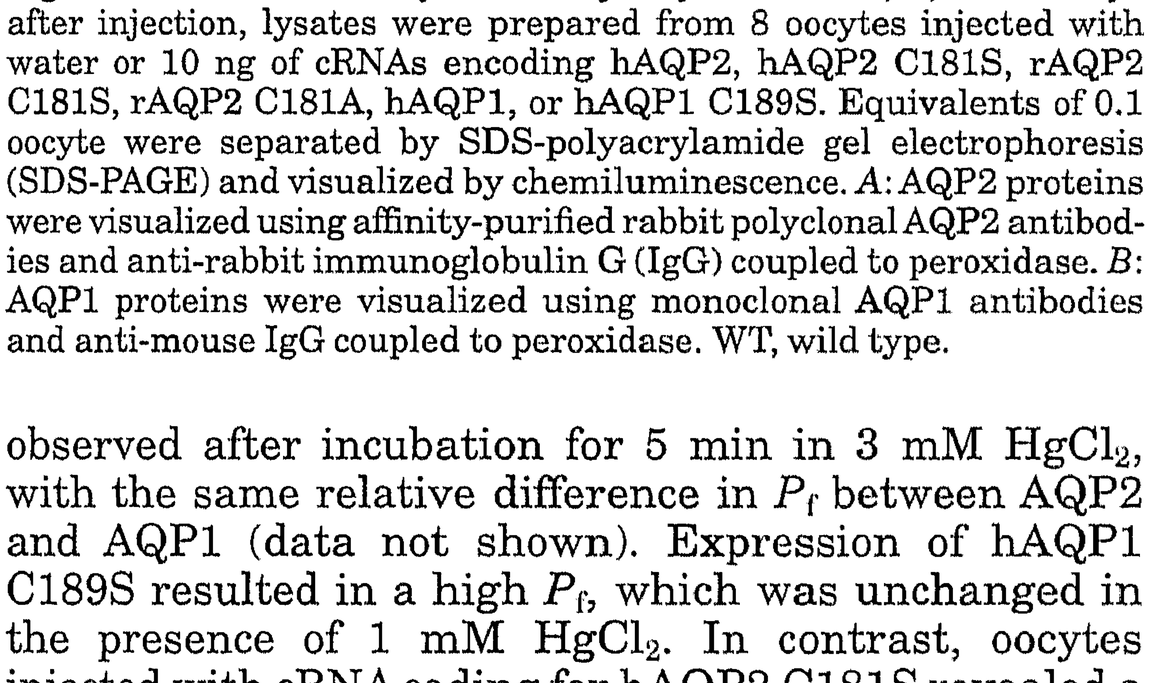

Fig. 4. Immunoblot analysis of oocyte lysates after endoglycosidase H (endo H) digestion. Lysates of oocytes expressing wt-AQP2, hAQP2 C181S, rAQP2 C181S, or rAQP2 C181A were incubated in the presence (+) or absence (−) of endo H. Equivalents of 0.1 oocyte were separated by SDS-PAGE and immunoblotted. AQP2 proteins were detected as described in Fig. 3.

