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Schizosaccharomyces pombe Pmr1p Is Essential for Cell Wall Integrity and Is Required for Polarized Cell Growth and Cytokinesis

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The *cps5-138* fission yeast mutant shows an abnormal lemon-like morphology at 28°C in minimal medium and a lethal thermosensitive phenotype at 37°C. Cell growth is completely inhibited at 28°C in a Ca^{2+}-free medium, in which the wild type is capable of growing normally. Under these conditions, actin patches become randomly distributed throughout the cell, and defects in septum formation and subsequent cytokinesis appear. The mutant cell is hypersensitive to the cell wall-digesting enzymatic complex Novozym234 even under permissive conditions. The gene SPBC31E1.02c, which complements all the mutant phenotypes described above, was cloned and codes for the Ca^{2+}-ATPase homologue Pmr1p. The gene is not essential under optimal growth conditions but is required under conditions of low Ca^{2+} (<0.1 mM) or high temperature (>35°C). The green fluorescent protein-tagged Cps5 proteins, which are expressed under physiological conditions (an integrated single copy with its own promoter in the *cps5*Δ strain), display a localization pattern typical of endoplasmic reticulum proteins. Biochemical analyses show that 1,3-β-D-glucan synthase activity in the mutant is decreased to nearly half that of the wild type and that the mutant cell wall contains no detectable galactomannan when the cells are exposed to a Ca^{2+}-free medium. The mutant acid phosphatase has an increased electrophoretic mobility, suggesting that incomplete protein glycosylation takes place in the mutant cells. These results indicate that *S. pombe* Pmr1p is essential for the maintenance of cell wall integrity and cytokinesis, possibly by allowing protein glycosylation and the polarized actin distribution to take place normally. Disruption and complementation analyses suggest that Pmr1p shares its function with a vacuolar Ca^{2+}-ATPase homologue, Pmc1p (SPAPB2B4.04c), to prevent lethal activation of calcineurin for cell growth.

A transient increase in the intracellular Ca^{2+} concentration ($[Ca^{2+}]$) plays a key role in transmitting signals that regulate a variety of cellular functions in eukaryotes. A substantial body of knowledge has been accumulated concerning the roles of Ca^{2+} as a second messenger in various types of eukaryotic cells (5, 11). In yeasts, Ca^{2+} plays an essential role in the mating process (9, 26), and the Ca^{2+} calmodulin-dependent protein phosphatase calcineurin plays crucial roles in a variety of cellular functions, including ion homeostasis, cytokinesis, and transcriptional regulation (14, 55, 63). In the budding yeast *Saccharomyces cerevisiae*, *FKS1*, which codes for a putative catalytic subunit of 1,3-β-D-glucan synthase, is predominantly expressed under optimal growth conditions in a cell cycle-dependent manner, while the transcription of *FKS2*, an alternative gene for the putative glucan synthase, is completely dependent on calcineurin in the absence of a functional Fks1p and in the presence of mating pheromone or a high extracellular $[Ca^{2+}]$ (42, 74). In the fission yeast *Schizosaccharomyces pombe*, 1,3-β-D- and 1,3-α-D-glucan synthases, both of which contribute to the mechanical strength of the cell wall (12, 23, 29, 32), are presumed to be the downstream targets of Pck2p, a protein kinase C homologue (4, 8, 32). The overexpression of *pck2*$^+$ (OP-*pck2*$^+$) has been shown to increase 1,3-β-D-glucan synthase activity to a significant extent, as well as to induce an extremely high intracellular $[Ca^{2+}]$ (4, 8). The effects of OP-*pck2*$^+$ were reported to be abolished in the absence of Ehs1p, a homologue of the calcium channel component Mid1p of *S. cerevisiae* (9, 25). The *ehs1-1* mutant displays several cell wall-related defective phenotypes, and these defects are suppressed by moderate OP-*pck2*$^+$ levels, suggesting that Pck2p contributes, along with the Ehs1p calcium channel, to the integrity of the cell wall (9). More recently, another component, Cta4p, a cation P_5-type ATPase that is also required for calcium homeostasis, has been identified (45). The null mutant displayed pleiotropic phenotypes, including defects in cytokinesis and microtubule dynamics, similar to the calcineurin null mutant phenotypes (73). These findings suggest that the regulation of intracellular $[Ca^{2+}]$ is critical to cell wall integrity, cytokinesis, and cytoskeletal organization, all of which are essential for fungal-cell morphogenesis. Transient and spatial changes in intracellular $[Ca^{2+}]$ are thus critical for generating signals that regulate cellular events or maintain ion homeostasis; the processes are mediated by Ca^{2+} channels, Ca^{2+} antiporters, and Ca^{2+}-transporting ATPases, which are localized in the plasma or vesicle membranes to allow the transport of ions into or out of the cell or organelles via the membranes. However, at present, little is known concerning the molecular mechanisms of calcium signaling required for these cellular processes during the cell cycle. To address these issues, the molecular characterization of the mutants that show calcium-sensitive phenotypes might be useful.

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TABLE 1. Strains used in this study

Strain	Genotype	Source
972	h$^-$ wild type	NCYC[a]
903	h$^-$ *leu1-32*	This study
218	h$^+$ *cps1-12 leu1-32*	J. Ishiguro et al. (29)
CP13-8	h$^-$ *cps5-138*	J. Ishiguro et al. (30)
CP13-8-4D	h$^+$ *cps5-138 leu1-32*	This study
A3-2D-2A	h$^+$ *ura4-Δ18 leu1-32 cps5*::*ura4*$^+$	This study
B2B4#6	h$^-$ *ura4-Δ18 leu1-32 pmc1*::*ura4*$^+$	This study
733	h$^+$ *cps1-12 cps5-138 leu1-32*	This study
#16	h$^-$ *ehs1-1 leu1-32*	Carnero et al. (9)
JY741	h$^-$ *ade6-M216 ura4-Δ18 leu1-32*	C. Shimoda
JY746	h$^+$ *ade6-M210 ura4-Δ18 leu1-32*	C. Shimoda

[a] National Collection of Yeast Cultures, AFRC Institute of Food Research, Norwich, United Kingdom.

Fourteen genes, named *cps1* to *cps14*, were identified as mutant alleles that confer hypersensitivity to the mitotic poison isopropyl chlorophenyl carbamate (CIPC) (30). Among these, *cps1*$^+$ was found to code for a putative β-1,3-D-glucan synthase subunit, and the *cps1-12* mutation causes cells to lyse in the presence of cyclosporin A (CsA), a potent inhibitor of calcineurin (29). *cps8-188* encodes a mutated actin molecule (G273D), and the mutant shows a depolarized and multiseptated morphology with a disorganized cell wall structure (28, 31, 35). These findings suggest that in fission yeast, both calcium signaling and the actin organization are crucial to the integrity of the cell wall. We have recently found that the *cps5-138* mutant is incapable of growing in a medium depleted of calcium or at high temperature, under which conditions the cells show an abnormally rounded shape. In the present study, we demonstrate that *cps5* is a mutant allele of *pmr1*$^+$, a gene encoding a putative Ca^{2+}/Mn^{2+}-ATPase (SPBC31E1.02c) that plays a crucial role in the maintenance of cell wall integrity and cytokinesis. In addition, we show that the *S. pombe* Pmr1p is required for intracellular Ca^{2+} homeostasis, cooperating with a vacuolar Ca^{2+}-ATPase homologue, Pmc1p (SPAPB2B4.04c), to prevent the lethal activation of calcineurin for cell growth.

MATERIALS AND METHODS

Strains and culture conditions. The strains used in this study are listed in Table 1. Routine yeast extract (YE) medium and selected Edinburgh minimal medium (EMM) supplemented with the appropriate amino acids and sporulation medium have been described elsewhere (2, 20). Ca^{2+}-free EMM is EMM from which $CaCl_2$ was omitted and in which calcium panthothenate was replaced by sodium pantothenate. In some experiments, 0.1 and 10 mM EGTA was added to Ca^{2+}-free liquid and solid EMM, respectively. The yeast cells were cultured at 28°C, unless otherwise specified, on a plate or in liquid medium with continuous shaking. The *Escherichia coli* strain DH5α was used for the routine propagation of plasmids, as described elsewhere (56).

