Characterization of a fission yeast P₅-type ATPase homologue that is essential for Ca²⁺/Mn²⁺ homeostasis in the absence of P₂-type ATPases

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(Received 26 September 2008, accepted 17 October 2008)

In the fission yeast *Schizosaccharomyces pombe*, three P-type ATPases, namely Cta4p, Pmr1p, and Pmc1p, have been shown to be essential for Ca²⁺ homeostasis and are required for specific cellular functions as well. Here, we show that the simultaneous deletion of *pmc1*⁺ and *SPAC29A4.19c*, which encodes a putative P₅-type ATPase, causes a hypersensitive growth to either high concentrations of Ca²⁺ in a medium, or the antiarrhythmic drug amiodarone, which has been known to cause a disruption of Ca²⁺ homeostasis. On the other hand, simultaneous deletion of *pmr1*⁺ and *SPAC29A4.19c* causes a hypersensitive growth to Mn²⁺ depletion in a medium. The green fluorescent protein-tagged *SPAC29A4.19c* protein reveals a typical localization pattern of the Golgi proteins, but the *SPAC29A4.19c* protein is not exchangeable in function with Pmr1p, which is required for Ca²⁺/Mn²⁺ homeostasis in secretory pathways. These results suggest that the putative P₅-type ATPase encoded by *SPAC29A4.19c* is essential for Ca²⁺ and Mn²⁺ homeostasis in the absence of P₂-type ATPases, Pmc1p or Pmr1p, respectively. According to the precedent nomenclature of calcium/cation transporting ATPase in fission yeast, *SPAC29A4.19* was named *cta5*⁺ in this study.

Key words: Ca²⁺/Mn²⁺ homeostasis, cell growth, *cta5*⁺, fission yeast, P₅-type ATPase, *SPAC29A4.19c*

INTRODUCTION

The concentration of cytosolic calcium ions ([Ca²⁺]_i) in eukaryotic cells is precisely regulated to maintain a very low level, at around 10⁻⁷ M, by the pumping of Ca²⁺ out of the cell and/or into organelles. A transient and local increase in the [Ca²⁺]_i, which is mobilized via channels by extra- or intra-cellular stimuli, transmits signals that modulate a variety of cellular functions, including secretion, gene expression, and cell proliferation (for reviews, see Means, 1994; Clapham, 1995; Berridge, 1995; Tse and Tse, 1999; Berridge et al., 2003). A Ca²⁺ transporting system composed of channels, pumps (ATPases) and exchangers thus plays a crucial role in Ca²⁺ homeostasis to generate the signals. In the fission yeast, *Schizosaccharomyces pombe*, at least three P-type ATPases, namely Cta4p, Pmr1p, and Pmc1p, have been shown to be required for Ca²⁺ homeostasis as well as specific cellular functions (Okorokova-Façanha et al., 2002; Maeda et al., 2004; Cortés et al., 2004). The deletion mutant of *cta4*⁺

displays an aberrant microtubule organization and cytokinesis (Okorokova-Façanha et al., 2002), and the *cta4* mutation, *sev4-L5*, causes defect in forespore membrane formation (Yoshida et al., 2005). The mutation of *pmr1*⁺ (*cps5-138*) causes defects in actin organization and protein glycosylation, by which cell wall integrity and cytokinesis are impaired (Cortés et al., 2004). The *pmr1* mutants exhibit Ca²⁺-dependent growth, while the deletion mutant of *pmc1*⁺ is hypersensitive to high concentrations of Ca²⁺ in a medium (Cortés et al., 2004). In both *S. cerevisiae* and *S. pombe*, Pmr1p shares its function with a vacuolar Ca²⁺-ATPase, Pmc1p, to prevent lethal activation of a Ca²⁺/calmodulin-dependent protein phosphatase, calcineurin (Cunningham and Fink 1994a, 1994b; Cortés et al., 2004).