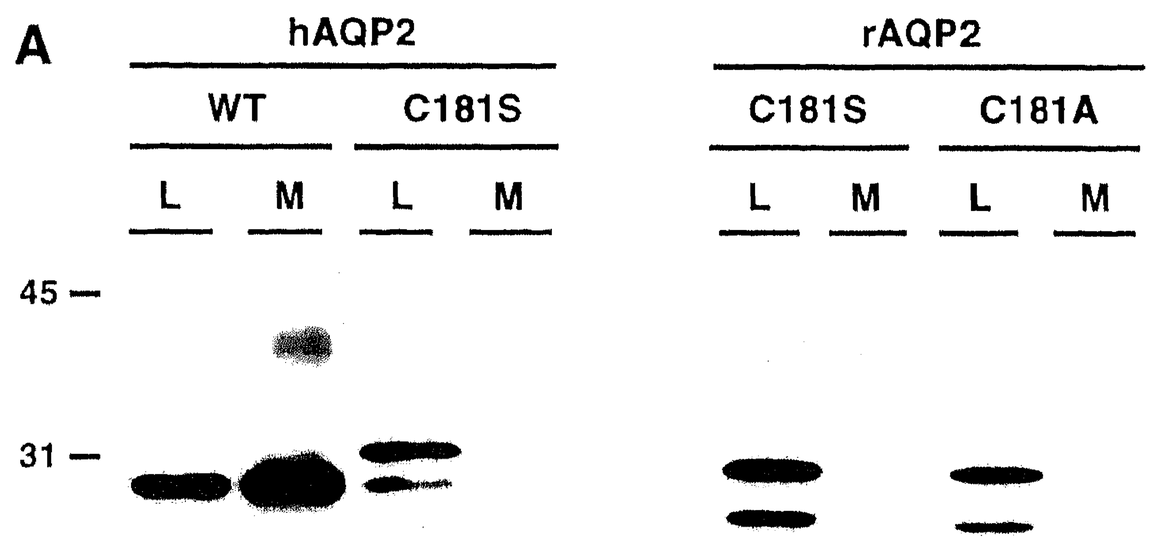

Fig. 5. Immunoblot analysis of oocyte lysates (L) and plasma membranes (M) of AQP2 (*A*) or AQP1 (*B*) expressing oocytes. Three days after injection of 10 ng of cRNAs encoding hAQP2, hAQP2 C181S, rAQP2 C181S, rAQP2 C181A, hAQP1, or hAQP1 C189S, oocyte lysates and plasma membranes were prepared. Equivalents of 0.1 oocyte (L) or 8 oocytes (M) were separated by SDS-PAGE and visualized as described in Fig. 3.

