The endoplasmic reticulum cation P-type ATPase Cta4p is required for control of cell shape and microtubule dynamics

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Here we describe the phenotypic characterization of the cta4¹ gene, encoding a novel member of the P4 family of P-type ATPases of fission yeast. The cta4Δ mutant is temperature sensitive and cold sensitive lethal and displays several morphological defects in cell polarity and cytokinesis. Microtubules are generally destabilized in cells lacking Cta4p. The microtubule length is decreased, and the number of microtubules per cell is increased. This is concomitant with an increase in the number of microtubule catastrophe events in the midzone of the cell. These defects are likely due to a general imbalance in cation homeostasis. Immunofluorescence microscopy and membrane fractionation experiments revealed that green fluorescent protein-tagged Cta4 localizes to the ER. Fluorescence resonance energy transfer experiments in living cells using the yellow cameleon indicator for Ca²⁺ indicated that Cta4p regulates the cellular Ca²⁺ concentration. Thus, our results reveal a link between cation homeostasis and the control of cell shape, microtubule dynamics, and cytokinesis, and appoint Ca²⁺ as a key ion in controlling these processes.

Introduction

The P-type ATPases are present in all living cells where they mediate ion transport across membranes on the expense of ATP hydrolysis. Among the ions transported by these pumps are protons, calcium, sodium, potassium, and heavy metals such as manganese, iron, copper, and zinc. The formation of a phosphorylated intermediate during the catalytic cycle is a characteristic of P-type ATPases that distinguishes them from V ATPases and F ATPases (Pedersen and Carafoli, 1987a,b).

Within the protein family of P-type ATPases, the Ca²⁺-ATPases are of special interest, since calcium plays a key role in signal transduction in eukaryotic cells (Clapham, 1995). The transient elevation in cytosolic-free calcium that act to elicit downstream signaling pathways. Ubiquitous effectors of calcium signaling are Ca²⁺/calmodulin (CaM)⁻dependent protein kinases and Ca²⁺/CaM-dependent phosphatase, calcineurin, which act by modulating the phosphorylation state of diverse proteins including transcription factors (Stull, 2001). There is a growing body of evidence that the cellular response to a rise in calcium depends on the amplitude, frequency, duration, and location of the Ca²⁺ signal (Sanders et al., 1999). The calcium signal is terminated when cytosolic-free Ca²⁺ concentration is reduced to basal levels by Ca²⁺-ATPases and Ca²⁺/H⁺ exchangers that transport calcium from the cell or sequester it in organelles. In the last case, refilling of intracellular compartments safeguards the Ca²⁺ release during subsequent signaling events and provides a luminal space with specific Ca²⁺ concentration required for diverse biochemical reactions taking place in those compartments (Corbett and Michalak, 2000).

Two main classes of Ca²⁺-ATPases have been described: sarco/ER Ca²⁺-ATPases (SERCA) and plasma membrane Ca²⁺-ATPases, which differ from one another in their subcellular distribution, biochemical characteristics, and mode of regulation (Guerini and Carafoli, 1999; Carafoli and Brini, 2000). In addition, the secretory pathway Ca²⁺-ATPases initially characterized in budding yeast (Rudolph...
et al., 1989) has emerged as a separate class (Guenteski-Hamblin et al., 1992; Sorin et al., 1997).

The fission yeast *Schizosaccharomyces pombe* is an excellent model system for eukaryotic cell biology. Several components of Ca\(^{2+}\)-mediated signaling of animal cells have been identified and characterized in fission yeast. A temperature-sensitive Ca\(^{2+}\)-binding site CaM mutant exhibits broken spindles and defects in chromosome segregation (Moser et al., 1997). CaM is localized to the spindle pole bodies and sites of polarized cell growth in *S. pombe* (Moser et al., 1997). In cells undergoing cytokinesis, CaM was found on both sides of septum. Similar CaM redistribution at the cell equator was observed in dividing animal cells where CaM activation by elevation of free Ca\(^{2+}\) was proposed to trigger the formation of the cleavage furrow (Li et al., 1999). Thus, there are several indications that Ca\(^{2+}\) and CaM have an important role in regulating aspects of the cytokinesis both in animal cells and fission yeast, but more direct evidence for Ca\(^{2+}\) affecting this process is lacking.

