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A calcineurin-like gene *ppb1*$^+$ in fission yeast: mutant defects in cytokinesis, cell polarity, mating and spindle pole body positioning

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SUMMARY

A calcineurin (type 2B)-like protein phosphatase gene designated *ppb1*$^+$ was isolated from the fission yeast *Schizosaccharomyces pombe*. The predicted amino acid sequence was 57% identical to rat PP2Bα. *ppb1* null mutant could form colonies at 33°C but the size of the colonies was small at 22°C. Cytokinesis was greatly delayed at 22°C, and a large number of multi-septate cells were produced. The cell polarity control was impaired, causing branched cells. *ppb1* null was virtually sterile. These phenotypes were rescued by a plasmid carrying the *ppb1*$^+$ gene. Multi-septate cells were also produced in wild type at 22°C by cyclosporin A, an inhibitor of calcineurin. This drug effect was enhanced in *stst1* null mutant, which was hypersensitive to various drugs and cations. *ppb1* null was not affected by cyclosporin A, consistent with the hypothesis that ppb1 is its target. Double-mutant analysis indicated that ppb1 had a function related to that of two other phosphatases, type 1-like dis2 and 2A-like ppa2. *ppb1* null-*sts1* null showed the severe multi-septate phenotype in the absence of cyclosporin A. *ppb1*$^+$ and *sts1*$^+$ gene functions are related. The double mutant *ppb1-sts5* was lethal, indicating that the *ppb1*$^+$ gene shared an essential function with the *sts5*$^+$ gene. Overexpression of *ppb1*$^+$ caused anomalies in cell and nuclear shape, microtubule arrays and spindle pole body positioning in interphase cells. Thus the *ppb1*$^+$ gene appears to be involved in cytokinesis, mating, transport, nuclear and spindle pole body positioning, and cell shape.

Key words: calcineurin, protein phosphatase, cytokinesis, fission yeast, immunosuppressant, microtubule

INTRODUCTION

Calcineurin, Ca^{2+}/calmodulin-regulated type 2B protein phosphatase, initially identified in muscle and brain (Stewart et al., 1982, 1983), is ubiquitous from yeast (Cyert et al., 1991; Liu et al., 1991a) to mammals (Klee et al., 1988; Ito et al., 1989). It consists of a heterodimer of a 61 kDa calmodulin-binding catalytic subunit, calcineurin A, and a 19 kDa Ca^{2+}-binding protein, calcineurin B. A fully active, trimeric phosphatase is formed by reversible association of calmodulin with the heterodimer in the presence of Ca^{2+}. Among protein serine/threonine phosphatases (Cohen, 1989), calcineurin is the only class of phosphatase that requires Ca^{2+} and calmodulin for activity. This phosphatase also exhibits a more restricted substrate specificity in vitro than do other serine/threonine phosphatases.

Calcineurin is involved in various physiological functions. It appears to control highly specialized events, such as *Paramecium* exocytosis (Momayezi et al., 1987) and sperm flagellar movement (Tash et al., 1988). Calcineurin was found to be the target of immunosuppressive agents (Liu et al., 1991b; reviewed by McKeon, 1991) such as cyclosporin A and FK506, which suppress the T cell-mediated immune response. These agents interact with a group of proteins called immunophilins (Handschumacker et al., 1984; Harding et al., 1986, 1989), cyclophilin and FKBP (FK506-binding protein), and the associated complexes are bound to calcineurin and inhibit the activity. Cyclosporin A effectively inhibits the induction of cytokine gene transcription in T cell activation by blocking nuclear translocation of a cytoplasmic subunit of NF-AT, a transcription factor implicated in the activation of the cytokine gene (Flanagan et al., 1991). Hence calcineurin is thought to be required for an early step in this transcriptional activation.

Although calcineurin has been extensively characterized in vitro and in vivo, much less is known about its gene functions in cell viability or cell division cycle control. In the budding yeast *Saccharomyces cerevisiae*, two calcineurin A-like genes have been identified (*CNA1/CMP1* and *CNA2/CMP2*; Cyert et al., 1991; Liu et al., 1991a). The predicted CNA1/CMP1 gene product is 54% identical to rat PP2Bα. Haploid cells containing single or double null mutations were viable, indicating that these genes are not essential for normal cell growth. These genes do, however, appear to antagonize the mating-pheromone response pathway; MATα *cna1 cna2* mutants failed to resume growth during continuous exposure to a-factor (Cyert et al., 1991). Type 2B-like phosphatases in budding yeast are also involved in adaptation to high salt stress conditions (Nakamura et al., 1993).

We here report the isolation and initial characterization of a calcineurin A-like gene *ppb1*$^+$ in the fission yeast *Schizosaccharomyces pombe*. Although *ppb1*$^+$ is non-essential for viability, pleiotropic cellular phenotypes were produced by null or overexpressed mutations. Furthermore, *ppb1* null became lethal or semi-lethal when it was combined with other mutations so that the in vivo role of *ppb1*$^+$ could be investigated.

MATERIALS AND METHODS

Yeast strains and media

A haploid *Schizosaccharomyces pombe* strain HM123 (*h*$^-$ *leu1 ura4*) was used as wild type. Standard genetic methods (Gutz et al., 1974; Moreno et al., 1991) were followed. Rich YPD (1% yeast extract, 2% bactopeptone and 2% glucose), sporulating SPA (1% glucose, 0.1% KH_2PO_4, and vitamins; Gutz et al., 1974), and synthetic EMM2 (Mitchison, 1970) were employed. Cyclosporin A (Sigma) was purchased. FK506 was a gift from the Fujisawa Pharmaceutical Co.

Plasmids

The vector plasmid used for the expression of the *ppb1*$^+$ gene in the *S. pombe* transformant was Bluescript SK(+) containing the *S. cerevisiae LEU2* and the *S. pombe* ARS sequence. pREP1 (Maundrell, 1990) was used for the induced overexpression of the *ppb1*$^+$ gene in *S. pombe.* The plasmid used for the production of fusion protein in *Escherichia coli* was pAR3038 (Studier and Moffatt, 1986). Transformation of *S. pombe* was done by the lithium method (Ito et al., 1983).

Cloning and gene disruption of the *ppb1*$^+$ gene

A mixed oligonucleotide 5′-CCANGTPuTCCATPuAA-3′ corresponding to the non-coding strand for an amino acid stretch FMDVFTW was made, then ^{32}P end-labeled and used to screen a cosmid library of *S. pombe* (Mizukami et al., 1993). Resulting hybridized cosmids were subcloned, and the 2.5 kb *Eco*RI fragment was found to contain the *ppb1*$^+$ gene. Southern hybridization under a non-stringent condition indicated that no other genomic DNA fragment was hybridized; only 2.5 kb *Eco*RI, 4.5 kb *Hin*dIII and 7.8 kb *Bgl*II bands, which corresponded to the obtained clone, were seen. The accession number of the nucleotide sequence is D28955. Gene disruption of the *ppb1*$^+$ gene was performed by one-step gene replacement (Rothstein, 1983). The *S. pombe ura4*$^+$ gene was inserted at the essential catalytic domain in the 2.5 kb *ppb1*$^+$ gene.

