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pmp1$^+$, 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^{2+}- 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^-)-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^- 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 *pmk1*$^+$ suppresses the Cl^--sensitive growth defect of *ppb1* null. Thus, calcineurin and the Pmk1 MAP kinase pathway may play antagonistic functional roles in the Cl^- homeostasis.

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

Introduction

Calcineurin is a Ca^{2+}- 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 (Δ*ppb1*). In this study, we show that Δ*ppb1* exhibits hypersensitivity to chloride ion (Cl^-) and that multicopy plasmids are capable of suppressing the Cl^- 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, Spk1, 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^- homeostasis by attenuating the Pmk1 MAP kinase pathway. Moreover, Pmp1 and calcineurin phosphatases share an essential function in Cl^- 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$^-$

Δ*ppb1* (deletion of the fission yeast calcineurin homolog) cells grow almost normally in rich YPD media (Figure 1). We found that Δ*ppb1* exhibited a severe growth defect in YPD which contained a high concentration of Cl^-. A concentration of 0.15 M of $MgCl_2$ was sufficient to arrest growth in Δ*ppb1* (Figure 1), while wild-type cells were able to grow on plates containing up to 0.6 M $MgCl_2$ (Figure 2). Similar hypersensitivity was seen in YPD

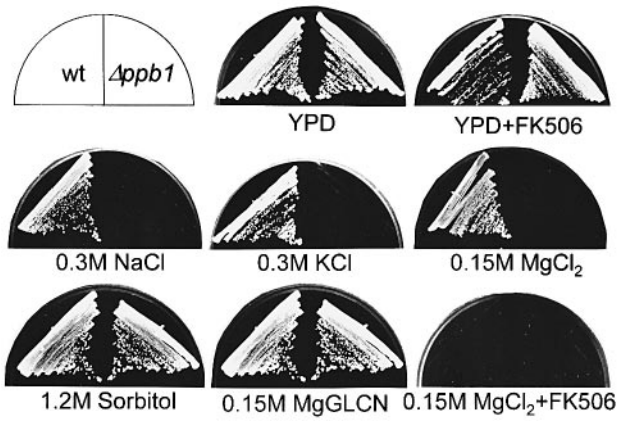

Fig. 1. The Cl^- sensitivity of Δ*ppb1*. Wild-type (wt) and Δ*ppb1* were streaked onto each plate, containing YPD, YPD + 0.5 μg/ml FK506, 0.3 M NaCl, 0.3 M KCl, 0.15 M $MgCl_2$, 1.2 M sorbitol, 0.15 M magnesium gluconate (MgGLCN) or 0.15 M $MgCl_2$ + 0.5 μg/ml FK506.

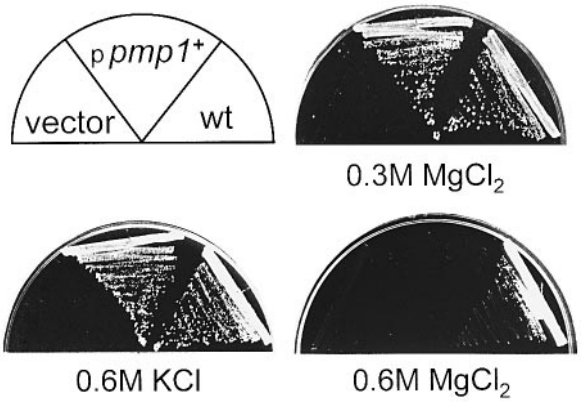

Fig. 2. Suppression of the Cl^--sensitive phenotype of Δ*ppb1* by a plasmid carrying the *pmp1*$^+$gene. Δ*ppb1* cells transformed with multicopy plasmid pDB248 carrying the wild-type *ppb1*$^+$ (wt) or the *pmp1*$^+$ gene (p *pmp1*$^+$) and with the control vector were streaked onto the YPD plates containing 0.3 M $MgCl_2$, 0.6 M KCl or 0.6 M $MgCl_2$, and incubated for 3 days at 33°C.

containing 0.3 M NaCl, 0.3 M KCl (Figure 1) or 0.15 M $CaCl_2$ (data not shown). The sensitivity is due to Cl^- rather than to high osmolarity or high anion concentration, as neither 1.2 M sorbitol nor 0.15 M magnesium gluconate affected growth of Δ*ppb1* cells (Figure 1). Ppb1 is thus required for the normal Cl^- sensitivity. The addition of FK506 to YPD led to no retardation effect on the growth of either wild-type or Δ*ppb1* (Figure 1) (Yoshida *et al.*, 1994). If, however, wild-type cells were treated with Cl^- in the presence of FK506, they did not grow (Figure 1). These results are consistent with a hypothesis that the loss of calcineurin phosphatase activity is the direct cause for the Cl^- hypersensitivity.