Recently, the gene *ppb1*\(^{+}\) encoding for the catalytic subunit of calcineurin was isolated from *S. pombe* (Yoshida et al., 1997). In *S. pombe* and other temperature-sensitive and cold sensitive lethal. Microtubules are generally destabilized in cells lacking Cta4p. The microtubule length is decreased, and the number of microtubules per cell is increased concomitant with an increase in the number of microtubule catastrophe events in the midzone of the cell. Fluorescence resonance energy transfer (FRET) experiments in living cells using the fluorescent yellow cameleon indicator for Ca\(^{2+}\) indicated that a deletion of *cta4* causes an elevation of cellular calcium levels. Our results reveal a link between control of cell shape, microtubule dynamics, cytokinesis, and cation homeostasis, and Ca\(^{2+}\) as a key regulatory ion. Hence, our data points to a new level of control over these important processes.

### Results

#### Analysis of the *cta4* gene

The full-length *SPAC2E11.07C* gene (sequence data available from GenBank/EMBL/DDBJ under accession no. O14072) referred to here as *cta4*\(^{+}\) encodes a 1211-amino acid–long protein. Twelve transmembrane spanning domains (TMDs) and three defined hydrophilic cytosolic domains linking TMDs 2 and 3, 4 and 5, and 6 and 7 were predicted linking TMDs 2 and 3, 4 and 5, and 6 and 7 were predicted.

#### Identification of P4-ATPase signatures in Cta4

<table>
<thead>
<tr>
<th>P4-ATPases signatures*</th>
<th>Cta4 sequence</th>
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<tr>
<td>(\alpha_{1}) (\gamma_{1}) (\delta_{1}) (\epsilon_{1}) (\zeta_{1}) (\xi_{1}) (\psi_{1}) (\chi_{1}) (\iota_{1}) (\kappa_{1}) (\lambda_{1}) (\mu_{1}) (\nu_{1}) (\delta_{2})</td>
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<td>980(\text{DASAAAPT}_{980})</td>
</tr>
</tbody>
</table>

Underline denotes conservative replacement, indicated by • in the consensus. Bold denotes conserved amino acids in the P4-ATPase specific sequences. Highly conserved amino acids in P-type ATPases are indicated by * in the consensus.

*Catty et al., 1997.*

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### Table I. Cta4 ATPase belongs to the P4 family of P-type ATPases

<table>
<thead>
<tr>
<th>P-type consensus*</th>
<th>Cta4 sequence</th>
<th>Putative function in Cta4</th>
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</tr>
<tr>
<td>(\iota_{1}) (\delta_{1}) (\epsilon_{1}) (\zeta_{1}) (\xi_{1}) (\psi_{1}) (\chi_{1})</td>
<td>484(\text{FDKTGTLT}_{484})</td>
<td>Phosphorylation site</td>
</tr>
<tr>
<td>KGA--</td>
<td>614(\text{KGAP}_{614})</td>
<td>ATP binding</td>
</tr>
<tr>
<td>M•TGD</td>
<td>704(\text{MITGD}_{704})</td>
<td>ATP binding</td>
</tr>
<tr>
<td>GDD•ND</td>
<td>823(\text{CGDT}_{823})</td>
<td>Coupling ATP-binding domain to domain involved in ion transport</td>
</tr>
</tbody>
</table>

Underline denotes conservative replacement, indicated by • in the consensus.

