**pmp1**<sup>+</sup>, a suppressor of calcineurin deficiency, encodes a novel MAP kinase phosphatase in fission yeast

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Calcineurin is a highly conserved and ubiquitously expressed Ca<sup>2+</sup>- and calmodulin-dependent protein phosphatase. The *in vivo* role of calcineurin, however, is not fully understood. Here, we show that disruption of the calcineurin gene (*ppb1*) in fission yeast results in a drastic chloride ion (Cl<sup>–</sup>)-sensitive growth defect and that a high copy number of a novel gene *pmp1* suppresses this defect. *pmp1* encodes a phosphatase, most closely related to mitogen-activated protein (MAP) kinase phosphatases of the CL100/MKP-1 family. *Pmp1* and calcineurin share an essential function in Cl<sup>–</sup> homeostasis, cytokinesis and cell viability. *Pmp1* phosphatase dephosphorylates Pmk1, the third MAP kinase in fission yeast, *in vitro* and *in vivo*, and is bound to Pmk1 *in vivo*, strongly suggesting that *Pmp1* negatively regulates Pmk1 MAP kinase by direct dephosphorylation. Consistently, the deletion of *ppb1* suppresses the Cl<sup>–</sup>-sensitive growth defect of *ppb1* null. Thus, calcineurin and the Pmk1 MAP kinase pathway may play antagonistic functional roles in Cl<sup>–</sup> homeostasis.

**Keywords:** ion homeostasis/MAP kinase/protein phosphatase/Schizosaccharomyces pombe

**Introduction**

Calcineurin is a Ca<sup>2+</sup>- and calmodulin-dependent serine/threonine protein phosphatase consisting of a catalytic subunit and a regulatory subunit (Klee *et al.*, 1979). Although it is highly enriched in the mammalian central nervous system (Klee *et al.*, 1979), it is also present in lower eukaryotic organisms (Cyert *et al.*, 1991; Higuchi *et al.*, 1991; Kuno *et al.*, 1991; Liu *et al.*, 1991a; Rasmussen *et al.*, 1994), suggesting that it may have an evolutionarily conserved function. Calcineurin is the target of immunosuppressive drugs such as FK506 and cyclosporin A (Liu *et al.*, 1991b), which have been used to prevent graft rejection after organ transplantation. These drugs inhibit calcineurin phosphatase activity after forming complexes with cytoplasmic binding proteins. An increasing number of calcineurin-dependent cellular processes are being identified using these specific inhibitors and molecular biological techniques (Clipstone and Crabtree, 1992; Fruman *et al.*, 1992; Jain *et al.*, 1993; Sharkey and Butcher, 1994; Garrett-Engele *et al.*, 1995; Shibasaki *et al.*, 1996), but the exact signaling functions of calcineurin are still obscure in many cellular processes.

The fission yeast *Schizosaccharomyces pombe* is an excellent model system for studying eukaryotic cell signaling and the division cycle. It has a gene encoding a catalytic subunit of calcineurin, *ppb1*, that has been shown to be implicated in cytokinesis, mating response and cell shape (Yoshida *et al.*, 1994). To understand the role of calcineurin in cellular signaling, we undertook to identify the gene products which interact with Ppb1, re-examining phenotypes of the calcineurin disruption mutant (*Δppb1*). In this study, we show that *Δppb1* exhibits hypersensitivity to chloride ion (Cl<sup>–</sup>) and that multicopy plasmids are capable of suppressing the Cl<sup>–</sup> hypersensitivity. One such plasmid contains a gene designated *pmp1*, encoding a protein closely related to dual-specificity MAP kinase phosphatases, such as mammalian CL100/MKP-1, PAC-1 and budding yeast MSG5 (Sun *et al.*, 1993; Doi *et al.*, 1994; Ward *et al.*, 1994). Three MAP kinases, Stp1, Sty1/Spc1 and Pmk1/Spm1, have been identified in fission yeast to date (Toda *et al.*, 1991, 1996; Gotoh *et al.*, 1993; Millar *et al.*, 1995; Shiozaki and Russell, 1995; Kato *et al.*, 1996; Zaitsevskaya-Carter and Cooper, 1997). Two protein tyrosine phosphatases, Pyp1 and Pyp2, negatively regulate Sty1/Spc1 MAP kinase (Millar *et al.*, 1995; Shiozaki and Russell, 1995). However, no MAP kinase phosphatase structurally related to the CL100/MKP-1 family has been shown to regulate any of the MAP kinases in fission yeast. We show that Pmp1 phosphatase directly dephosphorylates Pmk1 MAP kinase and is involved in Cl<sup>–</sup> homeostasis by attenuating the Pmk1 MAP kinase pathway. Moreover, Pmp1 and calcineurin phosphatases share an essential function in Cl<sup>–</sup> homeostasis, cytokinesis and cell viability. This is the first demonstration that the calcineurin-mediated pathway and the MAP kinase signaling pathway play antagonistic roles.

