Calcineurin, a protein phosphatase required for Ca\(^{2+}\) signaling in many cell types, is a heterodimer composed of catalytic and regulatory subunits. The fission yeast genome encodes a single set of catalytic (Ppb1) and regulatory (Cnb1) subunits, providing an ideal model system to study the functions of these subunits in vivo. Here, we cloned the cnb1\(^+\) gene and showed that the cnb1\(^+\) knock-out (Δcnb1) exhibits identical phenotypes with Δppb1 and that overexpression of Ppb1 failed to suppress the phenotypes of Δcnb1. Interestingly, overexpression of the C-terminal-deleted Ppb1 (Ppb1ΔC), the constitutively active form of Ppb1, also failed to suppress the phenotypes of Δcnb1. FK506 caused MgCl\(_2\) sensitivity to the wild-type cells in an FKBP12-dependent manner. Co-overexpression of Ppb1 and Cnb1 suppressed the FK506-induced MgCl\(_2\) sensitivity, but the suppression was only partial, suggesting that an excess amount of the Ppb1-Cnb1 complex cannot compete out the FKBP12-FK506 complex. Although overexpression of Ppb1ΔC alone had little effect on cell growth, co-overexpression of Ppb1ΔC and Cnb1 caused a distinct growth defect. FK506 suppressed the growth defect when Cnb1 was co-expressed using the attenuated nmt1 promoter, but it failed to suppress the defect when Cnb1 was co-expressed using the wild-type nmt1 promoter. Knock-out of the prz1\(^+\) gene, encoding a downstream target transcription factor of calcineurin, suppressed the growth defect irrespective of the promoter potency. These results suggest that Cnb1 is essential for the activation of calcineurin and that the activated calcineurin is the pharmacological target of the FKBP12-FK506 complex in vivo.

Calcineurin, a Ca\(^{2+}\)/calmodulin-dependent protein phosphatase, plays key roles in various Ca\(^{2+}\)-mediated cellular processes (1, 2), and inhibition of calcineurin by specific immunosuppressive drugs such as cyclosporin A or tacrolimus (FK506) underlies the molecular mechanisms of these drugs used in organ transplantation (3). Calcineurin is a heterodimer of a catalytic (calcineurin A) subunit and a Ca\(^{2+}\)-binding regulatory (calcineurin B) subunit. In known mammalian cells, three genes encode the catalytic subunits, and two genes encode the regulatory subunits (4). The physiological significance of the gene multiplicity encoding these subunits is unknown, and this makes it very difficult to study the role of each subunit of calcineurin in vivo.

Genes encoding calcineurin subunit proteins are highly conserved among species from yeast to man (5). The fission yeast Schizosaccharomyces pombe genome contains the smallest number of protein-coding genes yet recorded for a eukaryote (6) and has only a single gene encoding the catalytic subunit of calcineurin, Ppb1 (7), whereas the budding yeast Saccharomyces cerevisiae has two genes encoding the catalytic subunit (8, 9). The small genome size and gene number of fission yeast as well as its relevance to a higher system makes it a highly suitable model system in which to study the calcineurin signaling pathway by a molecular genetic approach. We have previously shown that Ppb1 plays an essential role in maintaining chloride ion homeostasis (10). We have also shown that calcineurin acts antagonistically with the Pmk1 MAP\(^1\) kinase in chloride ion homeostasis (10–12). In addition, analyses of mutants that require calcineurin activity for their growth revealed that calcineurin is implicated in cytokinesis, the septation initiation network, and membrane trafficking in fission yeast (13–17). Recently, we cloned the prz1\(^+\) gene, which encodes a Crz1/Tcn1 transcription factor homolog, and results have suggested that calcineurin activates at least two distinct signaling branches, i.e. the Prz1-dependent branch that regulates the expression of the Pmc1 Ca\(^{2+}\) pump and an unknown pathway that functions antagonistically with the Pmk1 MAP kinase pathway (18).