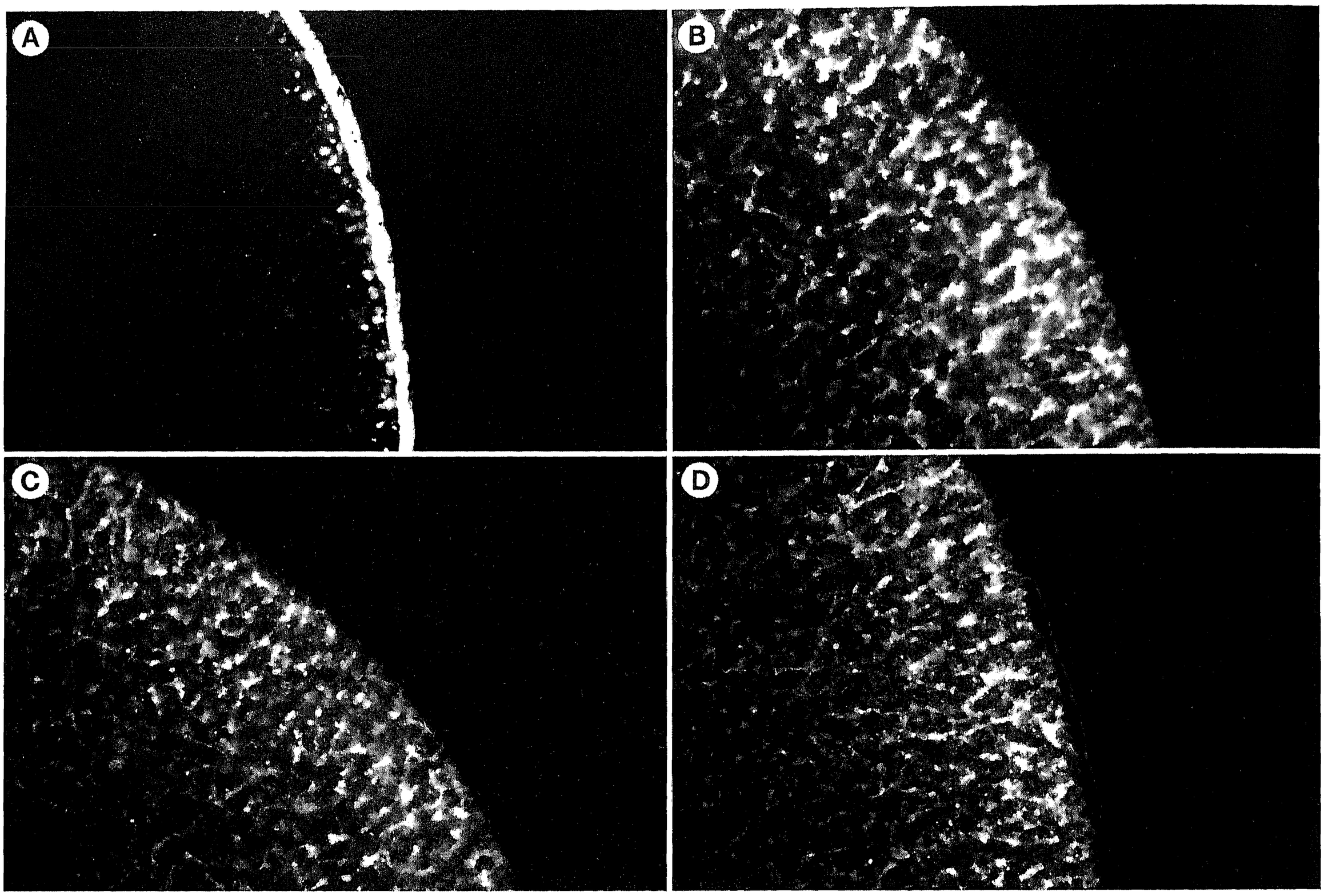

Fig. 6. Sections of oocytes expressing hAQP2 (*A*), hAQP2 C181S (*B*), rAQP2 C181S (*C*), or rAQP2 C181A (*D*). AQP2 proteins were visualized with affinity-purified polyclonal AQP2 antibodies and anti-rabbit IgG conjugated with fluorescein isothiocyanate.

band of ~31 kDa (Fig. 3*B*). Endoglycosidase H (endo H) digestion of oocyte lysates expressing AQP2 proteins and subsequent immunoblotting revealed that the additional bands of hAQP1 C189S (not shown), hAQP2 C181S, rAQP2 C181S, and rAQP2 C181A were not detectable anymore (Fig. 4). No AQP1 or AQP2 signals were obtained in lanes loaded with lysates from water-injected oocytes.

To determine the plasma membrane expression of wild-type and mutant aquaporins, a fraction enriched for plasma membranes was subjected to immunoblotting (Fig. 5). Chemiluminescence revealed that wt-hAQP2 was clearly present in the plasma membrane, whereas hC181S, rC181S, or rC181A mutant proteins could not be detected in this fraction (Fig. 5*A*). Wild-type AQP1 and AQP1 C189S were expressed in the plasma membrane to the same extent (Fig. 5*B*). In the plasma membrane fraction of oocytes expressing AQP1 C189S, a relatively higher amount of glycosylated AQP1 (40–45 kDa) was present than in the plasma membrane fraction of wt-AQP1.

Immunocytochemical analysis of oocytes expressing wt-hAQP2 (Fig. 6*A*), wt-AQP1 (data not shown), or AQP1 C189S (not shown) showed a clear, intense staining of the plasma membrane with a weak staining of the cytoplasm. In contrast, oocytes expressing hAQP2 C181S, rAQP2 C181S, or rAQP2 C181A showed an intense staining of the cytoplasm and only a very faint staining of the plasma membrane (Fig. 6, *B–D*). No AQP2 or AQP1 labeling was found in water-injected control oocytes (not shown).

To check the expression system, the P_f measurements and immunoblots were repeated with oocytes isolated from *Xenopus laevis* from an unrelated source. These experiments yielded identical results (data not shown).

DISCUSSION

To drag NDI-related AQP2 mutants to the plasma membrane of *Xenopus* oocytes to obtain information on the structure-function relationship of AQP2, two requirements had to be fulfilled: *1*) oocytes expressing wt-AQP2 should reveal a large decrease in P_f on incubation with mercurials, and *2*) the mutation of cysteine 181 to serine in hAQP2 should not affect the expression and function of the protein.