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*Okorokov et al., 2001.* Although the genes encoding for several putative calcium ATPases were identified by the *S. pombe* genome-sequencing project, no genetic analysis has been performed on the corresponding null mutants. Thus, the involvement of each individual pump in calcium homeostasis and the role of the pumps in signal transduction and diverse cellular functions have not been established. Toward this end, we have here determined the subcellular localization of the putative calcium ATPase *SPAC2E11.07C* and analyzed the physiological consequences of its gene deletion. To follow the preexisting nomenclature of P-type ATPases in fission yeast, *SPAC2E11.07C* was named *cta4*\(^{+}\) (calcium/cation transporting ATPase). The *cta4*\(^{+}\) gene encodes a novel member of the P4 family of P-type ATPases that is localized in the ER. *cta4*\(^{+}\) is not an essential gene, but *cta4*\(^{Δ}\) mutants display several morphological defects, an imbalance in cation homeostasis and are temperature sensitive and cold sensitive lethal. Microtubules are generally destabilized in cells lacking Cta4p. The microtubule length is decreased, and the number of microtubules per cell is increased concomitant with an increase in the number of microtubule catastrophe events in the midzone of the cell. Fluorescence resonance energy transfer (FRET) experiments in living cells using the fluorescent yellow cameleon indicator for Ca\(^{2+}\) indicated that a deletion of *cta4*\(^{+}\) causes an elevation of cellular calcium levels. Our results reveal a link between control of cell shape, microtubule dynamics, cytokinesis, and cation homeostasis, and appoint Ca\(^{2+}\) as a key regulatory ion. Hence, our data points to a new level of control over these important processes.
Cta4p has all five highly conserved domains characteristic of ion transporting P-type ATPases (Tables I and II). These include the potential phosphorylation site represented by Asp^485 and the domains involved in ATP binding, all located in a large hydrophilic loop between TMDs 6 and 7. Search for homologues in the GenBank database revealed that Cta4p is related to the S. cerevisiae ORF Yel031p, which encodes the Spf1 ATPase that belongs to the family of P4-ATPases with unknown substrate specificity (Catty et al., 1997). The Cta4p sequence shares specific amino acid sequence motifs intrinsic for P4 ATPases with the Spf1 amino acid sequence. These include one Cys residue preceding the consensus motif GDG^ND and the ××S4×FTS14×GR××LV×× sequence (Tables I and II). Whereas the highest sequence identity was obtained with the SPF1 gene product in amino acid sequence comparisons (49% overall identity), other S. cerevisiae cation ATPases, such as Na^+^-ATPase ENA1 and Ca^{2+}-ATPases PMR1 and PMC1, showed a relatively low sequence similarity with Cta4p (14.2, 15.1, and 12.6% overall amino acid identities, respectively). The Cta4p sequence showed low

Figure 1. Cellular localization of Cta4p. (A and B) Yeast cells were grown to early log phase, fixed and stained with antibodies, and examined by IF microscopy (as described in Materials and methods). (A) GFP-tagged Cta4p cells transformed with plasmid-encoding Sec61-myc fusion protein (Broughton et al., 1997) were subjected to IF and stained with anti-GFP (green), anti-myc (red), and DAPI (blue). The cell to cell variation in expression of Sec61-myc is due to unequal partitioning of the multicopy plasmid. Bar, 10 µm. (B) Wild-type cells expressing GFP-tagged Cta4p stained with anti-GFP (green), anti-BiP (red), and DAPI (blue). The merged images show colocalization of Cta4p with Sec61 and BiP (yellow) in the deconvolved image. (C) Cta4p is comigrating with ER membranes. Total membranes were isolated from the cells expressing both Cta4-GFP and Sec61-myc fusion proteins, fractionated on a sucrose density gradient, and submitted to immunoblot analysis. Dot blots of individual fractions were used for quantification of the relative levels of Cta4, Sec61, and BiP in membrane fractions. A fraction with higher immunoblot response was considered as 100% in each case.
overall identity (13.7%) with Cta3p of *S. pombe* (Ghislain et al., 1990). Besides SPF1 of *S. cerevisiae*, human KIAA1825 protein expressed in brain (sequence data available from GenBank/EMBL/DDBJ under accession no. AB058728) (Nagase et al., 2001) had a high sequence similarity to Cta4p (37.7% overall amino acid identity).

**Cta4-GFP is localized to the ER**

To investigate the intracellular localization of the Cta4 ATPase in fission yeast, a fusion protein between Cta4p at its COOH terminus and the green fluorescent protein (GFP) was generated (see Materials and methods). The Cta4-GFP gave a weak signal in live cells, but the fusion protein was readily detected by indirect immunofluorescence (IF) microscopy using antibodies against GFP (Fig. 1 A). The Cta4-GFP fusion protein is replacing the endogenous *cta4* allele and is thus expressed from the natural *cta4* promoter and is fully functional (see below). The localization pattern of the Cta4-GFP was found to be similar to that of Sec61, a known ER marker (Broughton et al., 1997) as revealed by IF. To test for colocalization of these two proteins, Sec61 fused to the c-myc epitope tag was expressed from a plasmid in the strain carrying Cta4-GFP (Fig. 1 A). Digital confocal microscopy revealed a partial colocalization of both proteins, suggesting that the Cta4 ATPase at least in part resides in the ER.