Biochemical studies using recombinant mammalian proteins have suggested that the regulatory subunit as well as calmodulin is required for the high calcineurin activity in vitro (19, 20) and that the regulatory subunit, but not calmodulin, is required for the binding of calcineurin to the drug-immunophilin complex (21). However, there is no in vivo molecular genetic study on the functions of calcineurin subunits. As described above, S. pombe has a single set of catalytic and regulatory
subunits, thus making it an ideal model system to study the role of these subunits in vivo using the molecular genetic approach. Here, we cloned the fission yeast cnb1+ gene, which encodes the regulatory subunit of calcineurin. Our present results suggest that the regulatory subunit is essential for the activation of calcineurin in vivo and that the activated calcineurin is the pharmacological target of the FKBP12-FK506 complex in vivo.

EXPERIMENTAL PROCEDURES

Strains, Media, and Miscellaneous Procedures—S. pombe strains used in this study are listed in Table I. The complete medium YPD and the minimal medium EMM have been described previously (22). Standard methods for S. pombe genetics were followed according to Moreno et al. (23). FK506 was provided by the Fujisawa Pharmaceutical Co. (Osaka, Japan).

Gene disruptions are denoted by lowercase letters representing the disrupted gene followed by two colons and the wild-type gene marker used for disruption (for example, cnb1::ura4+). Also, gene disruptions are denoted by an abbreviation of the gene preceded by Δ (for example, Δcnb1). Proteins are denoted by Roman letters, and only the first letter is capitalized (for example, Cnb1).

Data base searches were performed using the National Center for Biotechnology Information BLAST network service (www.ncbi.nlm.nih.gov) and the Sanger Center Gene Data Base (www.sanger.ac.uk). Protein data base at the Sanger Center (www.sanger.ac.uk). FK506 was provided by the Fujisawa Pharmaceutical Co. (Osaka, Japan).

Gene Cloning and Expression—The cnb1+ gene was amplified by PCR with the genomic DNA of S. pombe as a template. The sense primer used for PCR was 5′-GAAAAGCTGCTAGATGTTG-3′, and the antisense primer was 5′-CCCTCGGAGATCCGCTG-3′. The amplified product containing the cnb1+ gene was digested with HindIII and PstI, and the resulting fragment was cloned into Bluescript SK+.

For ectopic expression of proteins, we used the thiamine-repressible nmt1 promoter (24). Expression was repressed by the addition of 4 µg/ml thiamine to EMM and was induced by washing and incubating the cells in EMM lacking thiamine. Genes either tagged or non-tagged were subcloned into the pREP1, pREP41, or pREP81 vectors to express the cells in EMM lacking thiamine. Genes either tagged or non-tagged were subcloned into the vector containing the promoter at various levels. Maximum expression of the fused gene was obtained using pREP1, whereas pREP81 contained the most attenuated version of the nmt1 promoter (24).

When the pbpl+ and cnb1+ genes were co-expressed, the pbpl+ gene was integrated into the chromosome and the cnb1+ gene was harbored in the plasmid. To obtain the chromosome-borne tagged or non-tagged genes instead of the plasmid-borne genes, the genes with the nmt1 promoter at various levels were subcloned into the vector containing the urad4+ marker and were integrated into the chromosome at the urad4+ gene locus of KP1248 (h− leu1−32 urad4−294) (25).

Deletion of the cnb1+ Gene—A one-step gene disruption by homologous recombination (26) was performed. The cnb1::ura4+ disruption was constructed as follows. The cloned open reading frame of the cnb1+ gene in the Bluescript vector was digested with HindIII and PstI, and the resulting fragment containing the cnb1+ gene was subcloned into the HindIII/PstI site of pGem-5ZF (+) (Promega). Then, an EcoRI fragment containing the urad4+ gene was inserted into the EcoRI site of the previous construct, causing the interruption of the open reading frame. The fragment containing the disrupted cnb1+ gene was transformed into haploid cells. Stable integrants were selected on medium lacking uracil, and disruption of the gene was checked by genomic Southern hybridization (data not shown).

