Purification and characterization of two protein kinases acting on the aquaporin SoPIP2;1

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Abstract

Aquaporins are water channel proteins that facilitate the movement of water and other small solutes across biological membranes. Plants usually have large aquaporin families, providing them with many ways to regulate the water transport. Some aquaporins are regulated post-translationally by phosphorylation. We have previously shown that the water channel activity of SoPIP2;1, an aquaporin in the plasma membrane of spinach leaves, was enhanced by phosphorylation at Ser115 and Ser274. These two serine residues are highly conserved in all plasma membrane aquaporins of the PIP2 subgroup. In this study we have purified and characterized two protein kinases phosphorylating Ser115 and Ser274 in SoPIP2;1. By anion exchange chromatography, the Ser115 kinase was purified from the soluble protein fraction isolated from spinach leaves. The Ca^{2+}-dependent Ser274 kinase was purified by peptide affinity chromatography using plasma membranes isolated from spinach leaves. When characterized, the Ser115 kinase was Mg^{2+}-dependent, Ca^{2+}-independent and had a pH-optimum at 6.5. In accordance with previous studies using the oocyte expression system, site-directed mutagenesis and kinase and phosphatase inhibitors, the phosphorylation of Ser274, but not of Ser115, was increased in the presence of phosphatase inhibitors while kinase inhibitors decreased the phosphorylation of both Ser274 and Ser115. The molecular weight of the Ser274 kinase was approximately 50 kDa. The identification and characterization of these two protein kinases is an important step towards elucidating the signal transduction pathway for gating of the aquaporin SoPIP2;1.
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1. Introduction

Aquaporins are water channel proteins that are present in many different organisms, ranging from bacteria and mosses to higher plants and animals. In plants, aquaporins are present both in the plasma membrane and in the tonoplast [1,2], where they passively facilitate water transport, thereby changing the osmotic pressure across the plasma membrane and tonoplast, respectively [3].

In the flowering plant *Arabidopsis thaliana*, there are 35 different genes coding for aquaporin homologues [4] and in maize (*Zea mays*) there are 33 full-length aquaporin-encoded sequences [5]. Based on sequence similarities, the higher plant aquaporins are divided into four subfamilies; Plasma membrane Intrinsic Proteins (PIPs), Tonoplast Intrinsic Proteins (TIPs), NOD26-like Intrinsic Proteins (NIPs), and Small basic Intrinsic Proteins (SIPs). The large number of plant aquaporin isoforms implies that there is a need for dynamic and specific regulation of aquaporins both in time and in space.

There are several reports in the literature on aquaporin phosphorylation [6–16] and some aquaporins have been shown to be post-translationally regulated, i.e., gated, by phosphorylation [8,10,15]. The effect of the phosphorylation seems to vary, ranging from stimulation of water transport activity, e.g., for plant SoPIP2;1, formerly known as PM28A [10], to inhibition of water transport activity, e.g., for human HsAQP4 [11,12]. In the human aquaporin HsAQP2, which is phosphorylated on Ser256 in the C-terminal region [13], the phosphorylation is believed to be hormone regulated and cAMP-dependent, resulting in targeting of

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HsAQP2-containing periplasmic vesicles to the plasma membrane, thereby increasing the water permeability of the membrane.

SoPIP2;1 is one of the major aquaporins of spinach (*Spinacia olerecea*) leaf plasma membranes [14]. In the *Xenopus laevis* oocyte expression system, when assaying point-mutated forms of the protein in combination with protein kinase and phosphatase inhibitors, SoPIP2;1 was shown to be regulated by phosphorylation at two sites; Ser115 in the cytosolic B-loop and Ser274 in the C-terminal region. The effects of phosphorylation at these two sites were additive. When SoPIP2;1 was phosphorylated at one or both of these sites, the water transport activity increased. In vivo, Ser274 was phosphorylated in a turgor dependent manner [10].

Recently, an X-ray crystallography study implicates the phosphorylation of both Ser115 and Ser274 in the gating mechanism responsible for opening the channel [15]. Furthermore, the gating mechanism also confirms the importance of a fully conserved His residue, in the cytosolic D-loop of all thirteen PIP isoforms, as being responsible for closing the channel when protonated upon anoxia during flooding [17].

In the present study, we have characterized two protein kinases acting on the phosphorylation sites Ser115 and Ser274 in SoPIP2;1. The protein kinase acting on Ser115 was cytosolic and Mg^{2+}-dependent and it had a pH-optimum at 6.5. It has previously been shown that Ser274 was phosphorylated by a plasma membrane-bound Ca^{2+}-dependent protein kinase (CDPK) with a pH-optimum at 7.5 [10]. We found that the phosphorylation status of Ser274 was increased in the presence of phosphatase inhibitors and that the molecular weight of the CDPK was approximately 50 kDa. The two protein kinases were partly purified. The cytosolic kinase acting on Ser115 was purified by anion exchange chromatography. The kinase acting on Ser274 was associated with the plasma membrane and could be washed off with salt and further purified using peptide affinity chromatography with a peptide containing the consensus phosphorylation sequence around Ser274 covalently attached to the column matrix.