Plasmids and DNA techniques. DNA manipulations were carried out according to the standard methods described in Sambrook et al. (56). The plasmids pAL-KS and pREP1 are described elsewhere (29, 41, 65). The plasmids pDB248X-*pck1*$^+$, pDB248X-*pck2*$^+$, pREP3X-*pck1*$^+$, and pREP3X-*pck2*$^+$ were obtained from P. Pérez (4).

Gene cloning and mutation site determination. The mutant strain CP13-8-4D was transformed with an *S. pombe* genomic library constructed in the pAL-KS vector (pTN-L1; prepared by T. Nakamura). Four transformants capable of growing on both EMM and YE plates containing 280 μM isopropyl *N*-3-chlorophenyl carbamate (CIPC; Sigma) were isolated, and the plasmids were recovered. Nucleotide sequence determination revealed that all four plasmids contained only one complete open reading frame (ORF), SPBC31E1.02c. The plasmid called pCP5-15, which contained an 8.8-kb genomic DNA insert, was used for further analysis. PCR was carried out to detect the mutation site, using the *cps5-138* mutant genome as a template. The primers used were 5′-GGACT AGTCCTAAGAAACCAGCGGAAA GC-3′ (forward, with an SpeI site at the 5′ end) and 5′-CCCCCGGGGGTCGTTTTTGTTTTTGTATGA-3′ (reverse, with an SmaI site at the 5′ end). The amplified fragments were digested with SpeI/SacI, EcoRI/SacI, and EcoRI/SmaI, respectively, and the nucleotide sequence of each generated fragment (1.1, 1.1, and 0.7 kb, respectively) was determined by means of a Hitachi SQ5500E sequencer using the RPN2444 premixed cycle-sequencing kit (Amersham Pharmacia Biotech UK).

Gene disruption and overexpression. The one-step gene disruption method (53) was employed to construct the *cps5*Δ and *pmc1*Δ (SPAPB2B4.04cΔ) strains. The *cps5*$^+$ gene, amplified from the wild-type genome by using the same primers described above, was digested with SpeI/SmaI and ligated into pBluescript II KS(+) plasmids (Stratagene). The *ura4*$^+$ gene was inserted into the BamHI site of the *cps5*$^+$ ORF, and the SpeI/SmaI disruption fragment from the propagated plasmid was used for diploid (JY741XJY746) transformation. After diploid sporulation, tetrad analysis and PCR were performed on the haploid cells to check the uracil requirement phenotype and the correct *ura4*$^+$ insertion, respectively. For *pmc1*$^+$ disruption, a 1.5-kb fragment, which was amplified by PCR using the primers 5′-CGGGATCCCGTCGGTGTTAATTCATTTAAC-3′ (forward, with a BamHI site at the 5′ end) and 5′-CCCAAGCAATTGCTTTCAC A-3′ (reverse), was digested with BamHI/SalI (0.6 kb) and ligated into pBluscript II KS(+) plasmids. The *ura4*$^+$ gene was inserted into the HindIII site of the amplified fragment, and the BamHI/SalI disruption fragment (2.4 kb) from the propagated plasmid was used for diploid transformation. Gene disruption in the haploid cells was checked by tetrad analysis and PCR, as described above. For *cps5*$^+$ overexpression, the SPBC31E1.02c ORF (also called pgak2) was amplified by PCR from pCP5-15 using the primers 5′-TCCCCCGGGGGAATTTAGGA ATCCTTTACA-3′ (forward, with an SmaI site at the 5′ end) and 5′-TCCCCC GGGGGACATTGGAATTTTGTATTC-3′ (reverse, with an SmaI site at the 5′ end) and inserted into the pREP1 plasmid at the SmaI site. The plasmids bearing the *cps5*$^+$ gene in the right orientation were selected and used to transform the strains CP13-8-4D, B2B4#6, and 903.

GFP tagging. Two methods were employed to express a *cps5*$^+$-*GFP* fused gene in *cps5*Δ cells; one was a plasmid-borne expression, and the other was a physiological level of expression using an integrated copy of the fused gene in the chromosome. The *cps5*$^+$ gene bearing its own promoter region was amplified by PCR using the primers 5′-TCCCCCGGGGGATAACCTTTCCCAACTTGT-3′ (forward, with an SmaI site at the 5′ end) and 5′-GAAGATCTTCCCTACATT CCTTAGCAGATA-3′ (reverse, with a BglII site at the 5′ end). The amplified fragment (3.3 kb), which lacked the stop codon, was digested with SmaI/BglII and ligated in frame to the BglII site just before the ATG codon of the *GFP* ORF that was generated by PCR from the pEGFP vector (BD Biosciences and Clontech). The resulting *cps5*$^+$-*GFP* fragment with a SacI site at the 3′ end of the *GFP* ORF was inserted between the SmaI and SacI sites of the pAL-KS plasmid and used to transform the *cps5*Δ strain, CP13-8-4D. To integrate a copy of *cps5*$^+$-*GFP* into the chromosome, the *cps5*$^+$-*GFP* fragment was inserted between the SmaI and SacI sites of pJK148 (12), and the plasmid was cut with XbaI at position −245 of the *cps5*$^+$ ORF. The linearized plasmid was used to isolate leucine-nonrequiring transformants from the A3-2D-2A strain. For green fluorescent protein (GFP) tagging of *pmc1*$^+$, essentially the same procedure was used. The primers used for the gene amplification were 5′-TCCCCCGGGGGA GACTTTTTGTCTTTTAAA-3′ (forward, with an SmaI site at the 5′ end) and 5′-CGGGATCCCGATGAACATTTGAGCTTTTTT-3′ (reverse, with a BamHI site at the 5′ end). The amplified fragment (4.4 kb) was digested with SmaI/BamHI and ligated in frame to the BamHI site just before the ATG codon of the *GFP* ORF. The resulting *GFP*-tagged fragment was inserted between the SmaI and SacI sites of the pAL-KS plasmid and used to transform the *pmc1*Δ strain, B2B4#6. For GFP tagging of *cps11*$^+$/*alg3*$^+$, the same procedure described in the case of *cps5*$^+$-*GFP* integrated in pAL-KS was used. The primers used for gene amplification were 5′-TCCCCCGGGGGACCAGGCGTAGTAATCTAG-3′ (forward, with a SmaI site at the 5′ end) and 5′-GAAGATCTTCCCGGGTTT TCTGTAGTCGGA-3′ (reverse, with a BglII site at the 5′ end).

Fluorescence microscopy. For septum visualization, cells were washed with phosphate-buffered saline (PBS) and stained with 100 μg of calcofluor (fluorescent brightener 28; Sigma)/ml for 30 min and then washed again with the same buffer. For F-actin visualization, cells were fixed with 4% formaldehyde (electron microscopy grade; Polysciences) and stained with rhodamine-conjugated phalloidin (R-415; Molecular Probes) as described elsewhere (2). F-actin and GFP-fused Cps5p were observed with a laser scanning confocal microscope system (Radiance 2100; Bio-Rad).

Analysis of acid phosphatase. Acid phosphatase was analyzed according to the method described by Huang and Snider (24) with minor modifications (72). Logarithmic-phase cells, grown in YE medium, were transferred to YES-P (yeast

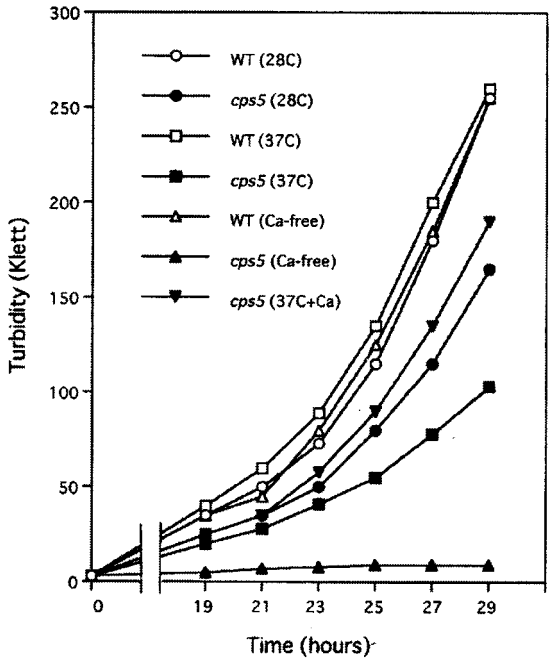

FIG. 1. Defective cell growth of the *cps5-138* mutant under restrictive culture conditions. Cell growth was monitored by measuring the turbidity at 640 to 700 nm (red filter) with a Klett-Summerson colorimeter. Wild-type (WT) (○) and *cps5-138* (●) cells in EMM at 28°C, wild-type (□) and *cps5-138* (■) cells in EMM at 37°C, wild-type (△) and *cps5-138* (▲) cells in Ca^{2+}-free EMM at 28°C, and *cps5-138* cells in EMM plus 50 mM $CaCl_2$ at 37°C (▼).