Cell Extract Preparation and Immunoblot Analysis—For the analysis of the expression of the tagged proteins, whole-cell extracts were prepared from cultures of cells grown at 30 °C to mid-log phase. Cells were resuspended in 450 µl of ice-cold homogenizing buffer, 50 mM Tris-HCl, pH 7.8, containing 2 mM EDTA, 1 mM dithiothreitol, and a mixture of protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 0.1 mM benzamidine, 0.1 mM sodium metabisulfite, chymostatin 0.1 µg/ml, aprotonin 2 µg/ml, pepstatin A 1 µg/ml, phosphoramidon 1 µg/ml, and leupeptin 0.5 µg/ml). Glass beads (0.2 g) were then added, and the cells were homogenized using a Mini-Beadbeater (BioSpec Products) at 5,000 rpm for 30 s, after which the tubes were placed on ice for 1 min. Homogenization and cooling were repeated twice, after which the glass beads and cellular debris were removed by centrifugation at 15,000 rpm for 5 min. Protein extracts (10–20 µg/sample) were subjected to immunoblot analysis with anti-GFP, anti-GST, or anti-FLAG antibodies. Antibody to fission yeast Cdc4 protein was prepared by immunizing rabbit with the purified Cdc4 protein and was used for the detection of endogenous Cdc4 as a loading control.

Mobility Shift Assay—Pbpl-deleted cells transformed with pREP1-FLAG-Prl were cultured for 20 h at 27 °C in EMM without thiamine to induce the expression of FLAG-Prl1. Cells were resuspended in ice-cold homogenizing buffer and homogenized using Mini-Beadbeater as described above. FLAG-Prl1 was immunoprecipitated with an agarose-immobilized anti-FLAG antibody as described previously (27). The immunoprecipitates were washed three times with 50 mM HEPES (pH 7.5) containing 150 mM NaCl, 10% glycerol, and 0.1% Triton X-100. To the washed beads, 0.4 µl of lysates from the Δcnb1 cells expressing both Pbpl1 and Cnb1p, Pbpl alone, or Pbpl·ΔC alone were added together with 2 µg of bovine calmodulin and CaCl2 (final concentration 5 mM), and the mixture was incubated for 30 min at 30 °C. The reaction was terminated by washing three times with 50 µl of HEPES (pH 7.5) containing 150 mM NaCl, 10% glycerol, and 0.1% Triton X-100 and by resuspension in an SDS sample buffer. After boiling for 5 min, the complexes were evaluated by immunoblot analysis.

Microscopic Analysis—Cells were grown to exponential phase in YPD or EMM medium and shifted to various conditions as indicated in the figure legends. Cells were washed with phosphate-buffered saline (pH 7.0), stained with Hoechst 33342 or Calcofluor to visualize the DNA or septum, respectively, and examined microscopically.

For F-actin staining, cells were fixed in 3% formaldehyde in phosphate-buffered saline for 30 min (28), and 1 µl of 100 µg/ml rhodamine-labeled phalloidin (Molecular Probes) was added to 50 µl of the fixed cell suspension. After 30 min at room temperature, the excess phalloidin was washed away with the saline.

Cells were examined under an Axioskop microscope (Carl Zeiss, Inc.). Photographs were taken with a SPOTZ digital camera (Diagnostic Instruments, Inc.). Images were processed with the CorelDraw software (Corel Corporation Inc.).

RESULTS

Identification of the S. pombe cnb1+ Gene—A BLAST program search using the peptide sequence of Cnb1 (YKL190W), the regulatory subunit of calcineurin of S. cerevisiae (29, 30), against the S. pombe protein data base at the Sanger Center revealed an open reading frame, SPCC30.06, which except for the N- and C-terminal domains, exhibits a significant similarity to the budding yeast Cnb1 (score = 680, p = 8.7e−69, identities = 142/174 (81%)). Hence, we named the gene cnb1+.

As shown in Fig. 1A, the cnb1+ gene encodes a protein of 174
Calcineurin Subunits in S. pombe

Molecular cloning and gene disruption of cnb1, encoding the regulatory subunit of fission yeast calcineurin. A, sequence alignment of the three regulatory subunits of calcineurin from fission yeast (Sp), budding yeast (Sc), and human (Hs). Residues conserved in the three sequences are indicated by asterisks, colons, and dots. Putative sequences for four EF-hands are underlined. B, the cnb1 deletion showed MgCl₂ hypersensitivity identical to that of the knock-out cells of the catalytic subunit Ppb1. Wild-type (wt), ppb1 knock-out (Δppb1), cnb1 knock-out (Δcnb1), and the double knock-out (Δppb1Δcnb1) cells were streaked onto a YPD plate and incubated for 3 days at 30 °C in the presence or absence of 0.1 or 0.12 M MgCl₂.

