Functional expression of the human CHIP28 water channel in a yeast secretory mutant

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Abstract The temperature-sensitive *Saccharomyces cerevisiae* mutant strain NY17, deficient in the secretory pathway (*sec6-4* mutation), is used for the heterologous expression of the human CHIP28 water channel. After a heat-shock, the protein is present in partially purified post-golgi secretory vesicles. Immunodetection and water transport studies, directly made on the vesicles, showed that CHIP28 is highly expressed and active in the yeast membranes.

Key words: AQP1; CHIP28; *Saccharomyces cerevisiae sec6-4* mutant; Post-golgi secretory vesicles; Water transport

1. Introduction

CHIP28 (channel-forming integral protein of 28 kDa) belongs to the growing MIP (major intrinsic protein) family of membrane proteins [1]. Several members of this family are water channels, and some others are transport proteins with different specificities. The structure-function relationships of the different MIP-like proteins are of primary importance for the molecular characterization of their transport properties.

The cDNA encoding the CHIP28 was recently isolated from a human fetal liver library [2]. CHIP28, also called AQP1 (for aquaporin-1), was the first water channel identified in human red cells and was also shown to be constitutively expressed in kidney proximal tubules, thin descending limbs of Henle's loops [3,4] and other water transporting tissues [5].

The functional characterization of CHIP28 has so far been essentially performed by micro-injecting in vitro-transcribed cRNA into *Xenopus laevis* oocytes [6] and by reconstituting purified CHIP28 into proteoliposomes [7–9]. In the present work, we investigated a new heterologous expression system for CHIP28 production and functional analysis: a *Saccharomyces cerevisiae* temperature-sensitive mutant strain, defective in the final step of the secretory pathway (*sec* mutant) [10], which has already been successfully used for the expression of membrane proteins [11–13]. The mutation induces, at a non-permissive temperature, the cytoplasmic accumulation of post-golgi secretory vesicles containing newly synthesized proteins, including exogenous proteins from expression plasmids, in transit to the plasma membrane. We sought to use the *sec6-4* mutant to express CHIP28 and directly use the purified CHIP28-containing secretory vesicles in functional studies. This project was made possible by the absence of endogenous water channels in the vesicles.

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2. Material and methods

2.1. Plasmid constructions

The human CHIP28 cDNA, provided by Dr. P. Agre (Baltimore, MD), was inserted into the yeast expression vector pYeDP1/8-10 [14] under the control of the inducible *GAL10-CYC1* hybrid promoter and the *PGK* (phosphoglycerate kinase) terminator. It is a 7 kb multicopy shuttle plasmid which contains origins of replication and selection markers for both *E. coli* and *S. cerevisiae*.

Plasmid pYeDP-CHIP (Fig. 1) was constructed by adding two complementary *Eco*RI-*Xmn*I oligonucleotide linkers (5′-GCTTCTTCTTGAACTCGCTAGCCATG-3′ and 5′-AATTCATGGCTAGCGAGTTCAAGAAGAAGC-3′) to the 5′ end of the 810 bp *Xmn*I-*Bst*EII (blunted) CHIP28 cDNA fragment. The CHIP28 translation initiation, absent in this fragment, was re-created in the linkers in order to be immediately adjacent to the *GAL10-CYC1* promoter. The 840 bp fragment obtained was inserted between the *Eco*RI and *Sma*I sites of the pYeDP1/8-10 polylinker. The pYeDP-CHIP plasmid was so constructed to provide high expression levels when transformants are grown on galactose-containing medium (expression is repressed in the presence of glucose [15]).

Blunt ends were made with the Klenow fragment of *E. coli* DNA polymerase I (New England Biolabs) and ligation with the T4 DNA ligase (Gibco BRL) according to standard protocols [16]. XL1-Blue competent cells (Stratagene) were used as host for plasmid manipulations. All restriction endonucleases were from New England Biolabs.

2.2. Yeast strains, transformation and culture

The temperature-sensitive *S. cerevisiae* NY17 strain (*MATa, ura3-52, sec6-4*) was provided by Dr. F. Kepes (CEA-Saclay, France). Transformation of yeasts was carried out on a BioRad Gene Pulser electroporator, according to BioRad transformation protocol, and selection was based on *ura3* complementation. Control and CHIP28 transformants, containing pYeDP1/8-10 and pYeDP-CHIP, respectively, were grown at the permissive temperature of 23°C with a 200 rpm shaking in minimal selective medium (0.67% (w/v) yeast nitrogen base without amino acids (Difco), 0.1% (w/v) casamino acids (Difco), 2% (w/v) glucose). To induce heterologous CHIP28 expression in pYeDP-CHIP transformants, cells were diluted to 0.1 OD in minimal inducible medium (0.67% (w/v) yeast nitrogen base without amino acids, 0.1% (w/v) casamino acids and 2% (w/v) galactose) and incubated 22 h at 23°C. The yeasts were finally placed at the non-permissive temperature of 37°C, to trigger secretory vesicle accumulation in the cytoplasm, and harvested after 2 h 30 (cultures reached 1–1.5 OD). Growth was determined by measurements of the yeast suspension absorbance at 600 nm.