Homology-based searches of ORFs deduced from the *S. pombe* genome database (http://www.sanger.ac.uk/Projects/S_pombe/) have identified fourteen putative P-type ATPase genes that are assigned into five phylogenetically distinct groups (Wood et al., 2002; Okorokova-Façanha et al., 2003). *cta4*⁺ belongs to a P₅-type ATPase family, which consists of two other homologue genes, *SPAC29A4.19c* and *SPCC1672.11c* (from herein, desig-

Edited by Akio Toh-e

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nated as *29A4.19c* and *1672.11c*, respectively). Homology-based searches have also revealed a subfamily of P₅-type ATPases including *29A4.19c* to be distributed in a wide range of eukaryotes, from fungi to mammalian cells (Schultheis et al., 2004). The *29A4.19c* protein is more closely related to the *S. cerevisiae* YOR291W protein (E-val: e-121) than Spf1p/Cod1p (E-val: 4e-52), which is orthologous to the Cta4p. The Spf1/Cod1p has been shown to be involved in secretory pathways and Ca²⁺ homeostasis (Suzuki and Shimma, 1999; Suzuki, 2001; Cronin et al., 2002), while the function of YOR291W protein remains unclear. Interestingly, a human P₅-type ATPase gene AFURS1 (also named *ATP13A3*), which is orthologous to *29A4.19c* (E-val: e-106), was identified as a gene that is overexpressed in senescent kidney parenchymal cells (Habtemichael and Kovacs, 2002). The role of the gene product has not yet been clarified but it is implied to be involved in cellular senescence and tumor suppression in mammalian cells. Recently, hereditary parkinsonism was reported to be caused by mutations in another human P₅-type ATPase gene, *ATP13A2*, that is also orthologous to *29A4.19c* (E-val: 1e-93) (Ramirez et al., 2006). However, at present, the substrate specificity and the physiological role of these presumable cation ATPases have not yet been clarified. We have characterized deletion mutants of two P₅-type ATPase homologue genes in *S. pombe*, and found that the gene product encoded by *29A4.19c* is essential for Ca²⁺ and Mn²⁺ homeostasis in the absence of P₂-type ATPases, Pmc1p or Pmr1p, respectively. We also show that the green fluorescent protein (GFP)-tagged *29A4.19c* protein reveals a typical localization pattern of the Golgi proteins, but the *29A4.19c* protein is not exchangeable in function with Pmr1p, which is required for secretory pathways.

MATERIALS AND METHODS

Strains and culture conditions The strains used in this study are listed in Table 1. Standard rich medium (YE), Edinburgh minimal medium (EMM) supplemented with the appropriate amino acids, and sporulation medium have been described elsewhere (Egel, 1984; Alfa et al., 1993). In some experiments, CaCl₂ was added to YE or EMM medium. Mn²⁺-free EMM was prepared from EMM medium by omitting MnCl₂ and MnSO₄ from Salt and Mineral Stocks, respectively. The yeast cells were cultured at 28°C on a plate or in liquid medium with continuous shaking. *Escherichia coli* strain DH5α was used for the routine propagation of plasmids, as described elsewhere (Sambrook et al., 1989).

DNA techniques and plasmids DNA manipulations were carried out according to standard methods as described previously (Sambrook et al., 1989). The plasmids pAL-KS, pJR1-3XL, and pJK148 are described elsewhere (Ishiguro et al., 1997; Moreno et al., 2000; Cortés et al., 2002).