2. Materials and methods

2.1. Plasma membrane isolation

Plasma membranes were isolated from spinach (*Spinacia oleracea*) leaves as previously described [18]. Spinach leaves were homogenized at 4 °C in 50 mM 3-(N-morpholino)propanesulfonic acid (MOPS)-KOH pH 7.5, 5 mM DTT, 5 mM ascorbate, 0.33 M sucrose, 0.6% (w/v) polyvinylpolypyrrolidone, 0.2% (w/v) casein (boiled enzymatic hydrolysate; Sigma-Aldrich, Stockholm, Sweden), 0.2% (w/v) protease free BSA, and 0.5 mM phenylmethylsulfonyl fluoride (added after filtration of the homogenate; [19]). Plasma membranes were isolated from the microsomal fraction ($10,000 \times g$, 15 min, then $50,000 \times g$, 30 min) by partitioning in an aqueous polymer two-phase system. The final plasma membrane pellet was resuspended in 0.33 M sucrose, 5 mM potassium phosphate pH 7.8, and 50 mM KCl and stored in liquid nitrogen until use.
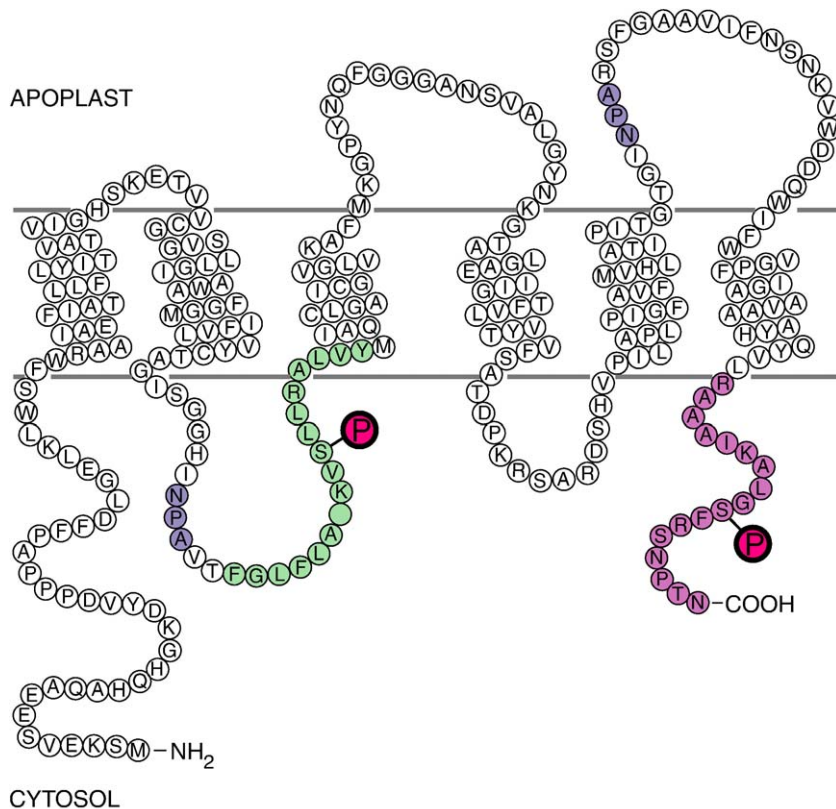

Fig. 1. Topology of the spinach leaf plasma membrane aquaporin SoPIP2;1. The aquaporin has six transmembrane helices and both the N- and C-terminal regions located in the cytosol. The two highly conserved NPA boxes are shown in grey. The peptide sequences corresponding to the region around the two phosphorylation sites Ser115 and Ser274, shown here in green and pink, respectively, were fused to the maltose binding protein (MBP). The peptides were expressed as fusion proteins in *E. coli* and purified on an amylose matrix. The fusion proteins were used as substrates for in vitro phosphorylation assays and for in-gel phosphorylation assays.

2.2. Soluble protein isolation

Spinach leaves were homogenized as described above. The homogenate was filtered and larger particles were removed by centrifugation (7500×g, 15 min). The obtained supernatant was further centrifuged (28,000×g, 50 min), yielding the soluble proteins in the supernatant.

2.3. Purification of a cytosolic protein kinase phosphorylating Ser115

The kinase activity phosphorylating Ser115 was purified from 10 mL, i.e., 72 mg, soluble spinach leaf proteins fractionated on a MonoQ anion exchange column (Pharmacia Biotech, Sweden). The running buffer used in the chromatography was 50 mM MOPS-KOH pH 7.5 and the bound proteins were eluted with 24 mL of the running buffer with a linear gradient from 0 to 1 M NaCl. 1.5 mL fractions were collected.