2.3. Secretory vesicle isolation

Secretory vesicles were isolated by yeast spheroplasting and differential centrifugations as described in [17] except that the S-1000 Sephacryl step was omitted. The final 100,000 × *g* pellet, consisting mainly of secretory vesicles, was washed once and resuspended in a 0.3 M sorbitol, 0.1 M KCl, 1 mM EDTA, 20 mM Tris-HCl pH 7.4 buffer containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 μg/ml leupeptin, antipain and benzamidine, Sigma Chemicals) so as to have a 15 mg/ml protein concentration. Vesicles were then frozen

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in liquid nitrogen and stored at −80°C. The protein concentration was determined according to Bradford [18] with bovine serum albumin as standard.

2.4. Immunodetection of expressed CHIP28

2.4.1. Immunoblotting. Proteins were separated on 12.5% SDS-PAGE gels [19] and electrotransferred onto PVDF membranes (Poly-Screen, DuPont, NEN products) [20]. Membranes were then incubated with a 1:800 dilution of a rabbit polyclonal anti-CHIP28 antibody raised against CHIP28 purified from human red blood cells according to [7]. Immunoreactive proteins were detected by the ECL Western blotting technique (Enhanced ChemiLuminescence, Amersham).

2.4.2. Indirect immunofluorescence. CHIP28 was localized by indirect immunofluorescence on frozen sections of yeasts containing the pYeDP plasmids. After vesicle accumulation, cells were fixed for 10 min in 2% paraformaldehyde, 10 mM sodium periodate and 75 mM glycine in pH 7.4 phosphate buffer and washed in PBS. Yeasts were then infiltrated in 2.3 M sucrose in PBS for 1 h and frozen in liquid nitrogen. 1 μm sections cut on an Ultracut-S ultracryomicrotome (Leica) were collected on Superfrost+ glass slides, blocked 10 min in 1% BSA in PBS and incubated for 90 min in a 1:100 dilution of the anti-CHIP28 antiserum. Sections were washed in PBS, incubated for 50 min in a 1:50 dilution of fluorescein-5-isothiocyanate (FITC)-conjugated goat anti-rabbit and washed again. Finally, sections were mounted in a 50% glycerol, 0.2 M Tris-HCl pH 8.0 buffer containing 2% *n*-propyl gallate and examined under a fluorescence microscope (Olympus, Vanox-T).

2.5. Freeze-fracture electron microscopy

Freeze-fracture electron microscopy was performed on secretory vesicles from yeasts containing the pYeDP constructions. Vesicles were cryoprotected in 30% glycerol, incubated 20 min at 4°C, frozen and fractured in a BAF301 apparatus (Balzers). Samples were then observed using an electron microscope (Philips, EM400).

2.6. Functional analysis

2.6.1. Stopped-flow experiments. Water transport measurements were performed at 8°C using a stopped-flow spectrophotometer (SFM3, Biologic, Claix, France). The instrument dead time is below 0.8 ms with a maximal rate of data acquisition of 10 kHz. The light of a 150 W mercury-xenon arc lamp is driven from the monochromator to the observation chamber (8 μl volume) by an optical fibre. Of diluted secretory vesicles (0.3 mg/ml in the sorbitol-free dilution buffer: 0.1 M KCl, 1 mM EDTA, 20 mM Tris-HCl, pH 7.4), 100 μl were submitted to a 125 mosmol/l inwardly directed sorbitol gradient by mixing with an equal volume of hyperosmotic solution. The increase of 90° scattered light intensity, corresponding to water efflux from the vesicles, was followed at λ_{ex} = 437 nm.