Gene disruption and overexpression The one-step gene disruption method (Rothstein, 1983) was employed to construct the deletion mutants of *pmr1*⁺ (*pmr1Δ*), *pmc1*⁺ (*pmc1Δ*), *29A4.19c* (*29A4Δ*) and *1672.11c* (*1672Δ*). *pmr1Δ* and *pmc1Δ* have already been described (Cortés et al., 2004). The primers used in this study are listed in Table 2. *29A4.19c* contains *Xba*I site (2,115 base from the start position of the ORF), *Xho*I site (2,422), *Nde*I site (2,558) and *Pst*I site (2,681), respectively. For disruption of the ORF, 1.8 kb *ura4*⁺ gene was inserted between the *Xba*I and *Xho*I sites, according to the following proce-

Table 1. *S. pombe* strains used in this study

Strains	Genotype	Source
903	h ⁻ <i>leu1-32</i>	J. Ishiguro
903-4D	h ⁺ <i>leu1-32 ura4-D18</i>	J. Ishiguro
JY741	h ⁻ <i>ade6-M216 ura4-D18 leu1-32</i>	YGRC ^{*1}
JY746	h ⁺ <i>ade6-M210 ura4-D18 leu1-32</i>	YGRC
<i>pmr1Δ</i>	h ⁺ <i>ura4-D18 leu1-32 pmr1::ura4</i> ⁺	J. Ishiguro ^{*2}
<i>pmc1Δ</i>	h ⁺ <i>ura4-D18 leu1-32 pmc1::ura4</i> ⁺	J. Ishiguro ^{*2}
<i>29A4Δ</i>	h ⁺ <i>ura4-D18 leu1-32 29A4.19c::ura4</i> ⁺	This study
<i>1672Δ</i>	h ⁺ <i>ura4-D18 leu1-32 1672.11c::ura4</i> ⁺	This study
<i>29A4Δ pmc1Δ</i>	h ⁻ <i>ura4-D18 leu1-32 29A4.19c::ura4</i> ⁺ <i>pmc1::ura4</i> ⁺	This study
<i>29A4Δ pmr1Δ</i>	h ⁺ <i>ura4-D18 leu1-32 29A4.19c::ura4</i> ⁺ <i>pmr1::ura4</i> ⁺	This study
<i>29A4Δ 1672Δ</i>	h ⁻ <i>ura4-D18 leu1-32 29A4.19c::ura4</i> ⁺ <i>1672.11c::ura4</i> ⁺	This study
<i>1672Δ pmc1Δ</i>	h ⁻ <i>ura4-D18 leu1-32 1672.11c::ura4</i> ⁺ <i>pmc1::ura4</i> ⁺	This study
<i>1672Δ pmr1Δ</i>	h ⁺ <i>ura4-D18 leu1-32 1672.11c::ura4</i> ⁺ <i>pmr1::ura4</i> ⁺	This study

^{*1} Yeast Genetic Resource Center <http://yeast.lab.nig.ac.jp/nig/>

^{*2} Eukaryot. Cell 3: 1124–1135 (2004).

Table 2. PCR primers used in this study

Name	Sequence
29A4-F	5'-GGGGTACCCCGCCTACAGAGTAGCTACATG-3'
29A4-R	5'-TCATAGCTGCTGACAGTGGA-3'
1672-F1	5'-GGAATTCCGTCATTGGGGATGTCTTTGA-3'
1672-R1	5'-GGGGTACCCCCATAGCTAATGAAGCACCT-3'
1672-F2	5'-CGGGATCCCGTGAAGCGTGAGCAAGTTGAA-3'
1672-R2	5'-GCTCTAGAGCTTGACGAGAGATGCTTCATCCG-3'
29A4-OPF	5'-TCCCCCGGGGACATTTTTCACCGATTGGA-3'
29A4-OPR	5'-TCCCCCGGGGGATGCACATAAAGCAAGTTT-3'
29A4-GFPF	5'-TCCCCCGGGGGAGCAACTAAATGCTAGCAATT-3'
29A4-GFPR	5'-CGGGATCCCGCATGTTTCAAGAATACTCGTAA-3'