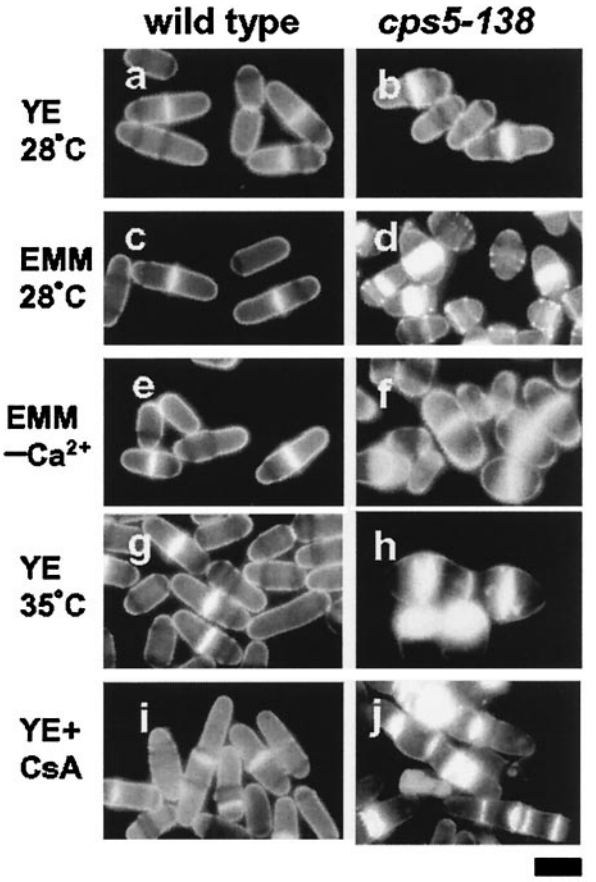

FIG. 2. Abnormal cell morphology and septum formation of the *cps5-138* mutant under restrictive culture conditions. Wild-type and *cps5-138* mutant cells were stained with calcofluor. Log-phase cells of the wild type (a) and *cps5-138* mutant (b) cultured in YE medium at 28°C and wild-type (c) and *cps5-138* mutant (d) cells in EMM at 28°C. Log-phase cells of the wild type (e) and *cps5-138* mutant (f) were transferred to Ca^{2+}-free EMM and cultured for 3 h at 28°C. Wild-type (g) and *cps5-138* mutant (h) cells cultured in YE medium at 35°C for 15 h. Wild-type (i) and *cps5-138* mutant (j) cells cultured in YE medium plus CsA (10 μg/ml) at 28°C for 15 h. Bar, 5 μm.

extract sugar minus phosphate) medium, which contains a reduced amount of inorganic phosphate, and further cultivated for 8 h. Samples prepared from the cell lysate were subjected to electrophoresis on a 6% nondenaturing acrylamide gel, and then activity staining of acid phosphatase was carried out as described by Schweingruber et al. (58).

Cell wall analysis and 1,3-β-D-glucan synthase assay. For the monitoring of enzymatic cell lysis, log-phase cells, grown at 28°C in YE, were washed with 50 mM citrate-phosphate buffer (pH 5.6), suspended in the same buffer containing 30 μg of Novozym234 (Novo Industries)/ml, followed by incubation at 30°C with shaking. The residual absorbance at 600 nm was monitored at hourly intervals, assuming an absorbance of 100% at time zero. Cell extracts for preparation of the enzyme and the 1,3-β-D-glucan synthase assay were carried out as described elsewhere (12). [^{14}C]glucose labeling and the fractionation of cell wall polysaccharides were carried out as described previously (29).

RESULTS

***cps5* mutants show defects in cell growth, morphology, and cytokinesis under low-extracellular-[Ca^{2+}] or high-temperature conditions.** *cps5-138* was originally identified as a mutant allele that confers hypersensitivity to the mitotic poison CIPC (30). *cps5-138* mutant cell growth was also found to be slower than wild-type cell growth under normal conditions and defective under low-[Ca^{2+}] or high-temperature conditions (Fig. 1). The mutant was found to be incapable of growing in the absence of Ca^{2+} (Ca^{2+}-free EMM), in which the wild-type strain is capable of growing normally. The growth defect in EMM (0.1 mM $CaCl_2$) at 37°C was significantly recovered by the addition of $CaCl_2$ (50 mM) to the medium. These results indicate that the *cps5-138* mutant requires a higher [Ca^{2+}] in the medium for normal growth than the wild type. The mutant grown in EMM or in YE at high temperature (37°C) became lemon-like in shape, showing abnormal staining patterns with the dye calcofluor, which has a specific affinity for septum glucans (Fig. 2a to h). The mutant septum was stained much more intensely and broadly than the wild type, suggestive of the excessive deposition of septum materials. A prolonged exposure to high temperature caused cell wall materials to disperse from the medial plane to the cell surface. The rounded shape and abnormal septum staining observed in EMM reverted to the wild-type morphology when the [Ca^{2+}] in the medium was increased to 10 mM (data not shown). The growth and morphology defects at 37°C can be restored to the wild-type phenotype in the presence of an osmotic stabilizer (1.2 M sorbitol), which suggests that the mutant has a cell wall defect (data not shown). Fluorescence-activated cell sorter (FACS) analysis showed that, when the mutant strain is exposed to Ca^{2+}-free EMM, 4C cells with an unseparated septum accumulate gradually (Fig. 3). These results indicate that cell separation becomes defective in the *cps5-138* mutant under restrictive conditions, because in *S. pombe*, DNA synthesis

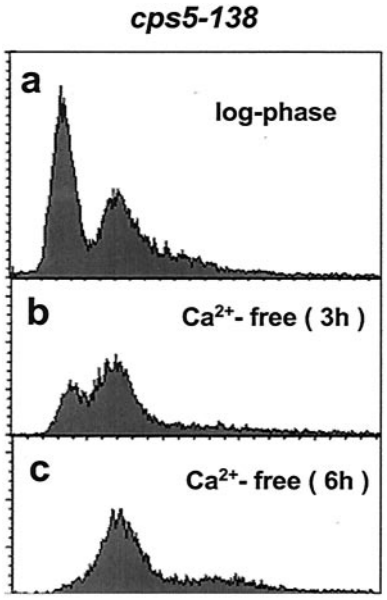

FIG. 3. Defective cytokinesis of the *cps5-138* mutant under restrictive culture conditions. The *cps5-138* mutant was cultured in YE medium at 28°C to the log phase (a), and the collected cells were transferred to Ca^{2+}-free EMM and cultured for 3 (b) and 6 (c) h. Each cell culture was stained with propidium iodide (57) and analyzed with an LSR flow cytometer (BD Biosciences). The horizontal and vertical axes show relative DNA content and cell number, respectively.

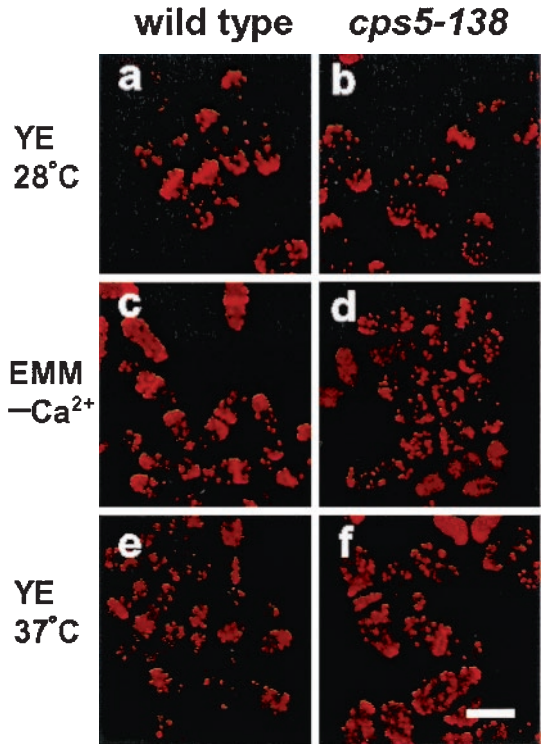

FIG. 4. Depolarized actin distribution of the *cps5-138* mutant under restrictive culture conditions. Wild-type and *cps5-138* mutant cells were stained with rhodamine-conjugated phalloidin. Log-phase cells of the wild type (a) and *cps5-138* mutant (b) cultured in YE medium at 28°C. Log-phase cells of the wild type (c) and *cps5-138* mutant (d) were transferred to Ca^{2+}-free EMM and cultured at 28°C for 3 h. Log-phase cells of the wild type (e) and *cps5-138* mutant (f) were transferred to YE medium and cultured at 37°C for 6 h. Bar, 5 μm.

occurs before cell division is complete. As in the cases of other septation mutants, the *cps5-138* mutant was found to be more sensitive to CsA, a specific inhibitor of calcineurin, than the wild type, giving rise to elongated cells with abnormally formed multisepta (Fig. 2i and j).