2.4. Purification of a plasma membrane-bound protein kinase phosphorylating Ser274

Purified spinach leaf plasma membrane vesicles containing 50 mg plasma membrane proteins were washed with 5 mL salt solution (10 mM Tris–HCl pH 7.5, 4 mM EDTA, and 0.5 M KCl). After centrifugation 100,000×g for 45 min at 4 °C, the protein kinase activity was present in the supernatant. The supernatant was desalted on a PD-10 column (Amersham Biosciences, Piscataway, NJ, USA). The desalted fraction was further purified on a SulfoLink peptide affinity column (SulfoLink Kit; Pierce, Rockford, IL, USA). In the column, a peptide containing the C-terminal Ser274 phosphorylation site (pink peptide in Fig. 1) with a Cys residue added to the N-terminal of the peptide was linked to the column matrix. The kinase activity was eluted with 0.5 M NaCl and 0.5 mL fractions were collected.

2.5. SDS-PAGE

SDS-PAGE was performed according to [20], with minor modifications. Gels composed of 5% stacking gel and 12% separation gel were used. Samples were solubilized in solubilization buffer [20] at room temperature for 20 min and gels were run at 200 V for 50 min. The gels were stained with Coomassie Brilliant Blue or silver [21].

2.6. Maltose binding protein fusions

The nucleotide sequences coding for the two peptides, corresponding to the sequence around the two phosphorylation sites Ser115 and Ser274 (Fig. 1), were cloned into the prokaryotic expression vector pMAL-c2 (New England Biolabs, Beverly, MA, USA). The peptides, called Ser115 and Ser274, were then expressed in *Escherichia coli* as fusion proteins linked to the maltose binding protein (MBP). The fusion proteins are called MBP-Ser115 and MBP-Ser274, respectively. Expression of the fusion proteins and subsequent purification on an amylose resin (New England Biolabs, Beverly, MA, USA) were performed according to the manufacturer's instructions.

2.7. In vitro phosphorylation assay

For visualization of the Ser274 kinase activity, 5 μL of kinase fractions were incubated in a phosphorylation buffer containing 5 mM Tris–HCl pH 7.5, 2 mM MgCl$_2$, 0.2 mM CaCl$_2$, 1 μg MBP-Ser274, and 10 μM ATP (including 10 μCi [γ-^{32}P]-ATP). The effects of sodium fluoride (NaF) and okadaic acid (OA) on the Ser274 kinase were analyzed by mixing 15 μg plasma membrane proteins in the same buffer as above with 15 mM NaF and 5 μM OA, respectively. When studying the pH optimum of the Ser115 kinase, 2 μL kinase fraction was mixed with 1 μg MBP-Ser115 and 10 μM ATP (including 10 μCi [γ-^{32}P]-ATP) in one of the following buffers: 5 mM MES-KOH for pH 5.0–6.5, 5 mM HEPES–KOH for pH 6.5–8.0 and 5 mM Tris–HCl for pH 7.5–9.0. The following Ser115 kinase experiments were performed in the same reaction mixture with HEPES–KOH buffer at pH 6.5. The effects of divalent cations on the Ser115 kinase were analyzed by addition of 1 to 20 mM Mg^{2+}, 5 to 20 mM Mn^{2+}, or 1 to 20 mM Ca^{2+}. 0 mM Mg^{2+}, Mn^{2+} and Ca^{2+} were obtained by adding 2.5 mM EDTA and 2.5 mM EGTA.

Consistently, the effects of a cyclic AMP analogue (8-Br-cAMP) and the two phosphatase inhibitors NaF and OA on the Ser115 kinase were analyzed by addition of 10^{-6} to 10^{-3} M 8-Br-cAMP, 25 mM NaF, and 0.5 μM OA. The total reaction volumes were 14 μL. The reactions were started by the addition of ATP, proceeded at room temperature for 30 min, and were then stopped by the addition of 6 μL 3.33×Laemmli solubilization buffer [20]. The samples were analyzed by SDS-PAGE and the radiolabelled polypeptides were visualized using a PhosphorImager (Amersham Biosciences, Little Chalfont, UK).

2.8. In-gel phosphorylation assay

SDS-PAGE gels were prepared as described above, containing 0.1 mg mL^{-1} MBP-Ser274 as the kinase substrate or 0.1 mg mL^{-1} MBP-Ser274Ala, having a disrupted phosphorylation site. After SDS-PAGE the gels were incubated in 50 mM Tris–HCl pH 7.5 and 20% (v/v) isopropanol at room temperature for 1 h and then in 50 mM Tris–HCl pH 7.5 and 5 mM β-mercaptoethanol at room temperature for 1 h. The proteins in the gels were denatured in 50 mM Tris–HCl pH 7.5, 5 mM β-mercaptoethanol, and 6 M guanidine–HCl at room temperature for 1 h. The proteins were then renatured in 50 mM Tris–HCl pH 7.5, 5 mM β-mercaptoethanol, and 0.04% (w/v) Tween-20 at 4 °C over night. The next day the gels were equilibrated in phosphorylation buffer without ATP (25 mM Tris–HCl pH 7.5, 10 mM MgCl$_2$, and 0.2 mM CaCl$_2$) for 30 min, then incubated in the same phosphorylation buffer containing 50 mM ATP and 5 μCi mL^{-1} [γ-^{32}P] ATP at room temperature for 1 h. The phosphorylation reaction was stopped by washing the gels extensively in 5% (w/v) trichloroacetic acid (TCA), and 1% (w/v) sodium pyrophosphate (Na$_4$P$_2$O$_7$) at room temperature for 2.5 h, to remove the unreacted ATP. The gels were dried and the radiolabelled polypeptides visualized using a PhosphorImager (Amersham Biosciences, Little Chalfont, UK).