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 Δ*ppb1*. The Δ*ppb1* 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 $MgCl_2$ at 33°C. The suppressing plasmids fell into three classes by restriction enzyme analyses. One class had in common restriction fragments indicative of the *ppb1*$^+$ gene. Another class contained a gene whose restriction map was different from that of the *ppb1*$^+$ gene, which has been designated *pmp1*$^+$ (*S.pombe* MAP kinase phosphatase 1, formerly *dsp1*$^+$, 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 Δ*ppb1* to the same extent as wild-type in YPD containing 0.3 M $MgCl_2$ or 0.6 M KCl (Figure 2). In YPD containing 0.6 M $MgCl_2$, Δ*ppb1* 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 (*p*NPP), 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–Pmp1^{C158S} produced in bacteria and purified was tested for its ability to dephosphorylate *p*NPP. The GST–Pmp1^{C158S} protein did not hydrolyze *p*NPP (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

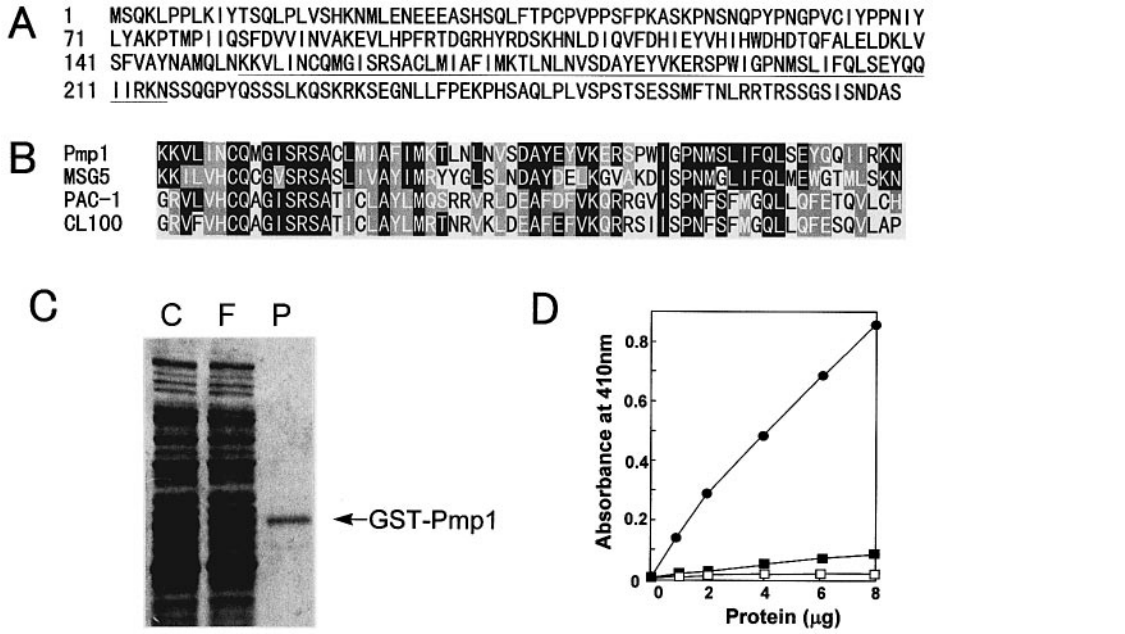

Fig. 3. Pmp1 is similar to MAP kinase phosphatases and possesses the phosphatase activity. (**A**) Predicted amino acid sequence of Pmp1. Amino acid numbers are shown on the left. The nucleotide sequence data reported here will appear in the DDBJ, EMBL and GenBank nucleotide sequence databases with the 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} (□) were expressed in bacteria and purified using glutathione–Sepharose CL-4B. Dephosphorylation of *p*NPP, 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 (■).

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 Δ*ppb1* by *pmp1*$^+$ is highly specific. Overproduction of other type 1 or 2A serine/threonine phosphatase genes such as *dis2*$^+$, *sds21*$^+$, *ppa2*$^+$ or *ppe1*$^+$ (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*$^+$ implicated in attenuation of Sty1/Spc1 MAP kinase (Millar *et al.*, 1995; Shiozaki and Russell, 1995) into Δ*ppb1* also failed to suppress the phenotype (Figure 4).