**Results**

*The calcineurin deletion mutant is hypersensitive to Cl<sup>–</sup>*

*Δppb1* (deletion of the fission yeast calcineurin homolog) cells grow almost normally in rich YPD media (Figure 1). We found that *Δppb1* exhibited a severe growth defect in YPD which contained a high concentration of Cl<sup>–</sup>. A concentration of 0.15 M of MgCl<sub>2</sub> was sufficient to arrest growth in *Δppb1* (Figure 1), while wild-type cells were able to grow on plates containing up to 0.6 M MgCl<sub>2</sub> (Figure 2). Similar hypersensitivity was seen in YPD...
containing 0.3 M NaCl, 0.3 M KCl, 0.15 M MgCl₂, 1.2 M sorbitol, 0.15 M magnesium gluconate (MgGLCN) or 0.15 M MgCl₂ + 0.5 μg/ml FK506.

Isolation of the pmp1⁺ gene as a multicopy suppressor for the Cl⁻ sensitivity of Δppb1

To identify genes the product of which interact with calcineurin, we isolated multicopy plasmids that suppressed the Cl⁻-sensitive growth defect of Δppb1. The Δppb1 cells were transformed with an S. pombe genomic library constructed in the multiple copy vector pDB248 (Beach et al., 1982). Transformants were obtained and subsequently screened for the ability to grow on YPD plates containing 0.15 M MgCl₂ at 33°C. The suppressing plasmids fell into three classes by restriction enzyme analyses. One class had in common restriction fragments indicative of the pmp1⁺ gene. Another class contained a gene whose restriction map was different from that of the pmp1⁺ gene, which has been designated pmp1⁺ (S. pombe MAP kinase phosphatase, 1, formerly aspp1⁺, Toda et al., 1996). Characterization of a gene contained in the third class will be reported elsewhere.

A plasmid carrying pmp1⁺ suppressed the Cl⁻-sensitive growth defect of Δppb1 to the same extent as wild-type in YPD containing 0.3 M MgCl₂ or 0.6 M KCl (Figure 2). In YPD containing 0.6 M MgCl₂, Δppb1 carrying pmp1⁺ failed to grow, while wild-type grew slowly, indicating that suppression by pmp1⁺ is partial (Figure 2).

Nucleotide sequence determination showed that pmp1⁺ encodes a protein of 278 amino acids (Figure 3A), and the C-terminal portion of Pmp1 shares significant amino acid sequence similarity with members of dual-specificity MAP kinase phosphatases (Figure 3B). This group of phosphatases, including CL100/MKP-1, PAC-1 and MSG5 (Sun et al., 1993; Doi et al., 1994; Ward et al., 1994), is known to possess phosphatase activity towards MAP kinases on its threonine and tyrosine residues.

