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Phosphorylation regulates the water channel activity of the seed-specific aquaporin α-TIP

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The vacuolar membrane protein α-TIP is a seed-specific protein of the Major Intrinsic Protein family. Expression of α-TIP in *Xenopus* oocytes confered a 4- to 8-fold increase in the osmotic water permeability (P_f) of the oocyte plasma membrane, showing that α-TIP forms water channels and is thus a new aquaporin. α-TIP has three putative phosphorylation sites on the cytoplasmic side of the membrane (Ser7, Ser23 and Ser99), one of which (Ser7) has been shown to be phosphorylated. We present several lines of evidence that the activity of this aquaporin is regulated by phosphorylation. First, mutation of the putative phosphorylation sites in α-TIP (Ser7Ala, Ser23Ala and Ser99Ala) reduced the apparent water transport activity of α-TIP in oocytes, suggesting that phosphorylation of α-TIP occurs in the oocytes and participates in the control of water channel activity. Second, exposure of oocytes to the cAMP agonists 8-bromoadenosine 3′,5′-cyclic monophosphate, forskolin and 3-isobutyl-1-methylxanthine, which stimulate endogenous protein kinase A (PKA), increased the water transport activity of α-TIP by 80–100% after 60 min. That the protein can be phosphorylated by PKA was demonstrated by phosphorylating α-TIP in isolated oocyte membranes with the bovine PKA catalytic subunit. Third, the integrity of the three sites at positions 7, 23 and 99 was necessary for the cAMP-dependent increase in the P_f of oocytes expressing α-TIP, as well as for *in vitro* phosphorylation of α-TIP. These findings demonstrate that the α-TIP water channel can be modulated via phosphorylation of Ser7, Ser23 and Ser99. To our knowledge, this is the first evidence of aquaporin regulation via phosphorylation and we propose this process as a mechanism for regulating water permeability of biological membranes.

Key words: germination/MIP/osmoregulation/plant/vacuole

Introduction

The functional characterization of the aquaporin family of proteins from animals and plants has recently provided a molecular clue to the puzzling efficiency of some specialized membranes in mediating water transport. Aquaporins are homologous 25–30 kDa intrinsic membrane proteins that form water-selective channels (for reviews see Chrispeels and Maurel, 1994; Engel *et al.*, 1994). The observation that expression of the erythrocyte protein AQP-CHIP in *Xenopus* oocytes increased the swelling rate of oocytes bathed in a hypotonic solution provided the first evidence of the water transport activity of such types of protein (Preston *et al.*, 1992). This property was later confirmed by reconstitution experiments in artificial membranes. These landmark experiments, together with the general use of *Xenopus* oocyte expression, have triggered the molecular identification of other aquaporins from animals and plants (Chrispeels and Maurel, 1994; Engel *et al.*, 1994). Aquaporins belong to the larger Major Intrinsic Protein (MIP) family of transmembrane channels, whose archetype is the Major Intrinsic Protein of lens fibers. Members of this family have been identified in many organisms, ranging from bacteria to mammals. Some of the homologs do not transport water, but solutes such as glycerol (Maurel *et al.*, 1994).

Aquaporins can increase the water permeability of biological membranes well above that expected from their lipid composition. However, the biological significance of these proteinaceous channels may reside primarily in their capacity to modulate membrane water permeability. Membrane lipid composition can determine to some extent the rate of water transport through a membrane (Finkelstein, 1987). Yet aquaporins give biological membranes a unique capacity to modulate, rapidly and with large amplitude, their permeability to water molecules.

In animals, inner medullary cells of the renal collecting ducts (IMCD) have emerged as a paradigm of water transport regulation by physiological signals (Harris *et al.*, 1991). Vasopressin receptors on the basal surface of these cells act through a cAMP transduction cascade to stimulate delivery of water channel proteins from subapical vesicles to the apical plasma membrane. Recently, Echevarria *et al.* (1993) described the functional expression in *Xenopus* oocytes of cAMP-regulated water channels from rat kidney medulla, but the mechanism underlying this regulation was not elucidated. The recent cloning of AQP-CD (Fushimi *et al.*, 1993), which is probably the vasopressin-regulated water channel of IMCD, will undoubtedly provide a new tool to investigate the membrane shuttle hypothesis (Nielsen *et al.*, 1993). To this short-term control mechanism allowing rapid and efficient modulation of water reabsorption, dehydration and vasopressin itself superimpose long-term controls of AQP-CD protein levels (Nielsen *et al.*, 1993; DiGiovanni *et al.*, 1994).

Plant cells have aquaporins in both their plasma and vacuolar (tonoplast) membranes and their abundance in these membranes is undoubtedly regulated at the transcriptional level (Ludevid *et al.*, 1992). As in animals (Engel *et al.*, 1994), plant aquaporins exhibit highly specialized developmental patterns. For instance, α-TIP is specifically

expressed in seeds and is present in the membranes of protein storage vacuoles during the late and early stages of seed maturation and germination (Johnson *et al.*, 1989; Melroy and Herman, 1991). Physiological conditions that require specific hydration adjustments can trigger the expression of specific aquaporins. Water deprivation in pea and *Arabidopsis* induces the expression of MIP homologs (Guerrero *et al.*, 1990; Yamaguchi-Shinozaki *et al.*, 1992) and the *Arabidopsis* homolog has recently been shown to be a plasma membrane aquaporin (Daniels *et al.*, 1994). Also, the expression of the vacuolar aquaporin γ-TIP correlates with cell elongation (Ludevid *et al.*, 1992; Maurel *et al.*, 1993) and is under the control of the plant hormone gibberellic acid (GA3), which promotes additional cell enlargement (Phillips and Huttly, 1994).