Construction of double mutants

ppb1 null cells carrying plasmid with the *ppb1*$^+$ gene were conjugated with various strains on SPA plates. For mating, *ppb1* null carrying the plasmid with the *ppb1*$^+$ gene was used. Resulting zygotes were sporulated and tetrads were dissected. As the marker gene for *ppb1* null was the *S. pombe ura4*$^+$, *ppb1* null could be distinguished by Ura$^+$.

Preparation of fusion protein

The method described by Studier and Moffatt (1986) was followed. The central to C-domain of Ppb1 protein (354 nucleotides long) was subcloned by the PCR method and ligated with the *Nde*I and *Bam*HI sites of pAR3038. Insoluble fusion protein was purified according to the procedure described by Watt et al. (1985).

Immunofluorescence microscopy

Cells were fixed by 3.7% formaldehyde at 33°C for 1 hour (Hagan and Hyams, 1988). For anti-tubulin TAT1 (Woods et al., 1989) and anti-sad1 antibodies (Funabiki et al., 1993), FITC-labeled sheep anti-rabbit IgG and rhodamine-conjugated goat anti-rabbit IgG were used, respectively, as the second antibodies.

Southern and colony hybridization, nucleotide sequencing and immunoblotting

The procedures described by Maniatis et al. (1982) for Southern and colony hybridization were followed. Oligonucleotide used for hybridization was end-labeled by T4 polynucleotide kinase using [^{32}P]dATP. It was hybridized at 37°C for 15 hours, and washed three times for 30 minutes each time. The dideoxy method (Sanger et al., 1977) and stepwise deletion (Yanisch-Perron et al., 1985) were employed for nucleotide sequencing.

RESULTS

ppb1$^+$ gene cloning

An oligonucleotide probe for the amino acid stretch FMDVFTM, conserved in rat calcineurin and budding yeast calcineurin-like gene products, was made and used for screening a previously constructed cosmid library of *S. pombe* (Mizukami et al., 1993). The probe sequence was not present in other types (1, 2A and 2C) of protein serine/threonine phosphatases.

A set of hybridization bands (7.8 kb *Bgl*II, 4.5 kb *Hin*dIII and 2.5 kb *Eco*RI) was obtained for the *S. pombe* genomic DNA when the ^{32}P end-labeled probe was used (data not shown). The sizes were consistent with those of the cloned *ppb1*$^+$ gene (described below).

Four cosmids (412, 638, 1059 and 1346) were obtained, derived from the same genomic region (Mizukami et al., 1993). By subcloning, we obtained a minimal hybridizing 2.5 kb *Eco*RI fragment, which was present in all the four cosmids. The sequence in this fragment was unique in the *S. pombe* genome; only a single hybridizing band was obtained under a non-stringent hybridization condition. The chromosomal locus of the cloned DNA was 200 kb from the *sts1*$^+$ gene (Shimanuki et al., 1992) in chromosome I, according to the ordered cosmid map (Mizukami et al., 1993).

Nucleotide sequencing and predicted amino acid sequence of *ppb1*$^+$

Nucleotide sequencing indicated the presence of a single coding region (designated *ppb1*$^+$) in the cloned 2.5 kb fragment. Nucleotides with the predicted amino acid sequence are shown in Fig. 1A. Two putative introns having the consensus sequences (Mertins and Gallwitz, 1987) are present near the N terminus: intron 1 (45 bp long) locates at amino acid position 47, while intron 2 (155 bp long) locates at position 116. The hypothetical Ppb1 protein consists of 554 amino acids (calculated molecular mass, 64 kDa).

The predicted amino acid sequence is similar to that of rat calcineurin CNAA/PP2Bα and budding yeast CNA1/CMP1 (Fig. 1B). The identity between Ppb1 and rat CNAAα was 57% higher than that between Ppb1 and CMP1/CNA1 (45%) or Ppb1 and CMP2/CNA2 (51%).

These three proteins have the highest similarity (~80% identity) in the central region. The catalytic domain resides in the NH_2-domain (amino acids 100-350), whereas the calcineurin/PP2B-specific domain spans the COOH-domain. The sequences for binding to the regulatory subunit B of calcineurin or to calmodulin and the phosphorylation site are also preserved in Ppb1.

