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The Major Integral Proteins of Spinach Leaf Plasma Membranes Are Putative Aquaporins and Are Phosphorylated in Response to Ca^{2+} and Apoplastic Water Potential

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We show that homologs of the major intrinsic protein (MIP) family are major integral proteins of the spinach leaf plasma membrane and constitute ~20% of integral plasma membrane protein. By using oligonucleotide primers based on partial amino acid sequences for polymerase chain reaction and screening of a spinach leaf cDNA library, we obtained two full-length clones of MIP homologs (*pm28a* and *pm28b*). One of these clones, *pm28a*, was sequenced, and it encodes a protein (PM28A) of 281 amino acids with a molecular mass of 29.9 kD. DNA gel blots indicated that PM28A is the product of a single gene, and RNA gel blots showed that *pm28a* is ubiquitously expressed in the plant. In vivo phosphorylation of the 28-kD polypeptide(s), corresponding to PM28A and PM28B, was dependent on apoplastic water potential, suggesting a role in regulation of cell turgor for these putative aquaporins. In vitro, only one of the homologs, PM28A, was phosphorylated. Phosphorylation of PM28A occurred on Ser-274, seven amino acids from the C terminus of the protein, within a consensus phosphorylation site (Ser-X-Arg) for vertebrate protein kinase C. In vitro phosphorylation of PM28A was due to a plasma membrane–associated protein kinase and was strictly dependent on submicromolar concentrations of Ca^{2+}.

INTRODUCTION

The major intrinsic protein (MIP) family is a group of channel-forming membrane proteins that has received much attention recently. Members of this ancient protein family have been found in a number of organisms, ranging from bacteria to animals and plants. The first member to be identified was MIP of bovine eye lens fiber cells (Gorin et al., 1984), which has given the family its name. In plants, NOD26 (a peribacteroid membrane protein in soybean nodules; Fortin et al., 1987) and tonoplast intrinsic protein (TIP; Johnson et al., 1989; Johnson and Chrispeels, 1992) were the first MIP homologs to be characterized. Some of the MIP homologs are indeed major intrinsic proteins of their respective membranes. This is particularly true for MIP itself, which comprises >60% of the membrane protein of eye lens fiber cells (Gorin et al., 1984). For plants, this has been shown to be the case for TIP, which is the major integral protein of the vacuolar membrane (tonoplast) in bean seeds (Johnson et al., 1989). Besides sequence homology, most members of the MIP family have similar molecular masses, ranging from 25 to 31 kD. They are all predicted to have a similar topology with six hydrophobic, membrane-spanning α-helices and with the N and C termini located on the cytoplasmic side of the membrane. Highly conserved regions are two NPA amino acid motifs (the NPA boxes), one in each half of the protein (reviewed in Reizer et al., 1993).

More recently, in two independent studies using immunoblotting and purified membrane fractions (Daniels et al., 1994; Kammerloher et al., 1994), MIP homologs were identified as constituents of plant (Arabidopsis) plasma membranes. Until then, plant MIP homologs had been located only to the vacuolar membrane and the peribacteroid membrane, although a number of cDNA clones encoding plant MIP homologs with unknown subcellular location had been identified using differential hybridization techniques (e.g., Guerrero et al., 1990; Yamaguchi-Shinozaki et al., 1992; Fray et al., 1994). The five Arabidopsis plasma membrane MIP homologs reported by Kammerloher et al. (1994) could be grouped into two subfamilies, PIP1 and PIP2 (for plasma membrane intrinsic proteins), based on sequence homologies. RD28 studied by Daniels et al. (1994) was originally identified by Yamaguchi-Shinozaki et al. (1992) as the product of a gene responsive to desiccation and is highly homologous with the members of the PIP2 subfamily.

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For some of the MIP homologs, the transport specificity has been determined. Thus, GlpF of *Escherichia coli* facilitates the uptake of glycerol and other small solutes (Heller et al., 1980), and NOD26 in the peribacteroid membrane probably transports dicarboxylates (Ouyang et al., 1991). However, in most cases in which transport specificity has been determined, the transported molecule has been found to be water. Thus, most of the MIP homologs seem to function as water-selective channels and are therefore termed aquaporins. Water channel activity is determined by injecting complementary RNA corresponding to the MIP homolog into Xenopus oocytes for transient expression of the protein in the plasma membrane. Incubation of the oocytes in hypotonic buffer leads to an increased rate of swelling of the oocytes if the protein coded for by the complementary RNA is a water channel. By using this method, a number of mammalian (e.g., the 28-kD channel-forming integral protein of human erythrocytes, CHIP28/AQP1, and the water channel of the collecting duct of rat kidney, WCH-CD/AQP2) and plant (e.g., α-TIP, γ-TIP, RD28, and the PIP1 and PIP2 subfamily members) MIP homologs have been classified as aquaporins (reviewed in Chrispeels and Agre, 1994; Agre et al., 1995).

Water supply as well as regulation of water loss are crucial for plants under conditions of osmotic stress. The discovery by Maurel et al. (1995) that the water channel activity of the vacuolar membrane aquaporin α-TIP is regulated by phosphorylation suggests that water loss may be regulated at the membrane level. In this study, we report that two MIP homologs, PM28A and PM28B, are truly major intrinsic proteins of spinach leaf plasma membranes, constituting ~20% of integral plasma membrane protein. We also demonstrate that the 28-kD polypeptide band, corresponding to these putative aquaporins, is the major phosphorylated polypeptide band of the spinach leaf plasma membrane both in vivo and in vitro and that the level of phosphorylation in vivo changes in response to osmotic stress. The gene for one of the homologs, *pm28a*, has been sequenced, and the corresponding protein, PM28A, is shown to be phosphorylated in vitro at Ser-274 close to the C terminus of the protein. In vitro phosphorylation of PM28A was dependent on submicromolar concentrations of Ca^{2+}, suggesting a role for Ca^{2+} in the cellular response to osmotic stress.

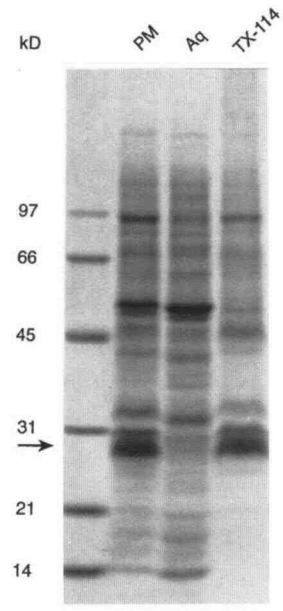

Figure 1. Polypeptide Pattern of Spinach Leaf Plasma Membranes and of the Aqueous and Detergent Phase Obtained by Triton X-114 Fractionation of Spinach Leaf Plasma Membranes.