The Pmp1 protein possesses an intrinsic phosphatase activity

To examine whether the pmp1⁺ gene product is an active protein phosphatase, a glutathione S-transferase (GST)–Pmp1 fusion gene was constructed and expressed in Escherichia coli (Figure 3C). Purified GST–Pmp1 fusion protein was found rapidly to hydrolyze p-nitrophenyl phosphate (pNPP), a chromogenic substrate structurally related to phosphotyrosine (filled circles, Figure 3D), and this phosphatase activity was inhibited by sodium orthovanadate, a specific inhibitor of protein tyrosine phosphatases (filled rectangles, Figure 3D). In addition, like CL100/MKP-1 (Alessi et al., 1993), Pmp1 is insensitive to high concentrations (up to 1 μM) of okadaic acid, a specific inhibitor of the type 1 and 2A serine/threonine protein phosphatases (data not shown).

Examination of whether the conserved cysteine residue in the protein phosphatase signature motif in Pmp1 is essential for phosphatase activity was done by site-directed mutagenesis to change the Pmp1 codon of Cys158 to serine (C158S). GST–Pmp1C158S produced in bacteria and purified was tested for its ability to dephosphorylate pNPP. The GST–Pmp1C158S protein did not hydrolyze pNPP (open rectangles, Figure 3D). These results established that GST–Pmp1 harbored an intrinsic phosphatase activity. Moreover, the cysteine residue (C158) conserved in all tyrosine phosphatases is an active site in Pmp1 phosphatase. The active site of Pmp1 is extremely unusual in that the invariant histidine within the highly conserved tyrosine phosphatase consensus sequence [I/V]HCXXGXXR[S/T] is replaced by asparagine. This histidine residue has been shown to stabilize the negative charge of the catalytic cysteine residue, making it a better leaving group and accelerating the breakdown of the phosphoenzyme intermediate. A systematic mutational analysis of the active site sequence in receptor-linked tyrosine phosphatase showed that substitution of asparagine for histidine at this
Fig. 3. Pmp1 is similar to MAP kinase phosphatases and possesses the phosphatase activity. (A) Predicted amino acid sequence of Pmp1. Amino acid numbers are shown on the left. The nucleotide sequence data reported here will appear in the DDBJ, EMBL and GenBank nucleotide sequence databases with the accession number D82022. (B) Amino acid sequence alignment of Pmp1, MSG5, PAC-1 and CL100. Amino acid residues identical to Pmp1 are indicated by filled boxes. Shaded boxes represent similar amino acids. (C) SDS–PAGE analysis of purified GST–Pmp1 fusion protein. C, crude extract of overexpressing E.coli; F, flow-through fraction; P, purified fusion protein. The GST–Pmp1 protein is indicated by an arrow. (D) Phosphatase activity of GST–Pmp1. GST–Pmp1 (o) and GST–Pmp1 C158S (u) were expressed in bacteria and purified using glutathione–Sepharose CL-4B. Dephosphorylation of pNPP, expressed as increased absorbance at 410 nm, was measured as a function of protein concentration. The GST–Pmp1 fusion protein was also incubated in the presence of 1 mM sodium vanadate (j). A position reduced the activity of the enzyme by up to 90% (Streuli et al., 1990). Pmp1 is, to our knowledge, the first active tyrosine phosphatase reported which contains a residue other than histidine at this position. This substitution is confirmed by repeated direct sequencing of genomic DNA (data not shown).

Genetic interactions with other protein phosphatases
We found that suppression of the Cl– sensitivity of Δppb1 by pmp1/H11001 is highly specific. Overproduction of other type 1 or 2A serine/threonine phosphatase genes such as dis2+/H11001, sds21+/H11001, ppa2+/H11001 or pep1+/H11001 (Ohkura et al., 1989; Kinoshita et al., 1990, 1993; Shimanuki et al., 1993) did not suppress the Cl–-sensitive phenotype. Introduction of multicopy plasmids carrying the tyrosine phosphatase gene pyp1/H11001 implicated in attenuation of Sty1/Spc1 MAP kinase (Millar et al., 1995; Shiozaki and Russell, 1995) into Δppb1 also failed to suppress the phenotype (Figure 4).