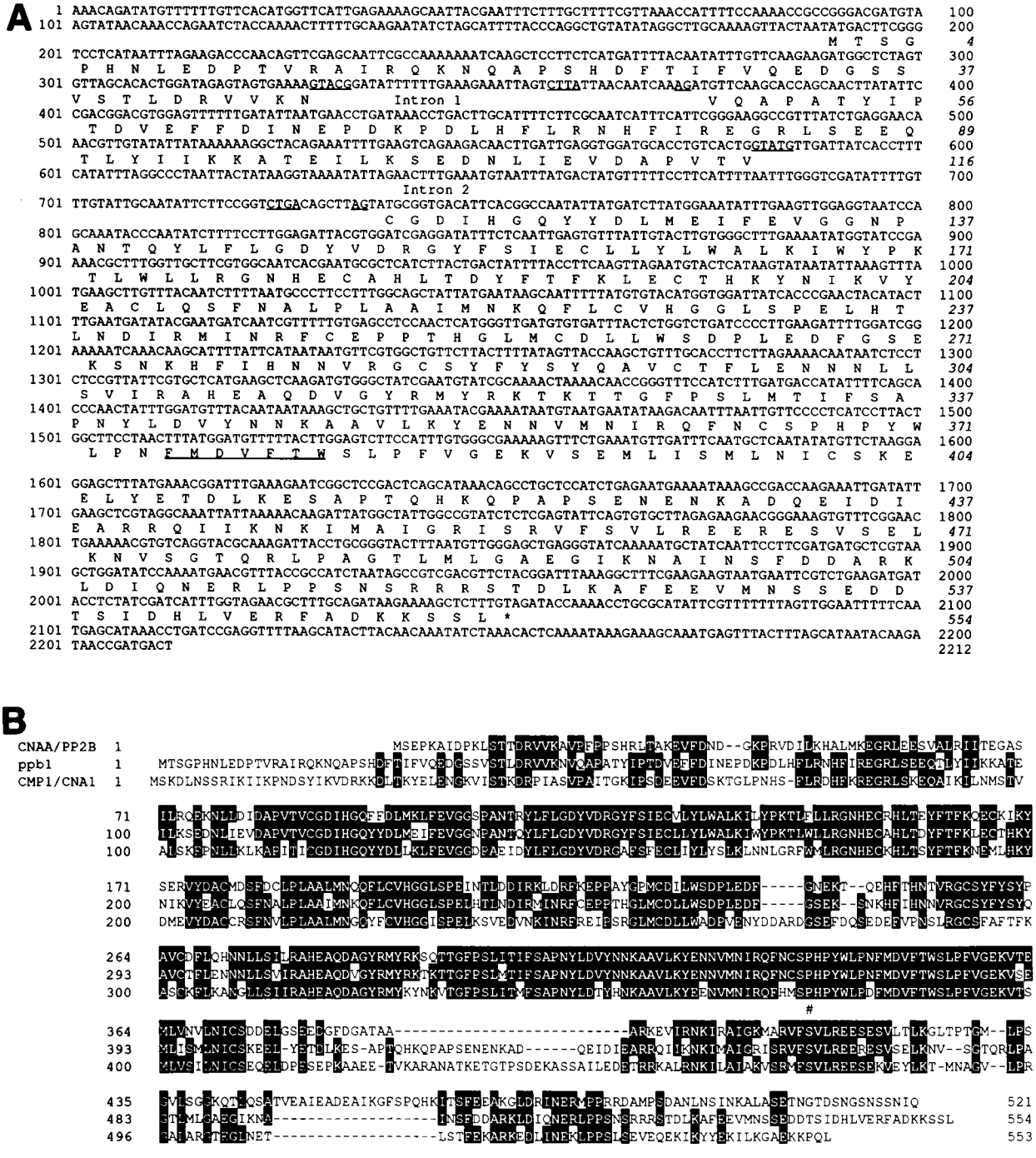

Fig. 1. Nucleotide and predicted amino acid sequences of the fission yeast *ppb1*$^{+}$ gene. (A) Assuming two introns in the N terminus, the predicted protein has 554 amino acids (calculated molecular mass, 64 kDa). The underlined nucleotides indicate the consensus for the introns in fission yeast. The underlined amino acid stretch FMDVFTW was used for making the probe for hybridization. (B) The *ppb1*$^{+}$ gene product is very similar to rat calcineurin CNAA, a Ca^{2+}-dependent type 2B protein phosphatase (PP2B) and the budding yeast CMP1/CNA1, one of the two calcineurin-like homologs present in this organism. Identical amino acids are boxed. The serine residue with the symbol # is a conserved phosphorylation site.

Disruption of the *ppb1*$^{+}$ gene

Disruption of the *ppb1*$^{+}$ gene was performed using the 1.8 kb *S. pombe ura4*$^{+}$ gene (Grimm et al., 1988), which was inserted into the essential catalytic domain in the 2.5 kb *ppb1*$^{+}$ gene (Fig. 2A). A linearized DNA fragment containing the interrupted *ppb1* gene was integrated into the chromosome of a diploid by homologous recombination. The resulting heterozygous diploid was Southern hybridized with the *ppb1*$^{+}$ gene probe: *Eco*RI bands with the expected sizes (2.5 and 4.3 kb) were obtained (Fig. 2B). The transformed diploids were then sporulated, and the resulting tetrads were dissected.

All the four spores produced colonies at 33°C, showing the

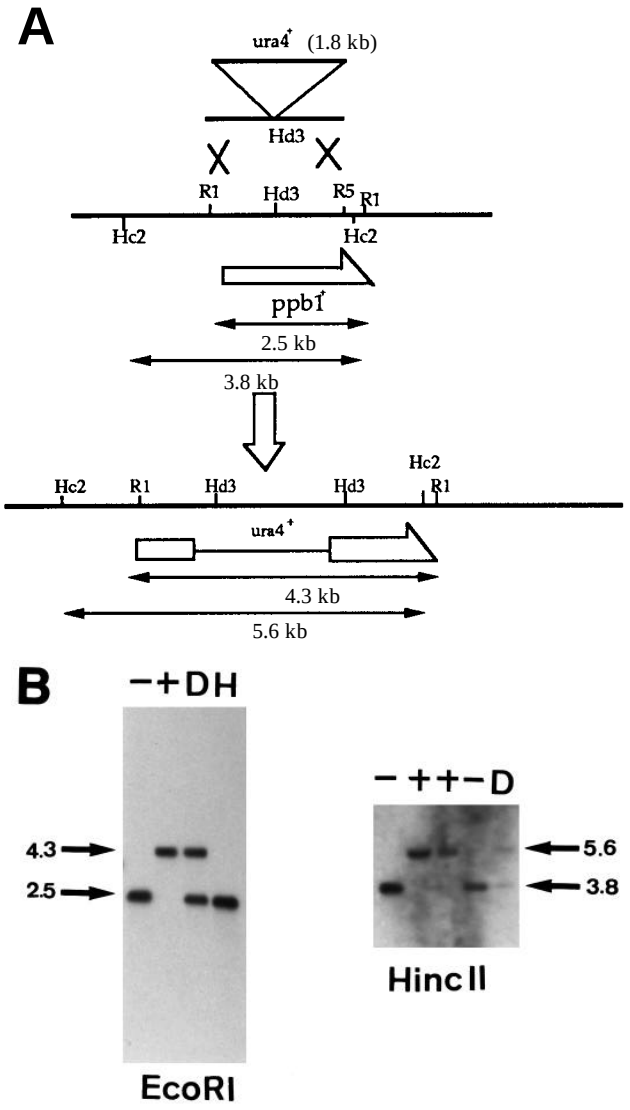

Fig. 2. Gene disruption of *ppb1*$^+$. (A) The *ppb1*$^+$ gene (the coding region indicated by the arrow) was disrupted by one-step gene replacement (Rothstein, 1983) using the *S. pombe ura4*$^+$ gene, which was inserted at the *Hin*dIII site in the coding region. The length (kb) of the restriction fragments expected to be produced by disruption is shown. Restriction sites: *Eco*RI, RI; *Hin*dIII, Hd3; *Eco*RV, R5; *Hin*cII, Hc2. (B) Genomic Southern hybridization of heterozygous diploid disruptant (D), haploid segregants with the Ura$^+$ (+) or Ura$^-$ (−) marker. The genomic DNAs were isolated and digested by *Hin*cII or *Eco*RI. The probe used for hybridization was the 2.5 kb *Eco*RI fragment. + and – represent, respectively, Ura$^+$ and Ura$^-$. D, heterozygous diploid; H, wild type.

Ura$^+$:Ura$^-$=2:2 segregation. Genomic hybridization of haploid segregants showed that Ura$^+$ segregants had the 4.3 kb *Eco*RI bands, whereas Ura$^-$ haploids had a 2.5 kb *Eco*RI band (Fig. 2B). Haploid segregants containing the disrupted *ppb1* gene were viable, indicating that the *ppb1*$^+$ gene was non-essential for viability.

Phenotype of *ppb1* null

Haploid segregants of *ppb1* null displayed a noticeable growth defect at low temperature: *ppb1* null cells on the rich YPD plates formed small colonies at 22°C. Normal-sized colonies were produced at 33°C.

Table 1. Cell length and the frequencies of multi-nuclear, branched cells in *ppb1* null at 22°C

	Wild type		*ppb1* null	
Strains	33°C	22°C	33°C	22°C
Cell length (μm)	10.9	11.3	12.4	16.1
Number of nuclei per cell (% frequency)				
1	0	79	60	33
2	20	21	39	49
>3	0	0	2	18
Branched cell (% frequency)	0	0	0.4	8.4

Wild-type and *ppb1* null cells were grown at 22°C or 33°C for 8 hours in rich YPD medium. More than 300 cells were counted for each measurement.

By 4′,6-diamidino-2-phenylindole (DAPI) staining, a small fraction of abnormal, multiply septate and branched cells (indicated by arrowheads) were observed at 33°C (Fig. 3A; bottom left). At 22°C, *ppb1* null cells were greatly elongated with septa (average cell length, 16 μm), occasionally (8.4%) branched, and contained abundant (67%) multiple nuclei (Fig. 3; bottom right). Each compartment formed by the septa usually contained a single nucleus, but not always. The size of compartments was often smaller than that of single wild-type cells; multiple septa were found adjacent. Thus not only was the progression of cytokinesis delayed, but also positioning of septation became abnormal in the null mutant. Such defects were enhanced at 22°C. The wild-type control at 33°C and 22°C is shown in the top panel of Fig. 3A. No branching or delay in cytokinesis was found at either temperature.