Spinach leaf plasma membranes (PM) were subjected to Triton X-114 fractionation, producing an aqueous (Aq) phase containing mainly peripheral membrane proteins and a detergent (TX-114) phase containing mainly integral membrane proteins. Polypeptides were separated by SDS-PAGE (50 μg of protein per lane). An arrow indicates the position of the 28-kD polypeptide band. Numbers at left refer to the molecular mass standards. The gel was stained with Coomassie blue.

RESULTS

Identification of MIP Homologs

When spinach leaf plasma membranes were analyzed by SDS-PAGE, a very dominant polypeptide band was seen at 28 kD. As shown in Figure 1, Triton X-114 fractionation of the plasma membranes led to a partitioning of the 28-kD polypeptide band to the detergent phase, suggesting that the 28-kD polypeptide band is due to integral membrane protein(s). Similar observations were made with other species. Thus, plasma membranes prepared from leaves of barley, wheat, pea, sunflower, and sugarbeet, as well as from Arabidopsis plants (roots omitted) and cauliflower inflorescences, all show a major polypeptide band at ~28 kD when analyzed by SDS-PAGE (data not shown). Triton X-114 fractionation of sugar beet and cauliflower plasma membranes indicates that the 28-kD polypeptide band also found with these species is due to integral membrane protein(s) (data not shown).

In spinach, the 28-kD band constitutes ~15% of total plasma membrane protein and ~20% of integral plasma membrane protein, as determined by Coomassie blue staining (Figure 1). To characterize this major plasma membrane polypeptide(s) further, the 28-kD band was cut from the gel and subjected to trypsin treatment. Five of the major resulting peptides were sequenced. Searches in sequence data bases showed significant homologies to proteins of the MIP family for all five peptides. Spinach plasma membrane MIP homologs banding at 28 kD after SDS-PAGE are referred to as PM28 proteins.

Cloning and Sequencing

The amino acid sequences of the five tryptic fragments were used to design degenerated oligonucleotides to be used as primers for polymerase chain reaction (PCR). Additional degenerated primers were based on highly conserved regions in proteins of the MIP family, namely, the NPA boxes and adjacent amino acids (Reizer et al., 1993). The combination of one tryptic fragment–based primer and one primer based on a conserved region (Figure 2) resulted in a 299-bp PCR product. This PCR product was cloned and sequenced to confirm its identity. The PCR clone was then used to screen a spinach leaf cDNA library. Four positive clones were identified, partially sequenced, and found to be identical.

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GAGAGCGAGAGAGAGAAACAAAACAGAGGAGAGAGAAAAAACCAGAGGAAAAATGTCTAAGGA   65
                                                  M  S  K  E

AGTAAGTGAAGAAGCACAAGCTCACCAACATGGAAAGGACTACGTAGATCCACCACCAGCACCAT  130
  V  S  E  E  A  Q  A  H  Q  H  G  K  D  Y  V  D  P  P  P  A  P

TCTTTGACTTAGGGGAGCTCAAATTGTGGTCTTTCTGGAGAGCTGCTATTGCTGAGTTTATTGCG  195
F  F  D  L  G  E  L  K  L  W  S  F  W  R  A  A  I  A  E  F  I  A

ACGCTTTTGTTTCTATACATAACCGTCGCCACTGTCATTGGTCACTCCAAGGAAACTGTTGTTTG  260
 T  L  L  F  L  Y  I  T  V  A  T  V  I  G  H  S  K  E  T  V  V  C

TGGTTCTGTTGGCCTTCTTGGTATTGCTTGGGCTTTTGGTGGCATGATCTTTGTCCTTGTTTACT  325
  G  S  V  G  L  L  G  I  A  W  A  F  G  G  M  I  F  V  L  V  Y

GCACCGCCGGTATCTCTGGAGGGCACATCAACCCAGCAGTGACTTTCGGACTATTCCTAGCAAGG  390
C  T  A  G  I  S  G  G  H  I  N  P  A  V  T  F  G  L  F  L  A  R

AAGGTGTCCTTACTAAGGGCACTAGTCTACATGATAGCCCAATGTTTAGGAGCCATATGTGGTGT  455
 K  V  S  L  L  R  A  L  V  Y  M  I  A  Q  C  L  G  A  I  C  G  V

AGGACTAGTGAAGGCCTTCATGAAGGGTCCTTACAACCAATTTGGTGGTGGGGCTAACTCTGTTG  520
  G  L  V  K  A  F  M  K  G  P  Y  N  Q  F  G  G  G  A  N  S  V

CTCTTGGATACAACAAAGGTACTGCCTTAGGAGCTGAGATTATCGGCACCTTTGTTCTTGTGTAC  585
A  L  G  Y  N  K  G  T  A  L  G  A  E  I  I  G  T  F  V  L  V  Y

ACCGTCTTCTCTGCTACTGACCCTAAGAGGAGCGCACGTGACTCTCACGTGCCTATCTTGGCCCC  650
 T  V  F  S  A  T  D  P  K  R  S  A  R  D  S  H  V  P  I  L  A  P

ACTTCCCATTGGTTTTGCTGTGTTCATGGTTCACTTGGCCACTATTCCCATCACTGGCACTGGTA  715
  L  P  I  G  F  A  V  F  M  V  H  L  A  T  I  P  I  T  G  T  G

TCAACCCTGCTAGAAGCTTTGGGCCTGCTGTTATCTTCAACAGCAACAAGGTTTGGGATGACCAA  780
I  N  P  A  R  S  F  G  P  A  V  I  F  N  S  N  K  V  W  D  D  Q

TGGATATTCTGGGTCGGACCATTCATTGGAGCAGCAGTAGCAGCAGCATACCACCAATATGTATT  845
 W  I  F  W  V  G  P  F  I  G  A  A  V  A  A  A  Y  H  Q  Y  V  L

GAGGGCAGCAGCAATTAAGGCGTTGGGATCCTTCAGAAGCAACCCTACCAATTAATTAAGGAAGA  910
  R  A  A  A  I  K  A  L  G  S  F  R  S  N  P  T  N

TATTACCAACTATGAAGAGATTATTCAGTGTTTCTCATCTCTTGTTCTTTTGTAATTCTTGGGTA  975

CTCTACTGTTTAATCAGAAATTAATGGAAGAAGGGAAAAAAATTGATTTGTTTTTTTGTGTGCAG 1040

TGTGAAGTAAATTATTGAGTTAAGTTAGCATCAATGGACAATGGTGTATTATTGTTGGTTCAAAA 1105

AAAAAA                                                              1111
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Figure 2. Nucleotide Sequence of the *pm28a* cDNA and the Deduced Amino Acid Sequence.