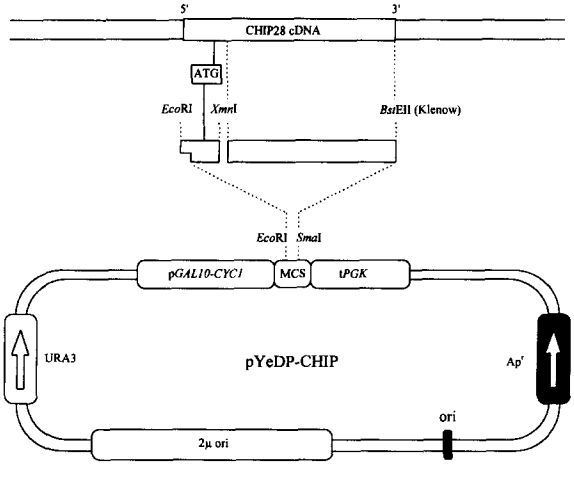

Fig. 1. Schematic construction of pYeDP-CHIP. White bars, *S. cerevisiae* sequences; black bars, *E. coli* sequences; ori, origin of replication; Ap^r, ampicillin-resistance gene; MCS, multicloning site; t*PGK*, phosphoglycerate kinase terminator; p*GAL10-CYC1*, hybrid *GAL10*-iso-1-cytochrome *c* promoter.

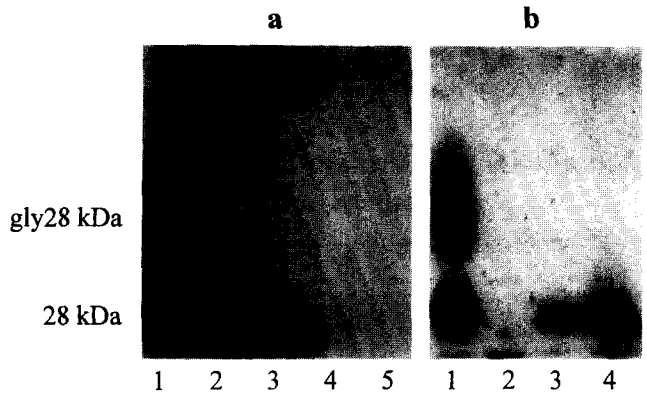

Fig. 2. Expression of human CHIP28 in yeast. 18 μg protein samples were separated by SDS-PAGE, transferred onto PVDF membranes and labelled with either anti-CHIP28 or pre-immune serum. (a) lane 1, KI-stripped vesicles from human red blood cells; lanes 2 and 3, vesicles from yeasts containing pYeDP1/8-10 and pYeDP-CHIP constructions, respectively; lanes 4 and 5, same samples incubated in pre-immune serum. (b) lane 1, KI-stripped erythrocyte vesicles; lane 2, vesicles from yeasts containing pYeDP-CHIP plasmid grown in glucose medium; lanes 3, total homogenate and lane 4, vesicles from yeasts containing pYeDP-CHIP plasmid.

2.6.2. Osmotic water permeability (Pf). Data obtained from at least 10 time-courses were averaged and analysed by the 'VOYONS' curve-fitting software [21]. The initial rate constant k (s^{-1}), calculated from single-exponential fits, was used to determine Pf (cm/s) according to the equation:

$$Pf = k/[(S/V_0) \cdot V_w \cdot \Delta osm],$$

where (S/V_0) is the vesicle surface area to initial volume ratio, V_w is the partial molar volume of water (18 cm^3/mol), Δosm is the osmotic difference between the initial intra- and extravesicular sorbitol concentrations.

In one experiment, the effect of various hyperosmotic sorbitol gradients (41–248 mosmol/l) was tested at 8°C on control vesicles. In another experiment, the effect of amphotericin B (Sigma Chemicals) was measured, at 21°C, by pre-incubating the vesicles 45 min in various concentrations of the antibiotic (0, 10, 50 and 100 μg/ml dimethylsulfoxide (DMSO); DMSO final concentration was 0.02%). The temperature dependence of the water efflux rate was also measured at 8, 12 and 15°C. Mercurial effect was tested on secretory vesicles pre-incubated 20 min at room temperature in the presence of various *p*-chloromercuribenzenesulfonate (pCMBS) concentrations in dilution buffer.

Results are given as means ± standard deviation (S.D.).