Underlines represent the sequences for the sites of restriction enzymes: *Kpn*I for 29A4-F and 1672-R1, *Bam*HI for 1672-F2 and 29A4-GFPR, *Eco*RI for 1672-F1, *Xba*I for 1672-R2, *Sac*II for 29A4-OPF, and *Sma*I for 29A4-OPR and 29A4-GFPF, respectively.

dures. A 1.2 kb fragment, which was amplified from the wild-type genome by PCR using the primers 29A4-F/29A4-R, was digested with *Kpn*I/*Pst*I and cloned in the plasmid pUC119. The *ura4⁺* gene was inserted into the *Xba*I/*Xho*I sites of the generated ORF in the plasmid. The construct propagated in *E. coli* DH5 α was digested with *Kpn*I/*Nde*I, and the 2.7 kb fragments were used for haploid (JY741) transformation. The uracil-nonrequiring (*Ura⁺*) transformants were isolated and *ura4⁺* insertion was checked by PCR using the same primers (29A4-F/29A4-R) and the transformant genome as a template. For the disruption of *1672.11c*, two fragments, 0.2 kb (Fragment A) and 0.25 kb (Fragment B), were amplified from the wild-type genome by PCR using the primers, 1672-F1/1672-R1 and 1672-F2/1672-R2, respectively. Fragment A was digested with *Eco*RI/*Kpn*I, and cloned into pUC119. The *ura4⁺* gene was inserted between the *Kpn*I site (3' end of fragment A) and *Bam*HI site in pUC119. Next, Fragment B was digested with *Bam*HI/*Xba*I, and cloned between the *Bam*HI site (3' end of *ura4⁺* ORF) and *Xba*I site in pUC119. A 2.3 kb fragment was amplified from the construct by PCR using the primers 1672-F1/1672-R2 and used for diploid (JY741 X JY746) transformation. After sporulation, the haploid *Ura⁺* transformants were selected by spore dissection, and *ura4⁺* insertion was checked by PCR using the primers 1672-F1/1672-R2 and the transformant genome as a template. All deletion mutants obtained were backcrossed with the strain bearing *ura4-D18 leu1-32* (903-4D) to remove the *ade6* marker, and the gene disruption was checked again by the segregation of the *ura4-D18* marker and PCR as described. The double deletion mutants were constructed by crossing each single mutant. The meiotic progenies obtained from the heterozygous diploids, which have both deletions, were identified by tetrad analysis and PCR. Overexpression of *Pmr1p* is

described elsewhere (Cortés et al., 2004). Overexpression of *29A4.19c* was carried out as follows. The 3.5 kb fragments that contain a complete ORF were amplified from the wild-type genome using the primers 29A4-OPF/29A4-OPR. The fragment was digested with *Sac*II/*Sma*I, and cloned into the overexpression vector pJR1-3XL. The *pmr1 Δ* strain was transformed with the construct, and the effect of the overexpression on cell growth was examined in Ca²⁺-free EMM medium in the absence of thiamine.

GFP tagging Two methods were employed to express a *29A4.19c-GFP* gene in *29A4 Δ* cells; a plasmid-borne expression and a physiological level of expression using an integrated copy of the fused gene in the chromosome. *29A4.19c* bearing its own promoter region was amplified from the wild-type genome by PCR using the primers 29A4-GFPF/29A4-GFPR. The amplified 4.0 kb fragment, in which the stop codon was replaced by the *Bam*HI site, was ligated in-frame to the *Bam*HI site just before the ATG codon of the *GFP* ORF that was generated by PCR from the pEGFP vector (BD Biosciences and Clontech). The *GFP*-fused *29A4.19c* with a *Sma*I site at the 5' end and a *Sac*I site at the 3' end was cloned into the multicloning sites of the plasmid pAL-KS, and used to transform the *29A4 Δ* cells. To integrate a copy of *29A4.19c-GFP* into the chromosome, the *GFP*-fused *29A4.19c* fragment was cloned in the plasmid pJK148, and the construct was cut with *Sph*I at position -321 of the *29A4.19c* ORF. The linearized plasmid was used to transform *29A4 Δ* .

