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., Delamarce, C., Pellerin, L., Bonnet, 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 Cys82 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) is 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 in oocytes for studying particular mutants of aquaporin.

To whom correspondence should be addressed. Fax: 33-2-99-28-14-77; E-mail: stephane.deschamps@univ-rennes1.fr.

EXPERIMENTAL PROCEDURES

Plasmid Construction and Mutagenesis—pSP-AQPcic corresponds to the full-length AQPcic coding sequence subcloned into plasmid pBSG-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′-GGG-TTTAATGCAGTATGAGCT-3′; Y2, 5′-CGCAAGCTTGAGCTCCTGGAGGCT-3′.

The polymerase chain reaction primers contain EcoRI and SacI 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 SacI site of pXPG-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-AQP-C134S 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 CaCl2, 1 mM MgCl2, 2 mM NaHCO3, 10 mM Hepes, pH 7.6) supplemented with 200 mM sorbitol.

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

\[
P_f = \frac{V_o \times d(V/V_o)/dt}{S \times V_o / (C_m - C_{out})} \quad (23)
\]

\( S \) is the oocyte surface area \((S = 0.045 \text{ cm}^2)\), \( V_o \) the initial volume \((V_o = 9 \times 10^{-4} \text{ cm}^3)\), \( V_{t} \) the oocyte volume at a given time \( t \), \( V_m \) the molecular volume of water \((V_m = 18 \text{ cm}^3/\text{mol})\), and \( d(V/V_o)/dt \) the initial rate of oocyte swelling. \( C_m \) 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 Saccaromyces cerevisiae—Studies were performed using the W303.1B strain of \( S. \) cerevisiae \((\alpha, leu2, his3, trpl, ura3, ade2-1, can1, cya1, cyr1)\) 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 \((0.7\% \text{ yeast nitrogen base})\) with or without aquaporins. The protein/lipid ratio of proteoliposomes was determined with an equal volume of buffer D containing mannitol, and the concentration of mannitol was sufficient to increase by 0.5 molar of water \((18 \text{ cm}^3/\text{mol})\), \( \Delta osm \) is the osmotic difference between the initial intra- and extravascular mannitol concentrations, and \( \sigma \) is the reflection coefficient of the mannitol \((\sigma_m = 1; \text{ see Ref. 29})\).

\[
\frac{SV_c}{S} = \frac{k}{S(V_m) \times V_o \times \Delta osm \times \sigma} \quad (2)\]

\( SV_c \) \((\text{cm}^3/\text{min})\) is the ratio of the vesicle surface area to the initial volume, \( V_o \) is the partial molar volume of water \((18 \text{ cm}^3/\text{mol})\), \( \Delta osm \) is the osmotic difference between the initial intra- and extravascular mannitol concentrations, and \( \sigma \) is the reflection coefficient of the mannitol \((\sigma_m = 1; \text{ see Ref. 29})\).

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, in the S. Cerevisiae XP-1 (24). Membranes were prepared by the method described in Ref. 24. Yeast cells were grown at 28 °C for 24 h in a minimal medium \((0.7\% \text{ yeast nitrogen base})\) with or without mannitol, and the concentration of mannitol was sufficient to increase by 0.5 molar of water. The concentration of mannitol was sufficient to increase by 0.5 molar of water.

\[
\frac{S}{V_o} = \frac{k}{S(V_m) \times V_o \times \Delta osm \times \sigma} \quad (2)\]

\( SV_c \) \((\text{cm}^3/\text{min})\) is the ratio of the vesicle surface area to the initial volume, \( V_o \) is the partial molar volume of water \((18 \text{ cm}^3/\text{mol})\), \( \Delta osm \) is the osmotic difference between the initial intra- and extravascular mannitol concentrations, and \( \sigma \) is the reflection coefficient of the mannitol \((\sigma_m = 1; \text{ see Ref. 29})\).

\[
\frac{S}{V_o} = \frac{k}{S(V_m) \times V_o \times \Delta osm \times \sigma} \quad (2)\]

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cells in a galactose culture medium for 16–20 h at 28 °C. In the pYeDP60 vector (yeast Y60, Y60-AQPcic, and Y60-C134S). Het-
vector (yeast Y10 and Y10-AQPcic) or constructs derived from cells were transformed with constructs derived from pYeDP10

proteins were expressed in the injected oocytes (Fig. 2B, 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. 2A). In contrast, no protein was present in the membrane (Fig. 2B, 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 Cys134 by a serine residue can alter expression, stability, or targeting of AQPcic protein (Fig. 4, electrophoretic mobility of which is similar to that of native polypeptide in Y10-AQPcic, Y60-AQPcic, and Y60-C134S membranes). The P _f _of oocyte expressing wild type or mutated AQPcic

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 Cys134 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 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...
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 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.

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 ± 0.1 10$^{-3}$ cm/s and 3.27 ± 0.15 10$^{-3}$ cm/s, respectively (Fig. 6B), similar to control liposomes. However, the addition of HgCl$_2$ had no effect on control liposomes water permeability (Fig. 6B).

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 are unlikely to be bound by mercury (see Fig. 1). Our data demonstrate that a single mutation of Cys$^{122}$ 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 hour-glass model (34), the residue Cys$^{122}$ of AQPcic and its equivalent Ala$^{73}$ in AQP1 are localized deep within the pore. The intramembranous 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$^{122}$ in AQPcic). The AQP4-G72C mutant presents a significantly greater sensitivity to HgCl$_2$ than AQPcic or AQP1 mutant. Similarly, replacement of Ala$^{110}$ by cysteine in AQP4 (the equivalent of Cys$^{118}$ 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 any change 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 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 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 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 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 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 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 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.