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A Yeast Recombinant Aquaporin Mutant That Is Not Expressed or Mistargeted in *Xenopus* Oocyte Can Be Functionally Analyzed in Reconstituted Proteoliposomes*

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We have recently identified AQPcic (for aquaporin cicadella), an insect aquaporin found in the digestive tract of homopteran insects and involved in the elimination of water ingested in excess with the dietary sap (Le Cahérec, F., Deschamps, S., Delamarche, C., Pellerin, I., Bonnec, G., Guillam, M. T., Gouranton, J., Thomas, D., and Hubert, J. F. (1996) *Eur. J. Biochem.* 241, 707–715). Like many other aquaporins, AQPcic is inhibited by mercury reagents. In this study, we have demonstrated that residue Cys^{82} is essential for mercury inhibition. Another mutant version of AQPcic (AQP-C134S), expression of which in *Xenopus laevis* failed to produce an active molecule, was successfully expressed in *Saccharomyces cerevisiae*. Using stopped-flow analysis of reconstituted proteoliposomes, we demonstrated that the biological activity and Hg sensitivity of yeast-expressed wild type and mutant type AQPcic was readily assessed. Therefore, we propose that the yeast system is a valid alternative to *Xenopus* oocytes for studying particular mutants of aquaporin.

The existence of molecules implicated in water transport across the cellular membranes has been postulated for many years. The high permeability of certain cell types (*i.e.* erythrocytes, epithelial cells of the kidney proximal tubules or collecting duct) are not readily explained by simple diffusion of water across the lipid bilayer (1). In 1992, Agre and co-workers identified AQP1 (for aquaporin 1, initially called CHIP28), which functions in the regulation of water transport across the membrane in human erythrocytes (2). In mammals, water channels have been characterized in cell membranes of a variety of tissues such as the kidney (AQP1 (Ref. 2), AQP2 (Ref. 3), or AQP3 (Refs. 4 and 5)), the brain (AQP4; Ref. 6), the salivary glands (AQP5; Ref. 7), and the testis (AQP7 and AQP8; Refs. 8 and 9) (reviewed in Refs. 10 and 11). In addition, aquaporins have also been described in plants (12–14) and *Escherichia coli* (15).

In a previous study, we characterized the first insect aquaporin, which we termed AQPcic (for aquaporin cicadella, initially called P25). AQPcic is present in an epithelial complex found in the digestive tract (the filter chamber) of an homopteran sap-sucking insect, *Cicadella viridis* (16–19). The filter chamber of this insect is highly specialized in water transport, permitting excess of ingested water to be rapidly transferred from the initial midgut to the terminal midgut through a transepithelial osmotic gradient (20). The cDNA encoding AQPcic was recently isolated and sequenced (18). Thus far, the functional characterization of AQPcic has been performed either by injecting native AQPcic-reconstituted proteoliposomes into *Xenopus* oocytes (19) or by microinjection of *in vitro* transcribed cRNA into *Xenopus* oocytes (18). Through this work, we demonstrated that the Hg sensitivity of AQPcic is lower than that of AQP1 (18). We postulated that this difference may be due to the intramembraneous localization of cysteine residue(s) implicated in mercurial inhibition.

In this study, we identify the mercurial inhibitory site of AQPcic by utilizing three AQPcic mutants, in which Cys^{82}, Cys^{90}, and Cys^{134} are replaced by serine residues.

The replacement of Cys^{82} in AQPcic completely abrogates Hg sensitivity of the protein, suggesting that Cys^{82} has a critical role in aquaporin function. We further demonstrate that certain mutants when expressed in *Xenopus* oocytes are not correctly processed and therefore propose an alternative system to analyze such mutants.

EXPERIMENTAL PROCEDURES

Plasmid Construction and Mutagenesis—pSP-AQPcic corresponds to the full-length AQPcic coding sequence subcloned into plasmid pXβG-ev1 (18). The yeast expression vectors (pYeDP10 and pYeDP60) were gifts of Dr. Pompon (21, 22). The wild type or mutated forms of AQPcic were placed under the control of a GAL10-CYC1 promoter and a phosphoglycerate kinase terminator. The coding region of AQPcic was amplified by polymerase chain reaction using two primers: Y1, 5′-GGGGAATTC**ATG**GCCGCCGACAAGT-3′; Y2, 5′-CGCAAGCTTGAGCTCGTACACTAGTGTCTGGAGCT-3′.