RESULTS

Cell growth of P₅-type ATPase deletion mutants under optimal conditions The deletion of P₂-type ATPase genes, *pmc1⁺* and *pmr1⁺*, does not noticeably affect cell growth under optimal growth conditions (in YE medium at 28°C), although the latter mutant shows somewhat slow growth compared with that of a parental wild-type strain, 903 (Fig. 1A). On the other hand, the deletion of *29A4.19c* exhibits better growth than that of the wild-type strain, and the deletion of *1672.11c* causes defect in cell growth to a significant extent. (Fig. 1A). To examine the genetic interactions among them, five double mutants (*29A4 Δ pmc1 Δ* , *29A4 Δ pmr1 Δ* , *1672 Δ pmc1 Δ* , *1672 Δ pmr1 Δ* , *29A4 Δ 1672 Δ*) were constructed and their growth was examined under the same conditions. It is appeared that the growth phenotype of *1672 Δ* is epistatic over those of *29A4 Δ* , *pmc1 Δ* , and *pmr1 Δ* respectively, because the double mutants bearing a deleted *1672.11c* exhibit almost the same growth rates as in the case of a single *1672 Δ* (Fig. 1B). The *29A4 Δ pmc1 Δ* double mutant shows the most defective growth among the mutants examined. These results suggest that P₅-type ATPases

Fig. 1. Cell growth of the P-type ATPase deletion mutants. The wild-type strain and deletion mutants were cultured at the same inoculum dose ($\approx 2 \times 10^5$ /ml) in YE medium at 28°C and their cell growth was monitored by measuring cell number with Coulter Z1 (BECKMAN COULTER). A: Cell growth of single deletion mutants: wild-type (903) (●), 29A4Δ (■), 1672Δ (—), *pmc1*Δ (▲) and *pmr1*Δ (◆). B: Cell growth of double deletion mutants: wild-type (903) (●), 29A4Δ *pmc1*Δ (—), 29A4Δ *pmr1*Δ (■), 1672Δ *pmc1*Δ (▲), 1672Δ *pmr1*Δ (■), 29A4Δ 1672Δ (◆). The averages obtained from triplicate experiments are plotted.

share their functions with P₂-type ATPases, particularly in assortment of 29A4.19c with *pmc1*, for normal cell growth.

The 29A4Δ *pmc1*Δ double mutant is extremely sensitive to high Ca²⁺ conditions The cell growth of four single mutants (29A4Δ, 1672Δ, *pmc1*Δ, *pmr1*Δ) and five double mutants (29A4Δ 1672Δ, 29A4Δ *pmc1*Δ, 29A4Δ *pmr1*Δ, 1672Δ *pmc1*Δ, 1672Δ *pmr1*Δ) was examined in a YE liquid medium containing 10 mM, 15 mM, and 30 mM CaCl₂, respectively. Both in *S. cerevisiae* and *S. pombe*, *pmc1*Δ has been shown to become hypersensitive to high concentrations of Ca²⁺ in media (Cunningham and Fink, 1994a; Cortés et al., 2004). *S. pombe pmc1*Δ is incapable

of growing in the presence of 30 mM CaCl₂ in a YE liquid medium, and the 29A4Δ *pmc1*Δ double mutant appears to be much more sensitive to the medium supplemented with 10 mM CaCl₂ (Fig. 2A). Recently, the antiarrhythmic drug amiodarone (AMD) has been reported to have antifungal activity (Courchesne, 2002), which causes a disruption of Ca²⁺ homeostasis with an abnormal rise in [Ca²⁺]_i (Courchesne and Ozturk, 2003; Gupta et al., 2003). The 29A4Δ *pmc1*Δ double mutant was also found to become hypersensitive to the drug; the cells are not able to grow on a YE plate supplemented with 60 μM AMD, while other deletion mutants can grow (Fig. 2B). These results strongly suggest that the 29A4.19c product is required, cooperating with Pmc1p, for the maintenance of Ca²⁺ homeostasis, which might be responsible for normal cell growth.