The results of quantitative measurements are summarized in Table 1. The average cell length of *ppb1* null at 22°C was 40% larger than that of wild type. The number of cells containing more than three nuclei was negligible in the wild type, but accounted for 18% in *ppb1* null at 22°C. The frequency of branching in *ppb1* null was unusually high, considering that the branched cell was not seen at all in wild-type culture.

The rate of the cell number increase of *ppb1* null at 22°C was significantly lower (approximately 50%) than that of wild type. On the other hand, the increase in the turbidity of cultures was similar between the two at 22°C after the shift from 33°C, supporting the conclusion that the frequency of cytokinesis, rather than cell mass increase, was severely affected in *ppb1* null at the temperature.

Another significant phenotype of *ppb1* null was its striking reduction in the mating efficiency at both 33°C and 22°C; *ppb1* null was virtually sterile. All crosses thus were done using *ppb1* null carrying multicopy plasmid with the *ppb1*$^+$ gene, which recovered normal mating frequency as well as other defects described above. Another noteworthy property of *ppb1* null was that, in liquid culture at 22°C, cells became sticky, and formed aggregates. The cultures were thereafter briefly sonicated before light microscopy and cell number counting. At 33°C, cells did not form aggregates.

Microtubule distribution in *ppb1* null cells

Examination of the microtubule architecture was done using anti-tubulin antibody. *ppb1* null (ppb1) and wild-type (wt) cells

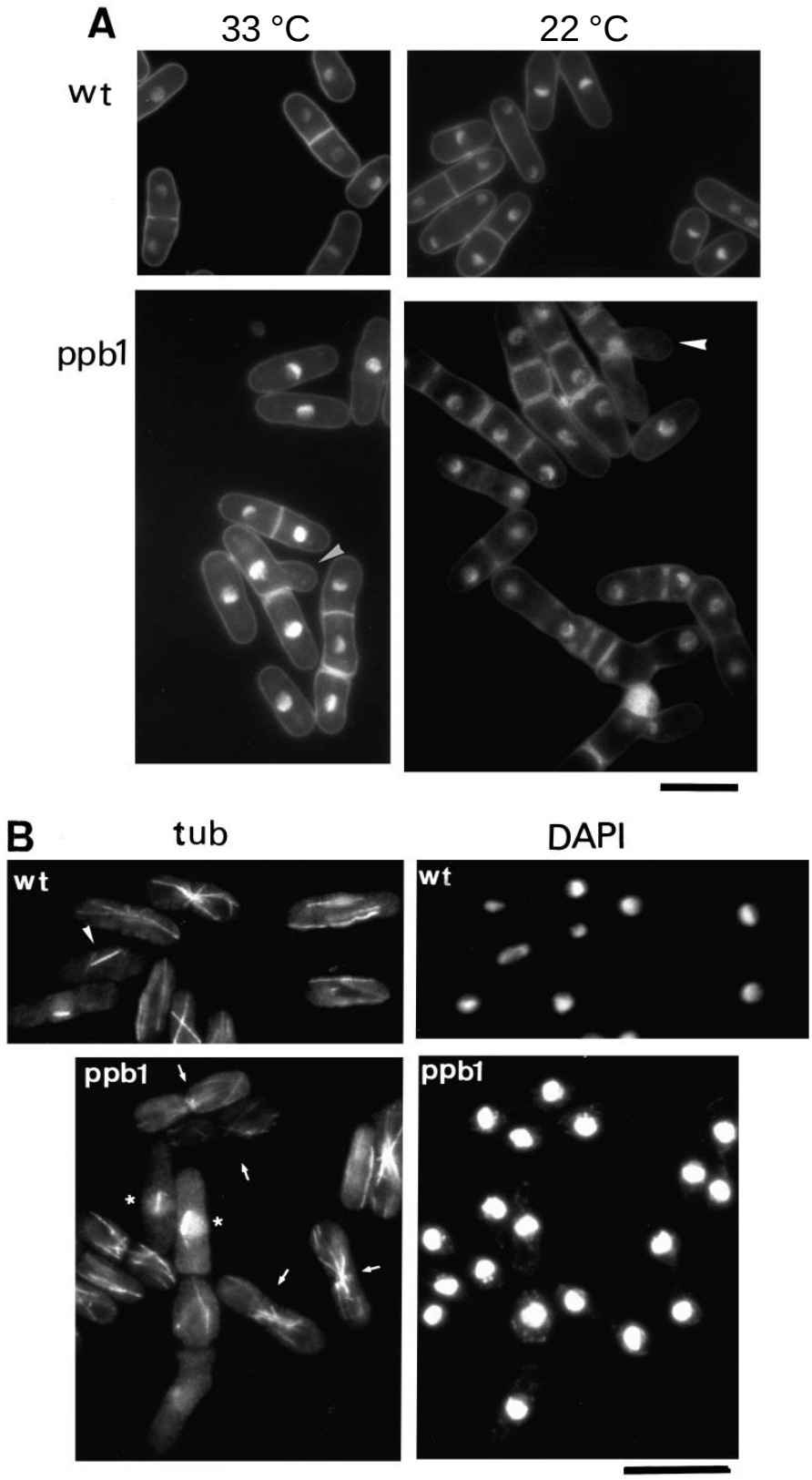

Fig. 3. Multi-septate and branched phenotypes of *ppb1* null mutant. (A) Wild-type (wt) and *ppb1* null (ppb1) cells grown at 33°C and 22°C were stained by DAPI. At 22°C, *ppb1* null cells were elongated, multi-nucleate and branched. At 33°C, *ppb1* null displayed similar but less frequent cells. The branched cells are indicated by the arrowheads. (B) *ppb1* null stained by anti-tubulin antibodies. Haploid *ppb1* null (ppb1) and wild-type (wt) cells were fixed and stained by anti-tubulin antibody TAT1 (Materials and Methods). Binuclear cells in *ppb1* null showed the post-anaphase microtubule arrays (indicated by the arrows) rather than the anaphase spindle seen in wild-type cells (arrowhead). Cells containing the short mitotic spindle are indicated by the asterisks. Bar, 10 μm.