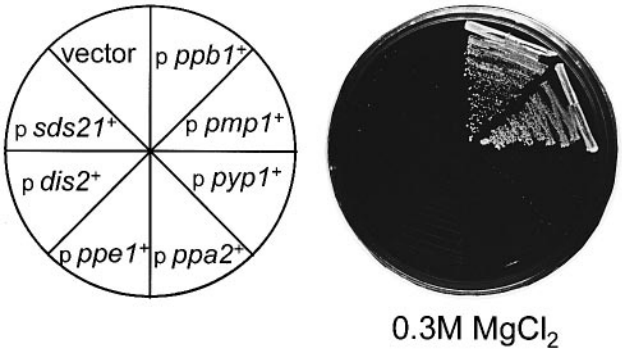

Fig. 4. Effect of high copy plasmids carrying *pmp1*$^+$ and other protein phosphatase genes on the Cl^--sensitive growth defect of Δ*ppb1*. Δ*ppb1* cells were transformed with each of the multicopy plasmids carrying *ppb1*$^+$, *pmp1*$^+$, *pyp1*$^+$, *ppa2*$^+$, *ppe1*$^+$, *dis2*$^+$, *sds21*$^+$, or with the control vector pDB248, streaked onto rich YPD plates containing 0.3 M $MgCl_2$ and incubated for 3 days at 33°C.

Calcineurin and Pmp1 share an essential function for cell viability

The *in vivo* role of the *pmp1*$^+$ gene product was investigated by creating a null allele (*pmp1::ura4*$^+$) by inserting a 1.8 kb *ura4*$^+$ fragment into the *Nco*I site of the *pmp1*$^+$ open reading frame (Figure 5A). This construct was used to disrupt the genomic copy of *pmp1*$^+$ in a *ura4/ura4* diploid strain by transformation. Genomic Southern hybridization of the resulting stable Ura$^+$ diploid transformants showed that the *pmp1*$^+$ 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*$^+$ gene was hence not essential for viability. However, Δ*pmp1* cells were hypersensitive to Cl^-. As shown in Figure 5C, these cells could not grow on the YPD plate containing 0.5 M $MgCl_2$, whereas wild-type cells grew normally. A concentration of 1.2 M sorbitol or 0.5 M magnesium gluconate did not affect growth of Δ*pmp1* (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 Δ*pmp1* (Figure 5C). We therefore assumed that inhibition of calcineurin activity in Δ*pmp1* led to the arrest of growth. To examine whether Δ*pmp1* and Δ*ppb1* were synthetically lethal, these disruption mutants were crossed. We did not obtain the

