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The vacuolar membrane protein γ-TIP creates water specific channels in *Xenopus* oocytes

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The vacuolar membrane (tonoplast) of higher plant cells contains an abundant 27 kDa protein called TIP (tonoplast intrinsic protein) that occurs in different isoforms and belongs to a large family of homologous channel-like proteins found in bacteria, plants and animals. In the present study, we identified and characterized the function of γ-TIP from *Arabidopsis thaliana* by expression of the protein in *Xenopus* oocytes. γ-TIP increased the osmotic water permeability of oocytes 6- to 8-fold, to values in the range $1-1.5\times10^{-2}$ cm/s. Similar results were obtained with the homologous human erythrocyte protein CHIP28, recently identified as the erythrocyte water channel. The bacterial homolog GlpF did not affect the osmotic water permeability of oocytes, but facilitated glycerol uptake, in accordance with its known function. By contrast, γ-TIP did not promote glycerol permeability. Voltage clamp experiments provided evidence showing that γ-TIP induced no electrogenic ion transport in oocytes, especially during osmotic challenge that resulted in massive transport of water. These results allow us to conclude that the various protein members of the MIP family have unique and specific transport functions and that the plant protein γ-TIP likely functions as a water specific channel in the vacuolar membrane.

Key words: *Arabidopsis thaliana*/glycerol transport/osmotic water permeability/tonoplast

Introduction

Most plant cells are characterized by a thin layer of cytoplasm sandwiched between the plasma membrane and the tonoplast, the membrane of the voluminous central vacuole. Both membranes are energized by H^+ pumps and function in the transport of mineral ions, metabolites and large water fluxes required for turgor generation and osmoregulation. The tonoplast contains an abundant 27 kDa integral protein, called tonoplast intrinsic protein (TIP) (Johnson *et al.*, 1990) that occurs in different isoforms with specific patterns of expression (Höfte *et al.*, 1992). TIP proteins belong to a large family of homologous membrane proteins found in bacteria, plants and animals (Pao *et al.*, 1991). This family, the MIP family, is named after the first member to be characterized, the major intrinsic protein (MIP) of bovine lens fibers (Gorin *et al.*, 1984).

All protein members of the MIP family have six putative membrane spanning domains, exhibit a channel-like structure, and a few have been shown to mediate transmembrane transport. The bacterial protein GlpF facilitates the transport of glycerol and other small straight chain polyols across the cytoplasmic membrane (Heller *et al.*, 1980; Sweet *et al.*, 1990). The human protein CHIP28 has recently been shown to form water channels (Preston *et al.*, 1992; van Hoek and Verkman, 1992; Zeidel *et al.*, 1992), and the bovine MIP protein itself forms ion channels when incorporated into lipid bilayers (Zampighi *et al.*, 1985; Ehring *et al.*, 1990).

No function has been reported yet for the plant members of the MIP family, although it has been suggested that the peribacteroid membrane protein NOD26 may be involved in malate transport (Ouyang *et al.*, 1991). The possibility that TIP proteins function as membrane transporters is supported by the expression in plants of different homologs under conditions and at developmental stages where pivotal and specialized transport processes must occur: during embryo development (Johnson *et al.*, 1989), cell elongation (Ludevid *et al.*, 1992), in *Rhizobium*–legume symbiosis (Fortin *et al.*, 1987) and as a response to water deprivation (Guerrero *et al.*, 1990; Yamaguchi-Shinozaki *et al.*, 1992).

The high efficiency with which *Xenopus laevis* oocytes translate injected mRNA and process the synthesized proteins, as well as their suitability for electrophysiological experiments have made these cells a system of choice for functional characterization of membrane transport proteins (Soreq and Seidman, 1992). The large size of these cells (i.e. low surface/volume ratio) and the low osmotic water permeability of their plasma membranes are additional features that make them well-suited for the study of water transport processes (Zhang and Verkman, 1991). Indeed, the use of this system led to the identification of the elusive water channels of erythrocytes, first as the translation products of mRNA pools (Zhang *et al.*, 1990; Tsai *et al.*, 1991), and recently as formed by intrinsic membrane protein CHIP28 (Preston *et al.*, 1992). Reconstitution studies with proteoliposomes containing purified CHIP28 have provided further evidence that this protein indeed forms the highly specific water channel of the red cell membrane (van Hoek and Verkman, 1992; Zeidel *et al.*, 1992).

In the present work, putative transport functions of the plant vacuolar membrane TIP proteins were investigated using the *Xenopus* oocyte system. We considered that, because of the absence of vacuoles from *Xenopus* oocytes, the TIP protein when synthesized in oocytes should be targeted to the plasma membrane, known to be a default destination of membrane proteins in animal cells. We characterized in parallel with *Arabidopsis* γ-TIP two other members of the MIP family: CHIP28 and GlpF, the glycerol facilitator of *Escherichia coli*. We present here evidence showing that the transport activity induced by γ-TIP expression in oocytes exhibits typical features for a specific

and protein-mediated transport of water. This leads to the proposal that beyond their identities in amino acid sequence, γ-TIP and CHIP28 share the same function: they are water specific channels. By contrast, γ-TIP is distinct from its homologs MIP and GlpF, for which other transport functions have been described.