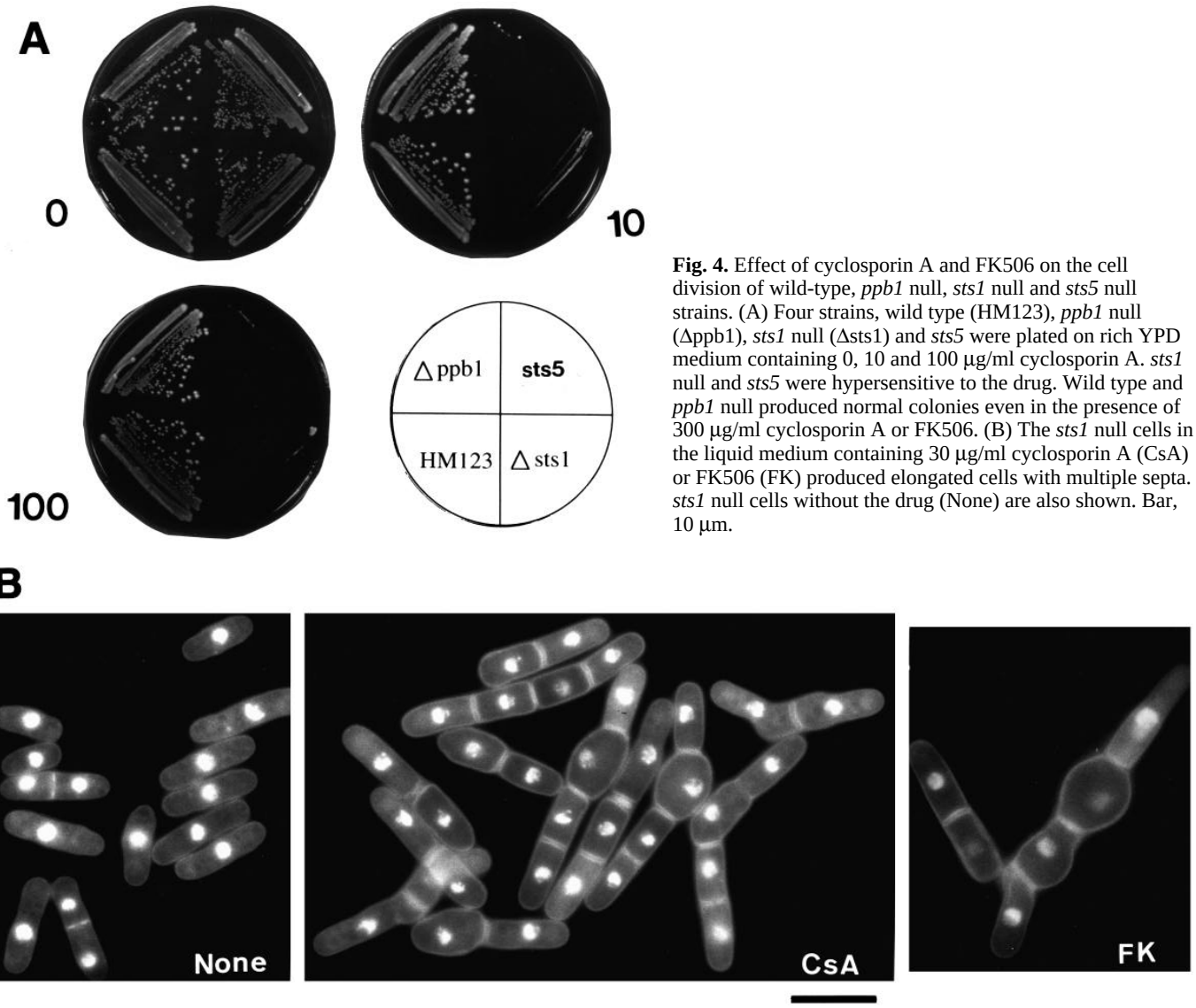

Fig. 4. Effect of cyclosporin A and FK506 on the cell division of wild-type, *ppb1* null, *sts1* null and *sts5* null strains. (A) Four strains, wild type (HM123), *ppb1* null (Δppb1), *sts1* null (Δsts1) and *sts5* were plated on rich YPD medium containing 0, 10 and 100 μg/ml cyclosporin A. *sts1* null and *sts5* were hypersensitive to the drug. Wild type and *ppb1* null produced normal colonies even in the presence of 300 μg/ml cyclosporin A or FK506. (B) The *sts1* null cells in the liquid medium containing 30 μg/ml cyclosporin A (CsA) or FK506 (FK) produced elongated cells with multiple septa. *sts1* null cells without the drug (None) are also shown. Bar, 10 μm.

were incubated at 22°C for 8 hours (Fig. 3B), then fixed and stained by monoclonal anti-tubulin antibody TAT1 (Materials and Methods). Binuclear cells in *ppb1* null cells displayed mostly the X-shaped post-anaphase microtubules (indicated by the arrows) that were characteristic of cells just prior to cytokinesis (Hagan and Hyams, 1988). The binuclear cells hence had finished nuclear division but not cytokinesis. *ppb1* null cells containing the short spindle of early mitotic stage were also plentiful. A wild-type control cell with the elongated spindle (top left) is indicated by an arrowhead; the extended, segregating chromosomes were seen by DAPI. Such anaphase cells were infrequent in *ppb1* null cells. Uni-nucleate cells displaying cytoplasmic microtubule arrays were abundant in both wild type and *ppb1* null; they represented the interphase cells. The microtubule distributions in *ppb1* null thus confirmed that cells were greatly delayed in cytokinesis. Early mitotic stage might also be slightly delayed.

Effect of cyclosporin A on fission yeast cell division

Because the *ppb1*$^+$ phosphatase gene product was a potential target of cyclosporin A, we examined whether the drug had any effect on cell division of wild-type fission yeast or *ppb1* null. From the size of colonies on plates containing cyclosporin A (0-300 μg/ml), no significant difference was seen between wild type and *ppb1* null in the size of colonies at 22°C and 33°C (Fig. 4A). Both could produce colonies even in the presence of 300 μg/ml cyclosporin.

Although the size of colony was normal, many multi-nucleate cells nearly identical to those found in *ppb1* null at 22°C were observed in wild type at 22°C in both liquid and plate cultures containing 30 μg/ml cyclosporin A, whereas uni-nucleate cells predominated in the same drug concentration at 33°C (about 60 and 20% multi-nucleate cells at 22°C and 33°C, respectively). The frequency of multi-nucleate cells at 22°C was also similar to the value obtained for *ppb1* null in the absence of the drug at 22°C. Cyclosporin A hence appeared to impair the normal process of cell division in wild type at 22°C. Interestingly, the frequency of multi-nucleate cells in *ppb1* null did not change with the addition of cyclosporin A. If the *ppb1*$^+$ gene product was the sole target of cyclosporin A, the growth of *ppb1* null would not be influenced by the drug.