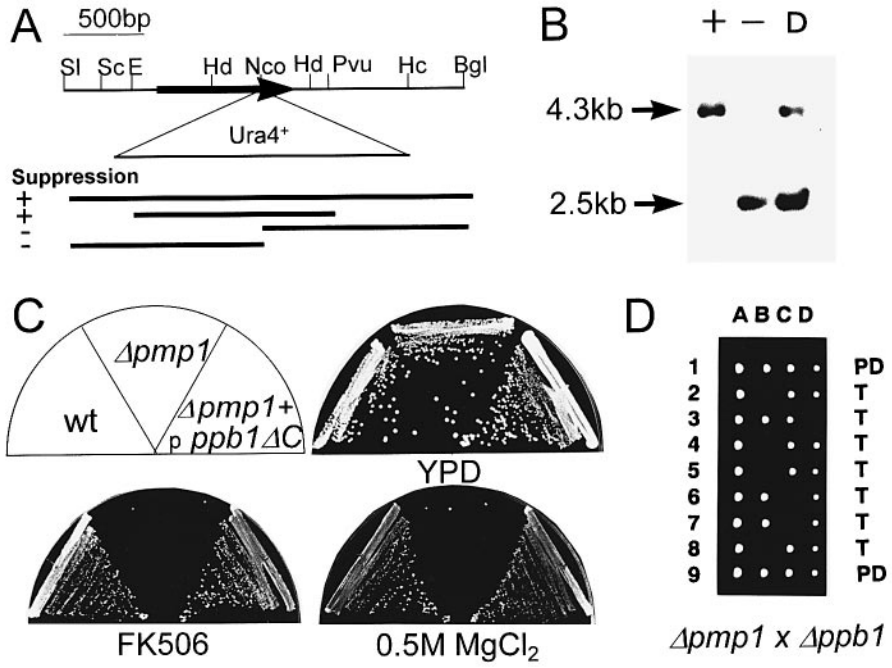

Fig. 5. Gene disruption of *pmp1*$^+$. (**A**) Restriction map and disruption of *pmp1*$^+$. The top line represents an abbreviated restriction map of the *pmp1*$^+$ region. Restriction fragments of the inserts were subcloned in pDB248 as shown, and their ability to complement Δ*ppb1* is indicated by (+) 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 *Nco*I site in the coding region. Restriction sites: Bg, *Bgl*II; E, *Eco*RI; Hc, *Hin*cII; Hd, *Hin*dIII; Nco, *Nco*I; Pvu, *Pvu*II; Sc, *Sac*I; Sl, *Sal*I. (**B**) Southern blot analysis of *pmp1*-deleted strains. *S.pombe* genomic DNA prepared from disruptant diploid cells (lane D), its haploid Ura$^+$ Δ*pmp1* cells (lane +) and Ura$^-$ progeny cells (lane –). Genomic DNAs were digested with *Eco*RI and *Bgl*II, separated by agarose gel electrophoresis and Southern blotted. The probe used for hybridization was a 0.5 kb *Hin*dIII 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 (*ppb1ΔC*) suppresses the phenotype of Δ*pmp1*. Δ*pmp1* cells transformed with vector (pDB248) or each of the multicopy plasmids carrying the wild-type *pmp1*$^+$(wt), *ppb1*ΔC, were streaked onto rich YPD plate containing 0.5 M $MgCl_2$ and 0.5 μg/ml FK506, and incubated for 3 days at 33°C. (**D**) Δ*ppb1*Δ*pmp1* is synthetic lethal. Two strains Δ*pmp1* and Δ*ppb1* were crossed, and asci formed were dissected on YPD medium at 27°C. Single mutants grew well, whereas Δ*ppb1*Δ*pmp1* double mutants cells were non-viable. T: tetratype, PD: parental ditype.

Δ*ppb1*Δ*pmp1* double mutant (Figure 5D), showing that Pmp1 and calcineurin shared an essential function for viability. The relationship between calcineurin and *pmp1*$^+$ was explored further by constructing a plasmid carrying a constitutively active calcineurin mutant gene (*ppb1*ΔC), and its effect on the phenotypes of Δ*pmp1* 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-Engele *et al.*, 1995). Overproduction of the C-terminal truncated Ppb1 by plasmid p *ppb1*ΔC, but not of the full-length Ppb1 (data not shown), dramatically improved the growth of Δ*pmp1*, allowing Δ*pmp1* 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.

Δpmp1 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 Δ*pmp1* 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 Δ*pmp1* (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.

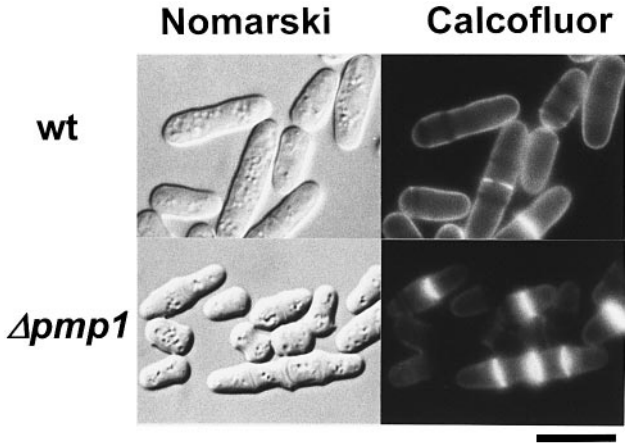

Fig. 6. Aberrant cell morphology of Δ*pmp1*. Exponentially growing wild-type (wt) and Δ*pmp1* cells are shown. Wild-type and Δ*pmp1* cells were cultured in the YPD liquid medium at 33°C. Cells were fixed with formaldehyde, washed in the phosphate saline buffer and stained with Calcofluor. The same cells were also observed by Nomarski. The bar indicates 10 μm.

Deletion of a MAP kinase homolog suppresses the Cl$^-$-sensitive phenotype of Δppb1

We considered the possibility that, if Pmp1 suppressed the Cl^- sensitivity of Δ*ppb1* through its phosphatase activity toward a MAP kinase, loss of that MAP kinase function might also suppress the phenotype of Δ*ppb1*. Three MAP

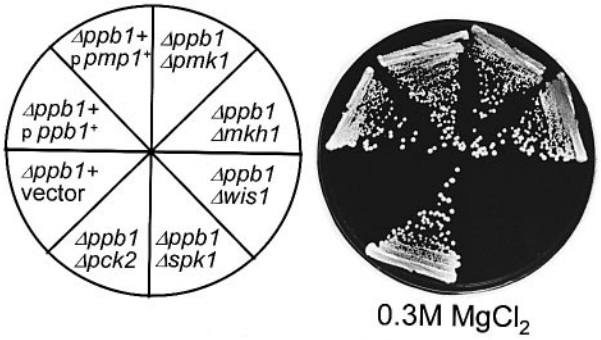

Fig. 7. Suppression of the Cl^- sensitivity of Δ*ppb1* by gene disruptions (Δ*pmk1*, Δ*mkh1* or Δ*pck2*) or elevated gene dosage of *pmp1*$^+$. Δ*ppb1* cells transformed with the control vector pDB248, each of the multicopy plasmids carrying *ppb1*$^+$or *pmp1*$^+$, and the indicated double mutants were streaked onto rich YPD plates containing 0.3 M $MgCl_2$ and incubated for 3 days at 33°C.