***cps5* mutants show a depolarized distribution of actin patches when exposed to low [Ca^{2+}] or high temperature.** The F-actin cytoskeleton is known to be important for cell wall integrity and cytokinesis. To determine whether F-actin is affected by *cps5* mutations, the cytoskeleton was visualized with rhodamine-conjugated phalloidin and observed during the cell cycle by fluorescence microscopy. The wild-type cells showed a polarized F-actin organization during the cell cycle; in interphase, actin patches were specifically localized to the growing end(s), and during late anaphase, they moved to either side of the actin ring, associating with the sites of septum wall formation (Fig. 4a). In the mutant cells, a polarized distribution was also observed under permissive conditions, although actin patches at the cell poles appeared to be somewhat dispersed compared with those in the wild type (Fig. 4b). When the mutant cells were exposed to low-[Ca^{2+}] or high-temperature (37°C) conditions, the actin patches became randomized throughout the cell (Fig. 4c, d, e, and f). Therefore, the altered morphology of the *cps5-135* mutant can be attributed to an altered actin distribution.

***cps5*$^+$ encodes a homologue of the *S. cerevisiae* Pmr1 ATPase.** The *cps5*$^+$ gene was cloned from a fission yeast genomic library by complementation of the CIPC-hypersensitive phenotype of the *cps5-138* mutant strain. Four clones, which were capable of growing in EMM as well as in YE medium containing 280 μM CIPC, were isolated by the screening of 7×10^4 transformants. A nucleotide sequence analysis of the DNA fragments from the clones revealed that only one complete ORF, SPBC31E1.02c (The *S. pombe* Genome Project, The Wellcome Trust Sanger Institute; http://www.sanger.ac.uk/Projects/S_pombe/), is common to all the obtained DNA clones. Plasmids bearing this single ORF complemented not only the hypersensitivity but the abnormal cell morphology and defective cell growth of the *cps5-138* mutant as well under restrictive conditions (data not shown). To determine whether the cloned gene was *cps5*$^+$ itself, the ORF was amplified by high-fidelity PCR from the *cps5-138* genome as a template, and the nucleotide sequence was determined. Analysis of three clones independently obtained by PCR showed that the guanine at nucleotide 1200 of the coding sequence had been replaced by adenine, resulting in the substitution of a nonsense codon (UGA) for the Trp codon. These results indicate that SPBC31E1.02c is *cps5*$^+$ and not an extragenic multicopy suppressor. The ORF was next disrupted in a diploid strain (JY741 × JY746) by replacing *cps5*$^+$ with a copy containing the *ura4*$^+$ gene inserted at the BamHI site that is situated near the middle of the ORF. The transformed diploid was sporulated, and the resulting tetrads were examined for viability. The *ura4*$^+$ disruptants grew normally on a YE plate (data not shown), indicating that *cps5*$^+$ is not essential for cell growth under optimal growth conditions. No phenotypic dif-

ferences between *cps5-138* and *cps5*Δ were noted with respect to cell shape, actin distribution, and cell growth under restrictive conditions (data not shown). These results are consistent with the finding that the *cps5-138* allele is a UGA opal mutation, predicted to encode only 399 amino acids instead of the 899 residues of SPBC31E1.02c. The effects of *cps5*$^+$ overexpression in the wild-type background were examined using the pREP1 plasmid in the absence of thiamine (derepressed conditions), but no remarkable phenotype was observed (data not shown).

The predicted amino acid sequence of the SPBC31E1.02c protein reveals significant homology to proteins of the Pmr1 ATPase family (overall identity with *S. cerevisiae* Pmr1p, 53%) (46). Because the SPBC31E1.02c protein was identified very recently as *S. pombe* Pmr1p (37), we use the name Pmr1p in the following description instead of Cps5p. The *S. pombe* Pmr1p protein was shown to play an important role in cell morphology, cooperating with an *Nramp*-related metal transporter via the control of Mn^{2+} homeostasis (37). In this study, the effects of Mn^{2+} on the defective cell growth and morphology of the *cps5-138* and *cps5*Δ cells under Ca^{2+}-free conditions were also examined. Supplying 0.5 mM $MnCl_2$ to the medium rescued the defective cell growth to a significant extent in both mutants (Fig. 5A) but did not suppress the aberrant cell morphology caused by the Ca^{2+}-free medium (Fig. 5B).

Pmr1p shows a localization pattern typical of endoplasmic reticulum (ER) proteins. The intracellular localization of *S. pombe* Pmr1p was examined by fluorescence microscopy using GFP-tagged Pmr1p. The proteins were observed under two different conditions, plasmid-borne and chromosomal (an integrated single copy with its own promoter) expression of the fused gene in a *cps5*Δ strain. Both transformants are capable of growing in the absence of calcium with an almost normal morphology (data not shown), suggesting that the GFP-tagged Pmr1p is functional with respect to its pump activity. The typical localization pattern is shown in Fig. 6 (top panel). Most of the Pmr1p-GFP was observed in the nuclear and plasma membranes throughout the cell cycle. This localization pattern is typical of the ER proteins, since in *S. pombe*, the ER is associated with the nuclear membrane and extends longitudinally to the cell ends, reaching the entire plasma membrane (6, 49, 50). To confirm this, the ER was also stained with a GFP-tagged Alg3 mannosyltransferase homologue encoded by *cps11*$^+$ (SPAC7D4.06c), because the protein is well established as localizing in the ER membrane. The Alg3p-GFP localization pattern was essentially the same as the case of Pmr1p-GFP (Fig. 6, middle panel). These results suggest that the fission yeast Pmr1p resides predominantly in the ER.

***cps5* mutants show decreased 1,3-β-D-glucan synthase activity with weakened cell walls.** In yeast, the cell wall plays an essential role in determining cell shape, and therefore, a large number of morphological mutants have defects in cell wall integrity and cytokinesis (27). In order to examine whether this is the case for the *cps5* mutant, physiological and biochemical analyses were carried out. Sensitivities to Nomozym234, a cell wall-digesting enzyme complex, in the mutant and wild-type strains were initially compared. The results are shown in Fig. 7. The *cps5-138* mutant grown in YE medium at 28°C was much more sensitive to Novozym234 than the wild-type strain, suggesting that the mutant cell wall is altered, even under permissive conditions. The *cps5*Δ mutant showed the same sensitivity as *cps5-138* (data not shown). The *cps1-12 cps5-138* double mutant was found to be more sensitive to Novozym234 than any of the single mutants, suggesting that Pmr1p and Cps1p/Bgs1p regulate cell wall integrity by different mechanisms.