3. Results

3.1. CHIP28 expression in yeast secretory vesicles

3.1.1. Immunoblotting. Yeasts were transformed with the expression plasmids pYeDP1/8-10 (control) and pYeDP-CHIP. Secretory vesicles were isolated as described in Methods and the protein expression was monitored by SDS-PAGE and Western blotting analysis (Fig. 2). A unique intense band was revealed in the secretory vesicle fraction from CHIP28 transformant (Fig. 2a, lane 3); this signal is at the same position as the 28 kDa non-glycosylated CHIP28 form of the KI-stripped erythrocyte membranes used as positive control (Fig. 2a, lane

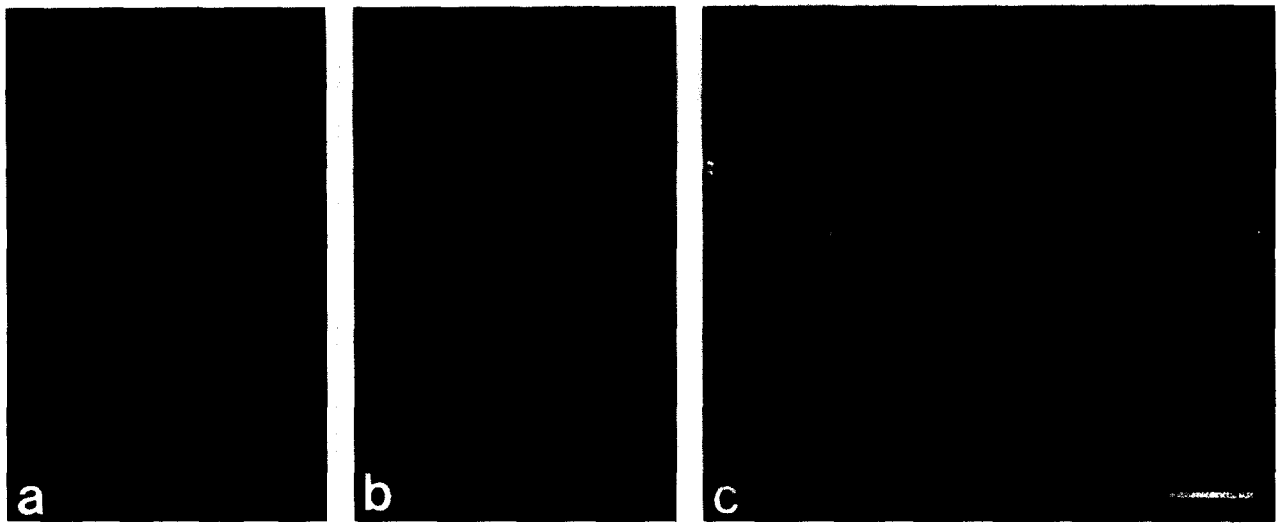

Fig. 3. Immunolocalization by indirect fluorescence of CHIP28 protein in yeast. (a) pYeDP1/8-10-containing yeasts probed with anti-CHIP28 antibodies. (b) pYeDP-CHIP-containing yeasts probed with pre-immune serum. (c) pYeDP-CHIP-containing yeasts probed with anti-CHIP28 antibodies. Bar = 10 μm.

1 , indicating that the CHIP28 protein is present in yeast secretory vesicles. No signal corresponding to the glycosylated form (gly28 kDa ranging from 40 to 60 kDa) can be observed. As expected, no band was detected in vesicle fraction from control transformant (Fig. 2, lane 2), indicating that the expression is specifically related to the presence of the CHIP28 cDNA. A ~5-fold CHIP28 enrichment was observed by comparing signals of the total homogenate (Fig. 2b, lane 3) and the vesicle fraction (Fig. 2b, lane 4) of yeasts containing pYeDP-CHIP. No 28 kDa band was revealed when yeasts were grown on glucose medium without any galactose induction (Fig. 2b, lane 2 , indicating strong control of CHIP28 expression by the glucose-repressible *GAL10* promoter.

3 1.2. Immunofluorescence. CHIP28 immunolocalization was performed on transformed yeasts. Immunofluorescence pictures of CHIP28-containing yeasts (Fig. 3c) revealed an intense cytoplasmic signal consistent with an endoplasmic reticulum and a vesicular localization. No fluorescence was detected in controls (Fig. 3a and b).

3 2. Expressed CHIP28 is functional

Purified secretory vesicles were directly used in functional studies. As illustrated in Fig. 4, freeze-fracture electron microscopy experiments revealed that these vesicles are predominantly spherical with an average diameter of 98.3 ± 34.9 nm (V = 158).

Preliminary stopped-flow experiments on control yeast vesicles indicated that: (a) no change in the intensity of the scattered light was observed when vesicles were mixed with an isoosmotic buffer (dilution buffer); (b) a linear relationship was found between ΔI, defined as the difference of scattered light intensity between zero time and maximal shrinkage values, and external/internal osmolality ratio (Fig. 5a); (c) Pf was greatly increased after vesicles were permeabilized with amphotericin B, in a dose-dependent manner, as depicted in Fig. 5b; (d) experiments not reported here gave high Arrhenius activation energy values (E_A > 10 kcal/mol) and an absence of inhibitory effects of mercurial agents. These experiments indicate intact transport characteristics of the vesicle membrane as well as the absence of endogenous water channels.