MIP homologs, including MIP itself, the soybean nodulin NOD26 and seed-specific α-TIP, have been described as undergoing *in vivo* and *in vitro* phosphorylation (see Lampe and Johnson, 1990; Johnson and Chrispeels, 1992; Miao *et al.*, 1992; Weaver and Roberts, 1992, and references therein). α-TIP is phosphorylated on Ser7 by a tonoplast-bound protein kinase, tentatively identified as a calcium-dependent protein kinase (CDPK) (Johnson and Chrispeels, 1992). However, the functional role of protein phosphorylation in the MIP family has been described only for the MIP ion channel. This channel reconstituted in artificial membranes showed altered voltage sensitivity of ionic current inactivation when phosphorylated (Ehring *et al.*, 1991). In the present paper we identify plant vacuolar α-TIP as a new member of the aquaporin family that exhibits properties distinctly different from that of its immediate homolog γ-TIP. By demonstrating that phosphorylation of α-TIP efficiently regulates its water channel activity, we describe a novel mechanism for the control of water permeability in biological membranes.

Results

α-TIP is an aquaporin

To assay the water channel activity of α-TIP and compare it with that of γ-TIP, complementary RNAs (cRNA) for α-TIP and γ-TIP were transcribed from cDNAs *in vitro* and injected into *Xenopus* oocytes. Osmotic water transport was investigated 3–4 days after cRNA injection by measuring oocyte volume change on exposure of the oocytes to a hypotonic bathing solution (160 mosmol/kg gradient). Individual osmotic water permeability coefficients (P_f) were calculated from the initial rate of oocyte swelling. In four experiments with independent oocyte donors, uninjected control oocytes swelled slowly (P_f = 0.095 ± 0.009×10^{-2} cm/s, n = 20), whereas oocytes injected with α-TIP or γ-TIP cRNAs showed higher swelling rates (α-TIP P_f = 0.447 ± 0.043×10^{-2} cm/s, n = 23; γ-TIP P_f = 1.057 ± 0.118×10^{-2} cm/s, n = 20). Osmotic responses of representative cells are shown in Figure 1. Voltage clamp experiments showed that, in contrast to P_f, the electrical conductance of the whole cell membrane was similar in control uninjected oocytes (1.8 ± 0.4 μS, n = 6) and in oocytes expressing α-TIP (2.1 ± 0.5 μS, n = 7). These results show that α-TIP, similarly to aquaporin γ-TIP (Maurel *et al.*, 1993), facilitated the diffusion of water molecules, but not of ions, across the oocyte plasma membrane and thus α-TIP appears to be another aquaporin in the plant cell tonoplast. However, the P_f of oocytes expressing α-TIP was consistently two to three times lower than the P_f of oocytes expressing γ-TIP. Metabolic labeling with [^{35}S]methionine showed that oocytes achieved similar expression levels for α-TIP and γ-TIP (data not shown).

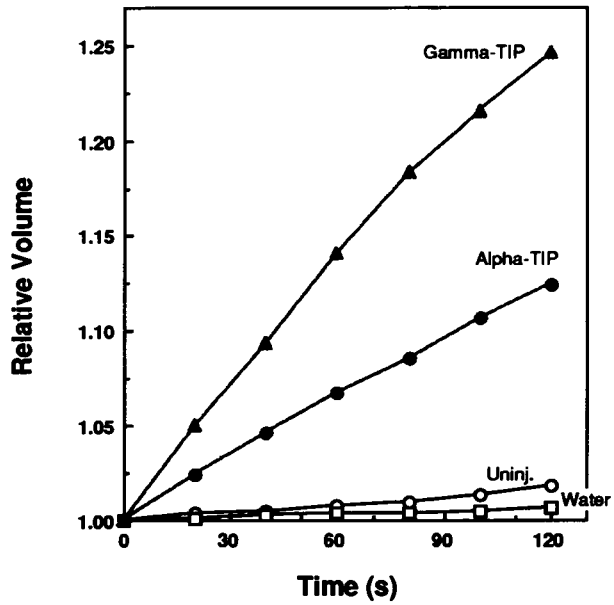

Fig. 1. Time course of osmotic swelling of individual oocytes. Oocytes were either uninjected (○) or injected with water (□), with cRNA encoding α-TIP (●) or γ-TIP (▲). After 3 days in isoosmotic Barth's solution, the oocytes were exposed from t = 0 to the same solution diluted 5-fold with distilled water. Osmotic water permeability coefficients (P_f) were derived, as described in Materials and methods, from the initial rate of cell swelling. P_f values are expressed in 10^{-2} cm/s: uninjected, P_f = 0.099; water, P_f = 0.035; α-TIP, P_f = 0.718; γ-TIP, P_f = 1.433.