The deduced amino acid sequence is shown below the nucleotide sequence. Numbering at right refers to nucleotides. The nucleotide sequences corresponding to the two degenerated oligonucleotides used for PCR are within boxes. The two peptide sequences obtained after trypsin digestion of the 28-kD polypeptide band and found to belong to PM28A are underlined. The *pm28a* nucleotide and predicted amino acid sequences have the GenBank, EMBL, and DDBJ accession number L77969.

The nucleotide and deduced amino acid sequence of the full-length clone is shown in Figure 2. The cDNA encodes a protein of 281 amino acids (predicted molecular mass of 29.9 kD), which we named PM28A. When we compared the amino acid sequence with the sequences of the tryptic fragments, only two of the five tryptic peptides belonged to PM28A (Figure 2), suggesting that the 28-kD polypeptide band consists of at least two proteins of the MIP family. This was confirmed recently when another PCR product was obtained by using oligonucleotides based on two of the tryptic fragments not found in PM28A. Sequencing of the corresponding full-length cDNA clone, *pm28b*, is in progress.

The degree of homology between PM28A and some other members of the MIP family is shown in Figure 3. The most homologous protein is RD28 of Arabidopsis (Yamaguchi-Shinozaki et al., 1992; 74% amino acid identity), which is very similar to PIP2b of Kammerloher et al. (1994), with only 13 amino acids differing between RD28 and PIP2b. Other homologs include the PIP1 subfamily proteins (PIP1c shows 68% identity), a tomato ripening–associated membrane protein (TRAMP; Fray et al., 1994), and clone 7a, the product of a water deficiency–induced transcript of pea (Guerrero et al., 1990). TRAMP and clone 7a show 66 and 64% amino acid identity to PM28A, respectively. The subcellular locations of TRAMP and clone 7a are not known, but sequence comparisons show highest homology with members of the PIP1 subfamily. Because RD28 and the PIPs are located in the plasma membrane (Daniels et al., 1994; Kammerloher et al., 1994), the relatively high homology between these proteins and PM28A is not surprising. The best-characterized plant MIP homologs, α-TIP and γ-TIP (Höfte et al., 1992), which are located in the vacuolar membrane, are more distantly related, and both show only 31% amino acid identity to PM28A. The sequence information we have on PM28B shows higher similarity to the members of the PIP1 subfamily than to PM28A and the members of the PIP2 subfamily (data not shown).

A DNA gel blot of genomic DNA digested with three different restriction enzymes (EcoRI, HindIII, and BamHI) and probed with the *pm28a* cDNA at high stringency showed a major single band with each enzyme, indicating that PM28A is the product of a single gene (data not shown).

Organ and Tissue Distribution

To gain some insight into the organ and tissue distribution of PM28A, we first compared transcript levels of the *pm28a* gene. Total RNA was isolated from roots, leaves, petioles, leaves devoid of midribs, and midribs. The division of leaves into leaves devoid of midribs and midribs was done to investigate whether PM28A was preferentially associated with vascular tissue. RNA gel blot analysis was performed at high stringency, using the 299-bp PCR clone of *pm28a* (Figure 2) as a probe. This demonstrated that *pm28a* is ubiquitously expressed in the plant, as shown in Figure 4A, although a stronger signal was obtained

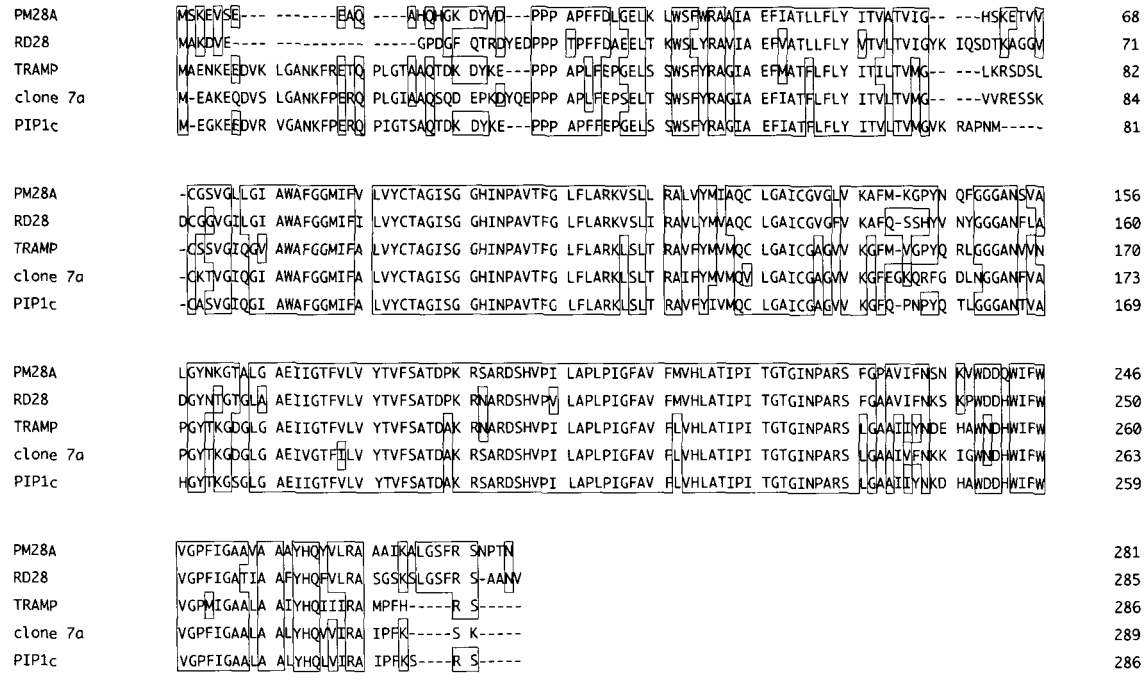

Figure 3. Alignment of the Deduced Amino Acid Sequences for PM28A and Closely Related Plant MIP Homologs.