The polymerase chain reaction primers contain *Eco*RI and *Sac*I restriction sites (underlined) used to clone into the yeast vectors' polylinker. These constructs were called pYeDP10-AQPcic and pYeDP60-AQPcic.

Mutagenesis on cysteine residues 82, 90, and 134 was performed with the CLONTECH mutagenesis kit (Promega) using pSP-AQPcic vector as a template. Primer PSE (Table I) contains a mutation that destroys the *Sca*I site of pXβG-ev1 (PSE, Table I), and the mutation primers (listed in Table I) contain the mutated codons. Mutations were confirmed by enzymatic nucleotide sequencing (U. S. Biochemical Corp.). The coding region of mutant C134S was then amplified by polymerase chain reaction using primers Y1 and Y2 and the pSP-AQPC134S construction as a template and subcloned in the yeast expression vector pYeDP60 (the construct was termed pYeDP60-C134S).

Water Transport Assays in Xenopus Oocytes—cRNA injections into oocytes were performed as described previously (18). Briefly, oocytes swelling was induced by a 5-fold dilution of extracellular buffer A (82.5 mM NaCl, 2.5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 2 mM $NaHCO_3$, 10 mM

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TABLE I
Oligonucleotides for site-directed mutagenesis of AQPcic

The mismatched bases are underlined, and codons in brackets represent the mutated amino acid residues. Superscript numbers represent the base pair number of the AQPcic cDNA coding sequence.

PSE	5′-CTTGGTT*GAATTC*TCAGCAGTCACAG-3′
C82S	^{234}5′-CGTGTCAGGG[TCC]CACATCAAC-3′^{255}
C90S	^{260}5′-CCGTCACC[TCA]GGCCTCCTCG-3′^{280}
C134S	^{387}5′-CAGGGGCACTCTT[TCT]ATGACGAG-3′^{410}

Hepes/NaOH, pH 7.4) and was monitored by videomicroscopy. The osmotic water permeability coefficient (P_f) was calculated by Equation 1.

$$P_f = V_0 \times d(V/V_0)/dt/[S \times V_w \times (C_{in} - C_{out}) \quad (23) \qquad \text{(Eq. 1)}$$

S is the oocyte surface area ($S = 0.045$ cm^2), V_0 the initial volume ($V_0 = 9 \times 10^{-4}$ cm^3), V the oocyte volume at a given time t, V_w the molecular volume of water ($V_w = 18$ cm^3/mol), and $d(V/V_0)/dt$ the initial rate of oocyte swelling. C_{in} is 176 mmol/kg and C_{out} 38 mmol/kg. For mercurial inhibition analysis, oocytes were incubated 15 min in 0.3 mM $HgCl_2$ prior to osmotic shock. For each experiment, *Xenopus* oocyte total membranes were prepared by the method described in Ref. 24.

Expression of Recombinant Wild Type or Mutated AQPcic in Saccharomyces cerevisiae—Studies were performed using the W303.1B strain of *S. cerevisiae* (α, *leu2, his3, trp1, ura3, ade2–1, can*^{R}, *cyr*$^+$) as described previously (25). Two culture conditions were used to overexpress the recombinant aquaporins. Yeast transformants containing pYeDP10 vector (called Y10) or pYeDP10-AQPcic (Y10-AQPcic) were grown at 28 °C for 24 h in a minimal medium (0.7% yeast nitrogen base without amino acids, 0.1% casamino acids, and 2% glucose) to $OD_{660\,nm} = 3$. To induce heterologous expression of AQPcic, the cells were diluted to 0.1 OD (660 nm) in galactose medium (0.7% yeast nitrogen base without amino acids, 0.1% casamino acids, and 2% galactose). Yeast transformants containing, respectively, pYeDP60 vector (Y60), pYeDP60-AQPcic (Y60-AQPcic), or pYeDP60-AQPC134S (Y60-C134S) were grown at 28 °C in a rich medium (1% yeast extract, 1% Bactopeptone, 0.5% glucose) for 36 h. Induction of protein expression was performed by direct addition of galactose in the culture medium (20 g/liter). The yeast cells were grown at 28 °C with continuous shaking for 16–20 h.