2.9. Sequence analysis

Protein sequences were aligned using ClustalW in DS Gene, with manual adjustments to optimize the fit around the phosphorylation sites.

3. Results and discussion

3.1. Background

By using site-directed mutagenesis in combination with inhibitors of protein kinases and phosphatases, we have previously shown that the spinach aquaporin SoPIP2;1 was phosphorylated on two sites, Ser115 and Ser274, when expressed in *Xenopus* oocytes [10]. When SoPIP2;1 was expressed in oocytes, the phosphorylation increased the water channel activity and the effect of phosphorylation at the two sites was additive. In vivo, Ser274 has been shown to be phosphorylated in response to increasing apoplastic water potential [14]. In the present study, we have purified and characterized two protein kinases acting on these two phosphorylation sites that are involved in the gating of the channel [15].

3.2. Purification of the Ser115 kinase

The protein kinase phosphorylating Ser115 was purified from soluble spinach leaf proteins fractionated on a MonoQ anion exchange column. The fraction size was 1.5 mL and the kinase activity was eluted in fraction 4, visualized by an in vitro phosphorylation assay (not shown). Further attempts to purify the protein kinase on a peptide affinity column similar to the one used for the Ser274 kinase, using a peptide containing the loop B consensus phosphorylation site (green peptide in Fig. 1) covalently attached to the column matrix, was not successful.

The peptide was attached to the matrix at the N-terminus in these experiments. Future attempts will be made to purify the kinase with the peptide attached at the C-terminus instead. This might mimic the in vivo structure of loop B better, since the C-terminal constitutes the beginning of the transmembrane helix 3. An alternative approach may be to attach a spacer in the N-terminus of the peptide and then attach it to the matrix. This could make the important recognition sequence of the peptide, around Ser115, more available to the kinase.

3.3. Purification of the Ser274 kinase

The protein kinase phosphorylating Ser274 was purified from spinach leaf plasma membranes. When the plasma membranes were washed with a salt solution (10 mM Tris–HCl pH 7.5, 4 mM EDTA, and 0.5 M KCl), the kinase activity was found in the soluble fraction. This soluble fraction was then desalted and in a representative experiment, the protein concentration was typically in the range of 5 mg mL^{-1}. Approximately 12 mg of protein was further loaded onto and purified on peptide affinity column, with the peptide containing the C-terminal consensus phosphorylation site (pink peptide in Fig. 1) covalently attached to the column matrix. The kinase activity bound to the column was eluted with 0.5 M NaCl. Fractions of 0.5 mL were collected and the kinase activity appeared in fraction 5 (Fig. 2). When purifying the Ser274 kinase on the peptide affinity column, the protein kinase activity could be detected by the in vitro phosphorylation assay, but no corresponding protein could be seen on a silver-stained SDS-
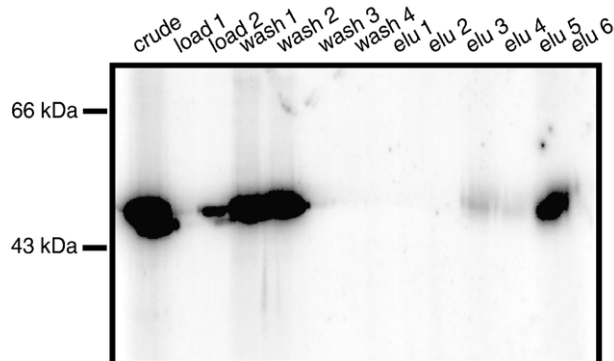