Calcineurin and Pmp1 share an essential function for cell viability
The in vivo role of the pmp1+/H11001 gene product was investigated by creating a null allele (pmp1::ura4+/H11001) by inserting a 1.8 kb ura4+/H11001 fragment into the NcoI site of the pmp1+/H11001 open reading frame (Figure 5A). This construct was used to disrupt the genomic copy of pmp1+/H11001 in a ura4/ura4 diploid strain by transformation. Genomic Southern hybridization of the resulting stable Ura+ diploid transformants showed that the pmp1+/H11001 gene was disrupted (Figure 5B). These heterozygous diploids were sporulated and tetrads were subsequently dissected. Four spores were viable in the tetrads, and the Ura marker was segregated in 2+:2– (data not shown). The pmp1+/H11001 gene was hence not essential for viability. However, Δpmp1+ cells were hypersensitive to Cl–. As shown in Figure 5C, these cells could not grow on the YPD plate containing 0.5 M MgCl2, whereas wild-type cells grew normally. A concentration of 1.2 M sorbitol or 0.5 M magnesium gluconate did not affect growth of Δpmp1 (data not shown). These results suggest that Pmp1 phosphatase is involved in Cl– homeostasis. Furthermore, the addition of FK506 to YPD resulted in severe growth inhibition of Δpmp1 (Figure 5C). We therefore assumed that inhibition of calcineurin activity in Δpmp1 led to the arrest of growth. To examine whether Δpmp1 and Δppb1 were synthetically lethal, these disruption mutants were crossed. We did not obtain the
Fig. 5. Gene disruption of \( pmp1^+ \). (A) Restriction map and disruption of \( pmp1^+ \). The top line represents an abbreviated restriction map of the \( pmp1^+ \) region. Restriction fragments of the inserts were subcloned in pDB248 as shown, and their ability to complement \( \Delta ppb1 \) is indicated by (+) or (–). The \( pmp1^+ \) gene (the coding region indicated by the arrow) was disrupted by one-step gene replacement (Rothstein, 1983) using the \( S. pombe \) \( ura4^+ \) gene which was inserted at the \( NcoI \) site in the coding region. Restriction sites: \( Bg, BglII; E, EcoRI; Hc, HinclII; Hd, HindIII; Nc, NcoI; Pvu, PvuII; Sc, SacI; Sl, SalI. \) (B) Southern blot analysis of \( pmp1 \)-deleted strains. \( S. pombe \) genomic DNA prepared from disruptant diploid cells (lane D), its haploid \( Ura^+ \) \( \Delta pmp1 \) cells (lane –) and \( Ura^- \) progeny cells (lane –). Genomic DNAs were digested with \( EcoRI \) and \( BglII \), separated by agarose gel electrophoresis and Southern blotted. The probe used for hybridization was a 0.5 kb \( HindIII \) fragment. The 2.5 kb band corresponds to the intact \( pmp1^+ \) allele and the 4.3 kb band to the disrupted allele. (C) The constitutively active calcineurin mutant (\( ppb1\Delta C \)) suppresses the phenotype of \( \Delta pmp1 \). \( \Delta pmp1 \) cells transformed with vector (pDB248) or each of the multicopy plasmids carrying the wild-type \( pmp1^+ \) (wt), \( ppb1\Delta C \), were streaked onto rich YPD plate containing 0.5 M MgCl\(_2\) and 0.5 \( \mu \)g/ml FK506, and incubated for 3 days at 33°C. (D) \( ppb1\Delta pmp1 \) is synthetic lethal. Two strains \( \Delta pmp1 \) and \( \Delta ppb1 \) were crossed, and asci formed were dissected on YPD medium at 27°C. Single mutants grew well, whereas \( \Delta ppb1\Delta pmp1 \) double mutants cells were non-viable. T: tetatype, PD: parental ditype.

\( \Delta pmpl \) cells are defective in cell shape control and cytokinesis

The effect of \( pmp1^+ \) gene disruption on cell growth was examined by microscopic observation, and the cell morphology of \( \Delta pmpl \) cells was found to be aberrant. Deformed cells (round or pear-shaped as seen by Nomarski) were found at a high frequency (Figure 6). Septa formed in \( \Delta pmpl \) (seen by Calcofluor staining) were often asymmetric, producing daughter cells of unequal size. Multiseptated cells were also observed (Figure 6). These results suggest that Pmp1 is implicated in cell shape control and cytokinesis.