Mutations at putative phosphorylation sites of α-TIP reduce its water transport activity in oocytes

Although the functional role of α-TIP phosphorylation at Ser7 in plant vacuolar membranes has remained elusive, we considered the possibility that the lower activity of α-TIP in oocytes might result from an inadequate phosphorylation of the protein in these cells. Indeed, the Arg–Arg–Tyr motif N-terminal to Ser7 defines a consensus site for phosphorylation by animal serine/threonine protein kinases such as cAMP-dependent protein kinase (PKA) and cGMP-dependent protein kinase (Pearson and Kemp, 1991). Further inspection of the α-TIP primary sequence revealed four additional consensus phosphorylation sites analogous to that defined for Ser7, with an arginine residue at the –2/–3 position of the serine/threonine phosphate acceptors. Figure 2 shows the position of these sites on a topological model of the α-TIP protein. This model of α-TIP was initially proposed by Johnson *et al.* (1990) and has remained consistent with most of the studies on the topology of the MIP homologs (Miao *et al.*, 1992; Preston *et al.*, 1994). According to this model, cytosolic protein kinases would have access to a limited number of sites, including Ser7, Ser23 and Ser99.

Four of the putative phosphorylation sites identified in the α-TIP amino acid sequence were changed by site-

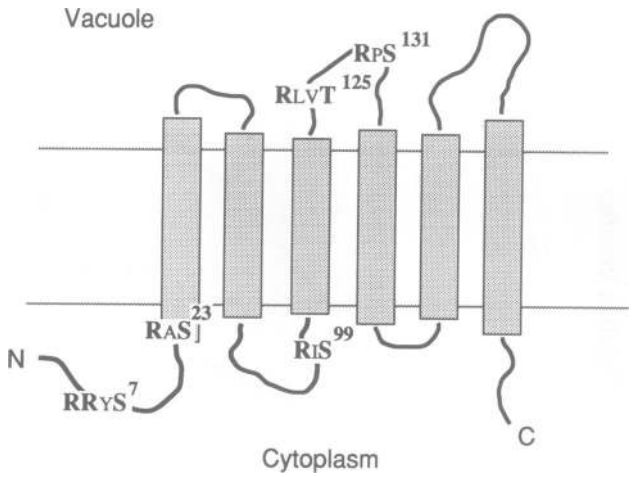

Fig. 2. Putative membrane topology and phosphorylation sites of α-TIP. Membrane-spanning domains are marked as boxes with the amino acid of origin and termination (not shown) as determined by Johnson *et al.* (1990), while connecting loops and N- and C-termini are figured with heavy lines. Putative phosphorylation sites are indicated with the serine/threonine phosphoacceptors, their position and the preceding basic residue(s) in bold. Upon expression in *Xenopus* oocytes, the cytoplasmic and vacuolar sides of the protein are thought to face the cytoplasm and the extracellular space respectively.

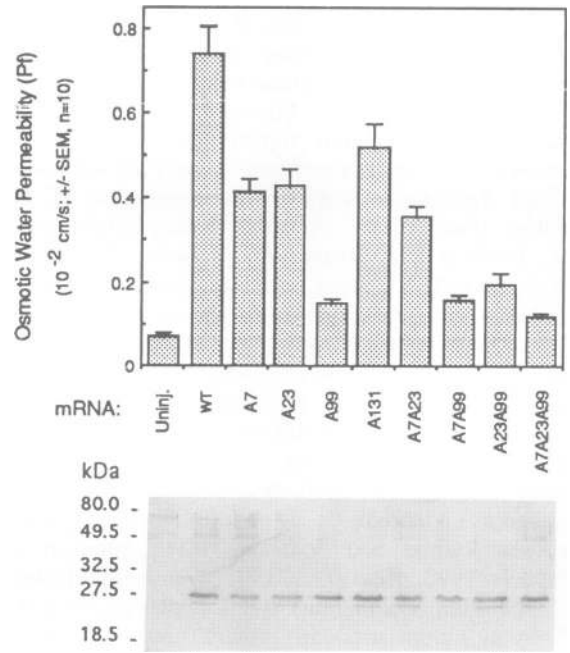

Fig. 3. Expression of Ser→Ala α-TIP mutants in oocytes. (Upper panel) Osmotic water permeability (P_f). P_f was measured 3–4 days after injection of 50 ng α-TIP cRNA, either wild-type (WT) or carrying mutations as indicated. Uninj., uninjected oocytes. (Lower panel) Corresponding immunodetection of wild-type and mutant α-TIP proteins. Total proteins were extracted from a pool of five oocytes, either uninjected or injected with the indicated cRNA. Equal amounts of proteins, corresponding to 0.1 oocytes, were separated by SDS–PAGE, transferred to PVDF membranes and probed with anti-α-TIP antibodies. Molecular mass markers are indicated on the left. The minor band revealed at 25 kDa by the antibodies probably represents the α-TIP degradation product described by Johnson *et al.* (1989).