Purification of AQPcic—Yeast cells were harvested, homogenized with glass beads, and shaken manually, and the total membrane fractions were prepared as described previously (26). AQPcic was purified using a method adapted from the purification of AQP1 (27, 28). Membrane proteins were solubilized by addition of one volume of 4% *N*-lauroylsarcosine, 4 mM $NaHCO_3$, 2 mM DTT[1] for 2 h at room temperature. The non-solubilized material was pelleted by centrifugation at 100,000 × *g* for 45 min at 10 °C. The pellet was resuspended in 0.1 volume of 1.2% OG (*n*-octyl β-D-glucopyranoside), 1 mM DTT, 20 mM Tris-HCl, pH 7.4, and AQPcic solubilization was performed overnight at 4 °C. Preparation was then centrifuged at 150,000 × *g* for 45 min at 10 °C, and the supernatant (containing AQPcic) was recovered and filtered through a 0.22-μm membrane (Millipore). The material was loaded onto a MonoQ HR 5/5 anion-exchange column (Amersham Pharmacia Biotech) equilibrated with chromatography buffer (1.2% OG, 20 mM Tris-HCl, pH 7.4, 1 mM DTT). The column was eluted with a 15-ml gradient of 0–0.4 M NaCl in the same buffer, and 0.5-ml fractions were collected. Highly pure AQPcic was eluted at 0.15 M NaCl.

Proteoliposome Reconstitution and Stopped Flow Experiments—To prepare proteoliposomes, lipid solution (23% cholesterol, 77% phosphatidylcholine/phosphatidic acid (9/1, w/w)) was dried to a thin film under a stream of nitrogen. The lipid film was dissolved either in 2 ml of 1.2% OG, 50 mM NaCl, 1 mM DTT, 20 mM Tris-HCl, pH 7.4, with or without aquaporins. The protein/lipid ratio of proteoliposomes was 1/150 (w/w). The solutions were dialyzed at 4 °C against buffer D (50 mM NaCl, 1 mM DTT, 20 mM Tris-HCl, pH 7.4) and filtered through a 0.2-μm Millex GV membrane (Nucléopore). AQPcic proteoliposomes or control liposomes were exposed to an osmotic gradient by rapid mixing with an equal volume of buffer D containing mannitol, and the P_f were measured. The concentration of mannitol was sufficient to increase by 2-fold the osmolarity of the mixed solution. Experiments were performed with a stopped-flow spectrophotometer (SFM3, Biologic, Claix, France) characterized by a dead time of 0.8 ms and a maximal rate of data acquisition of 10 kHz. The light of a 150-watt mercury-xenon arc lamp is driven from the monochromator to the observation chamber (8 μl) by an optical fiber. The increase of the 90° scattered light intensity, corresponding to water efflux from the liposomes, was followed at $\lambda_{ex} = 430$ nm. The data obtained from at least 10 determinations were averaged and fitted to single exponential curves using a software provided by Biologic (Claix, France). The fitting parameters were used to calculate the initial rate constant k (s^{-1}) and the P_f (cm·s^{-1}) was determined according to Equation 2.

$$P_f = k/[(S/V_0) \times V_w \times \Delta osm \times \sigma] \qquad \text{(Eq. 2)}$$

S/V_0 (cm^{-1}) is the ratio of the vesicle surface area to the initial volume, V_w is the partial molar volume of water (18 cm^3/mol), Δosm is the osmotic difference between the initial intra- and extravesicular mannitol concentrations, and σ is the reflexion coefficient of the mannitol ($\sigma_{man} = 1$; see Ref. 29).

Electrophoresis and Immunoblotting—Proteins resolved by SDS-PAGE (30) were either stained with Coomassie Blue or electrotransferred onto PVDF membrane (31). Immunodetection was performed using polyclonal antibodies raised against the native *Cicadella* AQPcic protein (17).