Amino acids that contains four EF-hands.

The cnb1 Knock-out Cells Showed Phenotypes Identical to Those of the Knock-out Cells of the Catalytic Subunit Ppb1—To further investigate the cnb1 gene function, we analyzed the Δcnb1 cells for phenotypes exhibited by the knock-out cells of the catalytic subunit, Ppb1, such as hypersensitivity to the Cl⁻ ion and aberrant cell morphology (7, 10). The growth of the Δcnb1 or Δppb1 cells was markedly inhibited by the presence of MgCl₂ in the media. Both Δcnb1 cells and ppb1 cells barely grew in the presence of 0.1 M MgCl₂, and both failed to grow in the presence of 0.12 M MgCl₂ on the YPD plate (Fig. 1B). Thus, the Cl⁻ sensitivity of the Δcnb1 cells was nearly equal to that of the Δppb1 cells. Notably, the Cl⁻ sensitivity of the Δppb1Δcnb1 double knock-out cells was also nearly equal to their single knock-out cells (Fig. 1B). These results indicate that cnb1 gene is required for the calcineurin activity in vivo. Microscopic analysis also revealed that the Δppb1, Δcnb1, and Δppb1Δcnb1 cells showed identical cell morphology with enlarged, multiseptated, and branched cells (Fig. 2). Furthermore, as shown in Fig. 3, the MgCl₂ sensitivity of the Δcnb1 cells was suppressed by overexpression of the pmp1 gene encoding a MAP kinase phosphatase, which dephosphorylates Pmk1, and were also suppressed by multicopy expression of the unphosphorylatable form of Pek1, an upstream kinase of Pmk1, to the same extent as that of the Δppb1 cells (10). These results also support the notion that Cnb1 is required for the calcineurin activity in vivo and that calcineurin acts antagonistically with the Pmk1 MAP kinase in Cl⁻ ion homeostasis (10–12).

Cnb1 Specifically Binds Ppb1—The Δppb1 cells expressing the GFP-Ppb1 fusion protein and the Δcnb1 cells expressing the GST-Cnb1 fusion protein grew in YPD medium containing 0.15 M MgCl₂, suggesting that these fusion proteins are fully functional (Fig. 4A). In the GST pull-down assay, GFP-Ppb1 associates with GST-Cnb1 but not with unfused GST (Fig. 4B), indicating that Cnb1 specifically binds Ppb1.

FIG. 1. Molecular cloning and gene disruption of cnb1, encoding the regulatory subunit of fission yeast calcineurin. A, sequence alignment of the three regulatory subunits of calcineurin from fission yeast (Sp), budding yeast (Sc), and human (Hs). Residues conserved in the three sequences are indicated by asterisks, colons, and dots. Putative sequences for four EF-hands are underlined. B, the cnb1 deletion showed MgCl₂ hypersensitivity identical to that of the knock-out cells of the catalytic subunit Ppb1. Wild-type (wt), ppb1 knock-out (Δppb1), cnb1 knock-out (Δcnb1), and the double knock-out (Δppb1Δcnb1) cells were streaked onto a YPD plate and incubated for 3 days at 30 °C in the presence or absence of 0.1 or 0.12 M MgCl₂.

FIG. 2. The ppb1 knock-out (Δppb1), the cnb1 knock-out (Δcnb1), and the double knock-out (Δppb1Δcnb1) cells showed identical phenotypes in cell morphology. Cells were grown in YPD medium to mid-log phase at 30 °C, washed with phosphate-buffered saline (pH 7.0), stained with Hoechst 33342 and Calcofluor to visualize the DNA or septum, respectively, and examined microscopically. Bar, 10 μm. DIC, differential interference contrast.