directed mutagenesis to study their role in α-TIP activity. These sites were individually disrupted by replacing the serine residues (S) in positions 7, 23, 99 and 131 by alanine residues (A). Mutant cRNAs were synthesized *in vitro* and injected into oocytes. Expression of each α-TIP mutant was characterized in at least three experiments with independent cRNA transcription and oocyte donor. Swelling assays revealed that each of the α-TIP mutants was able to increase the P_f of the oocyte membrane, but in all cases to a lesser extent than wild-type α-TIP. Specifically, the single mutations S7A, S23A, S99A and S131A caused a mean decrease in water transport activity of 42 ± 3, 26 ± 8, 69 ± 4 and 26 ± 7% ($n > 16$) respectively. Figure 3 shows a representative experiment where we also determined the response of double or triple mutants combining mutations S7A, S23A and S99A. Some of these mutants, in particular those containing mutation S99A, displayed a strong inhibition of water transport activity. In a parallel immunoblot experiment (Figure 3), whole oocyte proteins were probed with an anti-α-TIP antiserum. This and similar analyses performed over three independent experiments showed that all the mutant proteins studied were consistently expressed in oocytes at levels similar to those of the wild-type protein. These results indicate that the decrease in apparent water transport activity observed in the α-TIP point mutants is not the result of a deficiency in protein synthesis or stability. Nevertheless, these results suggest requirements of initial serine hydroxyl side chains for α-TIP channel structure and function. In particular, reversible phosphorylation may occur on some of these residues. We noticed that the most critical serine residues for activity are Ser7 and Ser99, which are likely to be the most accessible to cytosolic protein kinases. This indicates that phosphorylation of these residues may occur in resting oocytes and participate in activating the water channel.

α-TIP can be phosphorylated by cAMP-dependent protein kinase

The possibility that α-TIP can be recognized by animal protein kinases was investigated using an *in vitro* phosphorylation assay. A plasma membrane-enriched fraction was purified from oocytes, incubated in the presence of [γ-^{33}P]ATP and the labeled proteins were resolved by SDS–PAGE and visualized by autoradiography. In this procedure a faint labeling of membrane proteins resulted from the basal endogenous protein kinase activity. Identical phosphorylation patterns were obtained with membranes purified from control uninjected oocytes and from oocytes expressing wild-type α-TIP, the triple mutant S7A/S23A/S99A or the γ-TIP isoform (not shown). The addition of a purified PKA catalytic subunit to the phosphorylation assay strongly enhanced protein labeling and revealed a novel phosphorylation pattern (Figure 4B). In particular, the pattern obtained with oocytes expressing wild-type α-TIP differed from that of uninjected controls by the presence of an additional 27 kDa peptide. This peptide had the same mobility as α-TIP detected in immunoblots (Figure 4A and B), was immunoadsorbed by an anti-α-TIP immunoserum (Figure 4B and C) and was thus identified as PKA-phosphorylated α-TIP. α-TIP labeling could not be detected in membranes containing equivalent amounts of the S7A/S23A/S99A mutant (Figure 4). No additional labeling was detected in membranes from oocytes expressing γ-TIP (Figure 4B). This suggests that γ-TIP is not recognized by PKA, in agreement with the

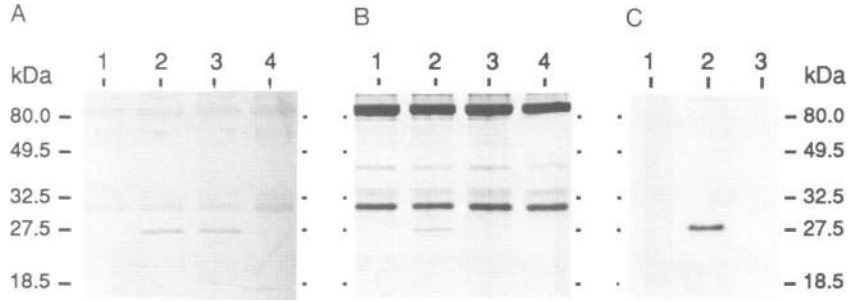

Fig. 4. *In vitro* phosphorylation of oocyte plasma membrane proteins by the cAMP-dependent protein kinase (PKA) catalytic subunit. A plasma membrane-enriched fraction was purified after 3 days from pools of oocytes previously uninjected (1) or injected with cRNAs encoding wild-type α-TIP (2), the S7A/S23A/S99A α-TIP mutant (3) or γ-TIP (4). *In vitro* phosphorylation of oocyte proteins was performed in the presence of exogenous [γ-^{33}P]ATP and the PKA catalytic subunit. P_f values determined on the same lot of oocytes were ($\times 10^{-2}$ cm/s ± SEM, n = 4): uninjected, 0.046 ± 0.012; α-TIP, 0.581 ± 0.107; S7A/S23A/S99A, 0.083 ± 0.018; γ-TIP, 0.356 ± 0.058. (**A**) Immunoblot of membrane oocyte proteins probed with anti-α-TIP antibodies. Equal amounts of protein corresponding to 0.3 oocytes were loaded in each lane. (**B**) Replicate autoradiogram of total labeled proteins from oocyte membranes. (**C**) Autoradiogram of labeled membrane proteins immunoprecipitated with anti-α-TIP antibodies. Proteins obtained from 2.5 oocytes were loaded in each lane. The high molecular weight band detected on top of the autoradiogram (C) corresponds to insoluble material that did not penetrate the running gel. Molecular mass markers are indicated on the left and on the right.

lack of any consensus phosphorylation site for PKA in the γ-TIP primary sequence (Höfte *et al.*, 1992).