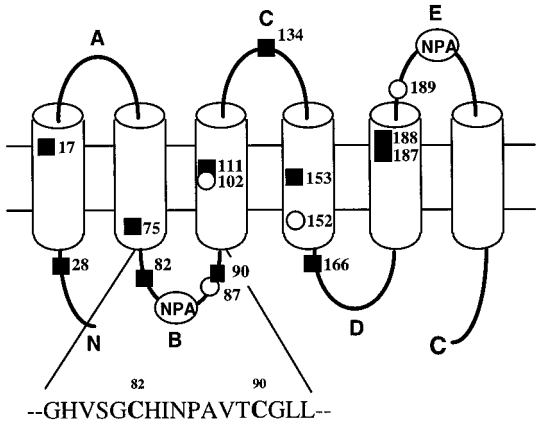

FIG. 1. **Localization of cysteines residues in AQPcic and AQP1.** Schematic representation of an aquaporin with the 6 putative transmembrane domains. The cysteine residues are indicated and numbered. ■ for AQPcic and ○ for AQP1. The amino acids sequence of the B loop of AQPcic is shown and the cysteines 82 and 90 are indicated in bold type.

RESULTS

Cysteine 82 Is the Mercurial-sensitive Site of AQPcic—In order to identify the mercurial inhibitory site(s) of AQPcic, we constructed three mutants, which contain substitutions to serine at positions Cys^{82} (AQP-C82S), Cys^{90} (AQP-C90S), and Cys^{134} (AQP-C134S) (Fig. 1). The mutant or wild type forms of AQPcic were *in vitro* transcribed and injected into *Xenopus* oocytes, and water permeability coefficients (P_f) of oocytes were measured after hypoosmotic shock with or without $HgCl_2$ pretreatment. As shown in Fig. 2*A*, the P_f values for wild type AQPcic and mutant types AQP-C82S and AQP-C90S are similar. In contrast, the replacement of Cys^{134} by a serine in AQP-C134S totally inhibited the aquaporin-induced water permeability (Fig. 2*A*).

As described previously, pretreatment of AQPcic-expressing oocyte with 0.3 mM $HgCl_2$ resulted in a total inhibition of the water permeability (Fig. 2*A*). Although the C90S substitution did not modify the $HgCl_2$ sensitivity of AQPcic, mutation of Cys^{82} totally abolished the inhibitory effect of mercurial reagent (Fig. 2*A*). These results clearly demonstrate that Cys^{82} is the Hg-sensitive site in AQPcic.

To verify the presence and the integrity of the expressed aquaporins, we performed a Western blot with oocyte membrane proteins, using an AQPcic antibody (Fig. 2*B*). The recombinant AQPcic or the two mutant AQP-C82S and AQP-C90S

[1] The abbreviations used are: DTT, dithiothreitol; OG, *n*-octyl β-D-glucopyranoside; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; SRP, signal recognition particle.

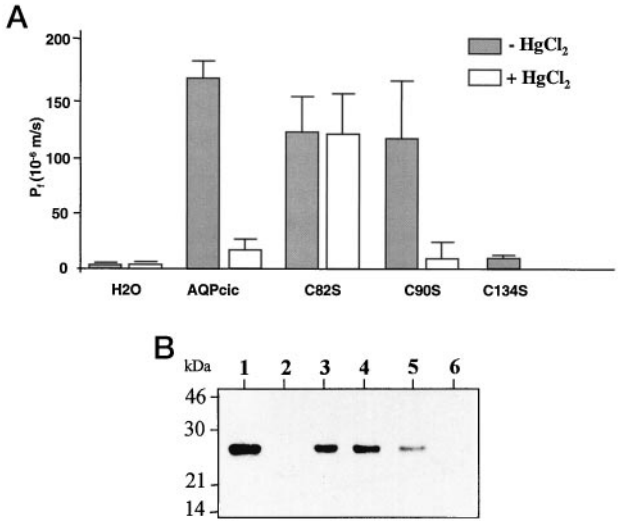

FIG. 2. **Expression of wild type or mutated AQPcic in *Xenopus* oocytes.** *A*, The P_f of oocyte expressing wild type or mutated AQPcic were measured under hypoosmotic condition with or without a pretreatment in 0.3 mM $HgCl_2$ for 15 min. The values represent an average of 10–15 measurements (± S.E.). *B*, Western blot analysis of total membrane proteins purified from injected oocytes. Total membrane proteins were prepared from 10–15 oocytes and amount of proteins equivalent to 1 oocyte was resolved by 12.5% SDS-PAGE. The proteins were transferred onto PVDF membrane and AQPcic was detected using a polyclonal antibody raised against the native aquaporin. Lane 1, native AQPcic; lane 2, water injected; lane 3, AQPcic; lane 4, AQP-C82S; lane 5, AQP-C90S; lane 6, AQP-C134S.