These data were further confirmed by subcellular fractionation of total membranes isolated from cells expressing both Cta4-GFP and Sec61-myc fusion proteins. Immunoblots of membrane fractions showed that Cta4-GFP comigrated with Sec61-myc at positions within the gradient corresponding to 41–47% sucrose where membrane vesicles enriched with ER were shown previously to migrate (Fig. 1 C) (Okorokov et al., 2001). However the Sec61-myc fusion protein showed a wider distribution than Cta4-GFP and was also detected in membrane fractions migrating at 32–41% sucrose corresponding to the Golgi and/or Golgi-like membranes. Thus, it is likely that this broader distribution of Sec61-myc corresponds to Golgi and that Cta4-GFP is more restricted to the ER. In animal cells, a Sec61 homologue was found in the ER-to-Golgi intermediate compartment in addition to the ER, thus supporting this idea (Greenfield and High, 1999; Kamhi-Nesher et al., 2001). Another possible explanation for the broader distribution of Sec61-myc might be its overexpression from a plasmid.

To verify the ER localization of Cta4 ATPase, the antibody against another ER organelle marker, chaperone BiP (Pidoux and Armstrong, 1993), was used. IF microscopy demonstrated colocalization of Cta4-GFP and BiP (Fig. 1 B). Distribution of BiP in membrane fractions was more restricted than that of Sec61-myc and was similar to that of Cta4. Both BiP and Cta4 proteins were concentrated in fractions 20–22 (Fig. 1 C). Therefore, it could be concluded from these results that Cta4 ATPase is localized to the ER compartment.

**cta4** regulates cation homeostasis

To further investigate the function of Cta4p in fission yeast, the *cta4* gene was disrupted with the *ura4* marker gene, and the resulting *cta4Δ* phenotype was compared with that of wild type. Tetrad analysis revealed that *cta4Δ* was not an essential gene; however, *cta4Δ* cells exhibited poor growth at 25°C compared with that at 30°C and died at 36°C, indicating that some...
aspect of cell cycle progression was impaired in these conditions (Fig. 2 A). Growth of Cta4-GFP cells was not impaired at 25 and 36°C, pointing out that this allele is fully functional.

Yeast P-type ATPase mutants commonly display phenotypic defects in response to variations in the concentration of cations in the growth media (Rudolph et al., 1989; Cunningham and Fink, 1996). Consistent with this idea, we found that growth of cta4Δ cells was completely inhibited by a high concentration, that is, 100 mM of CaCl₂. Next, growth inhibition of lower concentrations of CaCl₂ was investigated using liquid cultures (Fig. 2 B). It was clear that the growth of wild-type cells was unaffected by addition of up to 50 mM CaCl₂, whereas the growth of cta4Δ cells was partially inhibited already by the addition of 10 mM CaCl₂ and completely inhibited by the addition of 50 mM CaCl₂. The cta4Δ deletion mutant was also sensitive to 0.25 mM MnCl₂ (Fig. 2 A). Furthermore, the cta4Δ growth at 25°C was improved by high concentrations of Fe³⁺ (0.1 mM FeCl₃) (Fig. 2 A), and the lethality of cta4Δ at 36°C could be overcome by K⁺ (300 mM KCl), ruling out that Cl⁻ could be toxic to cta4Δ cells (unpublished data). Collectively, these results showed that the homeostasis of different ions is severely compromised in cta4Δ cells. Although the data did not elucidate which cation is transported by the cta4Δ gene product, they strongly suggest that Cta4 ATPase might have a crucial function in maintenance of cation homeostasis in fission yeast. This conclusion was further reinforced by the observation that overexpression of cta4Δ from the regulatable nmt1Δ promoter was deleterious to wild-type cells, but growth was restored by the addition of 100–200 mM CaCl₂ (unpublished data).