The osmotic water permeability coefficients of control and CHIP28-containing vesicles were measured at 8°C in the presence of a 125 mosmol/l inwardly directed sorbitol gradient. As illustrated in Fig. 6, which is a representative experiment, the rate of water efflux is 5 times faster in CHIP28-containing vesicles than in control vesicles (Fig. 6a and b). This experiment was repeated and the mean calculated Pf was $(4.15 \pm 2.4) \times 10^{-3}$ cm/s ($N = 7$) for CHIP28-containing vesicles versus $(1.35 \pm 0.26) \times 10^{-3}$ cm/s ($N = 7$) for control vesicles ($P < 0.01$). A significant inhibition was observed in the presence of 5 mM pCMBS (Fig. 6c). In the same experiment, the inhibitory effect of various pCMBS concentrations on Pf of control and

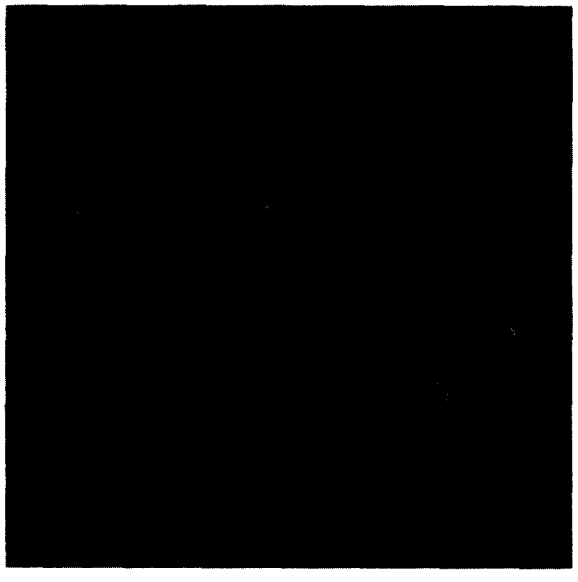

Fig. 4. Freeze-fracture electron microscopy of pYeDP1/8-10 secretory vesicles. Magnification: ×77000. Bar = 100 nm.

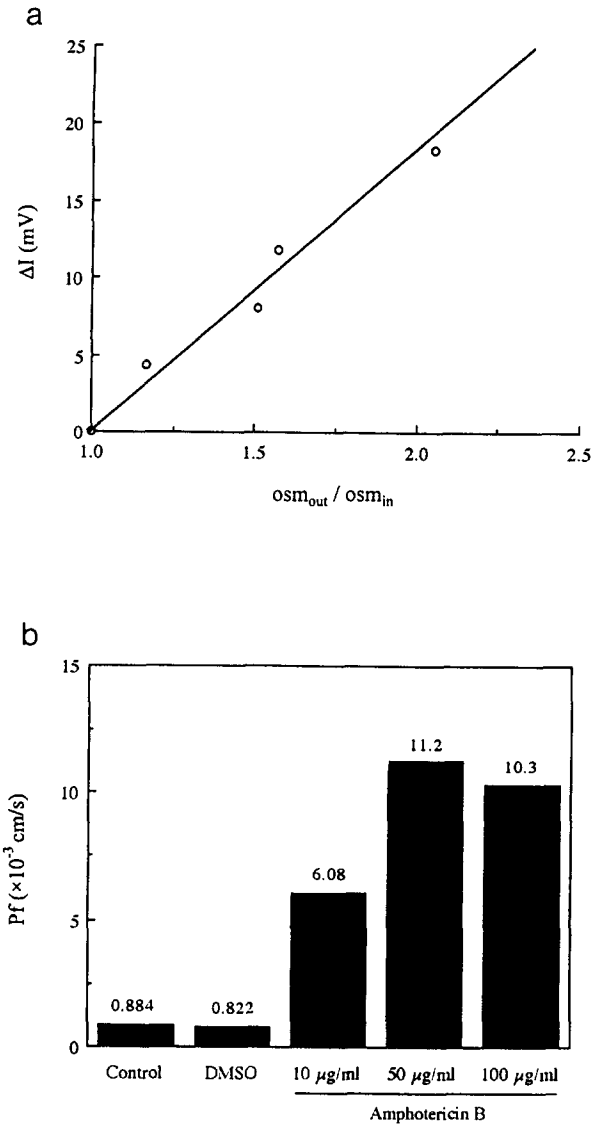

Fig. 5. (a) Dependence of ΔI, the amplitude of scattered light intensity signal, on external osmolality. Osm_{in} is the intravesicular osmolality before osmotic shock (237 mosmol/l) and osm_{out} the osmolality of the external solution (278, 359, 371.5 and 485 mosmol/l). Typical experiment performed on control vesicles. (b) Dependence of control vesicle Pf on amphotericin B concentrations at 21°C for a 268 mosmol/l inwardly-directed sorbitol gradient.