Results

In vitro transcribed mRNAs encoding γ-TIP, GlpF or CHIP28, each with the 5′ and 3′ untranslated regions of a β-globin gene from *X.laevis*, were injected into *Xenopus* oocytes and osmotic water transport was investigated 2−3 days following injection. For this, oocytes were exposed to hypoosmotic conditions (160 mosM/kg gradient) and initial changes in cell volume were measured. Control oocytes that were either uninjected or injected with water instead of mRNA, swelled very slowly during the first 5 min following the sudden decrease in osmotic strength (Figure 1A). This result illustrates a limited osmotic water flux through the oocyte plasma membrane whose permeability can be mainly accounted for by lipid-mediated water transport (Zhang and Verkman, 1991). In contrast, oocytes injected with CHIP28 mRNA rapidly increased their volume by up to 60% (Figure 1A) and ruptured in <3−4 min, displaying the appearance of a new pathway for facilitated diffusion of water into the hypertonic cell. Recent evidence indicates that the CHIP28 protein is responsible for the formation of pores highly permeable to water (Preston *et al.*, 1992; van Hoek and Verkman, 1992; Zeidel *et al.*, 1992).

When the hypoosmotic challenge was performed on oocytes injected with GlpF mRNA, the rate of cell volume adjustment was slow and comparable with that of control (water injected) oocytes (Figure 1A). In contrast, 42 oocytes, isolated from eight frogs, injected with γ-TIP mRNA rapidly swelled and all burst in <6 min, exhibiting a response similar to that of oocytes injected with CHIP28 mRNA (Figure 1A). Osmotic water permeability coefficients (P_f) were estimated from the rate of cell volume change upon osmotic challenge (Zhang and Verkman, 1991). Figure 1B shows that over a set of three independent experiments, control oocytes and those injected with GlpF mRNA displayed similar osmotic water permeability values (P_f = $0.15-0.2\times10^{-2}$ cm/s). In contrast, γ-TIP and CHIP28 mRNA injection elicited respectively, a 6- to 8-fold and an 8- to 10-fold increase in P_f over the control value. These results demonstrate that expression of γ-TIP in oocytes creates a transport pathway with a high permeability to water.

The lack of a swelling response of oocytes injected with GlpF mRNA could reflect a failure of the oocytes to express the corresponding protein. To investigate this possibility, proteins of oocytes injected with water or mRNA were labeled metabolically with [^{35}S]amino acids. Proteins were then extracted with 1.5% SDS, resolved by SDS−PAGE and visualized by fluorography. The autoradiogram shown in Figure 2 reveals that the labeling pattern of the oocytes injected with GlpF, γ-TIP or CHIP28 mRNA differed from the profile of water injected oocytes by the presence of additional polypeptides of 20−30 kDa mol. wt, labeled with similar intensities for the different mRNA preparations used. Furthermore, the mobility of these new labeled proteins was similar to that of the major products obtained by *in vitro* translation of the corresponding mRNA (Figure 2). These results indicate that *Xenopus* oocytes were capable of synthesizing GlpF, CHIP28 and γ-TIP with similar efficiencies.

GlpF has been shown to facilitate the diffusion of small polyols into *E.coli* cells (Heller *et al.*, 1980). The possibility that the prokaryotic GlpF protein is synthesized in oocytes but is not functionally expressed in the oocyte plasma membrane was investigated by performing a glycerol uptake assay. Oocytes were incubated in the presence of 100 mM [^{14}C]glycerol and intracellular radioactivity was measured after 1−30 min time periods. Figure 3 shows that a slow glycerol influx was detected in water injected oocytes.

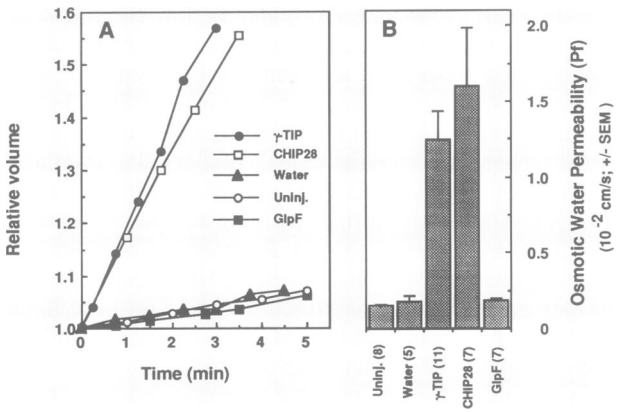

Fig. 1. Osmotic water permeability in mRNA injected oocytes. (**A**) Time course of osmotic swelling of individual oocytes uninjected (○) or injected with water (▲) or *in vitro* synthesized mRNA encoding γ-TIP (●), GlpF (■) or CHIP28 (□). Oocytes in Barth's buffer were perfused from t = 0 with a 5-fold dilution of Barth's buffer with distilled water. Measurements on oocytes injected with γ-TIP and CHIP28 mRNA stopped at the time of cell rupture. Representative data from the same batch of oocytes. (**B**) Osmotic water permeability (P_f) values. P_f values of individual oocytes were derived from volume change measurements made with three independent batches of oocytes. Data are expressed as the mean ± SEM; the number of cells is indicated in parentheses.