kinase pathways have been identified in fission yeast, namely Spk1 (Toda *et al.*, 1991; Gotoh *et al.*, 1993), Sty1/Spc1/Phh1 (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 Δ*ppb1* 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 Δ*ppb1*Δ*pmk1*, whereas it was not with Δ*ppb1*Δ*spk1* or Δ*ppb1*Δ*wis1* (*wis1*$^+$ encodes a MAP kinase kinase for Sty1/Spc1 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 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 Δ*ppb1* (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–Pmp1^{C158S} 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–Pmp1^{C158S} 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).

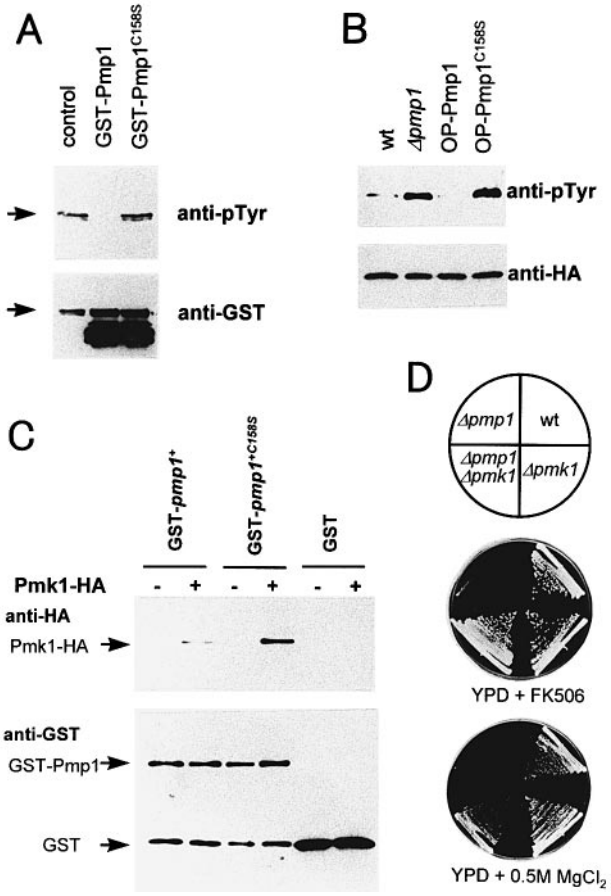

Fig. 8. Pmp1 dephosphorylates Pmk1 MAP kinase *in vitro* and *in vivo*. (**A**) Pmp1 phosphatase directly dephosphorylates Pmk1 *in vitro*. Log phase cultures of *S.pombe* cells expressing GST–Pmk1 (GST–Spm1, Zaitsevskaya-Carter and Cooper, 1997) were used. GST–Pmk1 was purified by glutathione–Sepharose and incubated either in the presence of no protein (control), 1 μg of purified GST–Pmp1 fusion protein or 1 μg of purified GST–Pmp1^{C158S} fusion protein for 30 min at 33°C. Immunoblotting was performed using antibodies against phosphotyrosine (anti-pTyr) or GST (anti-GST). The position of GST–Pmk1 is indicated by arrows. (**B**) Pmp1 regulates the level of tyrosine phosphorylation in Pmk1 *in vivo*. Wild-type cells (wt) containing pREP42-*pmk1*$^+$-HA/6His and either pREP1-GST vector, pREP1-GST–*pmp1*$^+$ or pREP1-GST–*pmp1*^{C158S} were grown in the minimal EMM medium. Δ*pmp1* cells were transformed with pREP41-*pmk1*$^+$-HA/6His. Pmk1 was isolated by Ni^{2+}-NTA affinity precipitation and immunoblotted using anti-phosphotyrosine (anti-pTyr) or anti-HA (anti-HA) antibodies. (**C**) Pmp1 associates with Pmk1 MAP kinase *in vivo*. Wild-type cells containing pREP42-*pmk1*$^+$-HA/6His (+ lanes) or pREP42 (control plasmid; – lanes) and either pREP1-GST–*pmp1*$^+$ (GST–*pmp1*$^+$), pREP1-GST–*pmp1*$^{+C158S}$ (GST–*pmp1*$^{+C158S}$) or pREP1-GST control plasmid (GST) were grown in EMM medium in the absence of thiamine. GST, GST–Pmp1 and GST–Pmp1^{C158S} were precipitated by glutathione beads, washed extensively and subjected to SDS–PAGE and immunoblotted using anti-HA or anti-GST antibodies. The ECL system was employed for detection (Amersham). (**D**) Suppression of the FK506 and Cl^- sensitivity of Δ*pmp1* by gene disruption of Δ*pmk1*. One set of tetra-type tetrad segregants derived from a cross between Δ*pmp1* and Δ*pmk1* was streaked on rich YPD plates containing 0.5 μg/ml FK506 or 0.5 M $MgCl_2$ and incubated at 33°C for 3 days.