α-TIP activity is enhanced by cAMP agonists

The functional significance of α-TIP phosphorylation by PKA was investigated using the endogenous PKA activity of oocytes. The membrane-permeable analog of cAMP, 8-bromoadenosine 3′,5′-cyclic monophosphate (8-Br-cAMP), in conjunction with forskolin, which activates cAMP production by adenylate cyclase, and 3-isobutyl-1-methylxanthine (IBMX), which blocks cAMP degradation by phosphodiesterases, was used to increase the cytosolic cAMP concentration and activate cellular PKA. Oocytes were pre-incubated for 10–120 min in isotonic bathing solution supplemented with a cocktail of these cAMP agonists, then transfered to a hypotonic solution and assayed for water transport. The cAMP pretreatment was without effect on the subsequent P_f of control uninjected oocytes (Figure 5). In contrast, cAMP induction increased the water transport capacity of oocytes expressing α-TIP by 30–50% after 10 min and by a maximum of >100% after 90–120 min (Figure 5). The P_f of oocytes expressing the γ-TIP isoform was unaffected by 1 h exposure to cAMP agonists (Figure 5). These results, in conjunction with the biochemical evidence for PKA-mediated phosphorylation of α-TIP but not γ-TIP, support the idea that the specific activation of α-TIP by cAMP agonists is mediated by phosphorylation of the protein by PKA.

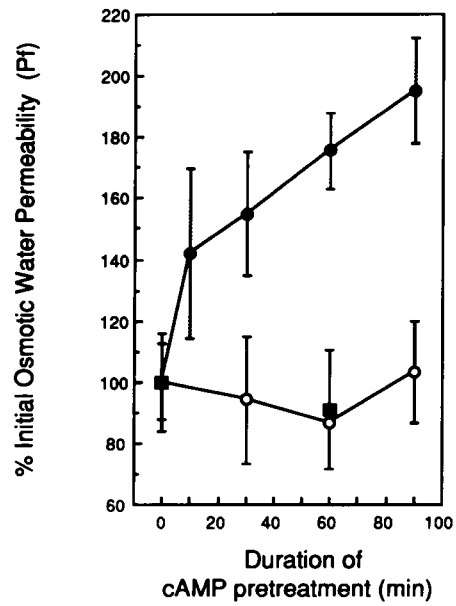

Fig. 5. Time course of the effect of cAMP agonists on the osmotic water permeability (P_f) of oocytes expressing α-TIP or γ-TIP. Control uninjected oocytes (○) or oocytes expressing α-TIP (●) or γ-TIP (■) were pre-incubated for the times indicated in isotonic Barth's medium complemented with 0.5 mM 8-Br-cAMP, 50 μM forskolin and 0.5 mM IBMX, and osmotic water permeability values (P_f) were measured over the following 2 min. P_f values (± SE, n = 5) are shown as a percentage of the initial P_f determined in the absence of agonist pretreatment.

Phosphorylation sites at positions 7, 23 and 99 individually mediate the cAMP agonist stimulation of α-TIP activity

To determine whether functional modulation of α-TIP by cAMP indeed occurs by direct phosphorylation of the protein, we characterized the sensitivity of oocytes expressing α-TIP Ser→Ala mutants to cAMP agonists. The P_f of oocytes expressing the single mutants S7A, S23A and S99A or the double mutants S7A/S23A, S7A/S99A and S23A/S99A were stimulated >2-fold in all cases after a 1 h cAMP induction (Figure 6). In contrast, most of the cAMP stimulation was absent in the triple mutant S7A/S23A/S99A (Figure 6). This mutant protein was not phosphorylated by PKA *in vitro* (Figure 4) and the slight residual activation of S7A/S23A/S99A by cAMP agonists in oocytes (Figure 6) remains unexplained. Nevertheless, our results show that optimal activation of α-TIP water transport activity by cAMP agonists is mostly mediated by phosphorylation of Ser7, Ser23 and Ser99. The cAMP activation of double mutants S7A/S23A, S7A/S99A and S23A/S99A, when compared with that observed for the triple mutant S7A/S23A/S99A, demonstrates the individual efficacy of respectively Ser99, Ser23 and Ser7 phosphorylation in channel activation (Figure 6). From these results it appears that each phosphorylation site at positions 7, 23 and 99 can independently modulate water transport by the α-TIP protein.