CHIP28-containing vesicle was tested (Fig. 7). A dose-dependent inhibition was observed for CHIP28-containing vesicles, with a maximal effect obtained in the presence of 5 and 10 mM pCMBS. However, in both cases, a slight 'non-specific' increase of Pf was observed in the presence of the inhibitor, especially at lower concentrations.

The temperature dependence of the rate of water efflux is shown on an Arrhenius plot (Fig. 8). The activation energy was 10.39 ± 1.48 kcal/mol ($N = 4$) for control vesicles and 3.10 ± 0.65 kcal/mol ($N = 3$) for CHIP28-containing vesicles.

4. Discussion

The *Saccharomyces cerevisiae sec6-4* mutant has already been used for the expression of a few eukaryotic membrane

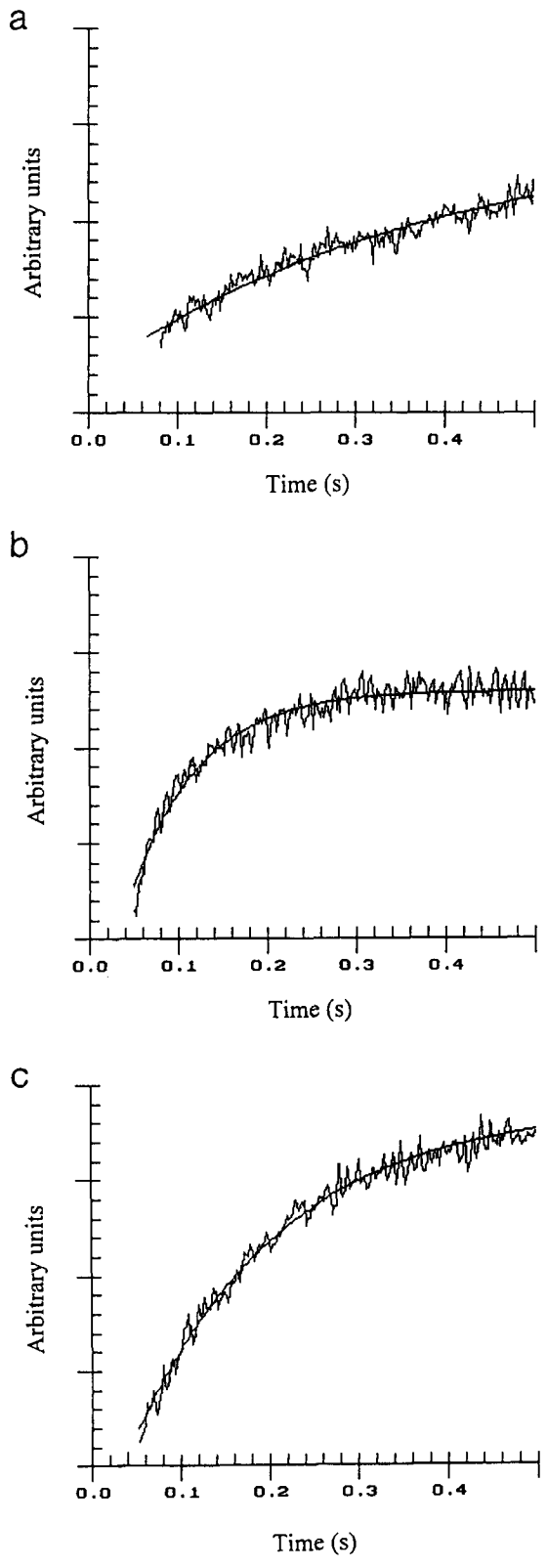

Fig. 6. Time-courses of control and CHIP28-containing vesicle shrinkage in the presence of a 125 mosmol/l 1 inwardly-directed sorbitol gradient at 8°C. Fitted single-exponential rate constants (k) were (a) 2.6 s^{-1} for control vesicles, (b) 12.9 s^{-1} for CHIP28-containing vesicles and (c) 5.9 s^{-1} for CHIP28-containing vesicles pre-incubated 20 min in 5 mM pCMBS. Each time-course was recorded during 2 s at a 1 kHz frequency.

proteins [11–13]. Here, we succeeded in expressing a functional water channel (human CHIP28) in this system, which was previously checked for the absence of endogenous water channels.