FIG. 3. The MgCl₂ hypersensitivity of the cnb1 deletion was suppressed by overexpression of the inhibitors of the Pmk1 MAP kinase pathway in an identical manner with that of the ppb1 deletion. The MgCl₂-sensitive phenotype of Δppb1 and Δcnb1 was suppressed by the plasmid carrying the pmp1 or pek1 gene. The Δppb1 or Δcnb1 cells transformed with the multicopy plasmid pDB248 vector (+vector) or the vector carrying the ppb1 (+ppb1), the cnb1 (+cnb1), the pmp1 (+pmp1), or the pek1 (+pekt1) genes were streaked onto YPD plates or the plate containing 0.15 M MgCl₂ and incubated for 3 days at 30 °C.
Calcineurin Subunits in S. pombe

indicates that the huge amount of the catalytic protein, Ppb1, has no physiological function without its regulatory partner.

It is thought that the C-terminal domain of the catalytic subunit has an autoinhibitory function to the phosphatase activity; hence the C-terminal-deleted calcineurin is used as a constitutively active mutant (10, 15, 18, 32). We then tested whether the C-terminal-deleted Ppb1ΔC can suppress the MgCl2 sensitivity of the Δcnb1 cells. As shown in Fig. 5D, the Δcnb1 cells overexpressing Ppb1ΔC by the pREP1 vector (pR1−) barely grew in the presence of 0.1 M MgCl2, and they failed to grow on the plate containing 0.15 M MgCl2. The growth defect in the presence of MgCl2 was identical with those of the Δcnb1 cells harboring vector or full-length Ppb1. This indicates that Ppb1ΔC has no physiological enzymatic activity in the absence of the regulatory subunit in vivo.

Together, overexpression of either full-length Ppb1 or Ppb1ΔC failed to complement the cnb1 knock-out. Consistently, the dephosphorylation-induced mobility shift of the calcineurin substrate Prz1 (18) was observed when it was incubated with both Ppb1 and Cnb1 in the mixture containing calmodulin and Ca2+, but this shift was not observed when it was incubated with either Ppb1 or Ppb1ΔC alone in the same reaction mixture (Fig. 5E).

Co-overexpression of Full-length Ppb1 and Cnb1 Only Partially Antagonized the Effect of FK506—Next, we examined the effect of co-overexpression of full-length Ppb1 and Cnb1 on the FK506-induced Cl− sensitivity of wild-type cells. As shown in Fig. 6 (lower panel), the wild-type cells overexpressing the full-length ppb1+ gene chromosomally borne under the control of the nmt1 promoter could not grow on an EMM plate containing FK506 and 0.15 M MgCl2. When Cnb1 was co-expressed with full-length Ppb1 using the pREP1 or pREP41 expression vectors, the cells were able to grow in the presence of FK506 and 0.15 M MgCl2 in a Cnb1 expression-dependent manner (Fig. 6, lower panel). The ability of the co-overexpression of full-length Ppb1 and Cnb1 to suppress the FK506-induced Cl− sensitivity suggested that the immunophilin-drug complex targets the calcineurin holoenzymes. However, the suppression was only partial. Wild-type cells failed to grow in the presence of FK506 and 0.2 M MgCl2 even when both full-length Ppb1 and Cnb1 were co-overexpressed using the pREP1 vector. Notably, the growth of FKBP12-deleted cells (Δfhk1) was not affected by the presence of FK506 in the medium. These results suggest that an excess amount of the full-length Ppb1-Cnb1 complex cannot compete out the FKBP12-FK506 complex.

Hyperactivity of Calcineurin Caused Growth Arrest and Aberrant Cell Morphology—From the above results, it is noted that the effect of calcineurin hyperactivity was seen when both the catalytic and the regulatory subunits were overproduced at the same time. To further examine this effect, the wild-type cells with the full-length (ppb1+) or truncated (ppb1ΔC) genes chromosomally borne under the control of the nmt1 promoter were transformed with pREP1-cnb1+ or pREP41-cnb1− in the presence of thiamine, and the transformants were shifted to the media without thiamine to induce gene expression. It should be noted that there was only a slight difference between the amounts of the Ppb1 and Ppb1ΔC protein expressed in the cells (Fig. 5A).