proteins were expressed in the injected oocytes (Fig. 2*B*, *lanes 3*, *4*, and *5*, respectively). Although there was a slight difference in the amount of AQP-C90S and AQP-C82S, the P_f values measured for these oocytes were similar (Fig. 2*A*). In contrast, no protein was present in the membrane (Fig. 2*B*, *lane 6*) or in the cytoplasm (data not shown) prepared from oocyte injected with C134S cRNA, suggesting a lack (or undetectable) of protein expression.

In order to understand the lack of AQP-C134S protein expression in oocytes, we analyzed translation ability of the C134S cRNA in an *in vitro* reticulocyte lysate system. Fig. 3 clearly shows that AQP-C134S cRNA was translatable (*lane 3*), as well as its wild type counterpart (*lane 1*).

These results indicate that a single replacement of Cys^{134} by a serine residue can alter expression, stability, or targeting of recombinant aquaporin in *Xenopus* oocyte.

AQP-C134S Mutant Can Be Overexpressed in Yeast—Yeast cells were transformed with constructs derived from pYeDP10 vector (yeast Y10 and Y10-AQPcic) or constructs derived from pYeDP60 vector (yeast Y60, Y60-AQPcic, and Y60-C134S). Heterologous protein expression was induced by transferring the cells in a galactose culture medium for 16–20 h at 28 °C. In minimal medium conditions, yeast concentrations reach values of $2–3.10^7$ cells/ml, whereas values of $9–10.10^7$ cells/ml are obtained in rich medium culture. To verify expression of wild type or mutated aquaporins, total membrane proteins were prepared. Western blot analyses revealed a single 25-kDa polypeptide in Y10-AQPcic, Y60-AQPcic, and Y60-C134S membrane fractions (Fig. 4, *lanes 3*, *4*, and *6*, respectively), the electrophoretic mobility of which is similar to that of native AQPcic protein (Fig. 4, *lane 1*). No immunoreactive band was observed in total membranes prepared from control yeast (Fig. 4, *lanes 2* and *5*). Interestingly, the mutation of Cys^{134} modified neither the expression nor the stability of the protein when it was expressed in yeast cells.

AQPcic and AQP-C134S Can Be Easily Purified—Treatment of the Y60-AQPcic (Fig. 5, *lane 3*) or Y60-C134S membranes

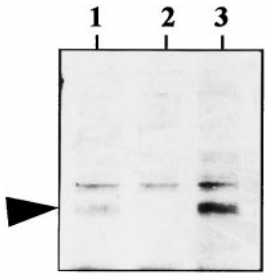

FIG. 3. ***In vitro* translation of AQPcic cRNA.** The cRNA molecules encoding wild type AQPcic or AQP-C134S were translated *in vitro* with the CLONTECH kit (Promega). Translation products were resolved on 12.5% SDS-PAGE, transferred onto PVDF membrane and revealed using AQPcic antibodies (1/1000). Arrow indicates the AQPcic recombinant protein. Lane 1, AQPcic; lane 2, Water control; lane 3, AQP-C134S.

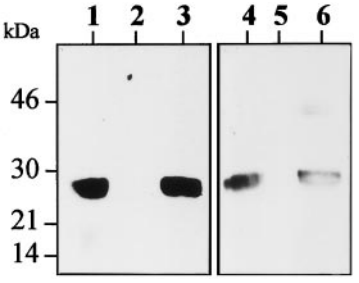

FIG. 4. **Expression of wild type or C134S AQPcic in *S. cerevisiae*.** Yeast cells were transformed with the recombinant expression vectors (yeast Y10-AQPcic and Y60-AQPcic) and Y60-C134S as described in "Experimental procedures". Control yeast cells were transformed with the non recombinant expression vector (yeast Y10 and Y60). Proteins from total membrane extracts were prepared and resolved on 12.5% SDS-PAGE. The gel was transferred on PVDF membrane and probed with antibodies anti-AQPcic. Lane 1, 2.2 μg of native AQPcic; Lane 2, 20 μg of control-yeast (Y10) membrane proteins; lane 3, 20 μg of Y10-AQPcic membrane proteins; lane 4, 15 μg of Y60-AQPcic membrane proteins; lane 5, 15 μg of control yeast Y60 and lane 6, 15 μg of Y60-C134S membrane proteins.