We demonstrated, in the first part of this paper, that the human CHIP28 cDNA inserted in the yeast vector pYeDP1/8-10 was expressed in the *S. cerevisiae* NY17 strain. Immunoblotting analysis of yeasts containing the pYeDP constructions allowed us to detect CHIP28 in the secretory vesicle fraction. The fact that the unique form of CHIP28, revealed in Western blots as the 28 kDa one, strongly suggests that the protein is not or very lightly glycosylated in the yeast secretory pathway. This apparent lack of glycosylation was already observed for the expression of a membrane phosphoglycoprotein, named P-glycoprotein, in the *sec6-4* secretory mutant [13]. However, we cannot exclude that the anti-CHIP28 antibodies did not recognize the yeast-specific glycosylated form of the protein. Immunofluorescence experiments confirmed the cytoplasmic localization of CHIP28.

In the second part of our work, we showed that the CHIP28-containing vesicles shrunk very rapidly after a hyperosmotic shock, suggesting a functional insertion of the water transport protein in the membrane. The estimated number of functional CHIP28 molecules per vesicle is 6–13, considering that the single water channel Pf is 6.8–16 $\times 10^{-14}$ cm^3/s [6,9], the vesicle surface area is 3.14×10^{-10} cm^2 and that ΔPf (defined as CHIP28-containing vesicle Pf–control vesicle Pf) is 2.8×10^{-3} cm/s. Several water channel characteristics were verified, in particular a low activation energy value and sensitivity to a classical mercurial sulfhydryl reagent. However, the osmotic water permeability of control vesicles ($(1.35 \pm 0.26) \times 10^{-3}$ cm/s) is relatively high when compared with a simple lipid bilayer membrane Pf ($\sim 10^{-4}$ cm/s [22]). This can be explained by the presence of multiple membrane proteins in secretory vesicles, able to permeate water (for review, see [23] and [24]). Treatments applied to yeasts during vesicle preparation can also alter membrane permeability properties. In the same way, the increased Pf value observed in pCMBS inhibition experiments is probably due to an interaction of the inhibitor with endogenous proteins and/or the lipid components of the membrane.

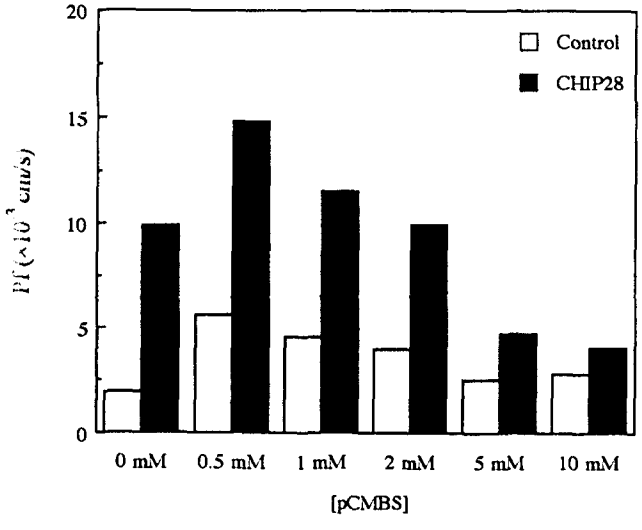

Fig. 7. pCMBS effect on the Pf of control vesicles (white bars) and CHIP28-containing vesicles (black bars). Measurements were performed at 8°C, in the presence of a 125 mosmol/l inwardly-directed sorbitol gradient and after a 20 min incubation in the presence of the inhibitor at room temperature.