The amino acid sequence deduced from the *pm28a* cDNA (PM28A) is compared with the deduced amino acid sequences for RD28 (Yamaguchi-Shinozaki et al., 1992), TRAMP (Fray et al., 1994), clone 7a (Guerrero et al., 1990), and PIP1c (Kammerloher et al., 1994). GeneWorks (IntelliGenetics) was used for sequence analysis. Sequence identity with PM28A is indicated by boxes. Dashes indicate gaps introduced to maximize sequence identity.

with root and petiole compared with leaf RNA. Furthermore, Figure 4A shows that leaves devoid of midribs contained a lower amount of *pm28a* transcript as compared with midribs. RNA from whole leaves confirmed this transcript distribution by showing an intermediate hybridization signal. This result may indicate that PM28A is more abundant in cells associated with the vascular tissue. Because the existence of at least one other transcript similar to *pm28a* was confirmed by the cloning of *pm28b*, the specificity of the probe may be questioned. However, a dot blot experiment using the *pm28a* and *pm28b* clones and the respective ^{32}P-labeled PCR clones as probes showed <15% cross-reactivity (data not shown), indicating a high specificity of both probes under the hybridization conditions used.

Because the level of gene transcript is usually assumed to correlate with the amount of the corresponding protein, we analyzed the polypeptide composition of plasma membranes, obtained from the same material as used for RNA preparation, to determine whether the 28-kD band showed similar variation. As shown in Figure 4B, the 28-kD band was a major polypeptide band in all plasma membrane preparations analyzed and did not show the strong variation observed for the *pm28a* transcript.

In Vivo and in Vitro Phosphorylation

For some of the plant MIP homologs, gene expression has been reported to be affected by water deficiency (*clone 7a*, Guerrero et al., 1990; *rd28*, Yamaguchi-Shinozaki et al., 1992) or salt stress (*MipA*, *MipB*, and *MipC*, Yamada et al., 1995). Therefore, we studied the effect of water deficiency on the level of *pm28a* transcript and amount of PM28 proteins. Spinach plants were not watered until they showed signs of wilting, leaves were harvested at intervals, and RNA as well as plasma membranes were isolated. RNA gel blots probed with the *pm28a* PCR clone showed no changes in transcript level upon desiccation; moreover, SDS-PAGE indicated no changes in density of the 28-kD band (data not shown). Thus, neither the expression of *pm28a* nor the amount of PM28 proteins in the leaf plasma membrane seemed to change when plants were subjected to drought stress. Rather, *pm28a* seems to be constitutively expressed, and the PM28 proteins are present at high levels in nonstressed plants. Assuming that PM28A acts as a water channel similar to its closest relative RD28 (Daniels et al., 1994), its high abundance in the plasma membrane suggests that the activity of PM28A may be

post-translationally regulated so that it can respond quickly to osmotic stress. PM28A contains several potential phosphorylation sites. This fact combined with the recent findings that the water channel activities of α-TIP and WCH-CD/AQP2 are regulated by phosphorylation/dephosphorylation of serine residues (Kuwahara et al., 1995; Maurel et al., 1995) prompted us to perform in vivo and in vitro phosphorylation studies to determine whether PM28A is phosphorylated.

The in vivo phosphorylation experiment was performed with pieces of spinach leaves vacuum-infiltrated with a medium containing ^{32}P-labeled Pi followed by plasma membrane isolation. To determine the effect (if any) of osmotic stress on phosphorylation, infiltration media with different concentrations of sucrose were used. As seen in Figure 5, a heavily phosphorylated polypeptide band appeared at 28 kD, and the degree of phosphorylation decreased with increasing osmolarity of the infiltration medium. Similar results were obtained when sorbitol rather than sucrose was used as the osmoticum (data not shown), showing that the decrease in phosphorus-32 phosphorylation of the 28-kD polypeptide(s) was not due to the increase in sucrose per se but due to the decrease in water potential. Unfortunately, the 28-kD polypeptide(s) was not sufficiently labeled in vivo to permit identification of the phosphorylation site(s); therefore, the identity of the phosphorylated polypeptide could not be determined. Thus, we turned to in vitro phosphorylation using isolated plasma membranes and γ-^{32}P-ATP.

Many protein kinases are Ca^{2+} dependent, and in vitro phosphorylation of α-TIP is strictly dependent on Ca^{2+} (Johnson and Chrispeels, 1992). Therefore, a series of buffers

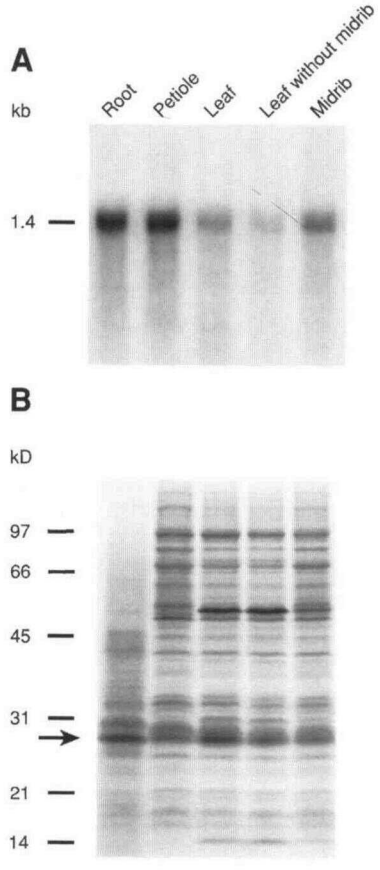

Figure 4. Transcript Levels of *pm28a* and Amounts of PM28 Proteins in Different Organs and Tissues of Spinach.

(A) Total RNA was extracted from roots, petioles, leaves, leaves without midribs, and midribs and probed with the ^{32}P-labeled, 299-bp PCR clone of *pm28a* (see Figure 2) at high stringency (15 μg of RNA per lane). **(B)** Polypeptide patterns of plasma membranes isolated from roots, petioles, leaves, leaves without midribs, and midribs after SDS-PAGE (80 μg of protein per lane). An arrow indicates the position of the 28-kD polypeptide band corresponding to the PM28 proteins. Numbers at left refer to molecular mass standards. The gel was stained with Coomassie blue.