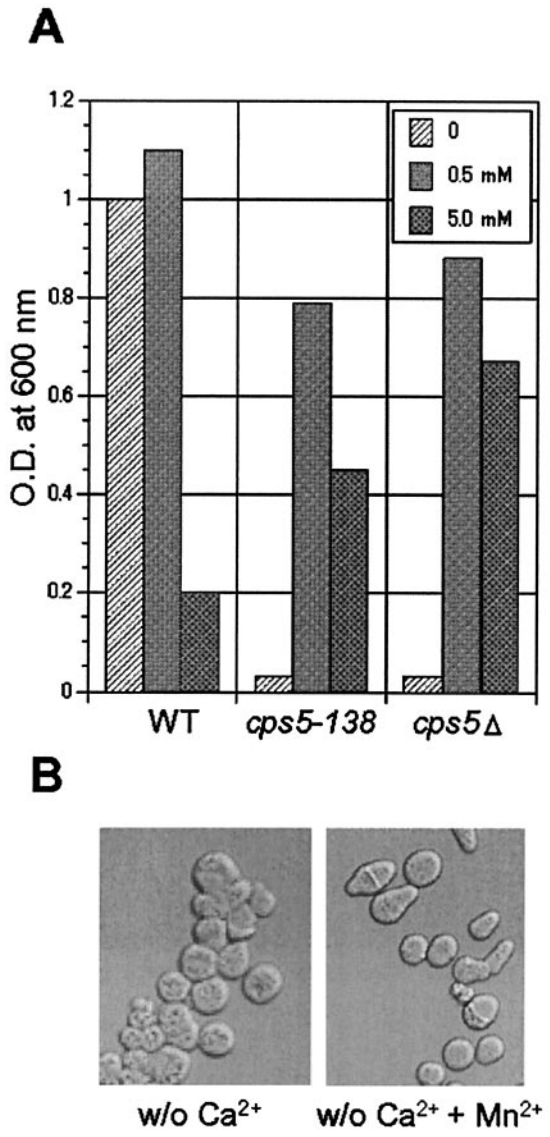

FIG. 5. Effects of $MnCl_2$ on cell growth and morphology of the wild-type (WT), *cps5-138*, and *cps5*Δ strains. A: Each strain was cultured in Ca^{2+}-free EMM and Ca^{2+}-free EMM containing 0.5 or 5.0 mM $MnCl_2$. After 48 h of cultivation, the optical density (O.D.) at 600 nm was measured to quantify the cell growth. B: The *cps5-138* cells were grown in Ca^{2+}-free EMM or Ca^{2+}-free EMM containing 0.5 mM $MnCl_2$ to the log phase and observed with a Nomarski interference microscope. w/o, without.

Next, 1,3-β-D-glucan synthase activities were determined for the wild-type, *cps5-138* mutant, and *cps5*Δ mutant strains grown under permissive and restrictive conditions (37°C in YE medium). As shown in Table 2, the specific activities of the mutant strains grown under permissive conditions in YE medium at 28°C were dramatically reduced by almost half for *cps5-138* (56%) or even more for *cps5*Δ (45%) compared to the

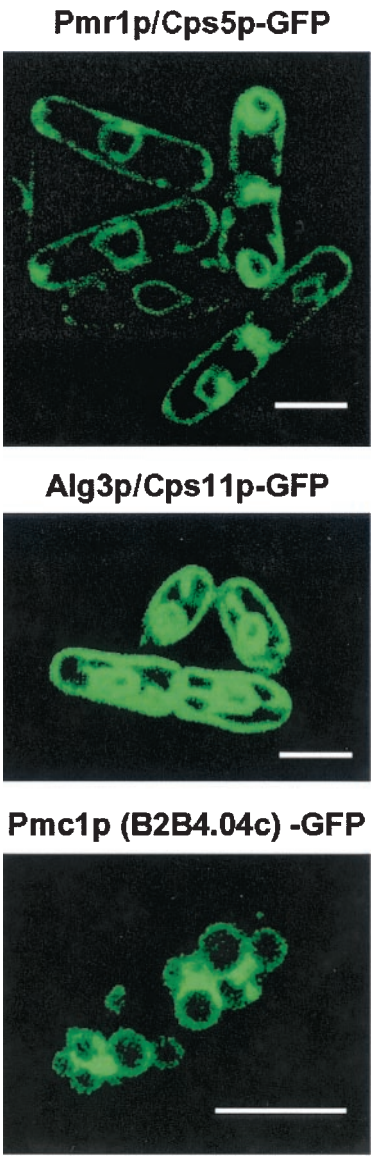

FIG. 6. Intracellular localizations of GFP-tagged Pmr1p/Cps5p, Alg3p/Cps11p, and Pmc1p. Pmr1p was observed in *cps5*Δ cells capable of growing in Ca^{2+}-free EMM with multicopy plasmids bearing the fused gene or the gene integrated in the chromosome. Almost the same fluorescence pattern was obtained in each case. The localizations of Alg3p and Pmc1p were examined in each disruptant with multicopy plasmids. Pmr1p-GFP (top panel) and Alg3p-GFP (middle panel) display a localization pattern typical of *S. pombe* ER proteins, and Pmc1p-GFP (bottom panel) shows the vacuole membrane localization. Bar, 5 μm.

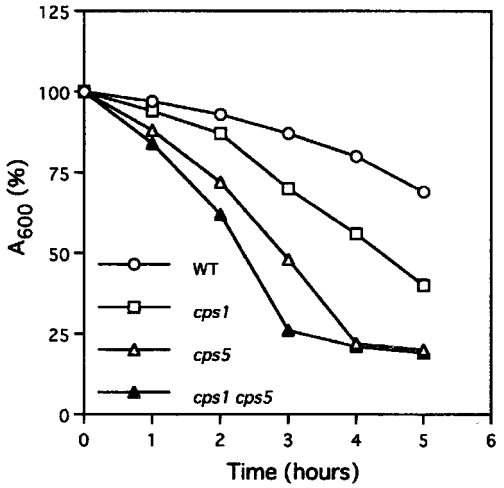

FIG. 7. The *cps5-138* mutant is hypersensitive to the cell wall-digesting enzyme complex Novozym234. Log-phase cells cultured in YE medium were incubated at 30°C with 30 μg of Novozym234/ml, and the residual absorbance was monitored at 600 nm. Average values obtained from triplicate experiments were plotted. Wild type (WT) (○), *cps1-12* mutant (□), *cps5-138* mutant (△), and *cps1 cps5* double mutant (▲).

wild-type strain. Similar results were obtained when the mutant strains were grown in YE medium plus 1.2 M sorbitol at 28°C. Both *cps5-138* and *cps5*Δ present a thermosensitive phenotype at 37°C that can be prevented by osmotic stabilization (1.2 M sorbitol), suggesting that it is due to a cell wall defect. The activity of 1,3-β-D-glucan synthase was assayed in both mutants grown at 37°C, either in the presence or in the absence of sorbitol. In the presence of sorbitol, the cells were normal in shape and growth, and the activity rose to almost 90% of that of the wild type. However, in the absence of sorbitol, in which the cells have a strong oval or rounded phenotype and grow slowly but are still viable (for 24 h cultivation), the activity increased considerably to >120% (*cps5-138*) and 140% (*cps5*Δ) that of the wild type (Table 2). Therefore, the thermosensitive morphological phenotype of the *cps5* mutants is not due to a defect in 1,3-β-D-glucan synthase activity but rather to other *cps5*-related defects.

The *cps5* mutants are unable to grow at 28°C in the absence of Ca^{2+} and grow normally but show an altered morphology in YE medium and EMM (0.1 mM Ca^{2+}). The rounded shape is suppressed when both media contain 10 mM Ca^{2+}. The *cps1-12* mutant also displays hypersensitivity to high tempera-

TABLE 2. Characterization of (1,3)-β-D-glucan synthase activities from *S. pombe* wild-type, *cps5-138*, and *cps5*Δ strains grown at 28 and 37°C in the absence or presence of 1.2 M sorbitol

Growth temp (°C)	Strain	Sp act (mean ± SD)[a]
28	Wild type	10.1 ± 0.1 (100)
	cps5-138	5.7 ± 0.2 (56)
	*cps5*Δ	4.5 ± 0.2 (45)
28 + sorbitol	Wild type	5.8 ± 0.2 (100)
	cps5-138	3.3 ± 0.4 (57)
	*cps5*Δ	2.3 ± 0.2 (40)
37[b]	Wild type	4.6 ± 0.1 (100)
	cps5-138	5.7 ± 0.0 (124)
	*cps5*Δ	6.8 ± 0.7 (148)
37 + sorbitol	Wild type	6.1 ± 0.1 (100)
	cps5-138	5.5 ± 0.1 (90)
	*cps5*Δ	5.2 ± 0.1 (85)

[a] Specific activity is expressed as milliunits per milligram of protein. The reaction mixture contained 150 μM GTP. The values were obtained from three independent experiments. Values in parentheses are percentages of specific activity compared to that of the wild type under the same conditions.

[b] The cells were grown for 24 h at 37°C. Under these conditions, the thermosensitive morphological phenotype is completely expressed in the absence of sorbitol, and the cells are still viable and able to grow.