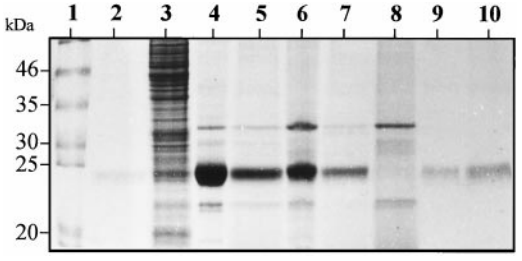

FIG. 5. **Purification of recombinant wild type or mutated AQPcic.** Total membrane extracts from control yeast (TMY), AQPcic expressing yeast (TMYcic) or AQPcic-C134S yeast (TMYC134S) were prepared and solubilized with 2% (w/v) *N*-lauroylsarcosine. Insolubilized material was recovered, resuspended in 1.2% OG (n-octyl-β-D glucopyranoside). The solubilized proteins were recovered as described in "Experimental procedures". Proteins from various stages were resolved on SDS-PAGE and analyzed by Coomassie blue staining. Lane 1, Molecular mass markers; lane 2, native AQPcic; lane 3, TMYcic proteins; lane 4, TMYcic proteins insolubilized with *N*-lauroylsarcosine; lane 5, *N*-lauroylsarcosine insolubilized TMYcic proteins solubilized with OG; lane 6, TMYC134S proteins insolubilized with *N*-lauroylsarcosine; lane 7, *N*-lauroylsarcosine insolubilized TMYC134S proteins solubilized with OG; lane 8, *N*-lauroylsarcosine insolubilized TMY proteins solubilized with OG; lane 9 FPLC purified AQPcic protein; lane 10, purified AQP-C134S protein.

with *N*-lauroylsarcosine solubilized most of the membrane proteins except the aquaporin, which remained in the insoluble fraction (Fig. 5, *lanes 4* and *6*). AQPcic or AQP-C134S were extracted from the insoluble fraction by addition of 1.2% OG (Fig. 5, *lanes 5* and *7*). Such procedure allowed us to purify

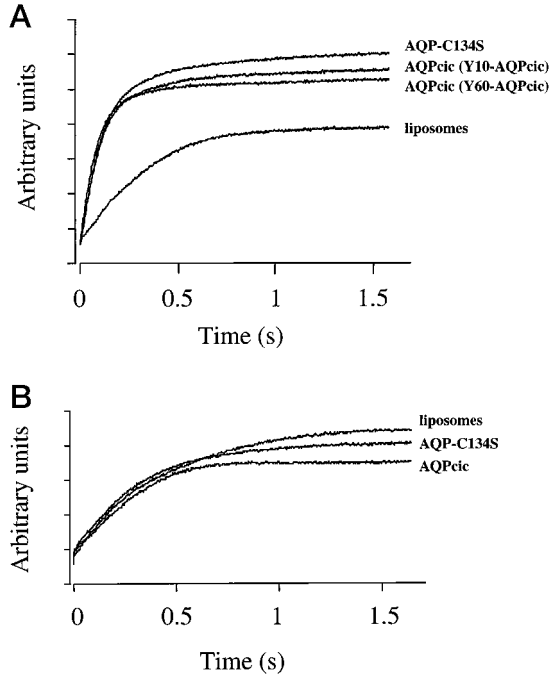

FIG. 6. **Water permeability of control, AQPcic, or AQP-C134S proteoliposomes and mercurial sensitivity.** Control liposomes, AQPcic proteoliposomes or AQP-C134S proteoliposomes were abruptly exposed to a two fold increase of osmolarity in a stopped-flow apparatus and the resulting time course increase of the light scattering intensity (indicating decreased volume) was monitored. Each curve is an average of ~10 measurements. The signal was fitted to an exponential function to calculate P_f. **A.** Stopped-flow analysis of control liposomes, AQPcic reconstituted proteoliposomes (with AQPcic prepared from Y10-AQPcic or Y60-AQPcic cells) or AQP-C134S reconstituted proteoliposomes (0.001 mg/ml of protein/ml). **B.** Mercurial sensitivity of recombinant wild type or mutated AQPcic. Stopped-flow experiments were performed on liposomes or proteoliposomes after treatment in 1 mM $HgCl_2$ for 15 min.