The 29A4Δ *pmr1*Δ double mutant is extremely sensitive to Mn²⁺-depleted conditions It has been shown that *pmr1*Δ is incapable of growing in a Ca²⁺-depleted medium, and the phenotype is suppressed to a significant extent by supplying excess Mn²⁺ in the medium (Maeda et al., 2004; Cortés et al., 2004). This suggests that *S. pombe* Pmr1p is also involved in Mn²⁺ homeostasis, as was shown in *S. cerevisiae* (Dürr et al., 1998). None of the single mutants examined in this experiment shows a sensitive growth to Mn²⁺-depleted medium, but 29A4Δ *pmr1*Δ and 1672Δ *pmr1*Δ double mutants were found to become hypersensitive to the medium (Fig. 3). These results suggest that 29A4.19c and 1672.11c products are required for the maintenance of Mn²⁺ homeostasis when Pmr1p function is lacking.

Transcriptional level of 29A4.19c is lower than that of *pmc1*⁺ or *pmr1*⁺ To compare the transcriptional level of 29A4.19c with that of *pmc1*⁺ and of *pmr1*⁺, RT-PCR was carried out. The logarithmic-phase cells cultured in YE medium were examined. Figure 4 shows that mRNA of 29A4.19c is less abundant than that of *pmc1*⁺ or *pmr1*⁺. It seems reasonable that *pmr1*⁺ is most efficiently transcribed among them, because Pmr1p is constantly required for mobilizing Ca²⁺/Mn²⁺ into the secretory organelle to maintain functions such as ER quality control, protein processing, and exocytosis (Rudolph et al., 1989; Antebi and Fink, 1992; Lapinskas et al., 1995; Sorin et al., 1997; Dürr et al., 1998; Okorokov and Lehle, 1998; Wei et al., 2000; Vashist et al., 2002). Pmc1p may be less important under optimal growth conditions, because there is no excess Ca²⁺ in the cytosol. Indeed, it has recently been reported in *S. pombe* that expression of *pmc1*⁺ is greatly increased under high Ca²⁺ conditions through a Ca²⁺/calmodulin-dependent transcription factor, Prz1p (Hirayama et al., 2003). The low mRNA level of 29A4.19c implies that this gene product has a rather minor role in modulating Ca²⁺ and Mn²⁺ con-

Fig. 2. (A) Effect of Ca^{2+} concentrations on cell growth of the P-type ATPase deletion mutants. The wild-type strain (903) and deletion mutants were cultured at the same inoculum dose ($\approx 8 \times 10^5/\text{ml}$) in YE medium supplemented with CaCl_2 (10 mM, 15 mM and 30 mM) for 48 h at 28°C and cell growth was monitored with a Klett-Summerson colorimeter. Strains and CaCl_2 concentrations are indicated in the figure. The averages and the standard deviations from triplicate experiments are shown. (B) Effect of amiodarone (AMD) on cell growth of the P-type ATPase deletion mutants. The single and double deletion mutants cultured in YE medium for 48 h were spotted on a YE plate containing $60 \mu\text{M}$ AMD, and incubated for 4 days at 28°C . Strains are indicated in the figure.

Fig. 3. Effect of Mn^{2+} depletion in EMM medium on cell growth. The wild-type strain and deletion mutants were cultured at the same inoculum dose ($\approx 8 \times 10^5/\text{ml}$) in Mn^{2+} -free EMM medium for 48 h at 28°C and cell growth was monitored with a Klett-Summerson colorimeter. Strains and the media are indicated in the figure. The averages and the standard deviations from triplicate experiments are shown.