Fig. 2. Partial purification of the Ser274 protein kinase using peptide affinity chromatography. After the protein kinase was washed off the plasma membrane with 0.5 M KCl, the fraction was desalted. The kinase activity was purified on an affinity matrix with the peptide RAAAIKALGSFRSNPTN, corresponding to the phosphorylation site around Ser274, bound to the matrix. The fractions were: crude (desalted fraction), load 1 and 2 (not bound to the column, coming out when loading the column), wash 1 to 4 (not bound to the column, coming out when washing the column with buffer to remove unbound molecules), and elu 1 to 6 (bound to the column and eluted with 0.5 M NaCl). 4.8 μL fraction was loaded in each lane, corresponding to the protein amounts 5.77 μg crude, 0 μg load 1, 0.10 μg load 2, 0.69 μg wash 1, 0.34 μg wash 2, 0 ng each of wash 3, wash 4 and elu 1, 4.93 μg elu 2, 5.15 μg elu 3, 0.91 μg elu 4, 1,16 μg elu 5 and 1.41 μg elu 6. The kinase activity was eluted in fraction 5. The kinase activities in the different fractions were detected by in vitro phosphorylation assays. The fractions were incubated with MBP-Ser274 and [γ-^{32}P]-ATP in the phosphorylation buffer for 30 min at room temperature, whereafter the reaction mixtures were separated by SDS-PAGE and the radiolabelled polypeptides visualized using a PhosphorImager.
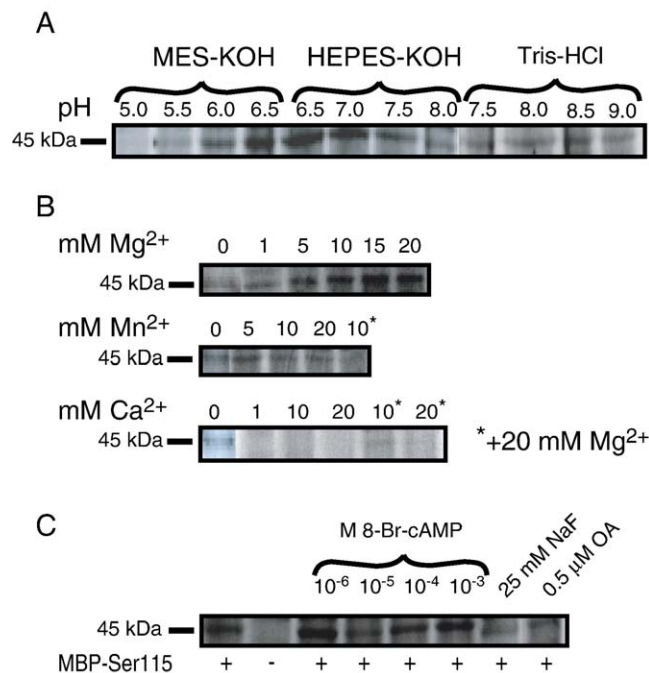

Fig. 3. Characterization of the Ser115 protein kinase. The different conditions were tested using in vitro phosphorylation assays, in which the kinase fractions were incubated with MBP-Ser115 and [γ-^{32}P]-ATP in the phosphorylation buffer for 30 min at room temperature. The reaction mixtures were separated by SDS-PAGE and the radio labelled polypeptides were visualized using a PhosphorImager. Additional weaker bands were also detected on the gels (not shown in the figure), which probably arise from other proteins in the fraction that are phosphorylated and/or autophosphorylated. (A) The protein kinase had a pH optimum at 6.5. (B) The kinase required Mg^{2+} for activity, and was inhibited by mM concentrations of Mn^{2+} and Ca^{2+}. (C) The phosphorylation was not increased by the cyclic AMP-analogue 8-Br-cAMP, or the phosphatase inhibitors sodium fluoride (NaF) and okadaic acid (OA).

PAGE gel (not shown). This may indicate that this protein kinase has a high activity and that it is present in low amounts.

3.4. Characterization of the Ser115 kinase

The protein kinase acting on Ser115 had a pH optimum of 6.5 (Fig. 3A). The kinase activity in the presence of the divalent cations, Mg^{2+}, Mn^{2+} and Ca^{2+}, was also analyzed. The kinase activity was Mg^{2+}-dependent and we observed the highest kinase activity at 20 mM Mg^{2+} (Fig. 3B). The kinase activity was inhibited by mM concentrations of Mn^{2+} and Ca^{2+} (Fig. 3B). We also tested whether the addition of 20 mM Mg^{2+} had any positive effect on the kinase activity in the presence of Mn^{2+} and Ca^{2+}, but this was not the case. Whether this is a direct inhibition of the kinase or an effect of Mn^{2+} and Ca^{2+} replacing Mg^{2+} in Mg-ATP remains to be investigated. The phosphorylation was not consistently affected by the cyclic AMP analogue 8-Br-cAMP (Fig. 3C). The phosphorylation of Ser115 was not increased by the phosphatase inhibitors NaF and OA, it even seemed to decrease slightly, which is difficult to explain (Fig. 3C). However, the lack of increased Ser115 phosphorylation due to the addition of the two phosphatase inhibitors is in full agreement with previous results [10] using the oocyte expression system, where kinase inhibitors

cause a dephosphorylation of Ser115 but phosphatase inhibitors have no effect on the phosphorylation status of Ser115.

The cytosolic kinase fraction phosphorylates Ser115 mainly and to a smaller extent also Ser274 in in vitro phosphorylation assays with the two different MBP-fused peptides (Fig. 1) as substrates (not shown). It is possible that some of the plasma membrane-associated kinase activity is released into the soluble fraction during homogenization and contaminates the soluble kinase fraction.