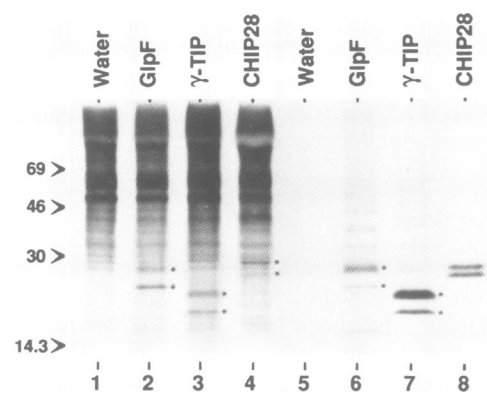

Fig. 2. Expression of γ-TIP, CHIP28 and GlpF in mRNA injected oocytes. *In vivo* labeled proteins of oocytes injected with water (lane 1) or specific mRNAs (lanes 2−4) and *in vitro* translation products obtained in the presence of water (lane 5) or specific mRNA (lanes 6−8) were prepared as described in Materials and methods. Asterisks indicate the polypeptides resulting from mRNA injection and their counterparts in the *in vitro* translation assay. In each case, the smaller of the two polypeptides may derive from translation initiation at an internal methionine residue. Arrowheads at left refer to the position of molecular size standards in kilodaltons. In each case, oocytes proteins were extracted from a pool of three oocytes.

However, oocytes expressing GlpF exhibited a 5- to 6-fold higher rate of glycerol uptake, which was sustained over the 30 min assay period. In contrast, γ-TIP mRNA injection did not modify the glycerol uptake capacity of the oocytes (Figure 3). These experiments, which allow precise monitoring of glycerol transport, confirm genetic and transport studies in bacteria showing that GlpF facilitates the diffusion of glycerol across the cytoplasmic membrane of these cells (Heller *et al.*, 1980; Sweet *et al.*, 1990). The failure of γ-TIP (Figure 3) and CHIP28 (data not shown) to create a glycerol transport system in the oocyte membrane indicates considerable specificity in the transport properties of different members of the MIP family.

Pores formed by amphotericin B and other antibiotics are highly permeable to water, but exclude other small neutral molecules (Finkelstein, 1987). However, these pores do allow the passage of massive ion fluxes. The ability of MIP to form water channels has not yet been examined, but when inserted into lipid bilayers, MIP forms high conductance ion channels (Zampighi *et al.*, 1985; Ehring *et al.*, 1990). To determine if γ-TIP could transport ions as well as water, we characterized the electrical conductance of the plasma membrane of oocytes expressing γ-TIP. Oocytes injected with water or *in vitro* synthesized mRNA encoding Xsha2, a K^+-channel present in adult *Xenopus* (Ribera, 1990), were used as controls. Oocytes were voltage clamped at a holding potential of −60 mV. A sequence of 1 s voltage steps from −130 to +30 mV was performed, the membrane potential being returned to the holding potential after each step. The traces of the electrical currents recorded in response to the 1 s pulses in oocytes injected with water or γ-TIP mRNA showed similar patterns, with small amplitudes and voltage dependence. In contrast, Xsha2 mRNA-injected oocytes displayed a strong outward rectifying current upon membrane depolarization to values higher than −40 mV. Figure 4 shows for each series of oocytes the deduced relationship between applied voltage and recorded current. After performing voltage clamp experiments, all oocytes were subjected to an osmotic change to confirm functional expression of γ-TIP.

Water channels produced by γ-TIP may be closed in the absence of osmotic gradient across the oocyte membrane. We therefore characterized the electrical response of osmotically challenged oocytes, under conditions were γ-TIP dependent water transport is known to occur. Membrane currents were recorded in oocytes voltage clamped at both 0 and −60 mV, to exclude the possibility that the oocytes were clamped at the reversal potential of putative currents specific for γ-TIP expression. Figure 5A and C shows that, upon transfer to hypoosmotic conditions, control water injected oocytes displayed a slight positive deflection in membrane current (+20−50 nA). Oocytes expressing γ-TIP showed a similar electrical response to control oocytes during the first minute of exposure to hypoosmotic conditions (Figure 5B and D). Microscopic observation confirmed that oocytes expressing γ-TIP swelled immediately and continuously after the osmotic challenge was imposed in accordance to the response recorded on non-voltage clamped oocytes. After the swelling of voltage clamped oocytes expressing γ-TIP had proceeded for several minutes, lesions could be observed at the sites of electrode insertion. These lesions coincided with the appearance of erratic membrane currents (Figure 5B and D). These currents were not reversible when the perfused medium was replaced by the initial isoosmotic buffer (Figure 5D). Similar results were obtained when the impermeant ions Tris and MES were substituted for Na^+ or Cl^-, respectively, in Barth's buffer. When oocytes were immersed in Barth's buffer at pH 4.2 to mimic the intravacuolar pH of plant cells, no electrical response specific for γ-TIP was detected (data not shown). Altogether, our electrophysiological data (see Figures 4 and

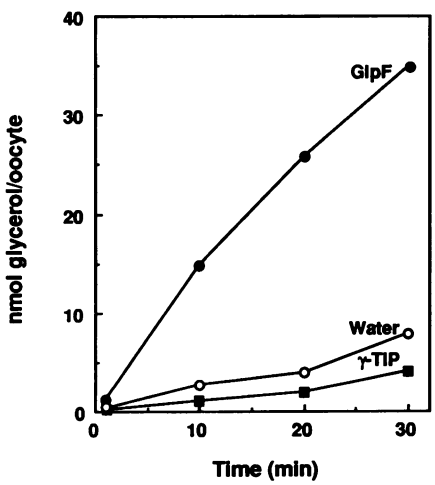

Fig. 3. Time course of uptake of 100 mM [U-^{14}C]glycerol into oocytes injected with water (○) or specific mRNAs for γ-TIP (■) or GlpF (●). Values are averaged from measurements on two oocytes at each time point. The cellular concentration of glycerol following 30 min of GlpF uptake in oocytes expressing GlpF was calculated to be 30 mM, indicating that no concentrative uptake occurred during the assay.