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 Δ*pmp1* cells,

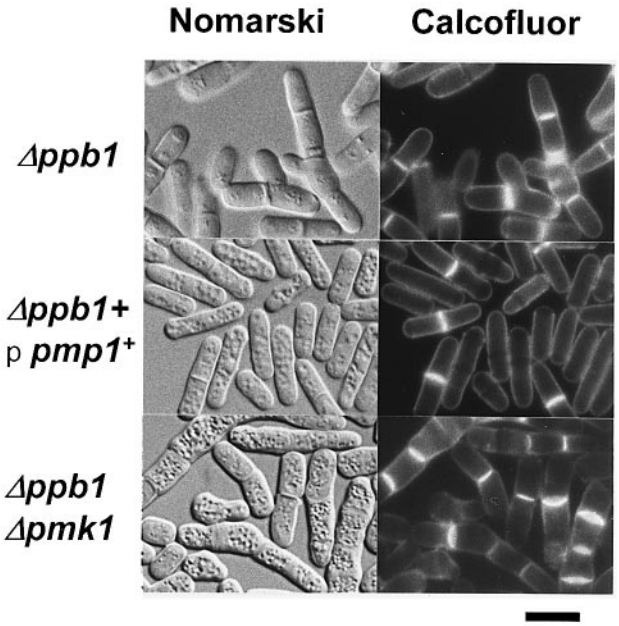

Fig. 9. Suppression of cytokinesis abnormality of Δ*ppb1* by multicopy plasmid carrying the *pmp1*$^+$ (p *pmp1*$^+$) but not by the deletion of *pmk1*$^+$ (Δ*pmk1*). Δ*ppb1* cells contain the control vector. Cells were fixed with formaldehyde, stained with Calcofluor and observed by either fluorescence microscopy (Calcofluor) or differential interference contrast microscopy (Nomarski). The bar indicates 10 μm.

but almost completely abolished in cells overproducing Pmp1. In sharp contrast, overproduction of catalytically inactive Pmp1^{C158S} resulted in an elevated level of tyrosine-phosphorylated Pmk1 (Figure 8B).

With the goal of establishing that Pmk1 MAP kinase is a direct target of Pmp1 phosphatase *in vivo*, we then asked whether Pmp1 and Pmk1 physically interacted. For this, hemagglutinin (HA) epitope-tagged Pmk1 and GST–Pmp1, GST–Pmp1^{C158S} or unfused GST were co-expressed in *S.pombe*, followed by precipitation with glutathione beads. Beads were washed thoroughly and analyzed by immunoblotting using anti-HA and anti-GST antibodies. The results obtained (Figure 8C) clearly show that HA-tagged Pmk1 co-precipitates with GST–Pmp1, but not with unfused GST. A much larger amount of Pmk1 co-precipitates with GST–Pmp1^{C158S}. Immunoblotting using anti-GST antibody confirmed that GST, GST–Pmp1 and GST– Pmp1^{C158S} were present at approximately equal levels (Figure 8C, lower panel). In contrast to the results obtained with CL100/MKP-1 (Sun *et al.*, 1993), Pmp1 phosphatase appears not to require mutation of the essential cysteine within the active site of the enzyme to form a physical complex with Pmk1 *in vivo* (Figure 8C). Similar results have been reported previously for the mammalian MAP kinase phosphatase Pyst1 (Groom *et al.*, 1996). Unlike CL100/MKP-1, the Pyst1 enzyme shows substrate selectivity for the classical MAP kinase isoforms. Taken together, these results suggest that the ability of the wild-type phosphatase to physically interact with a particular MAP kinase may be a more robust indicator of a *bona fide in vivo* substrate than substrate trapping experiments using the mutant phosphatase.