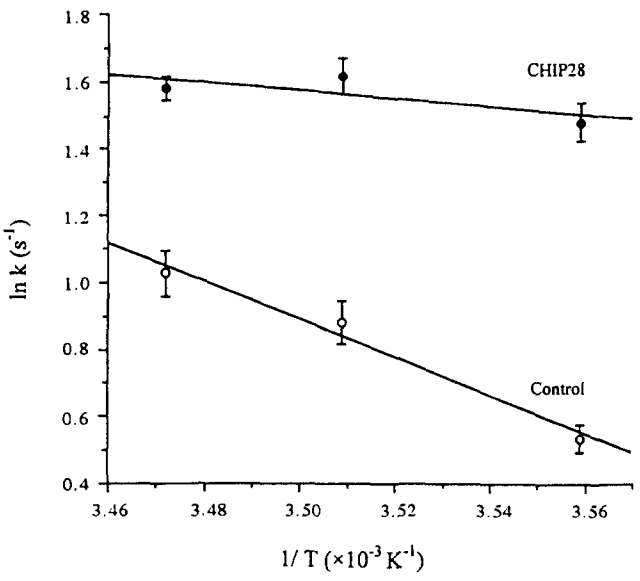

Fig. 8. Temperature-dependence of vesicle water efflux. Rate constant (k) were calculated from single-exponential fits obtained for vesicle shrinkage in the presence of a 125 mosmol/l inwardly-directed sorbitol gradient. Activation energy determined from linear regression was 10.39 ± 1.48 kcal/mol (N = 4) for control vesicles and 3.10 ± 0.65 kcal/mol (N = 3) for CHIP28-containing vesicles.

Altogether, our data demonstrate that the yeast expression system is a valuable approach for the study of AQP1, and probably other AQP-MIP related proteins. The protein is properly expressed, functional and can be directly studied in the purified secretory vesicles. Mutated aquaporins are under investigation in our laboratory by using this experimental approach. Last but not least, large amounts of interesting proteins will be easily purified from yeast cultures.

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References

[1] Gorin, M.B., Yancey, S.B., Cline, J., Revel, J.P. and Horwitz, J. (1984) Cell 39, 49–59.
[2] Preston, G.M. and Agre, P. (1991) Proc. Natl. Acad. Sci. USA 88, 11110–11114.
[3] Nielsen, S., Smith, B.L., Christensen, E.I., Knepper, M.A. and Agre, P. (1993) J. Cell Biol. 120, 371–383.
[4] Zhang, R., Skach, W., Hasegawa, H., Van Hoek, A.N. and Verkman, A.S. (1993) J. Cell Biol. 120, 359–369.
[5] Agre, P., Preston, G.M., Smith, B.L., Jung, J.S., Raina, S., Moon, C., Guggino, W.B. and Nielsen, S. (1993) Am. J. Physiol. 265, F463–F476.
[6] Preston, G.M., Carroll, T.P., Guggino, W.B. and Agre, P. (1992) Science 256, 385–387.
[7] Zeidel, M.L., Ambudkar, S.V., Smith, B.L. and Agre, P. (1992) Biochemistry 31, 7436–7440.
[8] Abrami, L., Tacnet, F. and Ripoche, P. (1995) Pflügers Arch. 430, 447–458.
[9] Van Hoek, A.N. and Verkman, A.S. (1992) J. Biol. Chem. 267, 18267–18269.

[10] Novick, P., Field, C. and Schekman, R. (1980) Cell 21, 205–215.
[11] Nakamoto, R.K., Rao, R. and Slayman, C. (1991) J. Biol. Chem. 266, 7940–7949.
[12] Centeno, F., Deschamps, S., Lompré, A.M., Anger, M., Moutin, M.J., Dupont, Y., Palmgren, M.G., Villalba, J.M., Møller, J.V., Falson, P.and Le Maire, M. (1994) FEBS Lett. 354, 117–122.
[13] Ruetz, S. and Gros, P. (1994) J. Biol. Chem. 269, 12277–12284.
[14] Pompon, D. (1988) Eur. J. Biochem. 177, 285–293.
[15] Guarente (1982) Proc. Natl. Acad. Sci. USA 79, 7410–7414.
[16] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
[17] Walworth, N.C. and Novick, P.J. (1987) J. Cell Biol. 105, 163–174.
[18] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[19] Laemmli, U.K. (1970) Nature 227, 680–685.
[20] Matsudaira, P. (1987) J. Biol. Chem. 262, 10035–10038.
[21] Thiéry, J.M. (1991) in: VOYONS, Programme de Simulations Conversationnelles en Physico-Chimie. Logiciels pour la Chimie (G.M. Come, E. Ducloy, E. Soulié and J.M. Thiéry eds.) pp. 292–293, Société Française de Chimie, Paris and Association Nat. Logiciel, Nancy.
[22] Finkelstein, A. (1987) in: Distinguished Lectures Series of the Society of General Physiologists, vol. 4, pp. 1–228, Wiley-Interscience, New York.
[23] Van Os, C.H., Deen, P.M.T. and Dempster, J.A. (1994) Biochim. Biophys. Acta 1197, 291–309.
[24] Fischbarg, J. and Vera, J.C. (1995) Am. J. Physiol. 268, C1077–C1089.