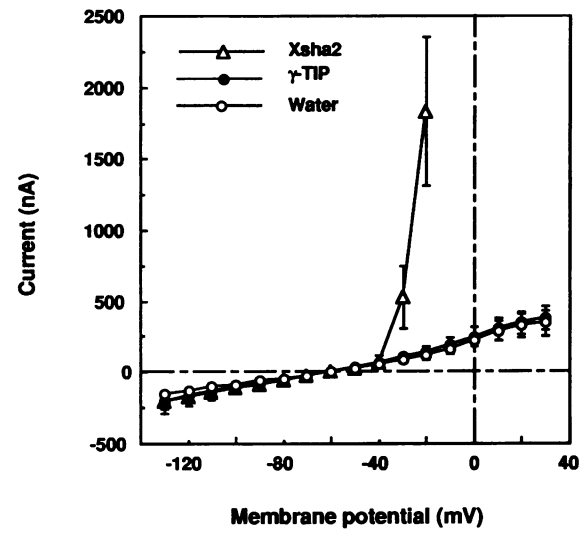

Fig. 4. Membrane current-to-voltage relationship in oocytes injected with water (○) or mRNA specific for γ-TIP (●) or Xsha2 (△). Membrane potential was held at −60 mV and stepped for 1 s to potentials from −130 to +30 mV in 10 mV increments with 10 s pauses at −60 mV between each pulse. Current amplitudes were measured 300 ms after the initiation of each pulse and corresponded to the peak current amplitude. Data are presented as the mean ± SEM of currents measured on four cells. Error bars not shown fall within a symbol. Corresponding osmotic water permeability values on the same lot of oocytes were (in 10^{-2} cm/s ± SEM): water, P_f = 0.124 ± 0.010 (n = 8); Xsha2, P_f = 0.126 ± 0.007 (n = 8); γ-TIP, P_f = 0.578 ± 0.061 (n = 5).