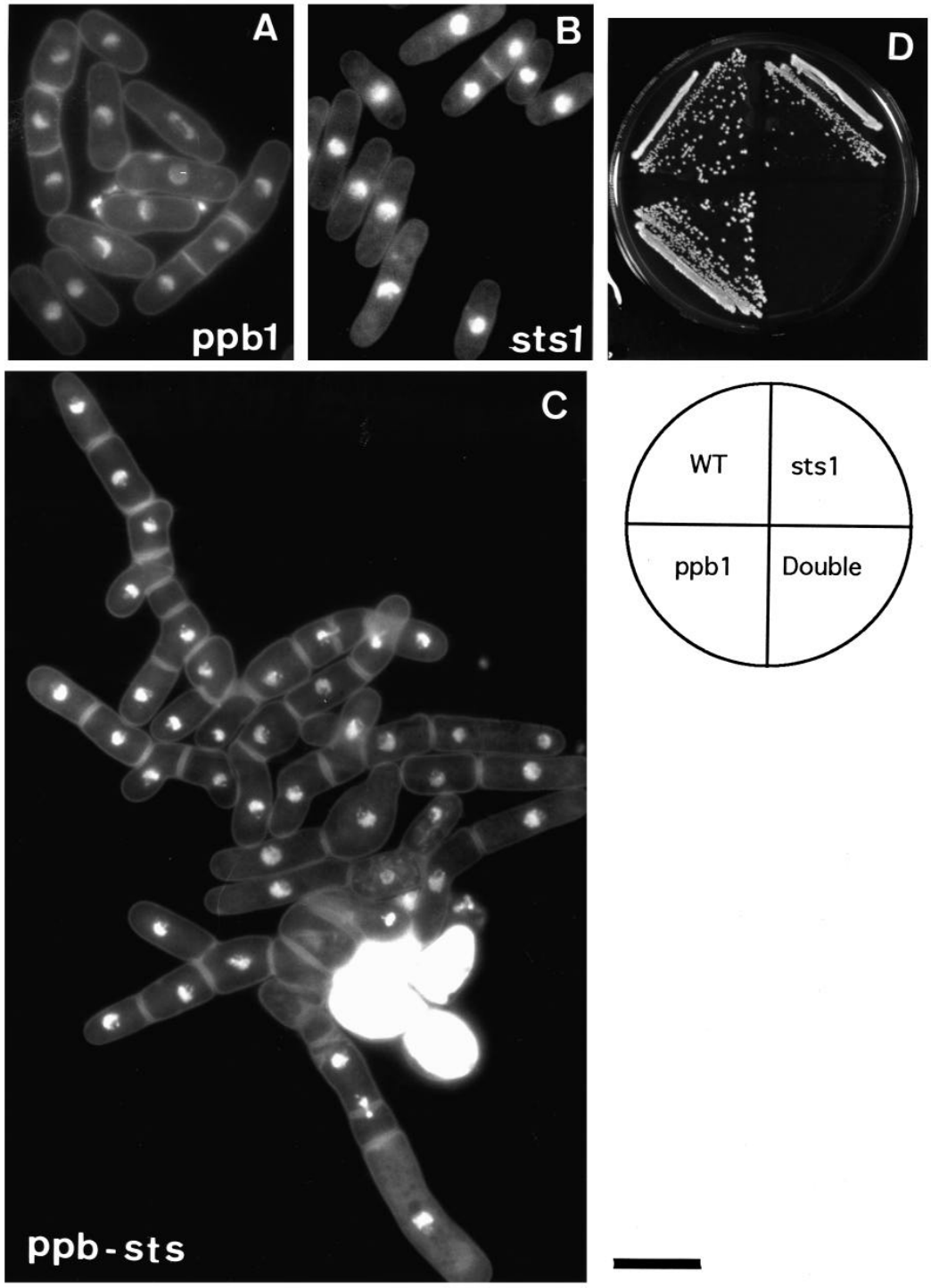

Fig. 5. Phenotype of *ppb1* null-*sts1* null double mutant. (A-C) DAPI-stained fluorescence micrographs of single *ppb1* null (A), *sts1* null (B) and double disruption mutants *ppb1* null-*sts1* null (C). Cells were grown in YPD liquid medium for 16 hours at 33°C. The cellular phenotypes were similar to that of single *sts1* in the presence of drug. Bar, 10 μm. (D) Wild type (WT), single *ppb1* null (ppb1) and *sts1* null (sts1), and double *ppb1* null-*sts1* null (Double) were plated on rich medium at 33°C without drug. The photograph was taken 3 days later. No colonies were seen on the double disruptant. Tiny colonies of the double mutant cells, however, were produced after 6 days.

A mutant hypersensitive to cyclosporin A

We examined whether a previously reported multi drug-sensitive strain, *sts1* (Shimanuki et al., 1992), was also hypersensitive to cyclosporin A. This strain was hypersensitive to various agents like staurosporine (a protein kinase inhibitor), sorbitol, SDS and cations such as Ca^{2+}, Mg^{2+}, Zn^{2+} and Mn^{2+}, but not to thiabendazole or hydroxyurea (Shimanuki et al., 1992). The *sts1*$^{+}$ gene encodes a polypeptide containing the membrane spanning domain and resembling chicken lamin B receptor. It was non-essential for viability, but *sts1* null became

cold-sensitive in the absence of drugs; it produced somewhat small colonies at 33°C but tiny ones at 22°C.

We found that *sts1* null was also hypersensitive to cyclosporin A at the permissive temperature (Fig. 4A). The colony formation of *sts1* null at 33°C was greatly reduced on the plates containing 10 μg/ml cyclosporin A. No colonies were formed on the plates containing 100 μg/ml cyclosporin A at 33°C. Among *sts* mutants examined, *sts5* mutant was also hypersensitive to cyclosporin A. Unlike *sts1*, *sts5* was hypersensitive to staurosporine but not to other drugs (Toda et al., 1991; Shimanuki et al., 1992). The gene product of *sts5*$^+$ has not been determined.

DAPI-stained *sts1* null cells incubated in the absence or presence of 30 μg/ml cyclosporin A or FK506 in the rich YPD medium at 33°C are shown in Fig. 4B. Cells are normal in the absence of drug (left, none). Elongated cells with septa were seen in the presence of cyclosporin A (CsA). A similar phenotype was obtained by FK506 (FK). Note that abnormal cell morphology produced by *sts1* null cells was dependent on the drug used. Short and round *sts1* cells were produced by staurosporine (Shimanuki et al., 1992). SDS caused rapid lysis of *sts1* null cells. Multi-septate cells were specific for cyclosporin A and FK506.

Phenotype of the double disruption mutant *ppb1* null-*sts1* null

To examine the genetic interaction between *ppb1*$^+$ and *sts1*$^+$, the double-mutant *ppb1* null-*sts1* null was constructed by crossing, and its phenotype was investigated (Fig. 5A-D). An enhanced defective phenotype was obtained. Tiny colonies were obtained at 33°C after 6 days in the absence of drug and no colonies at 22°C. The plate shown in D was taken after 3 days at 33°C. No colonies were seen for the double mutant, but tiny colonies appeared after 6 days at 33°C. Colony formation of single *ppb1* null and *sts1* null mutants is shown as control.

In liquid culture, the double-mutant cells, slowly grown at 33°C, were extremely elongated with multiple nuclei and septa with frequent branching (Fig. 5C). Control single-mutant cells of *ppb1* null and *sts1* null were normally grown under the same culture conditions (A and B). This double-mutant phenotype was produced in the absence of any drug including cyclosporin A. The *sts1*$^+$ and *ppb1*$^+$ gene products may be functionally related.

Interaction with other phosphatase genes

To explain the non-essential nature of Ppb1 phosphatase we considered that Ppb1 might share an essential function with other phosphatases. The possibility was examined by crossing *ppb1* null with one of the following five phosphatase mutant strains: namely, *dis2* null, *sds21* null, *ppe1* null, *ppa1* null or *ppa2* null (Ohkura et al., 1989; Kinoshita et al., 1990; Shimanuki et al., 1993). All the double mutants constructed formed colonies at 33°C (Table 2). *ppb1* null-*dis2* null, *ppb1* null-*sds21* null and *ppb1* null-*ppa1* null produced colonies even at 22°C, whereas *ppb1* null-*ppa2* null and *ppb1* null-*ppe1* null did not (*ppe1* null was cold-sensitive; Shimanuki et al., 1993; Matsumoto and Beach, 1993).

Cellular phenotypes of the double mutants at 33°C were examined. Normal cells were abundant in the double mutants with *ppa1* null and *sds21* null. The amounts of these phosphatases were shown to be minor compared with that of the major dis2 and ppa2 phosphatases (Kinoshita et al., 1990 and 1993). In double mutants with *ppa2* null and *dis2* null, however, the enhanced phenotype of *ppb1* null was observed. Single gene disruption of *dis2*$^+$ apparently caused no phenotype, while that of *ppa2*$^+$ produced a semi-wee (short cell size) phenotype (Kinoshita et al., 1993). In the double-mutant *ppb1* null-*ppe1* null, round cells were produced, the phenotype of which was similar to that of single *ppe1* null. Thus the double-mutant analyses indicated that the *ppe1* mutation was epistatic to *ppb1*.