\( \Delta ppb1\Delta pmpl \) double mutant (Figure 5D), showing that Pmp1 and calcineurin shared an essential function for viability. The relationship between calcineurin and \( pmpl^+ \) was explored further by constructing a plasmid carrying a constitutively active calcineurin mutant gene (\( ppb1\Delta C \)), and its effect on the phenotypes of \( \Delta pmpl \) was examined. Calcineurin deleted in the C-terminal portion is known to become a constitutively active Ca\(^{2+}\)/calmodulin-independent phosphatase (Parsons et al., 1994; Garrett-Engle et al., 1995). Overproduction of the C-terminal truncated Ppb1 by plasmid p \( ppb1\Delta C \), but not of the full-length Ppb1 (data not shown), dramatically improved the growth of \( \Delta pmpl \), allowing \( \Delta pmpl \) cells to grow on YPD containing FK506 or 0.5 M MgCl\(_2\) (Figure 5C). Thus, Pmp1 and Ppb1 have a similar and overlapping physiological function.

Fig. 6. Aberrant cell morphology of \( \Delta pmpl \). Exponentially growing wild-type (wt) and \( \Delta pmpl \) cells are shown. Wild-type and \( \Delta pmpl \) cells were cultured in the YPD liquid medium at 33°C. Cells were fixed with formaldehyde, washed in the phosphate saline buffer and stained with Calcofluor. The same cells were also observed by Nomarski. The bar indicates 10 \( \mu \)m.

Deletion of a MAP kinase homolog suppresses the Cl\(^{-}\)-sensitive phenotype of \( \Delta ppb1 \)

We considered the possibility that, if Pmp1 suppressed the Cl\(^{-}\} sensitivity of \( \Delta ppb1 \) through its phosphatase activity toward a MAP kinase, loss of that MAP kinase function might also suppress the phenotype of \( \Delta ppb1 \). Three MAP
kinase pathways have been identified in fission yeast, namely Spk1 (Toda et al., 1991; Gotoh et al., 1993), Sty1/Spcl/Pph1 (Millar et al., 1995; Shiozaki and Russell, 1995; Kato et al., 1996) and Pmk1/Spm1 (Toda et al., 1996; Zaitsevskaya-Carter and Cooper, 1997). Crossing was done to make double disruption mutants bearing Δppb1 and either of the null alleles for the genes encoding MAP kinase or MAP kinase kinase. The Cl− sensitivity was found to be suppressed in the double disruption Δppb1Δpck2, whereas it was not with Δppb1Δspk1 or Δppb1Δwis1 (wis1 encodes a MAP kinase kinase for Sty1/Spcl MAP kinase, Millar et al., 1995; Shiozaki and Russell, 1995) double disruption mutants (Figure 7).

Disruption of the mkh1+ gene, which encodes a putative MAP kinase kinase for Pmk1/Spm1 (Sengar et al., 1997), also suppressed the Cl− sensitivity. These data are consistent with a model whereby Mkh1 and Pmk1 act in a linear pathway. Ppb1 phosphatase and the Pmk1 MAP kinase pathway might thus play antagonistic roles in Cl− homeostasis. Pmk1 was reported to function coordinately with the protein kinase C (PKC) pathway (Toda et al., 1996). We then examined whether PKC interacted with Ppb1. The loss of pck2+, encoding a PKC-like protein (Toda et al., 1993), suppressed the Cl−-sensitive growth defect when combined with Δppb1 (Figure 7). The PKC pathway might also have an opposing role to the calcineurin signaling pathway in Cl− homeostasis.