significant amounts of 90% pure AQPcic. AQPcic and AQP-C134S were further purified by anion exchange chromatography (Fig. 5, *lanes 9* and *10*).

AQP-C134S Mutant Is Functional—Purified AQPcic or AQP-C134S were reconstituted into proteoliposomes, the radii of which were measured by electron microscopy after negative staining (the values are respectively 216 ± 46 nm and 199 ± 71 nm, $n = 40$). The osmotic water permeability coefficient (P_f) was determined by rapidly increasing the extravesicular osmolarity in a stopped-flow spectrophotometer. Experiments were performed on proteoliposomes reconstituted with recombinant proteins prepared from both Y10-AQPcic and Y60-AQPcic transformed cells. As shown on Fig. 6, no significant permeability difference was observed between the two AQPcic proteoliposomes ($P_f = 10.36 \pm 0.7\ 10^{-3}$ cm/s and $10.65 \pm 0.9\ 10^{-3}$ cm/s, respectively at 20 °C; Fig. 6*A* (mean of three independent experiments)). The P_f of the AQPcic reconstituted proteoliposomes was significantly increased when compared with the permeability of control liposomes ($P_f = 3.2 \pm 0.3\ 10^{-3}$ cm/s at 20 °C ($n = 3$), Fig. 6*A*). A similar increase of P_f was observed with the protein AQP-C134S reconstituted into proteoliposomes ($P_f = 9.1 \pm 0.3\ 10^{-3}$ cm/s at 20 °C ($n = 3$), Fig. 6*A*).

To investigate the effects of mercurial reagents on AQPcic permeability, some experiments were performed in the presence of $HgCl_2$. Addition of 1 mM $HgCl_2$ for 15 min dramatically reduced the P_f of AQPcic- or AQP-C134S proteoliposomes to values of $3.36 \pm 0.1\ 10^{-3}$ cm/s and $3.27 \pm 0.15\ 10^{-3}$ cm/s, respectively (Fig. 6*B*), similar to control liposomes. However, the addition of $HgCl_2$ had no effect on control liposomes water permeability (Fig. 6*B*).

Determination of P_f for the control liposomes or AQPcic proteoliposomes were then performed at varying temperatures (data not shown). Measurements of Arrhenius activation energies (E_a) indicated an E_a of 12.98 kcal/mol for liposomes and 4.49 kcal/mol for AQPcic-proteoliposomes. The low E_a value calculated for AQPcic-proteoliposomes clearly indicates that the incorporated proteins facilitate the water transport. These results provide additional evidence that the recombinant AQPcic is responsible of the water permeability in reconstituted proteoliposomes.

DISCUSSION

In this study, we have identified the cysteine residue involved in the mercurial sensitivity of AQPcic. We then showed that a single point mutation of aquaporin can abolish its expression in *Xenopus* oocyte. Furthermore, we have successfully expressed and purified recombinant aquaporin in yeast cells.