Interestingly, no double mutant was obtained at any temperature by a cross between *sts5* and *ppb1* null, indicating that the double mutant was synthetically lethal. These two gene products might have a close relationship, sharing an essential function for viability. It is unknown, however, whether the gene product of *sts5*$^+$ is related to protein phosphatase. The *sts5*$^+$ gene product has not been identified.

Table 2. Phenotype of double mutants at 33°C

Strains crossed	Gene product	Colony 33°	Colony 22°	Phenotype at 33°C
dis2	type 1-like PP	+	+	Multi-septate
sds21	type 1-like PP	+	+	Normal
ppa1	type 2A-like PP	+	+	Normal
ppa2	type 2A-like PP	+	-	Multi-septate
ppe1	SIT4-like PP	+	-	Round
sts5	Unknown	-	-	Lethal
sts1	Multi drug-sensitive	±	-	Supermulti-septate

Double mutants were made by crossing between *ppb1* null and the corresponding strains listed. Double mutants, except *ppb1-sts5*, could produce colonies at 33°C. Single *ppa1, dis2, sds21* and *sts1* null mutants produce apparently normal cells at 33°C. Single *ppa2* null mutant produces short-sized (semi-wee) cells at 33°C. Single *ppe1* and *sts5* mutant cells produce pear-shaped or round cells at 33°C.

Overexpression phenotype of *ppb1*$^+$

Since pleiotropic cellular phenotypes were produced by the loss of *ppb1*$^+$, it was of interest to examine the overexpression phenotype, if any, of the *ppb1*$^+$ gene. To this end, the *S. pombe* inducible promoter *nmt1* (Maundrell, 1992) was employed, which was repressed in the presence of thiamine (+T), and induced in the absence of thiamine (–T). The restriction site *Nde*I was introduced at the putative initiation codon of *ppb1*$^+$, and the resulting restriction fragment was cloned into plasmid pREP1 (Maundrell, 1990). Plasmid nmt-ppb1 thus made was introduced into wild type, and transformants were selected in +T. Cells were first grown in +T, and then transferred to –T in order to derepress the nmt1 promoter. nmt promoter-directed synthesis was observed 10 hours after the removal of thiamine (Maundrell, 1992).

Cells carrying plasmid nmt-ppb1 were fixed by glutaraldehyde and observed by fluorescence microscopy 16 hours after the transfer to –T (Fig. 6A). Average cell length increased 18% after overexpression. Elongated cells were slightly bent at the middle of the cell (indicated by the arrowhead). The shape of the DAPI-stained chromatin region was frequently altered, and a portion was sharply pointed or extruded from the remaining part (indicated by the arrow). Approximately 15% of the total cells displayed such pointed nuclear chromatin. Positioning of

the nucleus was also aberrant in many cells (see DAPI in B). The nuclei were displaced from the center of the cell (indicated by arrows in Fig. 6B).

Immunofluorescence microscopy was done using two antibodies, namely, *Trypanosoma* monoclonal anti-tubulin (TAT1; Woods et al., 1989), which displayed the *S. pombe* microtubules, and rabbit polyclonal anti-sad1 antibodies (Funabiki et al., 1993), which recognized the spindle pole body (SPB). Cells were fixed by glutaraldehyde/formaldehyde (Hagan and Hyams, 1988), and counter-stained by DAPI (Fig. 6B). No spindle structure was found for those cells that displayed the pointed or protruded nuclear chromatin. Instead, the cytoplasmic microtubule arrays were seen.

The SPB staining by the anti-sad1 antibodies showed that the single SPB dot was invariably positioned at the tip of the pointed nuclear chromatin region, indicating that the SPB was anomalously positioned (indicated by arrows). Note that the displaced SPB was kept close to the cytoplasmic microtubules.