During these experiments, we noted that overexpression of not only *pmp1*$^+$ but also *pmp1*$^{+C158S}$ was able to suppress the Cl$^-$ sensitivity of Δ*ppb1* (data not shown),

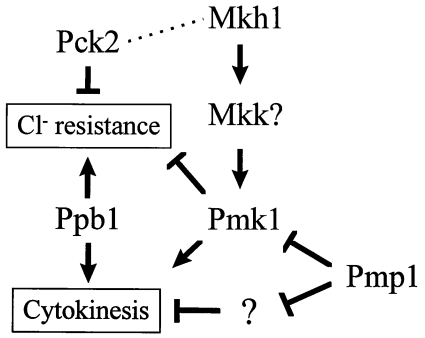

Fig. 10. A schematic presentation of interactions among Pmp1, Ppb1, Mkh1, Pmk1 and Pck2. Arrows indicate positive or activating interactions, and the lines with bars indicate negative or inhibitory interactions. '?' denotes a hypothetical target molecule of Pmp1. 'MKK?' represents an as yet unidentified MAP kinase kinase.

although the level of suppression by *pmp1*$^{+C158S}$ was less than that by wild-type *pmp1*$^+$. One possible interpretation is that, while overproduced Pmp1 can suppress the phenotype of Δ*ppb1* by reducing the level of tyrosine-phosphorylated Pmk1, the suppression by Pmp1^{C158S} may occur through a tight binding between the mutant Pmp1^{C158S} and phosphorylated 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 Pmp1^{C158S} 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 Δ*pmp1*Δ*pmk1* was no longer sensitive to FK506 or Cl$^-$ (Figure 8D). Second, the lethality of Δ*ppb1*Δ*pmp1* was overcome in the triple mutant Δ*ppb1*Δ*pmp1*Δ*pmk1* (data not shown). Third, introduction of the *pmp1*$^+$ gene into Δ*ppb1*Δ*pmk1* 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 Δ*ppb1* cells contained multiple septa due to incomplete cytokinesis (Yoshida *et al.*, 1994). Overexpression of *pmp1*$^+$ suppressed this cytokinesis abnormality of Δ*ppb1*, and cells with multisepta became negligible (Figure 9). Pmp1 and Ppb1 phosphatases therefore appear to share an essential function in cytokinesis. However, Δ*ppb1*Δ*pmk1* 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. If Pmk1 was the sole target of Pmp1, Δ*ppb1*Δ*pmk1* 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

Table I. Fission yeast strains used in this study

Strains	Genotypes	Derivations
HM123	*h⁻ leu1*	our stock
5A/1D	*h⁻/h⁺ leu1/leu1 ura4/ura4+/his2 ade6-M210/ade6-M216*	our stock
TP319-31A	*h⁻ leu1 ura4 pmk1::ura4⁺*	Toda *et al.* (1996)
TP114-9C	*h⁹⁰ leu1 ura4 spk1::ura4⁺*	Toda *et al.* (1991)
ED1011	*h⁻ leu1 his1 wis1::his1⁺*	obtained from Dr P.Fantes
TP170-2B	*h⁻ leu1 pck2::LEU2*	Toda *et al.* (1993)
TP193-1B	*h⁻ leu1 pck2::LEU2 ppb1::ura4⁺*	this study
KP119	*h⁺ leu1 ura4 ppb1::ura4⁺*	Yoshida *et al.* (1994)
KP251	*h⁻ leu1 ura4 pmk1::ura4⁺ ppb1::ura4⁺*	this study
KP252	*h⁻ leu1 ura4 wis1::his1⁺ ppb1::ura4⁺*	this study
KP253	*h⁺ his2 ura4 pmk1::ura4⁺ ppb1::ura4⁺*	this study
KP254	*h⁻ leu1 ura4 spk1::ura4⁺ ppb1::ura4⁺*	this study
KP264	*h⁻ leu1 ura4 pmk1::ura4⁺ pmp1::ura4⁺*	this study
KP266	*h⁺ his2 ura4 pmp1::ura4⁺*	this study
KP268	*h⁻ leu1 ura4 pmp1::ura4⁺*	this study
SDY1	*h⁻ leu1 ura4 mkh1::ura4⁺*	Toda *et al.* (unpublished)
KP468	*h⁻ leu1 ura4 mkh1::ura4⁺ ppb1::ura4⁺*	this study

phenotype of Δ*ppb1* was used to identify a novel MAP kinase phosphatase gene *pmp1⁺* as a multicopy suppressor. Pmp1 phosphatase is not essential for cell viability, but shares an essential function with Ppb1 in cell viability. They act synergistically in Cl^- homeostasis and cytokinesis. We also show that Pmk1, the third MAP kinase of fission yeast, is a physiological target for Pmp1.

Pmp1 is a Pmk1 MAP kinase phosphatase

We provide evidence that (i) Pmp1 directly dephosphorylates Pmk1 on tyrosine residue *in vitro*, (ii) Pmp1 influences the tyrosine phosphorylation state of Pmk1 *in vivo* and (iii) Pmp1 associates with Pmk1 *in vivo*. Genetic interactions are also consistent with our conclusion that Pmp1 negatively regulates Pmk1 by direct dephosphorylation. Pmp1 phosphatase may thus be implicated in Cl^- sensitivity through down-regulating the Pmk1 MAP kinase pathway.