With the exception of a few water channels (*e.g.* AQP4, Ref. 5), most aquaporins are inhibited by mercurial agents that bind the SH group of cysteine amino acids. Some of the cysteines involved in the Hg sensitivity were identified by site-directed mutagenesis (Cys^{189} on E loop for AQP1 (Ref. 24), Cys^{181} and Cys^{182} in the E loop for AQP2 and AQP5 (Refs. 32 and 7, respectively), or Cys^{118} in γ-TIP (Ref. 33)). Our previous results have shown that the sensitivity of AQPcic to $HgCl_2$ was lower compared with that of AQP1 and that the reversibility of AQPcic Hg inhibition by β-mercaptoethanol was partial (18). The absence of cysteine residue in the E loop area of AQPcic might explain the differential sensitivity of the two proteins. Among cysteine residues of AQPcic, Cys^{82}, Cys^{90} localized close to the NPA box in the B loop, and Cys^{134} in the C loop appeared most likely as potential Hg-binding sites. Other cysteine residues are localized in/or close to the transmembrane domains and thus are unlikely to be bound by mercury (see Fig. 1). Our data demonstrate that a single mutation of Cys^{82} in serine abolishes the $HgCl_2$ inhibition of AQPcic, identifying cysteine 82 as the Hg-binding site. Agre and collaborators (34) have constructed a double mutant of AQP1 (A73C/C189S), in which intracellular Ala^{73} from the B loop and extracellular Cys^{189} from the E loop were replaced, respectively, by a cysteine and a serine residue (Fig. 1). When expressed in *Xenopus* oocytes, the Hg sensitivity of this double mutant (A73C/C189S) was two-thirds that of wild type AQP1 (34). According to the hourglass model (34), the residue Cys^{82} of AQPcic and its equivalent Ala^{73} in AQP1 are localized deep within the pore. The intramembraneous position of these amino acids might explain the reduced accessibility of the Hg-binding site and, thus, the lower sensitivity of AQPcic or AQP1 mutant to a mercurial reagent. In contrast, Shi and Verkman (35) have mutated the Gly^{72} of AQP4 in a cysteine residue (in AQP4, Gly^{72} is the equivalent of Ala^{73} in AQP1 and Cys^{82} in AQPcic). The AQP4-G72C mutant presents a significantly greater sensitivity to $HgCl_2$ than AQPcic or AQP1 mutant. Similarly, replacement of Ala^{210} by cysteine in AQP4 (the equivalent of Cys^{189} in AQP1) did not confer mercury sensitivity (5). These results support the hypothesis that the structures of the channel apertures in AQPcic are closer to AQP1 than to AQP4 ones.

Our data showed that the C134S injected oocytes had a P_f value resembling to the water-injected oocytes. However, the absence of expression of the mutated protein and/or its instability in *Xenopus* oocytes might explain the lack of aquaporin function. To analyze the function of the AQP-C134S mutant, we have overexpressed wild type or mutated AQPcic in the yeast *S. cerevisiae*. The presence of a functional AQP-C134S in the yeast membranes as well as the wild type AQPcic attests that a single replacement of Cys^{134} in serine changes neither

the expression of the protein nor its stability in yeast. It thus appears that this substitution does not provoke the same changes in the expressing machinery of the two cells, which suggests that oocyte and yeast cells behave differently when overexpressing a foreign membrane protein. *Xenopus* oocytes have been largely used as an experimental system for expression and functional analysis of heterologous proteins (see, *e.g.*, review in Refs. 36–38), but the intracellular transport or plasma membrane targeting remains largely unexplained in these cells. In order to study aquaporin function, localization and quantification of the protein of interest are crucial steps. Unexpressed or misrouted aquaporin mutants in *Xenopus* oocyte system have been described previously for some AQP1 or AQP2 mutants. The only partial integration of several (potentially functional) AQP2 mutants in oocyte plasma membrane (39, 40), the mistargeting of some AQP1 mutants (34), or the low expression of AQP1-C189W (24) are responsible of the reduced P_f values of injected oocytes. Consequently, a functional analysis of such kind of aquaporin mutants in *Xenopus* oocytes is definitely not conceivable.

In contrast to the limited knowledge on membrane proteins targeting in higher eukaryote cells, the yeast intracellular transport of secretory or membrane proteins is extensively studied (see, *e.g.*, Refs. 41–43). Different observations suggest that, in *S. cerevisiae*, only a subset of preproteins that are translocated across the endoplasmic reticulum membrane require the function of a signal recognition particle (SRP) (44). For instance, yeast cells are viable in the absence of SRP and SRP receptor and may "adapt" over time and thereby gain the ability to translocate many proteins (45), indicating that SRP-mediated targeting is not the only route through which proteins enter secretory pathway. This "adaptation" of yeast cells possibly explain the easy production of high level of recombinant membrane aquaporins in *S. cerevisiae*.

In this paper, we propose a rapid and efficient way to successfully perform functional studies on wild type and/or mutant aquaporins by overcoming problems encountered by mistargeted aquaporins in *Xenopus* oocytes.

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