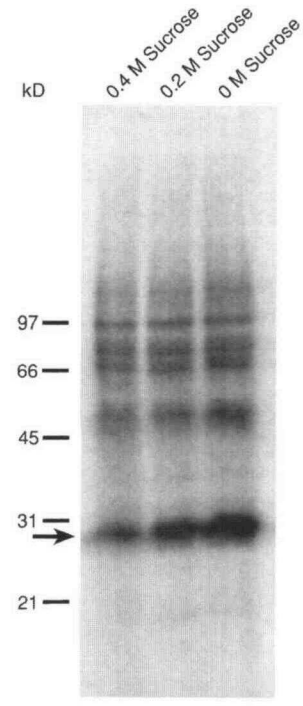

Figure 5. In Vivo Phosphorylation of Spinach Leaf Plasma Membrane Polypeptides at Different Apoplastic Water Potentials.

Pieces of spinach leaves were infiltrated with media containing ^{32}P-labeled Pi and different concentrations of sucrose to determine the effect of osmotic stress on protein phosphorylation. Plasma membranes were isolated, and polypeptides were separated by SDS-PAGE (100 μg of protein per lane). A PhosphorImager was used to visualize the ^{32}P-labeled polypeptides. The major labeled polypeptide band is located at 28 kD, corresponding to the position of the PM28 proteins (arrow). Numbers at left refer to molecular mass standards.

giving different concentrations of free Ca^{2+} (Askerlund, 1996) was used for the in vitro phosphorylation experiments. As shown in Figure 6, the phosphorylation of many of the plasma membrane polypeptides was Ca^{2+} dependent. This was particularly true for the phosphorylation of a polypeptide band at 28 kD, which was undetectable in the control lacking added Ca^{2+} but became the most heavily labeled band at submicromolar concentrations of Ca^{2+}. To determine whether this polypeptide was identical to PM28A, the band was excised from the gel and digested with protease. The resulting peptides were separated by reversed-phase HPLC and scintillation counted. After trypsin digestion, most of the radioactivity was confined to one peak. Unfortunately, this peak was not sufficiently pure to provide a sequence. However, the fact that only one major radioactive peak was obtained suggests that most of the radioactivity in the 28-kD band was due to one polypeptide.

Digestion with LysC (from *Achromobacter lyticus*) resulted in two major peaks (probably due to incomplete digestion) of 2800 and 1600 cpm, and in some minor peaks of below 200 cpm (data not shown). The 1600-cpm peak was again not sufficiently pure, but the peptide constituting the 2800-cpm peak gave the sequence ALGSFRSNPT. This sequence is identical to the C-terminal amino acid sequence of PM28A, except that the C-terminal Asn residue is missing (most probably it fell below the detection limit). Interestingly, the Ser residue at position 4 showed a much lower recovery than the Ser residue at position 7 (data not shown), indicating that the fourth serine was the phosphorylated amino acid residue. This was confirmed by measuring the radioactivity released during each sequencing cycle. As shown in Figure 7, >80% of the radioactivity applied was released with the fourth amino acid, that is, Ser-274 of PM28A, which resides in a consensus phosphorylation motif (Ser-X-Arg) for vertebrate protein kinase C (Ohno and Suzuki, 1995).

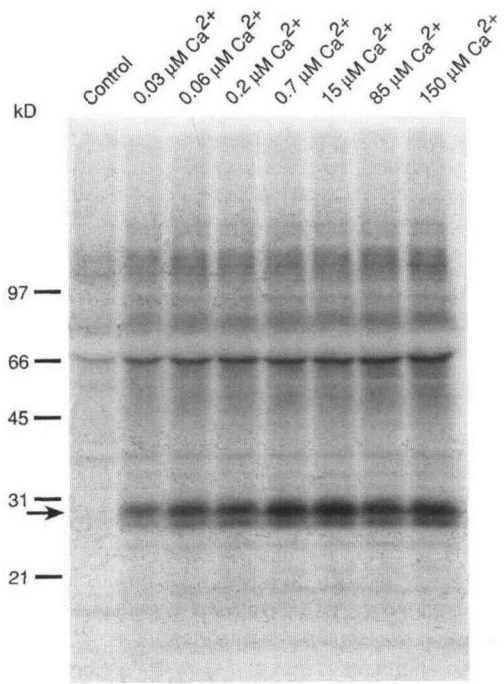

Figure 6. In Vitro Phosphorylation of Spinach Leaf Plasma Membrane Polypeptides at Different Ca^{2+} Concentrations.

Spinach leaf plasma membranes were incubated in media containing γ-^{32}P-ATP and no added Ca^{2+} (Control) or different concentrations of free Ca^{2+}. Polypeptides were separated by SDS-PAGE (50 μg of protein per lane), and ^{32}P-labeled polypeptides were visualized by using a PhosphorImager. The major labeled polypeptide band is located at 28 kD, corresponding to the position of the PM28 proteins (arrow). Numbers at left refer to molecular mass standards.

DISCUSSION

We show here that homologs of the MIP family constitute major integral proteins of the spinach leaf plasma membrane and indeed seem to be major constituents of most plant plasma membranes. Thus, the dominant polypeptide band of the spinach leaf plasma membrane, at 28 kD (Figure 1), is due to MIP homologs. By using oligonucleotide primers based on partial amino acid sequences obtained from this 28-kD polypeptide band, PCR, and a spinach leaf cDNA library, we obtained two full-length clones of MIP homologs and designated them *pm28a* and *pm28b*. One of these clones, *pm28a*, was sequenced, and it encodes a protein (PM28A) of 281 amino acids with a molecular mass of 29.9 kD (Figure 2). The presence of more than one MIP homolog in the 28-kD band agrees with the data of Kammerloher et al. (1994). Using Arabidopsis, they identified two subfamilies of plasma membrane MIP homologs, designated PIP1 and PIP2, which together comprise at least five homologs with predicted molecular masses of 30.4 to 30.7 kD.