There was no detectable change in the pattern of tyrosine-phosphorylated proteins in cell lysates prepared from Δ*pmp1* cells when compared with wild-type (data not shown), suggesting that Pmp1 phosphatase possesses a restricted substrate specificity. Our preliminary findings that Pmp1 was unable to dephosphorylate a model substrate, myelin basic protein, containing either phosphotyrosine or phosphoserine were also supportive of the substrate specificity of Pmp1 phosphatase (R.Sugiura, H.Shuntoh and T.Kuno, unpublished). Thus, Pmp1 displays properties that are hallmarks of known MAP kinase phosphatases. More definitive proof that Pmp1 is a dual-specificity phosphatase, however, will await further biochemical analysis.

Role of Pmp1 and calcineurin in Cl^- homeostasis

We show that both calcineurin and Pmp1 phosphatases are involved in Cl^- homeostasis, as Δ*ppb1* or Δ*pmp1* cells become hypersensitive to Cl^-. Three lines of evidence show that Ppb1 and Pmp1 act closely in Cl^- homeostasis. First, Pmp1 overexpression rescues the Cl^- sensitivity of Δ*ppb1*. Secondly, Δ*pmp1* is acutely sensitive to FK506, an inhibitor of calcineurin, consistent with the fact that the double mutant Δ*pmp1*Δ*ppb1* is lethal. Third, a constitutively active calcineurin suppresses the drug- and Cl^--sensitive phenotypes of Δ*pmp1*. Taken together, these results suggest that Δ*pmp1* 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 Cl^- homeostasis.

The demonstration that other type 1 and 2A serine/threonine phosphatases failed to suppress the Cl^- sensitivity of Δ*ppb1* suggested that Ppb1 might dephosphorylate a key functional component of Cl^- regulation as a specific substrate. Furthermore, the inability of *pyp1⁺*, which inactivates the Sty1/Spc1 MAP kinase (Millar *et al.*, 1995; Shiozaki and Russell, 1995), to suppress the Cl^- sensitivity, suggests that Pyp1 may not be involved in the regulation of the Pmk1 MAP kinase pathway.

In mammalian cells, Cl^- 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 Δ*ppb1* 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 Δ*ppb1* 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 Cl^- sensitivity of Δ*ppb1* is rescued by disruption of *pmk1⁺*, encoding a MAP kinase (Toda *et al.*, 1966), or of *mkh1⁺*, encoding a putative MAP kinase kinase kinase of Pmk1 (Sengar *et al.*, 1997). Calcineurin and the Pmk1 MAP kinase pathway might have opposing roles in Cl^- homeostasis. One hypothesis to explain this counteractive interaction is that calcineurin and Pmk1 kinase might regulate a common target, essential for Cl^- 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

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). pR4L-GSpm1 was generously provided by Dr J.Cooper (Zaitsevskaya-Carter and Cooper, 1997) and pREP42-*pmk1*$^+$-HA/6His was constructed by cloning the *Nde*I–*Bam*HI fragment of pREP41-*pmk1*$^+$-HA/6His (Toda *et al.*, 1996) into *Nde*I–*Bam*HI-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 *Bam*HI 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 was inserted into *Nde*I–*Bam*HI-digested pREP1 (Maundrell, 1990), creating pREP1-GST. A *Bam*HI fragment containing the coding sequence of *pmp1*$^+$ was inserted into the *Bam*HI site of pREP1-GST, creating pREP1-GST-*pmp1*$^+$. pREP1-GST-*pmp1*$^+$ fully suppressed the phenotypes of Δ*pmp1* cells. Site-directed mutagenesis was performed using the Sculptor mutagenesis kit (Amersham).

Phosphatase assay

Hydrolysis of *p*NPP was done in 200 μl of a solution containing 0.1% β-mercaptoethanol, 10 mM *p*NPP, 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 one-step gene disruption by homologous recombination (Rothstein, 1983) was performed. The *pmp1::ura4*$^+$ disruption was constructed as follows. The 3.5 kb *Sal*I–*Bgl*II fragment containing the *pmp1*$^+$ gene was subcloned into the *Sal*I–*Bam*HI site of pUC119 to form pUC-pmp1. Plasmid pUC-pmp1 was cleaved at the single *Nco*I site located in the *pmp1*$^+$ coding region and blunt ended with Klenow polymerase. The resulting DNA was then ligated to the 1.8 kb *Hin*dIII 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 *Eco*RI–*Hin*cII fragment containing disrupted *pmp1*$^+$ was transformed into diploid cells (5A/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

*ppb1*ΔC, a truncated version of *ppb1*$^+$ (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-Engele *et al.* (1995).

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