3.5. Characterization of the Ser274 kinase

The protein kinase phosphorylating Ser274 was associated with spinach leaf plasma membranes [14]. Using plasma membrane vesicles, the Ser274 kinase was previously shown to have a pH optimum at 7.5 and to be Ca^{2+}-dependent [14]. Since Ser274 is the major identified phosphorylated amino acid in PM28A in vivo, the Ca^{2+}-dependent phosphorylation is most likely linked to the amino acid Ser274. In contrast to the Ser115 site, where the level of phosphorylation is not increased by the two phosphatase inhibitors NaF and OA (Fig. 3C) [10], the Ser274 site shows a higher level of phosphorylation in the presence of NaF and OA (Fig. 4). This is also in agreement with results obtained using the oocyte expression system, site-directed mutagenesis and phosphatase inhibitors [10].

In an in-gel phosphorylation assay the size of the protein was determined to be approximately 50 kDa (Fig. 5). The molecular weight correlates with reported molecular weights of other CDPKs acting on aquaporins, which have been shown to be in the range of 45–60 kDa. A tulip plasma membrane aquaporin was phosphorylated by a 45 kDa CDPK [22] and a 52 kDa CDPK was reported to be responsible for the phosphorylation of two lentil (Lens culinaris) seed isoforms, LcTIP3;1 and LcTIP3;2 [23].

Many protein kinases have been reported to be auto-phosphorylated [24]. This may explain the phosphorylation activity seen in the in-gel phosphorylation assay with the MBP-Ser274Ala peptide, having a disrupted CDPK phosphorylation site, as substrate in the gel (Fig. 5). However, with the same exposure times, the 50 kDa band was much more prominent when MBP-Ser274 was used as substrate, indicating that this band in addition also contained the phosphorylated MBP-Ser274 protein, or resulted in a more efficient autophosphorylation.
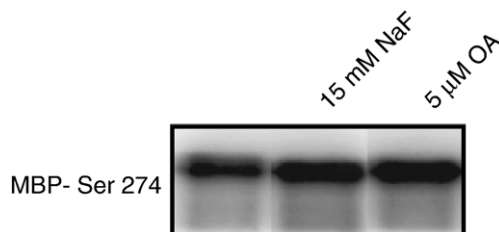

Fig. 4. The effects of phosphatase inhibitors on the Ser274 protein kinase. Plasma membrane fractions were incubated with MBP-Ser274 and $[\gamma\text{-}^{32}P]$-ATP together with phosphatase inhibitors sodium fluoride (NaF) and okadaic acid (OA) in phosphorylation buffer for 30 min at room temperature. The reaction mixtures were separated by SDS-PAGE and the radiolabelled polypeptides were visualized using PhosphorImager. The phosphatase inhibitors led to an increased phosphorylation of MBP-Ser274.
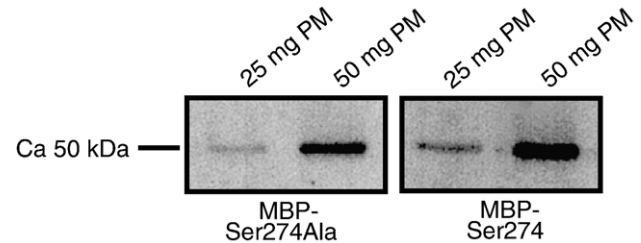

Fig. 5. Determination of the molecular weight of the Ser274 protein kinase using in-gel phosphorylation assay. Plasma membrane proteins were separated by SDS-PAGE on gels containing 0.1 mg mL^{-1} of MBP-Ser274Ala peptide (left) or MBP-Ser274 peptide (right). The proteins were renatured in the gels and the gels were incubated in phosphorylation buffer containing $[\gamma\text{-}^{32}P]$-ATP. The gels were dried and the radiolabelled polypeptides were visualized using a PhosphorImager. The 50 kDa band was much denser in the gel with the kinase substrate MBP-Ser274, as compared to the mutated kinase substrate MBP-Ser274Ala, indicating that this band contains the protein kinase responsible for phosphorylating the target peptide in the C-terminal region of SoPIP 2;1.

Other plant aquaporins, e.g., soybean (Glycine max) GmNOD26 [25], kidney bean (Phaseolus vulgaris) PvTIP3;1 [7], and lentil seed (Lens culinaris) LcTIP3;1 and LcTIP3;2 [23], have also been shown to be phosphorylated by membrane-bound CDPKs.

3.6. Phosphorylation sites

To our knowledge, the phosphorylated amino acid residues in aquaporins have so far been serine residues in all reported cases. The positions of the phosphorylated serine residues in the sequences have been located both to the N-terminal region, e.g., Ser7 in PvTIP3;1 [7], to the B-loop region, e.g., Ser115 in SoPIP2;1 [10], and to the C-terminal region, e.g., Ser274 in spinach SoPIP2;1 [10], Ser256 in human HsAQP2 [13], and Ser262 in soybean GmNOD26 [25].