Co-overexpression of Cnb1 and Ppb1ΔC caused a distinct growth defect as shown in Fig. 7. A and C. FK506 reversed the growth defect when Cnb1 was co-overexpressed using an attenuated nmt1 promoter (pREP41-cnb1−), but FK506 failed to reverse the growth defect when Cnb1 was co-overexpressed using the wild-type nmt1 promoter (pREP1-cnb1+). These results suggest that an excess amount of the Cnb1-Ppb1ΔC complex can compete out the FKBP12-FK506 complex. Together,
the findings that an excess amount of the Cnb1-Ppb1 full-length complex cannot compete out the FKBP12-FK506 complex and that an excess amount of the Cnb1-Ppb1/H9004 complex can compete out the FKBP12-FK506 complex suggest that the FKBP12-FK506 complex selectively binds to calcineurin in its active state. Notably, co-overexpression of Cnb1 and full-length Ppb1 caused no growth defect, suggesting that the activity of the enzyme is tightly regulated by calmodulin and the intracellular Ca²⁺ ion in its in vivo physiological condition.

the nmt1 promoter suppressed the MgCl₂ sensitivity of the ppb1 deletion. The wild-type (wt) or Δppb1 cells transformed with the attenuated version of the expression plasmid pREP81 vector alone or the vector carrying the ppb1 (pR1-ppb1) gene was streaked onto an EMM plate containing 0.2 μM MgCl₂ and 0.15 μg/ml thiamine to switch on the promoter (promoter ON) and incubated for 3 days at 30 °C. Cells were spotted in serial 10-fold dilutions starting with OD₆₆₀ = 0.3 of log-phase cells (5 μl).

FIG. 6. Co-overexpression of full-length Ppb1 and Cnb1 only partially antagonized the effect of FK506. The Δfkh1 cells, the wild-type cells overexpressing GFP-Ppb1 full-length from the gene chromosomally borne under the control of the nmt1 promoter (nmt1-ppb1), or the cells co-overexpressing GST-Cnb1 using pREP41 (nmt1-ppb1 + pREP41-cnb1) or pREP1 (nmt1-ppb1 + pREP1-cnb1) were spotted onto an EMM plate with or without the indicated additives in the absence of thiamine to switch on the promoter and incubated for 3 days at 30 °C. Cells were spotted in serial 10-fold dilutions starting with OD₆₆₀ = 0.3 of log-phase cells (5 μl).

Calcineurin Subunits in S. pombe

FIG. 5. Overexpression of the catalytic subunit of calcineurin failed to suppress the phenotypes of the cnb1 knockout. A and B, protein levels of GFP-tagged Ppb1 or Ppb1ΔC (A) and GST-tagged Cnb1 (B) examined by immunoblot analysis. Cells were transformed with the pREP1 (pR1), pREP41 (pR41), or pREP81 (pR81) expression vectors harboring DNA encoding GFP-Ppb1 or GFP-Ppb1ΔC (A) or GST-Cnb1 (B) and were grown to mid-log phase in EMM containing thiamine (4 μg/ml) at 30 °C. Cells were washed and incubated for 24 h in the presence (+) or absence (−) of thiamine and then analyzed by immunoblotting as described under “Experimental Procedures.” Endogenous Cdc4 was used as a loading control and was immunoblotted using anti-Cdc4 antiserum. C, the most attenuated expression of Ppb1 from the nmt1 promoter suppressed the MgCl₂ sensitivity of the ppb1 deletion. The wild-type (wt) or Δppb1 cells transformed with the attenuated version of the expression plasmid pREP81 vector alone or the vector carrying the ppb1 (pR1-ppb1) gene were streaked onto an EMM plate containing 0.2 μM MgCl₂ and 4 μg/ml thiamine to switch off the promoter (promoter OFF) and incubated for 3 days at 30 °C. D, overexpression of Ppb1 or its constitutively active form (Ppb1ΔC) failed to suppress the MgCl₂ sensitivity of the cnb1 deletion. The wild-type or Δcnb1 cells transformed with the most potent version of the expression plasmid pREP1 vector or the vector carrying the ppb1 (pR1-ppb1) gene or its truncated constitutive active form (pR1-ppb1ΔC) were streaked onto an EMM plate containing 0.1 or 0.15 μM MgCl₂ in the absence of thiamine to switch on the promoter (promoter ON) and incubated for 3 days at 30 °C. E, the dephosphorylation-induced mobility shift of the calcineurin substrate Prz1. Ppb1-deleted cells transformed with pREP1-FLAG-Prz1 were cultured for 20 h at 27 °C in EMM without thiamine to induce the expression of FLAG-Prz1. FLAG-Prz1 was immunoprecipitated and incubated with cell lysates from various cells as indicated and analyzed for the mobility shift as described under “Experimental Procedures.”
As shown in Fig. 7B, the arrested cells showed aberrant morphology, such as round, small, bent, or pear-shaped cells (Fig. 7B, DIC). Although the septum was the only structure that was strongly stained by Calcofluor in wild-type cells (data not shown), the aberrant structure probably derived from the cell wall was strongly stained in the arrested cells (Fig. 7B, CF). In addition, the arrested cells showed depolarized distribution of cortical F-actin patches and an abnormally concentrated structure that contains F-actin (Fig. 7B). The aberrant morphology caused by co-overexpression of Ppb1ΔC and Cnb1 was suppressed by the addition of FK506 to the media (Fig. 7B, lower panel), suggesting that hyperactivity of calcineurin also causes the aberrant morphology.