The plant MIP homologs identified thus far differ markedly in their organ and tissue distribution, as determined mainly from analysis of gene expression but also from a few immunolocalization studies on the proteins. Whereas α-TIP is seed specific (Höfte et al., 1992) and the expression of the tobacco homolog *tobRB7* is root specific (Yamamoto et al., 1991), several other MIP homologs have been found in all or most plant organs. *MipA* of the ice plant encodes a protein closely related to the PIP1 subfamily of Arabidopsis and is highly expressed in cells associated with vascular tissue, as shown by in situ hybridization (Yamada et al., 1995). The blue light–responsive *AthH2* gene of Arabidopsis (Kaldenhoff et al., 1995), which is identical to *PIP1b* of Kammerloher et al. (1994), is highly expressed in expanding and/or differentiating cells, including vascular bundle sheaths; AthH2 localization has been confirmed using immunocytochemistry (Kaldenhoff et al., 1995). Both MIPA and AthH2 have been shown to mediate water transport (Kaldenhoff et al., 1995; Yamada et al., 1995).

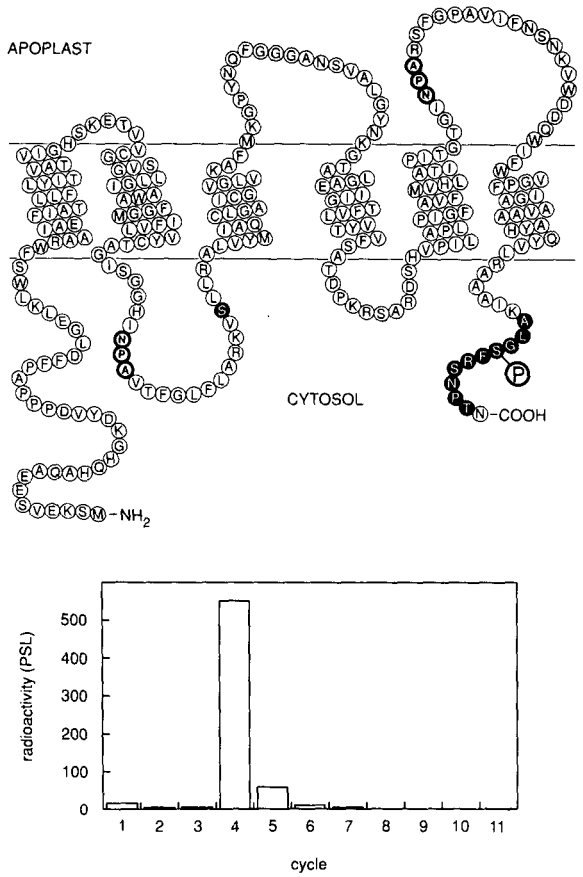

Figure 7. Topology of PM28A and Identification of the in Vitro–Phosphorylated Ser-274.

The topology of PM28A is based on a hydropathy plot according to Kyte and Doolittle (1982) (data not shown), taking into account previously published models for the topology of MIP homologs (Chrispeels and Maurel, 1994). The highly conserved NPA boxes are indicated in boldface. The amino acid residues at the C-terminal end shown in reverse typeface indicate the sequence derived for the ^{32}P-labeled peptide obtained after LysC digestion of the in vitro–phosphorylated 28-kD polypeptide. The graph below shows the radioactivity released for each cycle during sequencing of the labeled peptide, which identifies Ser-274 as the phosphorylated amino acid residue (indicated in the model). Ser-115, which resides within a phosphorylation consensus sequence for protein kinase A (RKXS) that is highly conserved among putative plasma membrane MIP homologs (Figure 3), is also shown in reverse typeface above. Only Ser-274 (and not Ser-115) was found to be phosphorylated in our study. PSL, photostimulated luminescence.

Pm28a is ubiquitously expressed in the plant, as shown by RNA gel blots (Figure 4A), although a large variation in transcript level was observed in the different organs and tissues. The fact that leaves devoid of midribs contained a lower amount of *pm28a* transcript compared with midribs may indicate that PM28A is more abundant in cells associated with the vascular tissue. Surprisingly, a much stronger signal was obtained with root and petiole RNA compared with leaf RNA. Judging from the peak sizes of the peptides corresponding to the oligonucleotides used for PCR, PM28A and PM28B should be the major (if not the only) MIP homologs of the spinach leaf plasma membrane banding at 28 kD. A high level of *pm28a* gene transcript was therefore expected in the leaf RNA preparation. In contrast to the strong variation observed for the transcript of *pm28a*, a major polypeptide band was found at 28 kD with plasma membranes from all organs and tissues (Figure 4B). One interpretation of this discrepancy is that the level of *pm28a* transcript does not reflect the level of PM28A protein but rather reflects the rate of turnover of the protein. However, because the 28-kD band is probably composed of two or more MIP homologs in all material analyzed in Figure 4B, the density of the 28-kD band does not reflect the amount of PM28A only. The density of the 28-kD band should, however, reflect the amount of PM28 proteins, which seems to be similar in the different organs and tissues. An analysis of the distribution of the different PM28 proteins must await the sequencing of all homologs and the production of homolog-specific antisera for immunolocalization.

In this work, we show that PM28A is a major phosphoprotein of the spinach leaf plasma membrane (Figures 5 and 6). A comparison with phosphorylated MIP homologs in other plant membranes (α-TIP in the vacuolar membrane and NOD26 in the peribacteroid membrane) and in animal plasma membranes (MIP of the eye lens fiber cell and WCH-CD/AQP2 of the kidney collecting duct cell) has revealed no characteristics common to all homologs. The phosphorylated amino acids detected thus far are not conserved or confined to a specific region of the proteins. Ser-274 in PM28A resides in a consensus sequence (Ser-X-Arg) recognized by vertebrate protein kinase C (Figures 3 and 7). In contrast, α-TIP and WCH-CD/AQP2 are, when expressed in oocytes, phosphorylated by cAMP-dependent protein kinases in consensus sequences (Arg-Arg-X-Ser and Arg-X-Ser, respectively) recognized by animal protein kinase A (Kuwahara et al., 1995; Maurel et al., 1995). In vitro, α-TIP is phosphorylated in a Ca^{2+}-dependent manner at one of the protein kinase A sites (Arg-Arg-X-Ser) (Johnson and Chrispeels, 1992). MIP is phosphorylated in vivo and in vitro by cAMP-dependent protein kinase at a protein kinase A site (Arg-X-Ser) (Johnson et al., 1986; Lampe and Johnson, 1990). NOD26 is phosphorylated by a Ca^{2+}-dependent, calmodulin-independent protein kinase that recognizes the sequence Lys/Arg-X-X-Ser/Thr (Weaver and Roberts, 1992). There are also differences with respect to which region of the molecule is phosphorylated. NOD26, MIP, and WCH-CD/AQP2 are phosphorylated within the C-terminal region, similar to PM28A. α-TIP, however, is phosphorylated in the N-terminal half of the molecule.