TABLE 3. Characterization of (1,3)-β-D-glucan synthase activities from *S. pombe* wild-type; *cps1-12*, *cps5-138*, and *cps5*Δ mutant; and *cps1-12 cps5-138* double mutant strains grown at 28°C in EMM in the presence or absence of calcium

$CaCl_2$, concn (mM)	Strain	Sp act (mean ± SD)[a]
10	Wild type	7.4 ± 0.4 (100)
	cps1-12	8.7 ± 0.7 (118)
	cps5-138	6.6 ± 0.6 (89)
	*cps5*Δ	6.1 ± 0.4 (82)
	cps1-12 cps5-138	6.2 ± 0.5 (84)
0.1	Wild type	9.8 ± 0.1 (100)
	cps1-12	12.4 ± 1.2 (127)
	cps5-138	8.7 ± 1.2 (89)
	*cps5*Δ	6.2 ± 0.8 (63)
	cps1-12 cps5-138	8.0 ± 0.5 (82)
0[b]	Wild type	15.0 ± 0.4 (100)
	cps1-12	12.3 ± 0.9 (82)
	cps5-138	8.0 ± 0.7 (53)
	*cps5*Δ	7.6 ± 1.3 (51)
	cps1-12 cps5-138	12.6 ± 1.1 (84)

[a] Values were obtained from three independent experiments as for Table 2. Values in parenthesis are percentages of specific activity compared to that of the wild type under the same conditions.

[b] The cells were grown for 4 h in Ca^{2+}-free EMM plus 0.1 mM EGTA. A longer incubation time produced the appearance of some cell lysis in the mutant cultures.

ture, to cell wall-digesting enzymatic complexes, to CsA (29), and to Ca^{2+}-free EMM. In addition, the thermosensitive phenotype is partially suppressed in the presence of 10 mM Ca^{2+}. To confirm the relationship between cell wall weakness and extracellular [Ca^{2+}] in these mutants, the 1,3-β-D-glucan synthase activity was analyzed under low (0.1 mM)- and high (10 mM)-[Ca^{2+}] conditions (Table 3). The activity of the putative 1,3-β-D-glucan synthase mutant *cps1-12* grown in the presence of 10 or 0.1 mM Ca^{2+} at 28°C was higher than that of the wild type (118 and 127%). A similar increase in activity was described for this mutant, based on an analysis of the thermosensitive phenotype (29). The increased activity may be the result of a compensatory mechanism taking place under conditions of cell wall stress (51). On the other hand, the absence of Ca^{2+} caused a decrease in the 1,3-β-D-glucan synthase activity of *cps1-12* cells to 82% of that of the wild type. The activities of single *cps5* and double *cps1 cps5* mutants were similar in a medium containing 10 and 0.1 mM Ca^{2+}. The *cps5*Δ mutant was more sensitive to [Ca^{2+}], as the activity in cells grown in 0.1 mM Ca^{2+} decreased to 63%. Nonetheless, in the absence of Ca^{2+}, both *cps5-138* and *cps5*Δ showed a dramatic decrease to 50% of the activity of the wild type. Under these conditions, the *cps1-12* mutant showed an epistatic phenotype, as the enzymatic activities were about the same in both single *cps1-12* and double *cps1-12 cps5-138* mutants (82 and 84%). These results led us to conclude that the Ca^{2+}-hypersensitivity of *cps5* mutants correlates with a decrease in 1,3-β-D-glucan synthase activity.

***cps5* mutant cells grown under Ca^{2+}-free conditions have no detectable cell wall galactomannan.** The results of both in vivo (sensitivity to Novozym234) and in vitro (1,3-β-D-glucan synthase activity) cell wall analyses of *cps5* mutants suggest that the mutant cell wall has an altered composition. To confirm this possibility, a quantitative analysis of cell wall polysaccharides from *cps5-138* and *cps5*Δ cells grown in the presence of 10 mM Ca^{2+} or in the absence of Ca^{2+} was carried out (Table 4). Even in the presence of a high [Ca^{2+}], the mutant cell walls showed an almost 30% decrease in galactomannan content compared with the wild-type cell wall. Under Ca^{2+}-free conditions, no detectable galactomannan appeared to be incorporated into the mutant cell walls. These results indicate that Pmr1p is involved in a general mechanism of wall protein galactosylation, and the process requires proper Ca^{2+} homeostasis brought about by Pmr1p. This is consistent with the finding that osmotic stabilization is not able to rescue the growth defect of the mutants under Ca^{2+}-free conditions (data not shown). A significant increase in cell wall α-glucan (30%) in the mutant cells grown under Ca^{2+}-free conditions could be explained by cell wall stress response. Indeed, it has been reported that when the level of Bgs3p, a putative 1,3-β-D-glucan synthase, is decreased using the 81X version of the repressible *nmt1*$^+$ promoter, the amount of α-glucan in the cell wall increases significantly (39).

Pmr1p is involved in protein glycosylation. *S. cerevisiae* Pmr1p has been shown to play a role in Golgi functions, including protein glycosylation and secretion (3, 19, 54). The analysis of cell wall galactomannan contents in *cps5* mutants also suggests the involvement of *S. pombe* Pmr1p in protein glycosylation. To confirm this, acid phosphatase was analyzed by means of nondenaturing acrylamide gel electrophoresis. Since acid phosphatase is highly glycosylated, activity staining of the enzyme shows a smeared broad band in the upper part of the gel, while the enzyme moves faster when it is incom-

TABLE 4. Incorporation of radioactivity from [^{14}C]glucose into cell wall polysaccharides of *S. pombe* wild-type, *cps5-138*, and *cps5*Δ strains grown at 28°C in EMM in the presence or absence of calcium

$CaCl_2$ Concn (mM)	Strain	% Incorporation of [^{14}C]glucose (mean ± SD)[a]			
		Cell wall	Galactomannan	α-Glucan	β-Glucan
10	Wild type	34.3 ± 0.7	4.8 ± 0.2 (14.0)	9.0 ± 0.4 (26.2)	20.5 ± 0.1 (59.8)
	cps5-138	34.0 ± 1.2	3.4 ± 0.1 (10.0)	8.0 ± 0.5 (23.5)	22.6 ± 0.6 (66.5)
	*cps5*Δ	34.1 ± 0.7	3.4 ± 0.2 (10.0)	8.2 ± 0.0 (24.0)	22.5 ± 0.9 (66.0)
0[b]	Wild type	34.3 ± 0.8	3.7 ± 0.0 (10.8)	9.1 ± 0.3 (26.5)	21.5 ± 0.9 (62.7)
	cps5-138	37.4 ± 1.2	<0.01 (0)	12.8 ± 0.6 (34.2)	24.6 ± 0.6 (65.8)
	*cps5*Δ	40.8 ± 0.8	<0.01 (0)	13.7 ± 0.4 (33.6)	27.1 ± 0.4 (66.4)

[a] Percent incorporation of [^{14}C]glucose is the counts per minute incorporated per fraction × 100/total counts per minute incorporated. The values were obtained from three independent experiments. Values in parentheses are percentages of the corresponding polysaccharide in the cell wall composition.

[b] The cells were grown for 4 h in Ca^{2+}-EMM plus 0.1 mM EGTA.

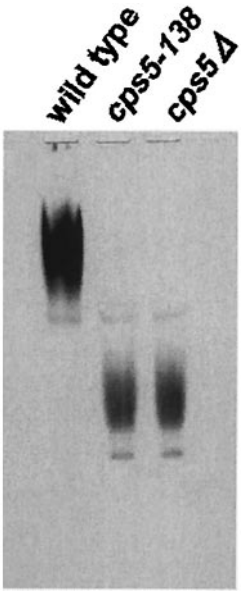

FIG. 8. Activity staining of acid phosphatase on a nondenaturing polyacrylamide gel. Lysates from wild-type, *cps5-138*, and *cps5*Δ cells induced for acid phosphatase expression were subjected to electrophoresis on a 6% gel and stained for acid phosphatase activity.