**Pmp1 is a Pmk1 MAP kinase phosphatase in vitro and in vivo**

The above results suggested that Pmp1 affected the Cl− homeostasis by inactivating Pmk1 MAP kinase. It was then of interest whether the Pmp1 directly dephosphorylated Pmk1 in vitro. Pmk1 was expressed as the GST fusion protein (GST–Pmk1) in *S. pombe* cells and was purified using a glutathione affinity column. Purified GST–Pmk1 was then incubated with GST–Pmp1 or GST–Pmp1C158S made in bacteria and purified. The phosphorylation state of Pmk1 was tested by immunoblot using a monoclonal antibody against phosphotyrosine. GST−Pmp1 was able to abolish phosphotyrosine on Pmk1, whereas inactive GST–Pmp1C158S failed to reduce the level of phosphotyrosine on Pmk1 (Figure 8A, upper panel). Duplicate samples probed using anti-GST antibody showed that the amount of GST–Pmk1 in each sample was approximately equal (Figure 8A, lower panel).

We next addressed the question of whether Pmp1 modulated the level of Pmk1 tyrosine phosphorylation in vivo. As shown in Figure 8B, Pmk1 tyrosine phosphorylation was significantly enhanced in Δppb1 cells,
but almost completely abolished in cells overproducing Pmp1. In sharp contrast, overproduction of catalytically inactive Pmp1C158S resulted in an elevated level of tyrosine-phosphorylated Pmk1, the suppression by Pmp1C158S may occur through a tight binding between the mutant Pmp1C158S and phosphatylated Pmk1, thereby preventing dephosphorylation of Pmk1 and also the ability of Pmk1 to transmit a downstream signal. This interpretation is consistent with the result that overproduction of Pmp1C158S leads to accumulation of tyrosine-hyperphosphorylated Pmk1 (Figure 8B).

The subsequent genetic experiments further supported the hypothesis that Pmp1 was a Pmk1 MAP kinase phosphatase. First, the double mutant Δpmp1Δpbb1 was no longer sensitive to FK506 or Cl⁻ (Figure 8D). Second, the lethality of Δpbb1Δpmp1 was overcome in the triple mutant Δpbb1Δpmp1Δpmmk1 (data not shown). Third, introduction of the pmp1⁺ gene into Δpbb1Δpmmk1 showed no effect on the Cl⁻ sensitivity (data not shown).

Role of Pmp1 in cytokinesis

We examined whether Ppb1 and Pmk1 MAP kinase pathways were functionally related in cytokinesis. A significant fraction of Δpbb1 cells contained multiple septa due to incomplete cytokinesis (Yoshida et al., 1994). Overexpression of pmp1⁺ suppressed this cytokinesis abnormality of Δpbb1, and cells with multisepta became negligible (Figure 9). Pmp1 and Ppb1 phosphatases therefore appear to share an essential function in cytokinesis. However, Δpbb1Δpmmk1 cells exhibit a drastic filamentous, multiseptate phenotype, which was more severe than that of the single mutant (Figure 9), suggesting that Pmk1 and Ppb1 acted synergistically in cytokinesis. In this case, the sole target of Pmp1, Δpbb1Δpmmk1 would be expected to show the minimal level of multiseptated cells. A possible explanation of this apparent discrepancy is that Pmp1 possesses a target other than Pmk1 which controls cytokinesis in a manner opposing the Pmk1 MAP kinase pathway (Figure 10).

Discussion

We show in this study that the fission yeast calcineurin gene ppb1⁺ is required for Cl⁻ homeostasis. The Cl⁻-sensitive
phenotype of \(\Delta p pb1\) was used to identify a novel MAP kinase phosphatase gene \(p mp1\) as a multicopy suppressor. \(p mp1\) phosphatase is not essential for cell viability, but shares an essential function with \(p pb1\) in cell viability. They act synergistically in \(C l^-\) homeostasis and cytotoxicity. We also show that \(p mk1\), the third MAP kinase of fission yeast, is a physiological target for \(p mp1\).

**Pmp1 is a Pmk1 MAP kinase phosphatase**

We provide evidence that (i) \(p mp1\) directly dephosphorylates \(p mk1\) on tyrosine residue in vitro, (ii) \(p mp1\) influences the tyrosine phosphorylation state of \(p mk1\) in vivo, and (iii) \(p mp1\) associates with \(p mk1\) in vivo. Genetic interactions are also consistent with our conclusion that \(p mp1\) negatively regulates \(p mk1\) by direct dephosphorylation. \(p mp1\) phosphatase may thus be implicated in \(C l^-\) sensitivity through down-regulating the \(p mk1\) MAP kinase pathway.