A detailed study of the topology of PM28A (Figure 7) revealed several potential phosphorylation sites in regions of the protein exposed to the cytosol. Only one of these potential phosphorylation sites, namely, the protein kinase A site in the loop between the second and third transmembrane helices

(Arg-Lys-X-Ser at Ser-115 of PM28A), is conserved in all plant MIP homologs, which show high homology with the PIP1 and PIP2 subfamilies and therefore probably are located in the plasma membrane (i.e., in addition to those listed in Figure 3: PIP1a, PIP1b, PIP2a, and PIP2b of Kammerloher et al. [1994]; and MIPA, MIPB, and MIPC of Yamada et al. [1995]). Notably, the corresponding Ser in α-TIP (Ser-99) is one of the three Ser residues suggested to be phosphorylated by protein kinase A in the study by Maurel et al. (1995). The protein kinase C site at Ser-274 of PM28A, shown to be phosphorylated in our study, is conserved among the homologs that may be grouped with the PIP2 subfamily (PM28A and RD28 in Figure 3; PIP2a and PIP2b; MIPC) but absent among those that may be grouped with the PIP1 subfamily (TRAMP, clone 7a, and PIP1c in Figure 3; PIP1a and PIP1b; MIPA and MIPB). The members of the PIP1 subfamily all have a shorter C-terminal region and therefore lack this phosphorylation site. The sequence information we have on PM28B thus far shows that it lacks the C-terminal phosphorylation site detected in PM28A and that it should be grouped with the PIP1 subfamily (data not shown).

The data from in vitro phosphorylation of spinach leaf plasma membranes suggest phosphorylation of one PM28 protein only, PM28A, and suggest one phosphorylation site only, Ser-274. However, other sites may be phosphorylated in vivo; these sites might be substrates for soluble protein kinase(s) lost during plasma membrane isolation, or the kinase(s) might be inactive under the conditions used for in vitro phosphorylation. There is also the possibility that a phosphorylation site is already occupied by a phosphate group with low turnover, thus preventing labeling with phosphorus-32. Nor can in vivo phosphorylation of PM28B or of additional yet-undetected PM28 proteins be excluded. Determination of in vivo phosphorylation sites is needed to clarify these points.

Phosphorylation of the vacuolar membrane aquaporin α-TIP was recently shown to increase strongly its water transport activity, and Maurel et al. (1995) suggested that "cells regulate the water permeability of their membranes by in situ phosphorylation of water channel proteins." Considering that abundant plant plasma membrane MIP homologs, such as PM28A and PM28B, are likely to be aquaporins, they should play an important role in regulation of cell turgor. Such a role is supported by our finding that in vivo phosphorylation of the 28-kD polypeptide(s), corresponding to the spinach leaf plasma membrane MIP homologs, PM28A and PM28B, was dependent on apoplastic water potential (Figure 5). The in vivo phosphorylation of the 28-kD polypeptide(s) decreased with decreasing apoplastic water potential, which, if analogous with the response of α-TIP to phosphorylation, suggests closing of plasma membrane water channels in response to water deficiency. Interestingly, it was shown recently that the yeast MIP homolog Fps1, which mediates the transport of glycerol, is closed under osmotic stress (Luyten et al., 1995). Whether opening/closing of Fps1 is controlled by protein phosphorylation/dephosphorylation is not known. In vitro phosphorylation of PM28A was due to a plasma membrane–associated protein kinase and was strictly dependent on submicromolar Ca^{2+} concentrations (Figure 6), that is, physiologically relevant Ca^{2+} concentrations. This finding agrees with the finding that in vitro phosphorylation of α-TIP is Ca^{2+} dependent (Johnson and Chrispeels, 1992) and suggests a role for cytosolic Ca^{2+} in regulating cell turgor.

METHODS

Plasma Membrane Isolation

Preparation of plasma membranes was as described by Kjellbom and Larsson (1984). Spinach (*Spinacia oleracea*) organs were homogenized at 4°C in 50 mM 3-(*N*-morpholino)propanesulfonic acid (Mops)-KOH, pH 7.5, 5 mM EDTA, 5 mM DTT, 5 mM ascorbate, 0.33 M sucrose, 0.6% polyvinylpolypyrrolidone, 0.2% casein (boiled enzymatic hydrolysate, C-0626; Sigma), 0.2% BSA (protease free, A-3294; Sigma), and 0.5 mM phenylmethylsulfonyl fluoride (added after filtration of the homogenate; Larsson et al., 1994). Plasma membranes were isolated from the microsomal membrane fraction (10,000 to 50,000*g* pellet) 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, 50 mM KCl and was stored in liquid nitrogen until use.

Triton X-114 Fractionation

Triton X-114 fractionation (Bordier, 1981) was performed essentially as described by Bricker and Sherman (1982), using Triton X-114 precondensed according to Bordier (1981). Briefly, plasma membranes were suspended at 2 mg of protein per mL in 150 mM NaCl, 10 mM Tris-HCl, pH 7.6, 1 mM EDTA, and 1.5% Triton X-114. The mixture was incubated at 0°C for 30 min with occasional vortexing. A temperature-induced two-phase system was obtained by incubating for 15 min at 37°C, followed by a low-spin centrifugation (10,000*g* for 1 min) to separate the phases. The detergent phase (containing mainly integral membrane proteins) was repartitioned twice with fresh buffer, and the aqueous phase (containing mainly peripheral membrane proteins) was repartitioned twice by adding fresh Triton X-114 to a final concentration of 1.0%. The final detergent phase was diluted to 0.5 mL (~5 mg protein/mL) in the NaCl-Tris-EDTA buffer. Both protein fractions were stored at −80°C until use.

SDS-PAGE

SDS-PAGE was performed essentially according to Laemmli (1970), using 8 to 15% gradient gels. Samples were solubilized at 22°C for 15 min in a standard sample buffer (Laemmli, 1970). Gels (1.5 mm thick) were run overnight at 9 mA and 14°C, and were stained with Coomassie Brilliant Blue R 250.

Protein Determination

Protein was assayed essentially according to Bearden (1978) or by using a BCA protein assay kit (Pierce, Rockford, IL). BSA was used as the standard in both assays.