Ser115 is located in the first cytoplasmic loop B and represents a typical protein kinase A recognition site (Arg–Lys–X–Ser, the phosphorylated serine in italics) [26], as well as a typical phosphorylation site for CDPKs (Leu–X–Arg–X–X–Ser) [27,28], and for protein kinase C (Arg–Lys–X–Ser–X–X–Arg) [29]. The Ser115 kinase may belong to the family of SNF-1 like kinases, which are calcium independent and not associated to the membrane [30,31]. The Ser115 phosphorylation site is conserved in most PIPs and a similar motif, usually with threonine instead of serine, is present in most TIPs (Fig. 6A).

The two Arabidopsis PIP2 isoforms AtPIP2;7 and AtPIP2;8, have almost identical sequences around Ser274 as compared to SoPIP2;1, differing in only 2 and 1 amino acid, respectively, within the peptide sequence high lighted pink in Fig. 1. Also, the C-terminal Ser274 phosphorylation site (Ser–X–Arg–Ser) is conserved in almost all members of the PIP2 subfamily and a similar motif is present in members of the NIP subfamily (Fig. 6B). Consequently, the MBP-Ser274 protein was also phosphorylated by an Arabidopsis plasma membrane protein fraction (not shown). Indeed, serine residues corresponding to Ser274 have been shown to be in-vivo phosphorylated in at least four PIP2 isoforms in Arabidopsis [32].

A

Loop B phosphorylation site

General	RKX**S**XXR	
SoPIP2;1	ARKV**S**LLRA	119
AtPIP1;1	ARKL**S**LTRA	132
AtPIP1;2	ARKL**S**LTRA	132
AtPIP1;3	ARKL**S**LTRA	132
AtPIP1;4	ARKL**S**LTRA	133
AtPIP1;5	ARKL**S**LTRA	133
AtPIP2;1	ARKV**S**LPRA	125
AtPIP2;2	ARKV**S**LIRA	123
AtPIP2;3	ARKV**S**LIRA	123
AtPIP2;4	ARKV**S**LVRT	125
AtPIP2;5	ARKV**T**LVRA	124
AtPIP2;6	ASKV**S**LVRA	124
AtPIP2;7	ARKV**S**LVRA	118
AtPIP2;8	ARKV**S**LPRA	116
AtTIP1;1	GGNI**T**LLRG	103
AtTIP1;2	GGNI**T**LLRG	104
AtTIP1;3	GGNI**T**LLRA	103
AtTIP2;1	GGQI**T**VITG	101
AtTIP2;2	GGNI**T**VITG	101
AtTIP2;3	GGNI**T**LITG	101
AtTIP3;1	GGRV**T**AIRA	111
AtTIP3;2	GGRI**S**VIRA	111
AtTIP4;1	GGHI**S**VFRA	97
AtTIP5;1	AGRI**S**VPTA	105
PvTIP3;1	GGRI**S**VIRA	103

B

C-terminal phosphorylation site

General	KXXX**S**XRS	
SoPIP2;1	AAIKALG**S**FRSNPTN	281
AtPIP2;1	SGSKSLG**S**FRSAANV	287
AtPIP2;2	SGSKSLG**S**FRSAANV	285
AtPIP2;3	SGSKSLG**S**FRSAANV	285
AtPIP2;4	AAIKALG**S**FGSFGSFRSFA	291
AtPIP2;5	GAIKALG**S**FRSQPHV	286
AtPIP2;6	GAMKAYG**S**VRSQLHELHA	289
AtPIP2;7	SAIKALG**S**FRSNATN	280
AtPIP2;8	AAIKALA**S**FRSNPTN	278
AtNIP1;1	REITKSG**S**FLKTVRIGST	308
AtNIP1;2	REITKSG**S**FLKTVRNGSSR	294
AtNIP2;1	PEFSKTG**S**SHKRVTDLPL	288
AtNIP3;1	RPNCNKV**S**SRDRQEASQDEICVLR	294
AtNIP4;1	RELTKSA**S**FLRAVSPSHKGSSSKT	283
AtNIP4;2	RELTKSA**S**FLRSVAQKDNASKSDG	283
AtNIP5;1	DPPRPVR**S**FRR	304
AtNIP6;1	EAPKERR**S**FRR	305
AtNIP7;1	CPSPVSP**S**VSSLLR	274
GmNOD26	SETTKSA**S**FLKGRAASK	271

Fig. 6. Conserved sequences around the two aquaporin phosphorylation sites in loop B and in the C-terminus, corresponding to Ser115 and Ser274, respectively, in SoPIP2;1. The potential phosphorylated amino acid in each sequence is marked in bold. (A) The sequence around the loop B phosphorylation site is well conserved the 13 Arabidopsis PIPs and a similar motif, usually with threonine instead of serine, is present in most TIPs. The residues Ser115 in SoPIP2;1 and Ser99 in PvTI P3;1, also shown in bold, have both been shown to be phosphorylated in oocytes [7,10]. (B) The sequence around the C-terminal phosphorylation site is conserved in all eight members of the Arabidopsis PIP2 group and a similar motif is present in members of the NIPs. The residues Ser274 in SoPIP2;1 and Ser262 in GmNOD26, also shown in bold, have both been shown to be phosphorylated in vivo and in vitro [14,41]. The C-terminal sequence of AtNIP3;1 extends with an additional 25 amino acids, which are not shown in this figure.