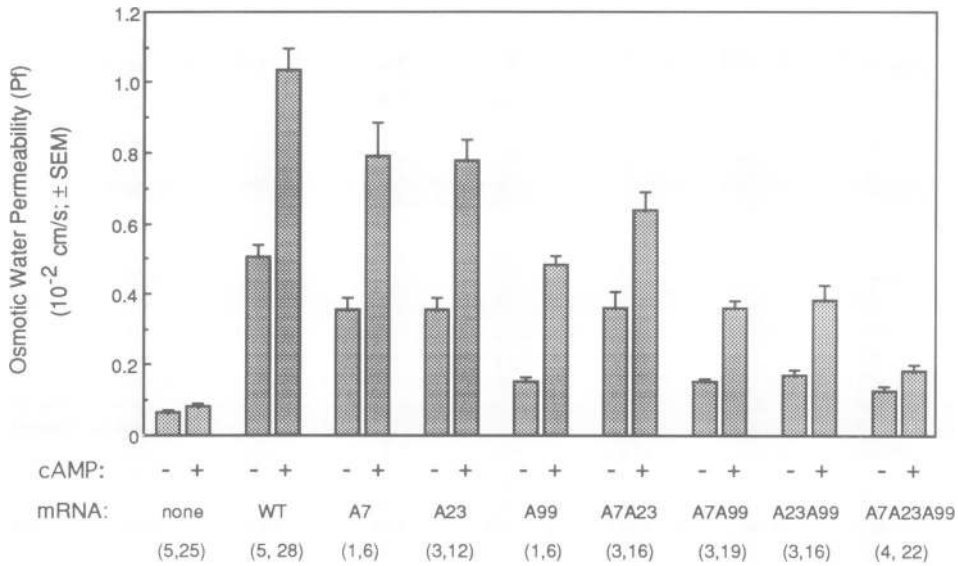

Fig. 6. Effects of cAMP agonists on the osmotic water permeability (P_f) of oocytes expressing Ser→Ala α-TIP mutants. Oocytes injected with the α-TIP cRNAs carrying the indicated mutations were vehicle-treated (–) or treated for 1 h with cAMP agonists (+) as described in the legend to Figure 5 and Materials and methods. P_f was measured over the following 2 min. The first and second numbers in parentheses indicate respectively the number of experiments with independent oocyte donors and the total number of oocytes tested under each condition.

Discussion

Expression of functional α-TIP in *Xenopus* oocytes indicates that this seed-specific protein is a new member of the aquaporin family. We suggest that α-TIP functions in plants by increasing the efficiency of water transport across the membrane of protein storage vacuoles.

Aquaporin phosphorylation, a novel mechanism for regulating transmembrane water transport

The finding that α-TIP is phosphorylated in plant vacuoles (Johnson and Chrispeels, 1992), together with the observation that consensus phosphorylation sites of some animal protein kinases can closely match the postulated recognition sites of plant protein kinases (Pearson and Kemp, 1991; Roberts and Harmon, 1992), led us to investigate the phosphorylation of plant α-TIP using heterologous oocyte expression together with exogenous and endogenous PKA activies.

The present work provides evidence that: (i) α-TIP, in contrast to its γ-TIP homolog, can be phosphorylated by PKA; (ii) cAMP induction in oocytes, which stimulates endogenous PKA, specifically increases the water transport activity of α-TIP. Both effects require the integrity of three PKA phosphorylation sites. Together, these findings demonstrate that the α-TIP water channel can be modulated via direct phosphorylation. To our knowledge, this is the first evidence of such a regulatory mechanism for aquaporins. We propose therefore that, in addition to modulating aquaporin populations and transport to their target compartments, cells can regulate the water permeability of their membranes by *in situ* phosphorylation of water channel proteins.

Multiple phosphorylation sites participate in α-TIP activation

Using a set of double and triple Ser→Ala mutants, we have demonstrated that each Ser residue at positions 7, 23 or 99 participates in α-TIP activation. The respective contribution of each site in maximal activation could not be precisely quantified, mostly because, in addition to impaired phosphorylation, unknown mutational effects may participate in reducing the activity of the mutant proteins. This is shown by the S131A mutation, which also caused a 26% reduction in α-TIP activity. The reason for this reduction is unclear at the moment. Nevertheless, we show that independent phosphorylation of Ser7, Ser23 or Ser99 stimulates water channel activity >2-fold and that these effects are additive.

Assuming that phosphorylation of α-TIP occurs at positions 7, 23 and 99, we place the N-terminus as well as the segment between the third and the fourth hydrophobic regions on the cytoplasmic side of the membrane (Figure 1), in agreement with the most widely accepted topological model of MIP homologs with six transmembrane segments (Miao *et al.*, 1992; Engel *et al.*, 1994; Preston *et al.*, 1994). Ser7 and Ser23 of α-TIP reside in the N-terminal domain, whereas Ser99 is thought to reside much deeper in the protein and may line the aqueous pathway (Engel *et al.*, 1994). Further structural studies are needed to show how phosphate moieties in these different positions interfere with aquaporin conformation and determine channel gating.

The fact that only phosphorylation of Ser7 was demonstrated *in planta* (Johnson and Chrispeels, 1992) legitimately questions the functional relevance of the two other sites in plants. In view of their efficacy for channel activation in our oocyte assay, equivalent to that of Ser7, we rather believe that the failure to detect phosphorylation of Ser23 and Ser99 in plants (Johnson and Chrispeels, 1992) could have resulted from the lack of specific protein kinases or of their activation. Alternatively, a very high stability of the phosphate residues at these positions could have limited phosphate substitution in radiolabeling experiments. In any case, this suggests that the three phosphorylation sites of α-TIP exhibit differential sensitivities to the protein kinases and protein phosphatases

active on the plant tonoplast. It also suggests that several phosphorylation pathways could converge on α-TIP and interact in the fine tuning of the channel.