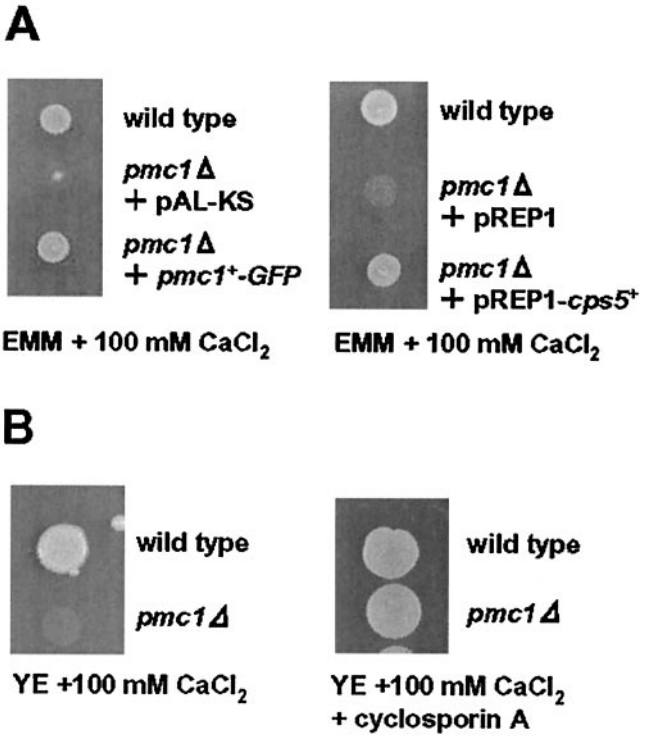

FIG. 9. Complementation of the high-Ca^{2+}-sensitive cell growth of the *pmc1*Δ strain. A: The cell suspension from the *pmc1*Δ strain transformed with pAL-KS, pREP1, pAL-KS bearing *pmc1*$^+$*-GFP*, or pREP1 bearing *cps5*$^+$ was spotted on an EMM plate containing 100 mM $CaCl_2$. B: The *pmc1*Δ cell suspension was spotted on a YE plate containing 100 mM $CaCl_2$ or 100 mM $CaCl_2$ plus CsA (10 μg/ml). The spotted plates were incubated at 28°C for 4 to 5 days.

pletely glycosylated (58). The acid phosphatase from the *cps5* mutants exhibited increased electrophoretic mobility compared with that from the wild type (Fig. 8), indicating that the mutants synthesized an incompletely glycosylated acid phosphatase, even under optimal growth conditions. The electrophoretic patterns are similar to those from *gmh3*Δ and *gms1*Δ strains, the genes of which encode a galactosyltransferase and UDP-galactose transporter, respectively (66, 72), consistent with the finding that *cps5* mutants contain no detectable galactomannan in the cell walls.

Pmr1p shares its function with the Pmc1p homologue to maintain intracellular Ca^{2+} homeostasis. In *S. cerevisiae*, it was shown that Pmr1p acts together with Pmc1p to deplete cytosolic Ca^{2+}, by which a lethal activation of calcineurin is prevented (15). To examine whether this is also true in *S. pombe*, the *PMC1* homologue, SPAPB2B4B.04c, was disrupted, and the phenotypes were examined. The *pmc1*Δ strain B2B4#6 was found to grow almost normally in YE medium but was incapable of growing in the presence of high [Ca^{2+}] in medium (>100 mM), conditions under which wild-type cells are able to grow normally (Fig. 9B). This result suggests that Pmc1p is not essential under normal growth conditions but is required for high-Ca^{2+} conditions. The high-Ca^{2+}-sensitive phenotypes of *pmc1*Δ were significantly suppressed either in the presence of CsA, a potent inhibitor of calcineurin, in the medium or by overexpression of the *cps5*$^+$ gene in *pmc1*Δ cells (Fig. 9A and B). These results suggest that the growth defect is due to an inappropriate activation of calcineurin, which is caused by an elevated intracellular [Ca^{2+}], and that Pmr1p also acts to deplete cytosolic Ca^{2+}, cooperating with the Pmc1p homologue. In *S. cerevisiae*, the double mutation of *PMR1* and *PMC1* is synthetic lethal (14). To examine whether this is also the case in *S. pombe*, a tetrad analysis of the heterozygous diploids (CP13-8-4D × B2B4#6) was carried out. Five sets of four meiotic progeny (PD type), 9 sets of two progeny (NPD), and 23 sets of three progeny (T) were obtained. None of the double mutants grew on YE plates. These results strongly suggest that the two Ca^{2+}-ATPase homologues, Pmr1p and Pmc1p, in *S. pombe* share an essential role in depleting cytosolic Ca^{2+} to maintain a proper level of calcineurin activity for cell growth, even under standard culture conditions.

In *S. cerevisiae*, Pmc1p, which is 40% identical to the plasma membrane Ca^{2+}-ATPase of mammalian cells, was shown to be localized to the vacuole membrane (15). To identify the intracellular localization of the Pmc1p homologue in *S. pombe*, GFP-tagged Pmc1p was observed by fluorescence microscopy. The Pmc1p-GFP fusion protein was capable of complementing high-Ca^{2+}-sensitive phenotypes (Fig. 9A), suggesting that the fusion protein is functional with respect to its pump activity. As shown in Fig. 6 (bottom panel), Pmc1p-GFP was essentially localized to the vacuole membrane, which is similar to the case of budding yeast.

DISCUSSION

In the fission yeast *S. pombe*, 14 putative P-type ATPase genes were identified by a homology search of ORFs deduced from the *S. pombe* genome project (46, 71). At least three putative calcium ATPases have been assigned, namely, Cta4p (45), Pmc1p (SPAPB2B4.04c), and Pmr1p/Cps5p (SPBC31E1.02c) (37). At

present, however, knowledge concerning the roles of the pump activity in cellular functions and how the activities are coordinately regulated in response to changes in intra- and extracellular [Ca^{2+}] is very limited. The SPBC31E1.02c protein shows significant sequence similarity to the Pmr1 family of ATPases, which are present in a wide range of fungi and animal tissues (the fission yeast Pmr1p protein is most closely related to that of *Yarrowia lipolytica* [48], with a CLUSTALW score of 52.8). It has been well established in *S. cerevisiae* that Pmr1p is required for a variety of cellular functions, including N-linked and O-linked protein glycosylation, protein sorting in secretory pathways, and ER-associated protein degradation via its Ca^{2+} Mn^{2+} transporting activity, and that the pump is localized primarily in the medial Golgi (3, 19, 36, 44, 54, 60). In *S. pombe*, however, GFP-tagged Pmr1p appears to be localized predominantly in the ER rather than in the Golgi apparatus. It has been shown that the budding yeast Pmr1p is required for ER functions, such as Ca^{2+} homeostasis in the ER lumen, oligosaccharide trimming, and ER quality control, despite its Golgi localization (19, 62, 70) and that Cod1p/Spf1p, a putative P-type ATPase, is involved in processing the outer chain of carbohydrates in the Golgi, despite its localization in the ER (13, 64). We have recently found that a mutation in the *cps11*$^+$ gene, which codes for the Alg3p homologue of *S. cerevisiae*, a Dol-P-Man-dependent mannosyltransferase residing in the ER (1, 59), causes defects of cell wall integrity and cell growth under the same restrictive conditions as in the case of *cps5* mutations (unpublished result). As expected, the GFP-tagged Cps11p/Alg3p showed essentially the same localization pattern as Pmr1p. These findings support the view that the fission yeast Pmr1p resides predominantly in the ER membrane. This notion does not exclude the possibility that the Pmr1p pump activity is required for the Golgi functions, because it is quite possible to supply Ca^{2+} and Mn^{2+} ions to the Golgi apparatus despite the ER localization or by a residual pump activity from a small number of Golgi-resident Pmr1 molecules (19). In *S. cerevisiae*, GFP-tagged Pmr1p was reported to show a typical Golgi localization pattern, even when expressed from multicopy plasmids (38). However, it has also been observed that hemagglutinin-tagged Pmr1p is localized in the Golgi but the GFP-tagged version is localized in the ER under certain experimental conditions. Therefore, the possibility should be noted that the fission yeast Pmr1p was mislocalized or accumulated in the ER owing to GFP tagging, although the fusion protein is able to rescue the *cps5* mutant phenotypes.