There was no detectable change in the pattern of tyrosine-phosphorylated proteins in cell lysates prepared from \(\Delta p mp1\) cells when compared with wild-type (data not shown), suggesting that \(p mp1\) phosphatase possesses a restricted substrate specificity. Our preliminary findings that \(p mp1\) was unable to dephosphorylate a model substrate, myelin basic protein, containing either phosphotyrosine or phosphoserine were also supportive of the substrate specificity of \(p mp1\) phosphatase (R.Sugiura, H.Shuntoh and T.Kuno, unpublished). Thus, \(p mp1\) displays properties that are hallmarks of known MAP kinase phosphatases. More definitive proof that \(p mp1\) is a dual-specificity phosphatase, however, will await further biochemical analysis.

**Role of Pmp1 and calcineurin in \(C l^-\) homeostasis**

We show that both calcineurin and \(p mp1\) phosphatases are involved in \(C l^-\) homeostasis, as \(\Delta p pb1\) or \(\Delta p mp1\) cells become hypersensitive to \(C l^-\). Three lines of evidence show that \(p pb1\) and \(p mp1\) act closely in \(C l^-\) homeostasis. First, \(p pb1\) overexpression rescues the \(C l^-\) sensitivity of \(\Delta p pb1\). Secondly, \(\Delta p mp1\) is acutely sensitive to FK506, an inhibitor of calcineurin, consistent with the fact that the double mutant \(\Delta p mp1\Delta p pb1\) is lethal. Third, a constitutively active calcineurin suppresses the drug- and \(C l^-\)-sensitive phenotypes of \(\Delta p mp1\). Taken together, these results suggest that \(\Delta p mp1\) cells require active calcineurin for cell viability. These genetic interactions are the first indications of a shared role for calcineurin and a MAP kinase phosphatase in the regulation of \(C l^-\) homeostasis.

The demonstration that other type 1 and 2A serine/threonine phosphatases failed to suppress the \(C l^-\) sensitivity of \(\Delta p pb1\) suggested that \(p pb1\) might dephosphorylate a key functional component of \(C l^-\) regulation as a specific substrate. Furthermore, the inability of \(p yp1\), which inactivates the Sty1/Spc1 MAP kinase (Millar et al., 1995; Shiozaki and Russell, 1995), to suppress the \(C l^-\) sensitivity, suggests that \(p yp1\) may not be involved in the regulation of the \(p mk1\) MAP kinase pathway.

In mammalian cells, \(C l^-\) is known to be involved in cell volume control and intracellular pH regulation (Szatkowski and Schlue, 1994; Strange et al., 1996). It is noteworthy that the average cell length and volume of \(\Delta p pb1\) cells was larger than that of wild-type (Yoshida et al., 1994). In budding yeast, null mutants lacking the calcineurin gene exhibit a salt-hypersensitive phenotype, but their sensitivity is dependent on cations such as \(Na^+\) or \(Li^+\) (Nakamura et al., 1993; Mendoza et al., 1994). The LiCl sensitivity of \(\Delta p pb1\) was compared with that of wild-type, but no marked difference was observed (data not shown). Thus, budding and fission yeasts show greatly different sensitivities to ions in the calcineurin null mutants.

**Calcineurin interacts with the Pmk1 signal transduction pathway**

An important finding in this study is that the \(C l^-\) sensitivity of \(\Delta p pb1\) is rescued by disruption of \(p mk1\), encoding a MAP kinase (Toda et al., 1996), or of \(mkh1\), encoding a putative MAP kinase kinase kinase of \(p mk1\) (Sengar et al., 1997). Calcineurin and the \(p mk1\) MAP kinase pathway might have opposing roles in \(C l^-\) homeostasis. One hypothesis to explain this counteractive interaction is that calcineurin and \(p mk1\) kinase might regulate a common target, essential for \(C l^-\) resistance, in an opposing manner, by influencing its phosphorylation state. Given the remarkable conservation of MAP kinase pathways and calcineurin, it is interesting to speculate that the counterparts

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**Table I. Fission yeast strains used in this study**

<table>
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<tr>
<th>Strains</th>
<th>Genotypes</th>
<th>Derivations</th>
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of Pmk1 MAP kinase in higher organisms may act antagonistically with calcineurin.