Plant cells harbor a large variety of CDPKs whose cellular localization and substrates remain largely unknown (Roberts and Harmon, 1992). In particular, tonoplast-bound CDPKs await molecular identification. Other classes of protein kinases might also target plant aquaporins. Plant signal transduction via cyclic nucleotides has been controversial for years (Brown and Newton, 1981), but recent studies indicate a role for a cAMP-dependent pathway in higher plants (Kurosaki and Nishi, 1993; Li *et al.*, 1994).

Protein phosphorylation and cellular osmoregulation

Previous studies have shown that α-TIP accumulates late in seed maturation (Johnson *et al.*, 1989; Melroy and Herman, 1991) and may play a key role in the early stages of seed germination. The rapid rehydration of seed tissues during this process, the production of osmolytes following storage product mobilization from protein storage vacuoles and the coalescence of the latter yielding the large central vacuole (Maeshima *et al.*, 1994) create an important osmotic challenge for the parenchyma cells of seeds. We believe that α-TIP phosphorylation may allow dynamic control of vacuolar swelling and fusion. In particular, highly selective (i.e. aquaporins) and poorly selective (Alexandre and Lassalles, 1991; Homblé and Very, 1992) pathways for water transport in the tonoplast might be counterbalanced in order to finely control the osmotic behavior of the protein storage vacuoles.

Johnson and Chrispeels (1992) observed that the capacity of α-TIP to be labeled in phosphorylation assays varied during seedling growth. In addition, α-TIP is progressively replaced during the germination process by its γ-TIP homolog (Maeshima *et al.*, 1994), the latter having a sensitivity to protein kinases (if any) clearly different from the former. These observations suggest that in germinating seedlings deposition and removal of aquaporin isoforms from the tonoplast and the differential phosphorylation of these proteins cooperate in a tight control of membrane water permeability by developmental and physiological factors.

Phosphorylation cascades have been shown as major pathways to mediate osmoregulation of yeast and mammalian cells (Brewster *et al.*, 1993; Galcheva-Gargova *et al.*, 1994). The present work and the finding that plant desiccation induces the expression of specific protein kinases (Anderberg and Walker-Simmons, 1992; Urao *et al.*, 1994) suggest similar mechanisms for turgor regulation in plants. In particular, drought induces in *Arabidopsis* seedlings the expression of two CDPKs (Urao *et al.*, 1994) the substrates of which remain to be discovered. Drought also induces the expression of RD28 (Yamaguchi-Shinozaki *et al.*, 1992), a MIP homolog recently identified as a plasma membrane aquaporin (Daniels *et al.*, 1994). These illustrate the variety of regulatory control levels for membrane water permeability and cell turgor during drought in plant cells.

Materials and methods

Plasmid construction, site-directed mutagenesis and in vitro cRNA synthesis

Point mutations S7A and S99A were introduced using the gapped duplex DNA approach of Stanssens *et al.* (1989) with single-stranded α-TIP cDNA (Johnson *et al.*, 1990) in a pMac5-8 vector as a template. Wild-type and mutated DNA fragments containing the entire α-TIP coding region were subcloned in the *Bgl*II site of pXβGev2, a pSP64T-derived Bluescript vector containing 5′ and 3′ untranslated sequences of a *Xenopus* β-globin gene (Preston *et al.*, 1992). The double mutant S7A/S99A was obtained by replacing the 650 bp *Nhe*I–*Bam*HI fragment of the S7A plasmid by its counterpart containing the S99A mutation. Point mutations S23A and S131A were introduced using the recombinant PCR technique described by Higuchi (1990). PCR amplifications were performed with Vent DNA polymerase (Biolabs, Beverly, MA). Wild-type, S7A- and S99A-mutated α-TIP cDNAs in pXβGev2 were used as templates. In the primary PCR reactions, oligonucleotides homologous to the sense and antisense sequences of the desired mutants were combined with oligonucleotides complementary to the T3 and T7 promoters respectively. PCR products were gel purified and combined in the secondary PCR reaction with both T3 and T7 oligonucleotides. Amplified DNA fragments were gel purified, digested by *Bam*HI and *Hin*dIII and cloned in the corresponding sites of pXβGev2. Each mutation was confirmed by DNA sequencing. The following mutagenic oligonucleotides were used (mismatch underlined).

S7A: 5′-CCTTCCAAAAGCATATCTTC-3′
S23A: 5′-CATGAGGGCCGCTTTGGCTG-3′ (sense)
5′-CAGCCAAAGCGGCCCTCATG-3′ (antisense)
S99A: 5′-ATACTGCACGGATCACAGCGATCCTCCCCCCA-3′
S131A: 5′-CATGAGGCCAGCAGGGTTCCAC-3′ (sense)
5′-GTGGAACCCTGCTGGCCTCATG-3′ (antisense)

Capped cRNA was synthesized *in vitro* and purified as previously described (Maurel *et al.*, 1993). Each introduced mutation was assayed by oocyte expression of cRNA transcribed from two independently mutagenized plasmids.