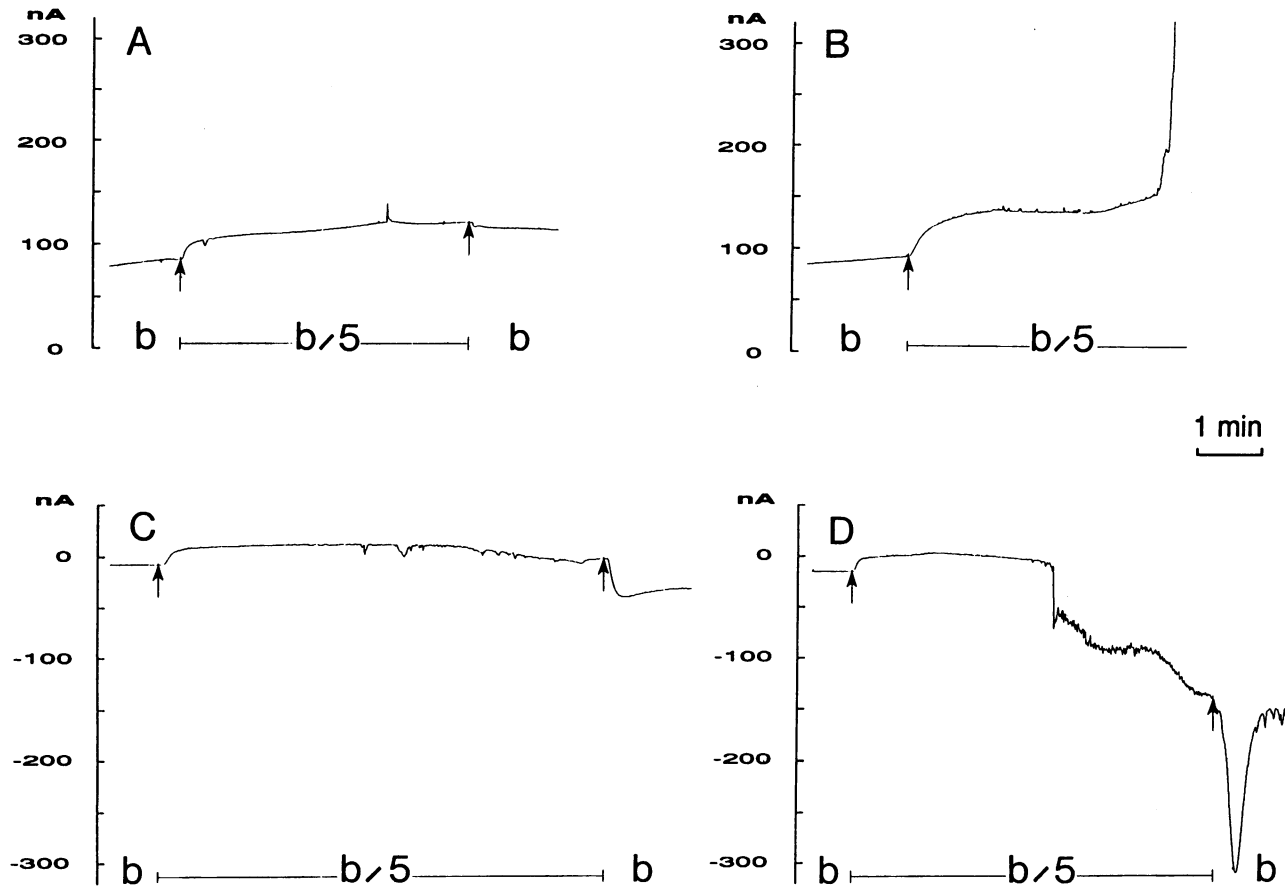

Fig. 5. Membrane current response of voltage clamped oocytes upon hypoosmotic challenge. Water injected oocytes are shown on the left (**A** and **C**). γ-TIP injected oocytes are shown on the right (**B** and **D**). Membrane potential was held at either 0 (A and B) or −60 mV (C and D). Ion currents across the plasma membrane of oocytes were recorded in cells perfused alternately with isotonic (b) or 5-fold hypotonic (b/5) Barth's buffers. Inward and outward currents correspond to the movement of positive charges into and out of the cytoplasm respectively and are displayed as negative and positive currents. In additional control experiments, oocytes treated with the pore forming antibiotic amphotericin B (500 μg/ml) and voltage clamped to 0 mV showed upon transfer to b/5 buffer immediate outward current >1000 nA.

5) suggest that the transport pathway induced in oocytes by γ-TIP possesses, at best, a very low permeability to mineral ions.

Water transport activity in oocytes expressing γ-TIP was probed using specific pharmacological compounds. We tested the effects of gadolinium ions (30 μM $GdCl_3$), which antagonize mechanosensitive and osmotic-pressure dependent channels (Morris, 1990; Alexandre and Lassalles, 1991; Chan and Nelson, 1992). We also used DCCD (50 μM), a hydrophobic carboxyl modifier which was shown to bind to a homolog of γ-TIP in radish (Maeshima, 1992), as well as mercury ions (1 mM $HgCl_2$), which inhibit the CHIP28 water channel (Preston *et al.*, 1992). $HgCl_2$ was the only one of these compounds that altered the osmotic water permeability (P_f) of oocytes expressing γ-TIP, with an inhibition of P_f of ~70% (Figure 6A). This effect was partially reversed by the reducing agent β-mercaptoethanol (Figure 6A). Glycerol uptake in oocytes expressing GlpF was also probed using the same compounds. $HgCl_2$ (1 mM) inhibited glycerol uptake by 70%, whereas 50 μM DCCD was without effect (Figure 6B).

Discussion

γ-TIP mediates specific transport of water

In the present work, the transport function of the vacuolar membrane γ-TIP protein was identified using functional expression in *Xenopus* oocytes and compared with the transport activities mediated by two homologous proteins: GlpF, the glycerol facilitator of *E.coli* and CHIP28, a water channel from human erythrocytes. Data presented here provide evidence that the expression of γ-TIP increased the osmotic water permeability of the oocyte plasma membrane to an extent comparable with CHIP28 expression (Preston *et al.*, 1992). In contrast, expression of GlpF or the potassium channel Xsha2 was without effect on water permeability. Yet, we showed that these two proteins are functional in oocytes for glycerol and ion transport respectively. This demonstrates the specificity of γ-TIP expression to enhance water transport through the oocyte plasma membrane.

Previous authors have reported a coupling of water and ion transport through tonoplast channels in plant cells. Streaming potential analysis of a large K^+ conductance in *Chara* vacuoles allowed Homblé and Véry (1992) to demonstrate the coupling of 29 molecules of water with one K^+ ion, during transport. Alexandre and Lassalles (1992) also described in red beet vacuoles an osmotic pressure sensitive channel that may mediate both cation and osmotically driven water fluxes. Detailed electrophysiological analysis suggests that, in contrast, γ-TIP mediates very little if any ion electrogenic transport in oocytes. In particular, osmotic fluxes of water occurring through the γ-TIP channel may drag charged molecules along (Alexandre

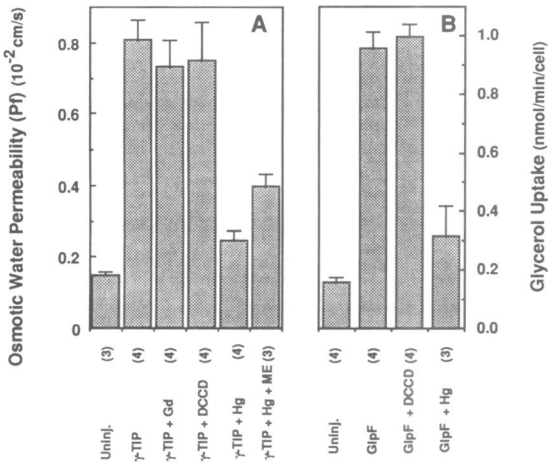

Fig. 6. Inhibition of osmotic water permeability (P_f) (**A**) or glycerol uptake (**B**) in oocytes. (A) P_f values were measured on oocytes either uninjected or injected with γ-TIP mRNA as described in the text and exemplified in Figure 1. When indicated, the assay was performed in the presence of gadolinium ions (Gd: 30 μM $GdCl_3$, 10 min preincubation), DCCD (50 μM, 30 min preincubation), mercury ions (Hg: 1 mM $HgCl_2$, 10 min preincubation) or mercaptoethanol after mercury ion pretreatment (Hg + ME: 5 min preincubation in the presence of 1 mM $HgCl_2$ followed by 15 min preincubation and the assay, both in the presence of 10 mM mercaptoethanol). Data are expressed as the mean ± SEM of data from the indicated number of cells. In an independent experiment, we found no effect of Hg and Hg + ME on the P_f value of uninjected oocytes (in 10^{-2} cm/s ± SEM; n = 3): uninj., P_f = 0.116 ± 0.016; uninj. + Hg, P_f = 0.144 ± 0.022; uninj. + Hg + ME, P_f = 0.151 ± 0.050. (B) Glycerol uptake was assayed over 30 min on oocytes either uninjected (uninj.) or injected with GlpF mRNA (GlpF) using the radiotracer technique described in Materials and methods. Inhibitor treatments and conventions as in (A).