Materials and methods

Nucleic acid preparation and manipulation
Standard molecular biology techniques were followed as described (Sambrook et al., 1989).

Strains, media, chemicals, plasmids and genetic techniques

The S.pombe strains used in this study are listed in Table I. FK506 was a generous gift from Fujisawa Pharmaceutical Co. Standard procedures for S.pombe genetics were followed according to Moreno et al. (1991). Fission yeast cells were transformed by lithium acetate as described by Ito et al. (1983). pRL-GSpn1 was generously provided by Dr J.Cooper (Zaitsevskaya-Carter and Cooper, 1997) and pREP42-pmp1::ura4+/HA::6His was constructed by cloning the Ndel–BamHI fragment of pREP41-pmp1::ura4+/HA::6His (Toda et al., 1996) into Ndel–BamHI-digested pREP42. These plasmids were used for the expression of epitope-tagged Pmk1.

Expression of Pmp1 in bacteria and yeast

In order to express Pmp1 in E.coli, the coding region for the pmp1+ gene was amplified using PCR and was subcloned into the BamHI site of pGEX-2T, a GST fusion protein expression vector, to produce pGEX-pmp1. The plasmid pGEX-pmp1 was used to express full-length Pmp1 protein as a fusion protein with GST, which was purified by glutathione agarose affinity chromatography. For expression in fission yeast, the coding region of GST was amplified using PCR from pGEX-2T and the resulting DNA was then ligated to the 1.8 kb HI fragment containing the coding sequence of pmp1+ into the BamHI site of pREP1-GST, creating pREP1-GST-pmp1+. pREP1-GST-pmp1+ fully suppressed the phenotypes of Δpmp1 cells. Site-directed mutagenesis was performed using the Sculptor mutagenesis kit (Amersham).

Phosphatase assay

Hydrolysis of pNPP was done in 200 μl of a solution containing 0.1% β-mercaptoethanol, 10 mM pNPP, 0.1 mg/ml bovine serum albumin, 25 mM HEPES (pH 7.4) and enzyme at 30°C for 30 min. The reaction was stopped by addition of 800 μl of 0.25 M NaOH. Absorbance at 410 nm was measured.

Disruption of pmp1+ gene

A disruption of pmp1+ gene disruption by homologous recombination (Rothstein, 1983) was performed. The pmp1::ura4+ disruption was constructed as follows. The 3.5 kb SalI–BglII fragment containing the pmp1+ gene was subcloned into the SalI–BamHI site of pUC119 to form pUC-pmp1. Plasmid pUC-pmp1 was cleaved at the single NcoI site located in the pmp1+ coding region and blunt ended with Klenow polymerase. The resulting DNA was then ligated to the 1.8 kb HincII fragment of the S.pombe ura4+ DNA which had been blunt ended using the Klenow polymerase to create pUC-pmp1::ura4+. The 2.0 kb EcoRI–HincII fragment containing disrupted pmp1+ was transformed into diploid cells (SA/1D, Table I). Stable integrants were selected on medium lacking uracil, and disruption of the pmp1+ gene was checked by genomic Southern hybridization.

Construction of constitutively active calcineurin mutant ppp1lac

A truncated version of ppp1+ (Yoshida et al., 1994) was created by changing amino acid 445 (a lysine) to a stop codon by site-directed mutagenesis as described by Garrett-Engel et al. (1995).

Acknowledgements

We thank Drs P.Fantes and J.Cooper for plasmids and strains, and S.Dhut for able technical assistance. This work was supported in part by research grants from the Ministry of Education, Science and Culture of Japan.

References


Received September 5, 1997; revised October 13, 1997; accepted October 14, 1997