Protein Digestion and Amino Acid Sequencing

The 28-kD polypeptide band, obtained by SDS-PAGE of the Triton X-114 detergent phase of spinach leaf plasma membranes, was used for protease digestion. Gel pieces were incubated with trypsin (Promega) or LysC from *Achromobacter lyticus* (Wako, Osaka, Japan), essentially as described by Rosenfeld et al. (1992). The eluted peptides were separated using a SMART chromatography station equipped with a μRPC SC C2/C18 2.1/10 column (Pharmacia, Sweden). The gradient was from 0.1% trifluoroacetic acid (TFA) in water to 50% acetonitrile/0.085% TFA for 75 min with a flow of 100 μL/min. Peaks were monitored at 214 and 280 nm, and automatic peak collection was used.

Amino acid sequencing was performed on a sequencer according to the manufacturer's instructions (model ABI 476A; Applied Biosystems/Perkin-Elmer, Foster City, CA). Determination of the phosphorylated amino acid was done by covalent sequencing of the radioactive peptide on arylactivated membranes (Millipore, Bedford, MA). After several washes with neat TFA in an ABI 477A sequencer, the AZT amino acid was extracted with neat TFA, collected, and concentrated by evaporation. Each fraction, corresponding to one released amino acid, was spotted on thin-layer chromatography plates, and radioactive spots were visualized by using a PhosphorImager (Fuji, Tokyo, Japan).

Polymerase Chain Reaction and Cloning of *pm28a*

The following degenerated oligonucleotides were used as primers for polymerase chain reaction (PCR). The forward primer 5′-CCGCTCGAGGAA/GGAA/GGCNCAA/GGCNCAT/CCAA/GCA-3′ corresponds to the protein sequence EEAQAHQH of a tryptic fragment of PM28A. The reverse primer 5′-CCGGAATTCAA/T/GA/T/GGTNACA/G/TGCA/G/TGGA/GTTNAC/T/GA/GTG-3′ corresponds to a conserved motif of MIP homologs, HI/M/VNPAVTF. To facilitate cloning, an EcoRI site was included at the 5′ end of the forward primer and a XhoI site at the 5′ end of the reverse primer. First-strand cDNA was synthesized from total RNA by reverse transcription. The conditions used for PCR were as follows: 35 mM Tris-HCl, pH 8.8, 1 mM $MgCl_2$, 8.3 mM $(NH_4)_2SO_4$, 3.3 mM EDTA, 5 mM β-mercaptoethanol, 25 μM of each deoxynucleotide triphosphates, 0.2 μg of each primer, 5 μL of cDNA (corresponding to 3 μg of total RNA) in a total volume of 100 μL. After 5 min of denaturation at 94°C, 2.5 units of Taq DNA polymerase (Boehringer Mannheim) was added. The amplification was performed for 30 cycles (1.5 min at 94°C, 2 min at 47°C, and 1.5 min at 72°C), after which 10 μL of the PCR product was used as template for another 30 cycles of amplification under identical conditions. The PCR product was cloned into pBluescript II SK+ (Stratagene) by using standard methods (Sambrook et al., 1989).

cDNA Library Screening and Sequencing

The cloned PCR product was used as a probe for screening of a spinach leaf cDNA library constructed in Uni-ZAP XR (Stratagene). Approximately 400,000 plaque-forming units were screened. Sequencing was performed according to Sanger et al. (1977), using Sequenase version 2.0 (U.S. Biochemical Corp., Cleveland, OH). Homology searches were done using the computer program BLAST (Altschul et al., 1990). Sequences were analyzed using the program GeneWorks (IntelliGenetics, Mountain View, CA).

DNA and RNA Gel Blot Analyses

Genomic DNA was isolated from spinach leaves, as described by Rogers and Bendich (1988). Aliquots of 20 μg were digested by EcoRI, HindIII, and BamHI and separated on a 0.8% agarose gel. Blotting onto GeneScreen membrane (Du Pont–New England Nuclear), prehybridization, hybridization, and washing were performed according to the manufacturer's instructions, using the 50% formamide/10% dextran sulfate method. Washing was at high stringency (0.1 × SSC [1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate], 0.1% SDS, at 68°C). An oligolabeling kit (Pharmacia, Sweden) was used to prepare a ^{32}P-labeled hybridization probe from the full-length *pm28a* cDNA. ^{32}P-labeled hybrids were visualized with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Total RNA was isolated using the method of Nato et al. (1988). Samples (15 μg per lane) were separated on a 1.5% denaturing agarose gel using standard methods (Sambrook et al., 1989). Hybridization and washing conditions were as for DNA. The ^{32}P-labeled PCR clone of *pm28a* was used as the hybridization probe.

In Vivo and in Vitro Phosphorylation

Spinach leaves (20 g) were cut in small pieces and incubated in 10 mM Mes-Tris, pH 6.0, 0.1 mM EDTA, 0.1 mM EGTA, 1 mCi ^{32}P-labeled Pi, and various concentrations of sucrose (0, 0.2, and 0.4 M, respectively). Vacuum was applied until the leaf pieces darkened and sank. The reaction mixture was then incubated for 15 min at room temperature, with occasional swirling. After removing the incubation buffer, plasma membranes were prepared from the leaf pieces, as described above, except that 50 mM NaF was included in the homogenization buffer. Polypeptides were separated by SDS-PAGE, the gel was dried, and ^{32}P-labeled polypeptides were visualized with a PhosphorImager.

For in vitro phosphorylation, plasma membranes (50 μg protein) were incubated in 25 mM Mops–1,3-bis(tris(hydroxymethyl)-methylamino)propane, pH 7.2, 0.33 M sucrose, 2.5 mM $MgCl_2$, 0.1 M KCl, 0.25 mM EGTA, 0.1% BSA, 10 μM ATP (including 10 μCi γ-^{32}P-ATP), and various concentrations of $CaCl_2$ to give final concentrations of free Ca^{2+} ranging from ~0.03 to 150 μM, as determined by Askerlund (1996). The total reaction volume was 50 μL. The reaction was allowed to proceed for 30 min at 30°C with gentle shaking and was stopped by the addition of 50 μL of solubilizing buffer (125 mM Tris-HCl, pH 6.8, 20% glycerol, 4% SDS, 10% β-mercaptoethanol, and 0.001% bromophenol blue; Laemmli, 1970). The samples were analyzed by SDS-PAGE, and phosphorylated polypeptides were visualized using a PhosphorImager.

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