Recently, Kulma and coworkers [33] identified a calcium-dependent protein kinase from Arabidopsis, AtCPK3, which phosphorylates the following motif: Ala–Ser–Gly–Ser–Phe–Arg. The corresponding phosphorylation motif in the C-terminus of spinach SoPIP2;1 is Ala–Leu–Gly–*Ser*–Phe–Arg. Thus, these two phosphorylation motifs are almost identical, and the CDPK acting on Ser274 in spinach SoPIP2;1 is probably an ortholog to AtCPK3 in Arabidopsis. Douglas et al. [34] purified a CDPK from spinach leaves that could phosphorylate a nitrate reductase on Ser543. The CDPK sequence was very similar to AtCPK3, suggesting that this CDPK might also phosphorylate Ser274 in SoPIP2;1. The size of AtCPK3 is reported to be 59 kDa [35], which is in the same range as determined here for the Ser274 protein kinase.

By GFP-tagging and proteomic studies, at least eight Arabidopsis CDPKs have been localized to the plasma membrane [36,37]. However, a mass spectrometry analysis of the active Ser274 kinase fraction could not detect any typical CDPK peptides. Matches obtained were to a putative leucine-rich repeat transmembrane protein kinase (At2g01210), and to calreticulin 1 (CRT1; At1g56340), and calreticulin 2 (CRT2; At1g09210) (not shown).

3.7. Calcium and protein phosphorylation

Many abiotic stresses trigger signal transduction pathways in cells. This often leads to transiently elevated cytosolic calcium levels inside the cells, which in turn triggers protein phosphorylation cascades, involving, e.g., CDPKs, which finally phosphorylate target proteins involved in responding to the stress [38,39].

Calcium regulates many responses in cells. Several calcium-binding proteins are known to influence metabolism and gene expression. In addition to the small Ca^{2+}-binding protein calmodulin that bind to and regulate protein kinases, plants and some protozoans contain CDPKs. The CDPKs participate in numerous aspects of plant growth and development. The CDPK family contains a large number of proteins, 34 CDPK isoforms in Arabidopsis [30], which implies that they have specialized regulatory roles.

3.8. pH-effect

A shift in pH has been shown to be involved in post-translational regulation, i.e., gating, of Arabidopsis PIPs [15,17]. This was shown to be due to a histidine residue in loop D. An acidification of the cytosol, e.g., due to anoxia, would cause a protonation of His193 in loop D of SoPIP2;1 thereby closing the channel. The pH optima for the two kinases basically reflects the normal cytosolic pH. Thus, inactivation of the kinases due to an acidification of the cytosol would lead to a dephosphorylation of Ser115 and Ser274 of SoPIP2;1 and represent an alternative closing mechanism [15]. This is in accordance with previous results using the oocyte expression system, site-directed mutagenesis and kinase inhibitors (instead of acidification), which cause a dephosphorylation of the two serine residues and closing of the aquaporin SoPIP2;1 [10].

4. Conclusions

In conclusion, we have purified and characterized two protein kinases phosphorylating the spinach leaf plasma membrane aquaporin SoPIP2;1. This is a first step towards dissecting the signal transduction pathway controlling the gating of the aquaporin SoPIP2;1. So far, only a few kinases and one phosphatase involved in aquaporin phosphorylation have been identified and characterized [7,22,23,25,40], but none has been completely isolated and cloned to date.

From our results, the Ser115 kinase is present in a soluble form in the cytosol and the Ser274 kinase is attached to the plasma membrane, from which it can be washed off with salt, indicating that the two protein kinases have different locations in the cell. In the SoPIP2;1 structure [15], Ser115 in loop B is located in the cytosolic vestibule, leading to the pore, and should be accessible to a soluble cytosolic kinase. Ser274 in the flexible C-terminal region, which in its non-phosphorylated state is interacting with and stabilizing the closed conformation of the cytosolic regulatory D-loop of a neighboring monomer of the homotetrameric aquaporin SoPIP2;1 [15], should be accessible to a plasma membrane bound kinase. This further underlines the need of two kinases with different characteristics to phosphorylate the two sites Ser115 and Ser274 in SoPIP2;1. Some future ideas for strategies to identify the two kinases would be to start with more material in the purification process, to then hopefully be able to get a sufficient amount of the kinase for further identification with mass spectrometry. Another suggestion, for the Ser274 kinase, would be to identify and isolate it using phosphor-Ser or CPK antibodies. We have previously tried to do this using several different antibodies, but have not been successful yet.

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