As mentioned, the results of our previous study have suggested that calcineurin activates at least two distinct signaling branches, one of which is Prz1-dependent transciptional regulation (18). To examine whether the growth arrest caused by co-overexpression of Ppb1ΔC and Cnb1 in Δprz1 cells is due to the functional hyperactivity of calcineurin is mediated by the activation of the zinc finger transcription factor Prz1 (18), Δprz1 cells with the truncated (ppb1ΔC) gene chromosomally borne under the control of the nmt1 promoter (KP1746) were transformed with pREP1-cnb1" or pREP41-cnb1" in the presence of thiamine, and the transformants were shifted to the media without thiamine to induce gene expression. Co-overexpression of Ppb1ΔC and Cnb1 in Δprz1 cells, as shown in Fig. 7C, did not cause the growth arrest that was observed in the wild-type cells, indicating that it is mediated by the activation of Prz1. As this defect was not suppressed by the FK506 treatment, these results suggest that hyperactivity of calcineurin caused the growth defect and likewise support the notion that the FKBP12-FK506 complex selectively binds to calcineurin in its active state.

DISCUSSION

In the present study, a molecular genetic approach was conducted to examine the in vivo functions of the calcineurin subunits in fission yeast as a simple model system. Here we report that the regulatory subunit is essential for the activation of calcineurin in vivo, and our results suggest that the FKBP12-FK506 complex selectively binds calcineurin in its active state and inhibits the activity in vivo.

Calcineurin, a Ca^{2+}/calmodulin-regulated type 2B protein phosphatase, is a heterodimer of the catalytic (calcineurin A, Ppb1 in S. pombe) and the regulatory (calcineurin B, Cnb1 in S. pombe) subunits. The catalytic subunit of calcineurin shows a high sequence homology to the catalytic subunit of other
protein phosphatases, types 1, 2A, and 5, and some of these catalytic subunits are reported to have functional enzymatic activity in the absence of their regulatory subunits (33). Higuchi et al. (34) reported that a recombinant catalytic A subunit of Neurospora crassa expressed in bacteria was stimulated 2–3-fold by calmodulin and was shown to have a p-nitrophenol phosphatase activity equal to that of the bovine brain calcineurin holoenzyme. Their findings suggest that the catalytic subunit of the fungal calcineurin can exhibit high activity in the absence of its regulatory subunit.

On the other hand, biochemical studies using a baculovirus-expressed mammalian catalytic subunit showed that the catalytic subunit alone has very low activity, and the regulatory subunit markedly increased the activity by lowering the $K_m$ value (19, 20). Therefore, it is important to answer the question of whether the catalytic subunit has functional activity in the absence of the regulatory subunit in vivo.

Here we demonstrate for the first time that the regulatory subunit is absolutely required for the full activation of calcineurin in vivo by showing that neither the overexpression of Ppb1 nor that of the constitutively active form, Ppb1ΔC, can suppress the phenotypes of the cnb1Δ knockout.