and Lassalles, 1992). To investigate this hypothesis, we have used continuous membrane current recordings on oocytes which were voltage clamped at 0 or −60 mV and transferred to hypoosmotic conditions. The resolution of our measurements indicates that a γ-TIP dependent current could not exceed 50 nA in osmotically challenged oocytes. This corresponds to a flux of 5×10^{-4} nmol charge per second while the rate of cell volume adjustment reflects a water flux across the oocyte membrane which is $\sim2\times10^5$ times larger, in the range 100 nmol/s (Zhang and Verkman, 1991). The possibility that electroneutral ion transport occurred in the various conditions tested (V = 0 mV and V = −60 mV in the presence of NaCl, Tris-Cl or Na-MES) appears very unlikely. The idea that TIP is essentially impermeable to charged molecules is consistent with the properties of water channels in red cells which are estimated to be at least 10^3-fold less permeable to ions including protons than to water molecules (Macey, 1984; Finkelstein, 1987). Preservation of the proton gradient which energizes numerous transport processes across the tonoplast would necessitate that TIP be largely impermeant to protons.

The selectivity of water transport with respect to the transport of glycerol was also investigated in oocytes expressing γ-TIP. We found that, in contrast to GlpF, γ-TIP does not transport glycerol. This and other reports showing that CHIP28 does not render membranes permeable to urea (van Hoek and Verkman, 1992; Zeidel *et al.*, 1992) suggest that channels like γ-TIP and CHIP28 exclude small neutral solutes while permitting water molecules to permeate the pore.

Table I. Binary comparison of the amino acid sequences of GlpF, γ-TIP, CHIP28 and MIP

	γ-TIP[a]	CHIP28[b]	MIP[c]
GlpF[d]	33 (79; 6)	32 (106; 8)	31 (107; 9)
MIP[e]	37 (218; 32)	44 (266; 65)	
CHIP28[f]	35 (229; 32)		

Sequence analysis was performed using the Fasta program of Pearson and Lipman (1988). Values which are not in parentheses represent percent identity in the aligned segments. First and second numbers in parentheses indicate, respectively, the number of residues in the compared segments and the identity score in SD values higher than those obtained with 100 comparisons of randomized sequences of these protein segments.
[a]Höfte *et al.* (1992); [b]Preston and Agre (1991); [c]Gorin *et al.* (1984); [d]Muramatsu and Mizuno (1989)

We conclude that the transport activity induced by γ-TIP in oocytes displays some of the functional features described for the protein-mediated transport of water in erythrocytes. This leads to the proposal that in addition to their identities in amino acid sequence, γ-TIP and CHIP28 share a similar function: they are water specific channels.

Transport specificities of the different members of the MIP family

The present work shows that γ-TIP and CHIP28 are functionally distinct from another member of the MIP family, the bacterial GlpF. γ-TIP and CHIP28 are likely to be distinct from MIP as well which has been shown to form ion channels (Ehring *et al.*, 1990). These four proteins display the features described for the MIP family, with the characteristic tandem sequence repeats, each carrying the highly conserved NPA box (Pao *et al.*, 1991; Reizer *et al.*, in preparation; Wistow *et al.*, 1991). Table I shows overall percent amino acid identities between γ-TIP, CHIP28, GlpF and MIP. GlpF appears to be only distantly related to the two water channels CHIP28 and γ-TIP. However, the highest similarity score is found between the two animal proteins MIP and CHIP28 and these proteins exhibit similar identity with respect to the plant protein γ-TIP. This and other sequence analyses (Reizer *et al.*, in preparation) suggest that overall sequence similarity between the various MIP family members may reflect evolutionary divergence rather than functional specialization.

The high selectivity of water channels has been attributed in part to a constriction in the pore formed by the protein that would exclude neutral molecules other than water (Macey, 1984; Finkelstein, 1987). Because GlpF was reported to be permeable to polyols and to small molecules like urea (Heller *et al.*, 1980) this protein could be thought of as having a wider pore than CHIP28 or γ-TIP. The surprising finding that GlpF has no water conductance implies that the transport selectivity of these proteins should not be considered solely in terms of size exclusion. In particular, GlpF may display a discontinuity in pore hydration, to allow separation of solute from water.

The inhibitory effects of mercurials on water channels in animal cells has been considered an important characteristic of these proteins (Macey, 1984; Finkelstein, 1987; Preston *et al.*, 1992). Here we show that mercury ions inhibit γ-TIP mediated water transport, as well as GlpF mediated glycerol uptake. These results suggest that cysteine residues

are crucial for transport activity in the MIP family but have no special relevance to water transport specificity. γ-TIP possesses only one Cys in the third membrane spanning domain (Cys118); the mercury-sensitive residue of CHIP28 was recently identified as Cys189, in the loop linking transmembrane domains 5 and 6 (Preston *et al.*, 1993).

We conclude that although the proteins of the MIP family share structural and functional similarities, they have nevertheless evolved divergent transport specificities. Further comparisons between GlpF, γ-TIP and CHIP28 should lead to a better understanding of the determinants of water transport specificity. Expression of the seed specific isoform of TIP (α-TIP) in oocytes led only to a small increase in osmotic water permeability (Maurel *et al.*, unpublished). We do not know yet if this protein behaves like a true water channel or instead possesses other as yet unidentified transport properties.