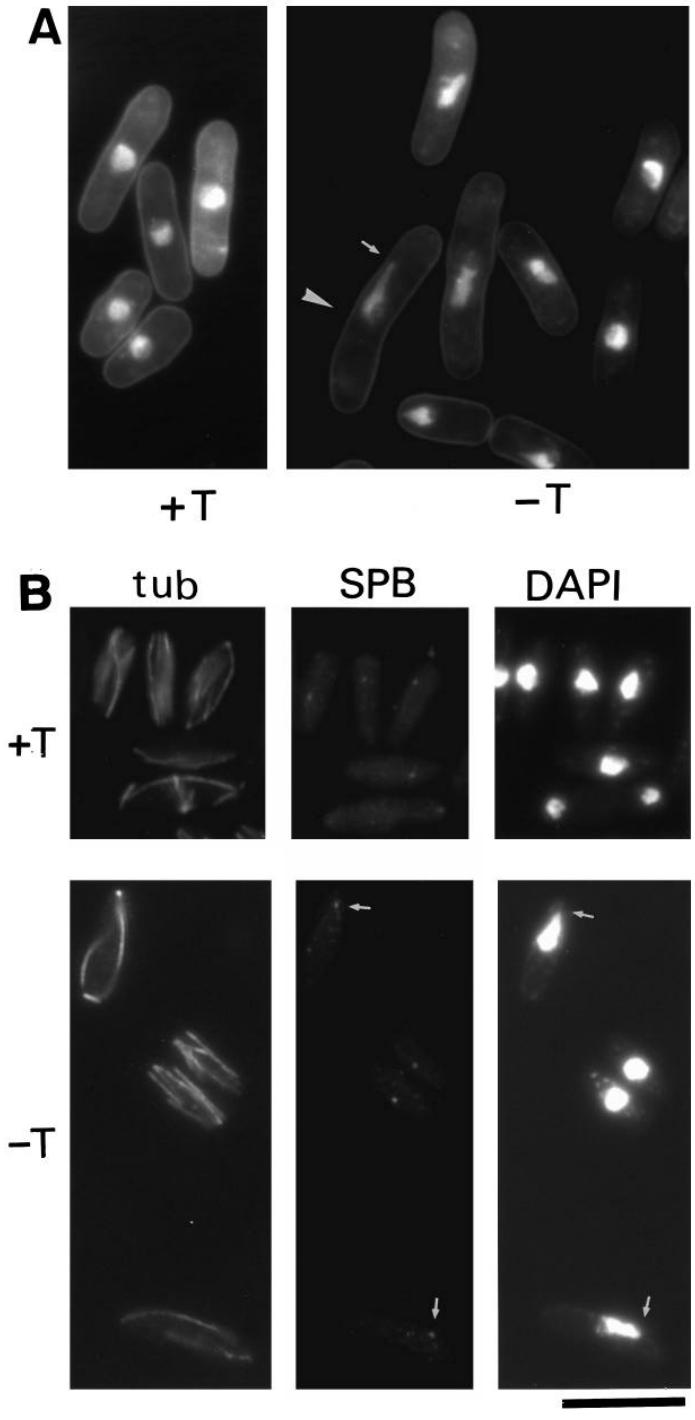

Fig. 6. Overexpression phenotype of the *ppb1*$^+$ gene. (A) Fluorescence micrographs of wild-type cells carrying plasmid with the *ppb1*$^+$ gene ligated with the inducible promoter nmt1. Gene expression was induced in the absence of thiamine (–T) but repressed in the presence (+T) of thiamine. Cells were first grown in the presence of thiamine (+T) and then in the absence of thiamine (–T) for 16 hours at 33°C. They were glutaraldehyde-fixed and stained with DAPI. A number of the nuclei were deformed, often having the pointed structure (indicated by the arrow). Approximately 15% of the cells displayed the deformed nuclear structure after 15 hours. A fraction of the elongated cells was slightly bent (indicated by the arrowhead). (B) Anti-tubulin (tub) and anti-SPB protein sad1 (SPB) antibody staining for wild-type cells overexpressing the *ppb1*$^+$ gene. Wild-type cells carrying nmt-ppb1 plasmid were grown first in the presence of thiamine (+T) and then in the absence of thiamine (–T) for 16 hours. After glutaraldehyde fixation, cells were double-stained with anti-tubulin (TAT1, Woods et al., 1989) and anti-sad1 (Funabiki et al., 1993), the latter of which was associated with the SPB. Chromosomal DNA was stained by DAPI. The tip of the pointed nuclei corresponded to the SPB (indicated by the arrow). Positions of the SPB were also located near the end of microtubule arrays. Bar, 10 μm.

DISCUSSION

We report the isolation and initial characterization of a calcineurin-like gene *ppb1*$^+$ of *S. pombe*. Southern hybridization of *S. pombe* genomic DNA under a low stringency condition using *ppb1*$^+$ as probe showed one set of hybridizing bands with sizes identical to those of the cloned *ppb1*$^+$ gene. Fission yeast thus appeared to have only one *ppb1*$^+$-like gene, although the presence of a gene weakly similar to *ppb1*$^+$ was still possible. Gene disruption experiments indicated that the *ppb1*$^+$ gene was not essential for viability. Two calcineurin-like genes are known in *S. cerevisiae* (Cyert et al., 1991; Liu et al., 1991b). They are also not essential for viability; cells are viable after the double gene disruption of *CNA1/CMP1* and *CNA2/CMP2*.

The predicted amino acid sequence of Ppb1 resembles rat calcineurin CNAA/PP2Bα slightly more than budding yeast CNA1/CMP1 or CNA2/CMP2, particularly in the NH_2 domain. The NH_2- to central domains, respectively, consist of the catalytic domain and the regulatory region bound to subunit B. As the sequence for calmodulin binding, which locates in the COOH-domain, is present, Ppb1 is likely to have the activity for a Ca^{2+}/calmodulin-regulated protein phosphatase. Direct biochemical evidence, however, is needed to establish the activity. A single calmodulin gene has been isolated in fission yeast (Takeda and Yamamoto, 1987), but the calcineurin B-like gene has not been identified.

The *ppb1* null mutant showed pleiotropic phenotypes. The cell number increase was strikingly reduced at 22°C, due to the delay in cytokinesis. Greatly elongated cells with multiple septa were observed. X-shaped microtubules at post-anaphase

were frequently seen. Cell polarity was also impaired, producing branched cells. The frequency of branched cells increased 20-fold by the temperature shift from 33° to 22°C. We suggest that microtubular functions are defective in *ppb1* null. Cold-sensitive β-tubulin mutation *nda3-311* showed the phenotypes of branching and multiple septa reminiscent of *ppb1* null at 22°C (Umesono et al., 1983; Hiraoka et al., 1984). Microtubule structures are destabilized in wild-type fission yeast cells at cold temperature (e.g. Funabiki et al., 1993). A number of microtubule mutants were isolated as cold-sensitive mutants (Toda et al., 1983, 1984; Umesono et al., 1983; Hiraoka et al., 1984), but none as temperature-sensitive. A fraction of *ppb1* null cells (approximately 10%) also showed short mitotic spindles, suggesting that spindle formation leading to metaphase might be slightly delayed in *ppb1* null.

A phenotypic similarity existed between the *ppb1* null mutation and the effect of cyclosporin A on wild-type cells, supporting the hypothesis that the *ppb1*$^+$ gene product seems to be the in vivo target of cyclosporin A. The fact that *ppb1* null was not influenced by the drug was probably due to the loss of cellular target. It is hence probable that Ppb1 phosphatase positively participates in cytokinesis. Cytokinesis is also greatly delayed in β-tubulin mutant *nda3-311* (Hiraoka et al., 1984).

The *sts1*$^+$ and *ppb1*$^+$ genes seemed to interact. Ppb1 phosphatase has a function related to that of Sts1 protein. Cells of *sts1* mutant were previously shown to be hypersensitive to multiple drugs and cations (Shimanuki et al., 1992). We found that they were also hypersensitive to cyclosporin A. The effect of *sts1* null and cyclosporin A was additive. Similarly, the phenotype of *ppb1* null-*sts1* null became increasingly severe; highly multi-septate cells were produced in the absence of cyclosporin. The *ppb1*$^+$ and *sts1*$^+$ gene functions thus appeared to be interrelated. The *sts1*$^+$ gene product should not simply act for efficient accumulation of cyclosporin A within cells; if so, the phenotype of *ppb1-sts1* cannot be explained. The loss of *sts1*$^+$ enhanced the phenotype by *ppb1* null. The gene product of *sts1*$^+$ might activate Ppb1 phosphatase.

The function of the *sts1*$^+$ gene product is little understood. It might be an integral membrane component, affecting transport of various substances across the cell membrane (Shimanuki et al., 1992). The delay in cytokinesis and other cellular defects in *ppb1* null-*sts1* null might be caused by the combined defects in Ca^{2+}/calmodulin-regulated protein dephosphorylation and the transport system. Budding yeast CMP/CNA mutant cells have recently been reported to be hypersensitive to high concentrations of cations (Nakamura et al., 1993).

It is not known how *ppb1* null causes sterility. The cell shape change during conjugation may not occur. It has not been determined whether cells fail to conjugate or conjugated cells fail to fuse the nuclei. In the budding yeast mutant, growth arrest was induced by hypersensitivity to the mating pheromone (Cyert et al., 1991). Although there is no direct link, the loss of a calcineurin-like gene in both yeasts affects the process of mating. Whether the budding and fission yeast calcineurin-like genes are functionally interchangeable remains to be determined. It was also not investigated whether cyclosporin A affected mating of fission yeast. The displacement of SPB by overexpression is not understood, but abnormality of the nuclear shape and positioning seemed to be affected by the dosage of Ppb1 phosphatase. A similar nuclear displacement phenotype was found by overexpression of the *sad1*$^+$ gene (I. Hagan and M. Yanagida, unpublished result).

Ppb1 phosphatase has a function related to that of two other phosphatases, type 2A-like Ppa2 and type 1-like Dis2. The double mutants *ppb1-ppa2* and *ppb1-dis2* showed the enhanced phenotype of *ppb1* at 33°C, and ppb1-ppa2 did not form colonies at 22°C. These phosphatases may share common substrates, or they may interact directly. Ppb1 phosphatase also has a shared function with Sts5 protein. The double mutant was lethal. *sts5* is one of the staurosporine hypersensitive alleles.

In summary, the fission yeast *ppb1*$^+$ gene, the product of which is highly similar to mammalian calcineurin, is not essential for viability but is involved in the regulation of a number of cellular processes such as cytokinesis, mating, transport, nuclear and SPB positioning and cell shape. It may be crucially important in morphogenesis of multicellular organisms.

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