Preparation of Xenopus laevis oocytes and cRNA injection

Stage V and VI oocytes were prepared as described (Maurel *et al.*, 1993), injected with 50 nl *in vitro* transcribed mRNA (0.5–1.0 mg/ml) and incubated at 19°C in Barth's solution [10 mM HEPES–NaOH, pH 7.4, 88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.33 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$] containing 50 mg/l gentamycin for 2–4 days prior to analysis.

cAMP stimulation of oocytes

Oocytes were incubated for the times indicated in Barth's solution containing 0.5 mM 8-Br-cAMP, 50 μM forskolin and 0.5 mM IBMX, all from Sigma (St Louis, MO). Unstimulated control oocytes were vehicle-treated in Barth's solution containing 0.5% ethanol and 0.05% dimethylsulfoxide.

Osmotic water permeability and electrical conductance measurements

Water transport was assayed by transfering individual oocytes from Barth's solution (Osm_{in} = 200 mosmol/kg) to the same solution diluted to 40 mosmol/kg (Osm_{out}) with distilled water and cell swelling was followed by video microscopy. Cell volume (V) was calculated from the cell section area measured using a NIH/Image program. Osmotic water permeability (P_f) was calculated from the initial rate of cell volume change using the following formula $P_f = V_0[d(V/V_0)/dt]/[S \times V_w (Osm_{in} - Osm_{out})]$ with initial oocyte volume $V_0 = 9 \times 10^{-4}$ cm^3, initial oocyte surface area $S = 0.045$ cm^2 and molar volume of water $V_w = 18$ cm^3/mol. Whole cell membrane currents were measured as described (Maurel *et al.*, 1994) using two-electrode voltage clamp techniques. Membrane conductance was calculated from currents recorded for membrane potentials between −50 and +50 mV.

Immunoblot analysis of oocyte proteins

Groups of four to six oocytes were homogenized in 100–200 μl medium H [30 mM Tris–HCl, pH 7.6, 100 mM NaCl, 5 mM EDTA, 5 mM EGTA, 1 mM 1,10-phenanthroline and protease inhibitors (1 mM phenylmethanesulfonyl fluoride, 2 μg/ml leupeptin and 1 μg/ml pepstatin)] complemented with 2% SDS. Proteins were denatured at 65°C for 20 min and solubilized proteins were designated as whole oocyte proteins. Whole oocyte proteins or membrane proteins (see below) were separated by SDS–PAGE and transferred to Immobilon PVDF membranes (Millipore, Bedford, MA). Proteins were visualized using a rabbit anti-α-TIP antiserum (Johnson *et al.*, 1989) and detected using goat anti-rabbit IgG coupled to alkaline phosphatase (Biosys, Compiègne, France)

with 5-bromo-4-chloro-3 indolyl phosphate and nitrobluetetrazolium reagents (BioRad, Hercules, CA).

In vitro phosphorylation of oocyte membranes

Ten to 15 oocytes were disrupted gently using a Gilson P-1000 Pipetteman by three to five slow strokes in 2–3 ml medium H. Oocyte ghosts were washed extensively by resuspension in medium H and homogenized in 1 ml medium H plus 10% (w/v) sucrose. The homogenate was loaded on a discontinuous gradient of 50 and 20% sucrose in medium H and centrifuged in a swing-out rotor for 30 min at 15 000 *g*. Membranes were collected at the 50–20% interface, washed in 10 ml medium M (66 mM HEPES, pH 7.5, 13 mM $MgCl_2$), sedimented for 30 min at 15 000 *g* and resuspended in 200 µl medium M. All these operations were carried out at 4°C. For *in vitro* phosphorylation, 0.2–0.3 mg membrane proteins were incubated for 15 min at 30°C in 100 µl 50 mM HEPES, pH 7.5, 10 mM $MgCl_2$, 0.2% Triton X-100, 1 mM DTT, 50 µM [γ-^{33}P]ATP (5 Ci/mmol; Isotopchim, Ganagobie-Peyruis, France) and, when indicated, 110 U of the catalytic subunit of bovine heart protein kinase (Sigma). The reaction was stopped with 7.2% TCA at 0°C. Membrane proteins were analyzed by SDS–PAGE immunoblotting as described above and autoradiographed. Alternatively, labeled membrane proteins were resuspended for subsequent immunoprecipitation in medium I (50 mM Tris–HCl, pH 7.6, 200 mM NaCl, 50 mM NaF, 5 mM EDTA, 5 mM EGTA, 5 mM β-glycerophosphate, 1 mM orthovanadate, 1 mM 1,10-phenanthroline, protease inhibitors as above, 50 nM okadaic acid, 1 µg/ml protein kinase A inhibitor fragment 6–22 amide) complemented with 1.5% SDS. Solubilized membranes were then incubated with anti-α-TIP antiserum in medium I plus 1% Triton X-100 and 0.2% SDS. Immunocomplexes were adsorbed onto protein A–Sepharose CL-4B (Sigma), washed repeatedly in medium I containing successively 1% Triton X-100 and 0.2% SDS, 0.2% Triton X-100 and finally no detergent. Immunocomplexes were eluted in denaturing buffer, separated by SDS–PAGE and exposed for autoradiography.

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