Why should plant vacuoles have water channels?

Previous work has shown that γ-TIP is located in the tonoplast of plant cells (Höfte *et al.*, 1992), and the present report suggests that water specific channels occur in this membrane. This proposal is consistent with the report of Kiyosawa and Tazawa (1977) who described in *Chara* cells a much higher water permeability for the tonoplast as compared with the plasma membrane. This feature suggests a central role for the vacuole in the osmoregulation of the cytoplasm.

The cytoplasm of a plant cell forms a thin layer delimited by the relatively large membrane surfaces of the tonoplast and the plasma membrane and this could expose it to drastic and sudden osmotic changes. Physiological osmotic challenges result, for instance, from water efflux upon water deprivation (drought) or from water influx during cell elongation. Non-limiting water transport through the tonoplast may allow the cell to use the vacuolar space to buffer rapid water exchanges with the extracellular space. This suggestion is in agreement with the specific pattern of γ-TIP mRNA expression in elongating root tissues (Ludevid *et al.*, 1992). TIP homologs are induced by water stress in pea (Guerrero *et al.*, 1990) and in *Arabidopsis* (Yamaguchi-Shinozaki *et al.*, 1992). If these proteins also turn out to be water channels, then a determination of their location in the cell, on the tonoplast or on the plasma membrane, will be of major interest to assess the role of water channels in plant cells.

Materials and methods

Plasmid constructions and in vitro RNA synthesis and translation

DNA fragments encoding respectively, γ-TIP (Höfte *et al.*, 1992) and GlpF (Muramatsu and Mizuno, 1989) were cloned into the *Bgl*II site of a pSP64T-derived BlueScript vector carrying 5′ and 3′ untranslated sequences of a β-globin gene of *Xenopus* (Preston *et al.*, 1992). pSP64T-derived vectors provide high mRNA stability and translation efficiency in *Xenopus* oocytes (Krieg and Melton, 1984). The cloned DNA fragments were (i) a *Bam*HI−*Xba*I fragment containing the entire γ-TIP coding sequence plus 7 bp and 1 bp at the 5′ and 3′ termini respectively, and with the *Xba*I end recessed and ligated to an 8 bp *Bam*HI linker (New England Biolabs, MA); and (ii) a *Bam*HI fragment containing the entire GlpF coding sequence plus 6 bp and 471 bp at the 5′ and 3′ termini, respectively. Capped RNA encoding γ-TIP, GlpF and CHIP28 were synthesized *in vitro* using T3 RNA polymerase and purified as described (Preston *et al.*, 1992). RNAs were translated *in vitro* using a rabbit reticulocyte system in the presence of [^{35}S]methionine (Jackson and Hunt, 1982).

Oocyte preparation and injection

Fully grown oocytes (stage V and VI) were isolated from *X.laevis* and incubated in Barth's solution [88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 10 mM HEPES−NaOH, 0.33 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$, pH 7.4] as described (Cao *et al.*, 1992). *In vitro* transcripts (0.5−1 mg/ml) or distilled water were injected in a volume of 50 nl.

Osmotic water permeability assay

Oocytes were transferred 2−3 days after mRNA injection from Barth's solution (200 mosM/kg) at room temperature to the same solution diluted to 40 mosM/kg with distilled water. Changes in cell volume were followed with a microscope by taking photographs at 30−45 s intervals. Oocyte diameters were measured four times along two sets of perpendicular axes. The volume V was estimated as the mean of two ellipsoid volumes. The osmotic permeability coefficient was calculated from $P_f = V_o[d(V/V_o)/dt]/[S \times V_w (Osm_{in} - Osm_{out})]$ with initial oocyte volume $V_o = 9 \times 10^{-4}$ cm^3, initial oocyte surface area $S = 0.045$ cm^2 and molar volume of water $V_w = 18$ cm^3/mol (Zhang and Verkman, 1991).

Labeling and SDS−PAGE of oocyte proteins

Oocytes were transferred 4−7 h after mRNA injection to Barth's solution supplemented with 95 MBq/ml [^{35}S]protein labeling mix (EXPRE^{35}S^{35}S, New England Nuclear, Boston, MA) containing mostly [^{35}S]methionine (68 MBq/ml; 37 TBq/mmol). After 14 h, oocytes were lysed in a TBS buffer (20 mM Tris−Cl, 500 mM NaCl, pH 7.5) containing 1 mM PMSF, 10^{-7} M pepstatin, 1 μg/ml leupeptin and 1% (w/w) Triton X-100. The lysate was heated for 10 min at 65°C, layered on a cushion of the same solution containing 25% (w/v) sucrose and centrifuged for 30 min at 14 000 *g*. The pellet was resuspended in TBS containing 1.5% (w/v) SDS, heated for 10 min at 65°C, and solubilized proteins were separated by SDS−PAGE and visualized by fluorography.

Glycerol uptake assay

Two to 3 days after water or mRNA injection, oocytes were incubated at 21°C in a half strength Barth's solution containing 100 mM [U-^{14}C]glycerol (final specific activity: 5 MBq/mmol; Amersham). At appropriate intervals, oocytes were rapidly rinsed five times in ice-cold Barth's solution, lysed for 5 min in 2% SDS at 100°C and the radioactivity measured by liquid scintillation.