A phenotypic relationship between loss of Pmr1p function and an abnormal cell wall morphology was first reported in *Kluyveromyces lactis* (67). *Klpmr1*Δ cells revealed several defective phenotypes, including incomplete protein glycosylation, aberrant chitin deposition, and a thickened cell wall with an unbalanced ratio of insoluble to soluble glucans. These results suggest that the cell wall and wall-related glycoproteins, including the 1,3-β-D-glucan synthases, are affected by *pmr1* mutations, resulting in changes in their enzymatic activities and/or in their subcellular distribution. Consistent with this hypothesis, we found that the in vitro 1,3-β-D-glucan synthase activity decreased by nearly 50% in *cps5* mutant cells, even those grown in a standard YE medium. In the wild type, specific activity levels were higher under calcium-free conditions than in the presence of 0.1 or 10 mM calcium, and the level of α- and β-glucans increased to some extent under Ca^{2+}-free conditions, particularly in the mutant cell walls. The reason for this is unclear, but it could be the result of a cell wall stress response, as described in Results. The 1,3-β-D-glucan synthase of the cell surface could be hyperactivated during the early stages of starvation as a survival mechanism and could be gradually inactivated during longer periods of starvation. Alternatively, the 1,3-β-D-glucan synthase activity measured in vitro may be different from the activity responsible for the synthesis of 1,3-β-D-glucan in vivo, as is the case in *S. cerevisiae* for differences between in vivo chitin synthesis and in vitro chitin synthase activities measured for Chs1p, Chs2p, and Chs3p (69). In *S. pombe*, four putative 1,3-β-D-glucan synthases have been identified, and at least three of them (Bgs1p/Cps1p, Bgs3p, and Bgs4p) are responsible for cell wall synthesis during the vegetative cell cycle (12, 18, 39; J. C. Ribas, unpublished results). We do not know yet which enzyme or enzymes are responsible for the activity detected in vitro and how they are regulated in response to changes in environmental conditions. However, it is clear that the specific activity levels of 1,3-β-D-glucan synthase were always much lower in the mutants than in the wild type under the same experimental conditions. An important finding is that there was almost no detectable galactomannan in the mutant cell walls, with an unbalanced ratio of α- and β-glucans, when the mutant cells were grown in the absence of calcium; the former could be caused by a defect in protein glycosylation, and the latter could be induced by changes in 1,3-β-D-glucan synthase activity. Cell wall weakness thus can be attributed to the dramatic changes in the composition of the mutant cell wall. It should be noted that deletion of *S. cerevisiae PMR1* does not result in a detectable change in cell wall composition, in spite of the glycosylation defects (67).

Very recently, the *S. pombe* Pmr1p was reported to play an important role in Mn^{2+} homeostasis, cooperating with Pdt1p (an *Nramp*-related metal transporter) to regulate cell morphogenesis (37). The *pmr1*Δ *pdt1*Δ double mutants displayed more severe morphological defects than each single mutant when they were grown in EMM. Supplying Mn^{2+} to the medium suppressed defects of the double mutant in both cell morphology and the glycosylation of acid phosphatase to a great extent (37), consistent with the fact that Mn^{2+} is an important cofactor for protein glycosylation. In this study, we also confirmed that Mn^{2+} has a rescuing effect on the defective growth of *cps5-138* and *cps5*Δ cells in Ca^{2+}-depleted medium, but it was incapable of restoring their cell morphology in the absence of calcium. As already mentioned, the growth defect in the absence of calcium was not rescued even in the presence of sorbitol. These findings suggest that, in addition to protein glycosylation, which requires Mn^{2+} homeostasis in the secretory pathway, Pmr1p plays an important role in cell growth and cell-wall morphogenesis via control of cytosolic and/or organelle Ca^{2+} concentrations.

In *S. cerevisiae*, it has been shown that cell wall synthesis requires the coordination of a variety of cellular events, including the recruiting of cell wall materials and the glucan synthases themselves to the plasma membrane, enzyme activation by the GTP-binding protein Rho1p, and glycosylation of the glycosyl phosphatidylinositol anchor and stress sensor proteins (7, 10, 17, 33, 47). Another important component is the actin cytoskeleton (27, 52). F-actin is required for maintenance of cell wall structure and development (21, 34), as well as the polarized localization of Fks1p (16). Actin patch motility is

also required for Fks1p movement, by which the structure and function of the cell wall are maintained (68). In *S. pombe*, the actin cytoskeleton was suggested to be involved in the secretion of cell wall materials, such as 1,6-β-glucan, α-glucan, and α-galactomannan (35). Although the molecular mechanisms for such interdependence of the actin cytoskeleton and cell wall synthesis remain unclear, the actin cytoskeleton is presumed to act as transporting machinery in the secretory pathway. Since Ca^{2+} is an important regulatory factor in the organization of F-actin, as well as in vesicle transport in mammalian cells, similar mechanisms could also function in fungal systems (43).

In most cases, cell wall mutants display a simultaneous defect in the formation of the septum (27). In *cps5* mutants, the early steps in septum formation may not be impaired, because an F-actin ring appears to form normally. The late steps requires enzymes responsible for the synthesis of glucans, as well as septum materials, to be properly transferred to the site where the septum is formed. It is easy to imagine that the unbalanced synthesis of polysaccharides observed in the mutant cell walls and/or the incomplete or missing glycosylation of wall proteins also impairs septum synthesis, resulting in a defect in cytokinesis followed by cell separation. Although an excessive deposition of septum materials was observed, the defective mechanism remains unclear. It may be worth noting that septated cells that accumulated in Ca^{2+}-free medium for 3 h were reduced in number by almost one-third when shifted back to permissive conditions within 3 h. This implies that ill-formed septa are reactivated to divide under permissive conditions when the cells are exposed for a short period to restrictive conditions.

In *S. cerevisiae*, it has been shown that Ca^{2+} signals generated by mating pheromones or a high-Ca^{2+} environment induce the expression of certain genes, including *PMR1* and *PMC1*, which encode the Golgi and vacuolar Ca^{2+} ATPases, respectively, and *FKS2*, which encodes a putative 1,3-β-D-glucan synthase catalytic subunit, via a calcineurin-dependent transcription factor, Tcn1p/Crz1p (40, 42, 61, 74). It was also reported that in *S. pombe* the expression of *pmr1*$^+$ and *pmc1*$^+$ is controlled through a calcineurin-dependent transcription factor, Prz1p (22, 37). In budding yeast, Pmr1p and Pmc1p cooperatively regulate cytosolic [Ca^{2+}] to a level appropriate for the activation of calcineurin, implying the existence of a feedback mechanism for the calcineurin pathway via modulation of cytosolic [Ca^{2+}] (15). In this study, we also observed the same functional interdependence between *S. pombe* Pmr1p and Pmc1p in preventing lethal activation of calcineurin. The reason why *pmr1*Δ causes cells to be low-calcium sensitive and *pmc1*Δ causes cells to be high-calcium sensitive is that Pmr1p is essential for the supply of Ca^{2+} and Mn^{2+} to the organelles but is not necessarily required for depleting excess cytosolic [Ca^{2+}], because Pmc1p is fully active in the latter process, although simultaneous loss of Pmr1p and Pmc1p functions causes fatal damage to the cell. We have observed that *pmc1*Δ cells become somewhat resistant to the cell wall-digesting reagent Novozym234 (unpublished results), suggesting that cytosolic [Ca^{2+}] may also affect cell wall integrity. Indeed, Ehs1p, a homologue of the Ca^{2+} channel component Mid1p of *S. cerevisiae*, is reported to be involved in the cell wall integrity pathway, functioning with Pck2p, a protein kinase C homologue (9). To determine whether Pmr1p pump activity is involved in the Pck2 pathway, *pck2*$^+$ was overexpressed in the *cps5* mutants. As a result, neither the *pck2*$^+$ overexpression-related phenotype nor the *cps5* mutant phenotypes were suppressed, consistent with the finding that *cps5*$^+$ failed to rescue the *ehs1-1* mutant phenotypes and vice versa (unpublished result). These results suggest that Pmr1p is not involved in the Ehs1p-Pck2p pathway.

cps1-12 mutants display hypersensitivity to CsA, a potent inhibitor of calcineurin, as well as to Ca^{2+}-free EMM, and an increase of exogenous [Ca^{2+}] (to 10 mM) is able to partially suppress the thermosensitive phenotype (reference 29 and unpublished results). CsA appears to aggravate *cps5-138* mutant phenotypes, and the *cps5-138 cps1-12* double mutant is more hypersensitive to Novozym234 than either of the single mutants. From these findings, it is tempting to speculate that the gene expression of a 1,3-β-D-glucan synthase homologue(s) other than Bgs1p/Cps1p could be controlled by a calcineurin-dependent mechanism and that Pmr1p is involved in the regulation of this pathway via the control of cytosolic and/or organelle [Ca^{2+}].

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