To address the first issue, mercury sensitivity of AQP2 was compared with that of AQP1. P_f studies revealed that the P_f values of oocytes expressing hAQP1 or hAQP2 were comparable (Fig. 2) but that the mer-

cury sensitivity of AQP2 was less than of AQP1 (40 and 70% inhibition, respectively). A full recovery of the P_f after incubation in β-mercaptoethanol was found for both AQP1 and AQP2 expressing oocytes indicative of specific mercury inhibition of AQPs rather than toxic effects of mercury chloride. The difference in mercury sensitivity suggests that the structure of the AQP2 pore is somewhat different from the water pore in AQP1, which is in line with mutagenesis studies from other groups (2, 11).

To address the second requirement, a C181S mutation was introduced into hAQP2. Expression of hAQP2 C181S, however, revealed no increase in P_f, whereas oocytes expressing wt-hAQP2, wt-hAQP1, or hAQP1 C189S revealed normal, high P_f values. The absence of functional expression for hAQP2 C181S was in complete contrast to the results reported for rat AQP2 C181S (1, 2). To rule out the possibility that a structural difference between human and rat AQP2 causes the discrepancy in functionality, hAQP2 C181S was expressed in parallel with rat AQP2 C181S and C181A mutants. Like hAQP2 C181S, however, both rat mutants did not confer water permeability to oocytes. To address the absence of expression in great detail, the sizes, stability, and cellular localization of these mutants were determined (Figs. 3–6).

Immunoblots revealed that hAQP2 C181S, rAQP2 C181S, and rAQP2 C181A proteins were as stable as wt-AQP2 but were, besides a wt-AQP2 protein form, also detected as an endo H-sensitive form (Figs. 3 and 4). In the endoplasmic reticulum (ER), chaperones guide the folding of proteins, and misfolding often leads to degradation and/or piling up of intermediates. The presence of endo H-sensitive, high-mannose AQP2 glycoproteins is a clear indication that the proteins are retarded in the ER, as has been shown for all missense AQP2 proteins involved in NDI (3). For all three AQP2 mutants, the signal of the ER-retarded form is more intense than the signal of the nonglycosylated form. In contrast, an ER-retarded band is hardly visible on immunoblots of oocytes expressing the functional hAQP1 C189S mutant. This indicates that the severity of the impairment of routing of AQPs is reflected by the relative expression levels of the ER form and the nonglycosylated form. Immunoblotting of fractions enriched for plasma membranes and immunocytochemistry confirmed the impaired routing of hAQP2 C181S, rAQP2 C181S, and rAQP2 C181A to the plasma membrane (Figs. 5 and 6). Identical results were obtained in oocytes isolated from *Xenopus laevis* of an unrelated source. Therefore, it is very unlikely that our results are a consequence of the batch of *Xenopi* used. Immunoblots of plasma membranes show that wt-AQP1 and AQP1 C189S are expressed at the plasma membrane at comparable levels. However, the amount of glycosylated AQP1 is higher for the mutated protein. A possible explanation can be that, because AQP1 C189S is somewhat retarded in the ER, more molecules are high-mannose glycosylated in the ER compared with wt-AQP1. Because ER glycosylation is essential for a change to complex glycosylation in the Golgi, a larger portion of the mutant AQP1 is consequently glycosylated.

Our results clearly show that in oocytes, human AQP2 C181S, rat AQP2 C181s, and rat AQP2 C181A are not functional because they are severely disturbed in their routing to the oolemma. The misrouting of hAQP2 C181S precluded our goal to use this mutation in AQP2 to discriminate between water permeability conferred by a truncation mutant and NDI-related AQP2 mutants. In addition, the water permeability obtained for rat C181S and C181A by Bai et al. (1, 2) was of critical importance for their conclusion that the water pore in AQP2 is different from the one in AQP1. They concluded that loops C and D are located near the pore in AQP2 and that loops B and E are not of critical importance in AQP2 as in AQP1. Our results with their clones and human AQP2 C181S makes this conclusion at least doubtful.

In conclusion, our results show that water transport through AQP2 is less sensitive to mercury inhibition than through AQP1 and that substitution of the cysteine residue in loop E for a serine completely disturbs proper folding, assembling, and/or routing of human and rat AQP2, whereas the same mutation has no effect on AQP1. This suggests that mutations in AQP1 are better tolerated than in AQP2 and thus that AQP1 and AQP2 might differ in their tertiary structure.

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