Fig. 4. Expression of the P-type ATPase genes in log-phase wild-type cells. RT-PCR was carried out to measure the mRNA levels of *pmr1*⁺, *pmc1*⁺, and *29A4.19c* in the wild-type cells. Total RNA was prepared from the log-phase cells and 1 µg of RNA was used for RT-PCR (Takara mRNA Selective PCR Kit ver.1.1). The *act1*⁺ gene was used to quantify the mRNA levels of the ATPase genes using Molecular Imager FX (BIO-RAD).

centrations under optimal growth conditions.

Localization of the GFP-tagged *29A4.19c* protein

The intracellular distribution of the *29A4.19c* protein was examined by fluorescence microscopy using the GFP-tagging method. The GFP-tagged proteins were observed under two different conditions, plasmid-borne and chromosomal expression of the fused gene (an integrated copy with its own promoter region) in *29A4Δ*. The plasmid bearing GFP-tagged *29A4.19c* was capable of recovering from the growth defect of the *29A4Δ pmc1Δ* double mutant under high Ca²⁺ conditions (data not shown), suggesting that the GFP-tagged protein is functional. The typical localization pattern is shown in Fig. 5. This pattern is very similar to that of Gma12 galactosyltransferase established to be present in the Golgi apparatus

Fig. 5. Intracellular distributions of GFP-tagged *29A4.19c* protein. The GFP-tagged *29A4.19c* protein was observed in *29A4Δ* cells cultured in EMM medium. Almost the same pattern of fluorescence was obtained in both *29A4Δ* cells transformed with multicopy plasmids bearing the fused gene and those with the gene integrated in the chromosome (see Materials and Methods). *29A4.19c*-GFP shows a localization pattern typical of the *S. pombe* Golgi proteins (Chappell et al., 1994; Cheng et al., 2002). Bar, 5 µm.

(Chappell et al., 1994; Cheng et al., 2002).

Functional relationships between *29A4.19c* protein, Pmc1p, and Pmr1p

In both *S. cerevisiae* and *S. pombe*, the *pmc1Δ pmr1Δ* double mutant is lethal even in a standard growth conditions, and death is prevented by inactivation of calcineurin with cyclosporin A (CsA), a potent inhibitor of calcineurin (Cunningham and Fink 1994a, 1994b; Cortés et al., 2004). One explanation for this is that Pmc1p becomes essential to prevent the lethal activation of calcineurin caused by excess cytosolic Ca²⁺, which is brought about by the loss of Pmr1p's functions (Cunningham and Fink 1994a, 1994b; Halachmi and

Fig. 6. (A) Effect of overexpression (OP) of the *pmr1*⁺ gene on cell growth of the *29A4Δ pmc1Δ* double mutant. OP-*pmr1*⁺ cells cultured in EMM medium were spotted on a EMM plate containing 20 mM CaCl₂ and incubated for 4 days at 28°C. (B) Effect of cyclosporin A (CsA) on cell growth of the single and double deletion mutants. The cells cultured in YE medium were spotted on a YE plate containing 20 mM CaCl₂ or 20 mM CaCl₂ plus 10 µM CsA and incubated for 4 days at 28°C.

Fig. 7. Effect of overexpression (OP) of 29A4.19c on *pmr1Δ* cell growth in Ca²⁺-free EMM medium. The *pmr1Δ* cells transformed with pCP5-15 (*pmr1*⁺ containing plasmid, see Cortés et al., 2004), pJR1-3XL (vector), or pJR1-3XL-29A4.19c (see Materials and Methods) were cultured in Ca²⁺-free EMM medium and cell growth was monitored at 24 h and 48 h with a Klett-Summerson colorimeter. The averages from duplicate experiments are shown.