As the stability or subcellular localization of Ppb1 was not affected by the presence or absence of Cnb1 (data not shown), our results suggest that the regulatory subunit is required for the conformational change to the active state after Ca$^{2+}$/calmodulin binding. Failure of Ppb1ΔC in suppressing the phenotypes of the cnb1Δ knockout indicates that the removal of the C-terminal inhibitory domain (35) is not sufficient to activate the enzyme and to dephosphorylate its physiological substrates.

We, as well as other researchers, have been using the C-terminal-deleted catalytic subunit of calcineurin (Ppb1ΔC, in the present study) as a constitutively active form to characterize the effects observed with the activation of calcineurin in various organisms and cells, and this has worked very well (36). Our present results indicate that these effects are dependent and limited by the presence and amount of the endogenous regulatory subunit, respectively. That is, the truncated version of the calcineurin catalytic subunit works only when it makes a complex with the pre-existing regulatory subunit. Upon expression, the truncated catalytic subunit may dominate the endogenous wild-type catalytic subunit, and part of the endogenous regulatory subunit will form a constitutively active complex with the truncated catalytic subunit. This complex may account for the effect obtained with overexpression of the constitutively active form.

In the present study, the wild-type cells treated with FK506 showed MgCl$_2$ sensitivity, whereas the FKBP12-deleted (Δph1) cells were not affected by the presence of FK506 in the medium. These results indicate that the FKBP12/FK506 immunophilin-drug complex binds and inhibits calcineurin that is required for Cl$^-$ homeostasis, thereby causing the FK506-induced Cl$^-$ sensitivity. Our results also suggest that neither Ppb1 nor Cnb1 alone can bind the FKBP12/FK506 complex and that only the Ppb1-Cnb1 complex can bind the inhibitor complex. Notably, wild-type cells co-overexpressing full-length Ppb1 and Cnb1 failed to grow in the presence of FK506 and 0.2 M MgCl$_2$, whereas Δph1Δ cells grew in the same medium, indicating that even though the amount of overproduced full-length Ppb1 and Cnb1 may be considerably higher than that of endogenous FKBP12; however, the excess amount of full-length Ppb1 and Cnb1 cannot compete out the FKBP12/FK506 complex, thereby suggesting that the FKBP12/FK506 complex selectively binds and inhibits the active Ppb1-Cnb1-calmodulin-Ca$^{2+}$ complex in vivo. Consistently, knock-out of the Prx1 transcription factor but not FK506 treatment suppressed the growth defect caused by co-overexpression of Ppb1ΔC and Cnb1 using pREP1-cnb1$^{+}$ and a potent wild-type nmt1 promoter. These results suggest that an excess amount of the Ppb1ΔC-Cnb1 complex can compete out the FKBP12-FK506 complex.

A biochemical study by Clipstone et al. (21) showed that the regulatory subunit but not calmodulin is required for the binding of calcineurin to the drug-immunophilin complex, and calcineurin activity has not been correlated with its binding to the complex. However, in our previous in vitro binding study using a rapid filtration assay, we showed that the FKBP12-FK506 complex binds the calcineurin-calmodulin-Ca$^{2+}$ complex but does not bind to the calcineurin-calmodulin-Mg$^{2+}$ complex (37), suggesting the correlation between calcineurin activity and its binding to the FKBP12-FK506 complex. In addition, we showed that although the calcineurin-Ca$^{2+}$ complex binds the FKBP12-FK506 complex, its binding affinity was ~8 times lower than that of the calcineurin-calmodulin-Ca$^{2+}$ complex (37). Together, these results suggest that the in vivo pharmacological target of the FKBP12-FK506 complex is the active calcineurin-calmodulin-Ca$^{2+}$ complex. Although the assembly of a calcineurin-FKBP12-FK506 complex was established by crystallographic studies (38), our studies suggest that the structure of the calcineurin-calmodulin-FKBP12-FK506-Ca$^{2+}$ complex needs to be clarified to uncover the underlying molecular mechanisms of drug action.

Acknowledgements—We thank Mitsuhiro Yanagida (Kyoto University, Japan), Takashi Toda, and Paul Nurse (Cancer Research UK London Institute, UK) for their generous gift of strains and plasmids.

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