Voltage clamp

Two-electrode voltage clamp experiments were performed at room temperature in a continuously perfused bath essentially as described (Cao *et al.*, 1992). Voltage pulse protocols and data acquisition were performed by a pCLAMP program (Axon Instruments, Inc., Burlingame, CA). No leakage current substraction protocol was used. Membrane currents in oocytes under hypoosmotic conditions were low-pass filtered at 3 Hz and recorded using an AXOTAPE program (Axon Instruments).

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References

Alexandre,J. and Lassalles,J.-P. (1991) *Biophys. J.*, **60**, 1326−1336.

Cao,Y., Anderova,M., Crawford,N.M. and Schroeder,J.I. (1992) *Plant Cell*, **4**, 961−969.

Chan,H.C. and Nelson,D.J. (1992) *Science*, **257**, 669−671.

Ehring,G.R., Zampighi,G., Horwitz,J., Bok,D. and Hall,J.E. (1990) *J. Gen. Physiol.*, **96**, 631−664.

Finkelstein,A. (1987) In *Distinguished Lecture Series of the Society of General Physiologists*. John Wiley & Sons, Inc., Vol. 4, pp. 1−228.

Fortin,M.B., Morrison,N.A. and Verma,D.P.S. (1987) *Nucleic Acids Res.*, **15**, 813–824.
Gorin,M.B., Yancey,S.B., Cline,J., Revel,J.-P. and Horwitz,J. (1984) *Cell*, **39**, 49–59.
Guerrero,F.D., Jones,J.T. and Mullet,J.E. (1990) *Plant Mol. Biol.*, **15**, 11–26.
Heller,K.B., Lin,E.C.C. and Wilson,T.H. (1980) *J. Bacteriol.*, **144**, 274–278.
Höfte,H., Hubbard,L., Reizer,J., Ludevid,D., Herman,E.M. and Chrispeels,M.J. (1992) *Plant Physiol.*, **99**, 561–570.
Homblé,F. and Véry,A.A. (1992) *Biophys. J.*, **63**, 996–999.
Jackson,R.J. and Hunt,T. (1982) *Methods Enzymol.*, **96**, 50–74.
Johnson,K.D., Herman,E.M. and Chrispeels,M.J. (1989) *Plant Physiol.*, **91**, 1006–1013.
Johnson,K.D., Höfte,H. and Chrispeels,M.J. (1990) *Plant Cell*, **2**, 525–532.
Kiyosawa,K. and Tazawa,M. (1977) *J. Membr. Biol.*, **37**, 157–166.
Krieg,P.A. and Melton,D.A. (1984) *Nucleic Acids Res.*, **12**, 7057–7070.
Ludevid,D., Höfte,H., Himelblau,E. and Chrispeels,M.J. (1992) *Plant Physiol.*, **100**, 1633–1639.
Macey,R.I. (1984) *Am. J. Physiol.*, **246**, C195–C203.
Maeshima,M. (1992) *Plant Physiol.*, **98**, 1248–1254.
Morris,C.E. (1990) *J. Membr. Biol.*, **113**, 93–107.
Muramatsu,S. and Mizuno,T. (1989) *Nucleic Acids Res.*, **17**, 4378.
Ouyang,L.J., Whelan,J., Weaver,C.D., Roberts,D.M. and Day,D.A. (1991) *FEBS Lett.*, **293**, 188–190.
Pao,G.M., Wu,L.-F., Johnson,K.D., Höfte,H., Chrispeels,M.J., Sweet,G., Sandal,N.N. and Saier,J.M.H. (1991) *Mol. Microbiol.*, **5**, 33–37.
Pearson,W.R. and Lipman,D.J. (1988) *Proc. Natl Acad. Sci. USA*, **85**, 2444–2448.
Preston,G.M. and Agre,P. (1991) *Proc. Natl Acad. Sci. USA*, **88**, 11110–11114.
Preston,G.M., Carroll,T.P., Guggino,W.B. and Agre,P. (1992) *Science*, **256**, 385–387.
Preston,G.M., Jung,J.S., Guggino,W.B. and Agre,P. (1993) *J. Biol. Chem.*, **268**, 17–20.
Ribera,A.B. (1990) *Neuron*, **5**, 691–701.
Soreq,H. and Seidman,S. (1992) *Methods Enzymol.*, **207**, 225–265.
Sweet,G., Gandor,C., Voegele,R., Wittenkindt,N., Beuerle,J., Truniger,V., Lin,E.C.C. and Boos,W. (1990) *J. Bacteriol.*, **172**, 424–430.
Tsai,S.-T., Zhang,R. and Verkman,A.S. (1991) *Biochemistry*, **30**, 2087–2092.
van Hoek,A.N. and Verkman,A.S. (1992) *J. Biol. Chem.*, **267**, 18267–18269.
Wistow,G.J., Pisano,M.M. and Chepelinsky,A.B. (1991) *Trends Biochem. Sci.*, **16**, 170–171.
Yamaguchi-Shinozaki,K., Koizumi,M., Urao,S. and Shinozaki,K. (1992) *Plant Cell Physiol.*, **33**, 217–224.
Zampighi,G.A., Hall,J.E. and Kreman,M. (1985) *Proc. Natl Acad. Sci. USA*, **82**, 8468–8472.
Zeidel,M.L., Ambudkar,S.V., Smith,B.L. and Agre,P. (1992) *Biochemistry*, **31**, 7436–7440.
Zhang,R. and Verkman,A.S. (1991) *Am. J. Physiol.*, **260**, C26–C34.
Zhang,R., Logee,K.A. and Verkman,A.S. (1990) *J. Biol. Chem.*, **265**, 15375–15378.

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