Eilam 1996). To see if 29A4.19c protein acts as a Ca²⁺-ATPase under such conditions, the effects of CsA and overexpression of *pmr1*⁺ on the defective growth of the 29A4Δ*pmc1Δ* double mutant were examined. The double mutant cells are capable of growing under high Ca²⁺ conditions, if *pmr1*⁺ is overexpressed (Fig. 6A), or 10 μM CsA is added to the medium (Fig. 6B). These results suggest that 29A4.19c protein is indeed functioning as a Ca²⁺-ATPase, cooperating with Pmc1p. In *S. pombe*, wild-type cells can grow in a Ca²⁺-free EMM medium almost normally, while the *pmr1Δ* cells cannot (Cortés et al., 2004). It has been well established in *S. cerevisiae* that Pmr1p is essential for a variety of cellular functions, including protein glycosylation, protein sorting in secretory pathways and ER quality control. These processes require the transport of Ca²⁺/Mn²⁺ into the secretory organelle. To clarify if 29A4.19c pumping activity is responsible for such secretory functions, the effect of overexpression of 29A4.19c on *pmr1Δ* cells was examined. Overexpression of 29A4.19c is not able to recover the defective growth of *pmr1Δ* cells in a Ca²⁺-free EMM medium completely (Fig. 7), suggesting that 29A4.19c protein is not exchangeable in function with Pmr1p, which is required for secretory pathways.

DISCUSSION

In this paper, three lines of evidence strongly suggest that a putative P₅-type ATPase encoded by 29A4.19c func-

tions as a potential Ca²⁺-ATPase. First, the 29A4Δ mutant becomes extremely hypersensitive to high Ca²⁺ conditions in the absence of a vacuolar Ca²⁺-ATPase, Pmc1p. Second, the 29A4Δ *pmc1Δ* double mutant also displays hypersensitivity to the antiarrhythmic drug amiodarone, which causes disruption of Ca²⁺ homeostasis with an abnormal increase in [Ca²⁺]_i in *S. cerevisiae*. Third, hypersensitivity of the double mutant to high concentrations of Ca²⁺ is suppressed by overexpression of Pmr1p or in the presence of CsA. In yeasts, Pmc1p becomes essential for cell growth to prevent lethal activation of calcineurin in the absence of Pmr1p. The 29A4Δ *pmr1Δ* double mutant displays almost no phenotype even under high Ca²⁺ conditions, suggesting that 29A4.19c protein is not necessarily required for preventing lethal activation of calcineurin, if Pmc1p is functioning. In addition, 29A4.19c protein might function as a Mn²⁺-transporter, because its loss of function shows hypersensitive growth to Mn²⁺-depleted conditions in the absence of Pmr1p. In this study, GFP-tagged 29A4.19c protein revealed a typical localization pattern of the Golgi proteins. The 29A4.19c protein, however, is not exchangeable in function with Pmr1p. Indeed, 29A4Δ cells are not defective in cell wall integrity and cytokinesis, both of which require Pmr1p functions (data not shown). This suggests that the 29A4.19c product may have other roles in secretory pathways than Pmr1p. Since the 29A4.19c product was shown to be involved in Ca²⁺/Mn²⁺ homeostasis, the ORF was named *cta5*⁺, according to the precedent nomenclature of calcium/cation transporting ATPase in fission yeast.

Together, these findings support the idea that Cta5p plays an important role, sharing its functions with P₂-type ATPases, in Ca²⁺ and Mn²⁺ homeostasis responsible for cell cycle progression under certain physiological conditions. Loss of *cta5*⁺ was found to cause rather better cell growth than that of the wild-type cells under optimal conditions, implying that the gene product functions as a negative regulator in cell growth. This is reminiscent of the finding that a human homologue of *cta5*⁺ was identified as a gene that is overexpressed in senescent kidney parenchymal cells (Habtemichael and Kovacs, 2002). Further physiological study using *S. pombe* P₅-type ATPase mutants would provide important clues to the mechanisms of human diseases, which could be caused by lack of P₅-type ATPase functions.

We thank J. C. Ribas for the kind supply of plasmids, and YGRC for the strains.

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