The Ca²⁺/CaM-dependent protein phosphatase 2B, calcineurin, was shown to be responsible for a maintenance of cation homeostasis in S. cerevisiae by regulating the expression of Ca²⁺ and Na⁺ ATPases (Nakamura et al., 1993; Cunningham and Fink, 1996; Mendoza et al., 1996). In S. pombe, the calcineurin A subunit-like protein encoded by the ppb1Δ gene is the target of cyclosporin A (CsA) (Yoshida et al., 1994). cta4Δ cells were found to be susceptible to 10 μg/ml CsA (Fig. 3), whereas wild-type strain remained insensitive to threefold higher drug concentration. This result indicates that cta4Δ acts in the same or parallel pathways as calcineurin, since ppb1Δ seems necessary for the viability of the cta4Δ mutant.

**Figure 4.** Loss of cta4Δ confers a resistance to *P. farinosa* killer toxin SMKT. The wild-type (Fy1180) and cta4 mutant cells (Hu285) were spread on the MB plates on which 5 μl of 100 μM SMKT solution was spotted (arrowhead). The *S. cerevisiae* strains used were CS202A and CS202B.

**Functional similarity with the *S. cerevisiae* Spf1 ATPase**

Because Cta4 ATPase shares 49% homology with *S. cerevisiae* Spf1p whose deletion confers resistance to *Pichia farinosa* killer toxin, SMKT (Suzuki and Shimma, 1999), we investigated the effect of SMKT on fission yeast wild-type and cta4Δ cells. In the halo assay, the wild-type strain displayed a clear sensitivity to SMKT, whereas cta4Δ was resistant to the toxin (Fig. 4). It was proposed that the resistance to killer toxin in spf1 null might be due to an alteration in glycosylation of the cell wall components (Suzuki and Shimma, 1999). The enzymes involved in the glycosylation process require Mn²⁺ for their activity (Kaufman et al., 1994). Therefore, the resistance to SMKT displayed by cta4Δ cells is not surprising, since the cta4Δ mutant also exhibits defects in Mn²⁺ homeostasis (Fig. 2 A). Thus, both Spf1 and Cta4 could have similar functions both with respect to Mn²⁺ homeostasis and the response to SMKT. It has been shown recently that SMKT interacts with the plasma membrane of wild-type but not mutant spf1 cells (Suzuki et al., 2001), raising a possibility that a structure and/or targeting of some membrane component, which binds the toxin, is similarly affected in *S. cerevisiae* spf1 and *S. pombe* cta4 mutant cells.

**cta4Δ is required for cytokinesis and microtubule integrity**

Next, the effect of cta4Δ gene disruption on cell morphology was investigated. cta4Δ cells were frequently multiseptated and formed hyphae-like structures, in which cells were not separated even when grown at the permissive temperature of 30°C. These multicellular structures were often branched and aggregated (Fig. 5 A). Lowering or increasing the temperature from 30°C or adding CaCl₂ to the cells aggravated the phenotype. The multiseptated phenotype suggested that Cta4p is required for the final stages of cytokinesis. The aberrant cell shapes exhibited by cta4Δ also suggested that the cells had defects in the cytoskeleton. To test if microtubule function was
perturbed in cta4Δ cells, growth in the presence of the microtubule destabilizing drug thiabendazole (TBZ) was assayed. cta4Δ cells were found to be sensitive to TBZ, indicating that Cta4p is normally required to stabilize microtubules (Fig. 5 B). To examine whether loss of cta4Δ leads to changes in microtubule assembly, microtubules in cta4Δ cells were visualized using monoclonal antitubulin antibodies by IF microscopy of cells grown at 25°C. Cytoplasmic microtubules in the mutant cells appeared abnormally short, and the number of microtubules per cell was increased in cta4Δ cells compared with wild-type cells (Fig. 6, A and B). Measurements of the microtubule length indicated that the microtubules in interphase cta4Δ cells were significantly shorter ($t = -3.573; P = 0.001$) than those of wild-type cells. The microtubules in cta4Δ cells rarely reached lengths greater than 4 μm, whereas those of wild-type cells extended up to 8 μm (Fig. 6 C). Furthermore, the number of microtubules per interphase cell was markedly increased in cta4Δ cells to three to six microtubules per cell, whereas wild-type cells had only one to three (Fig. 6 D). Thus, Cta4p generally stabilizes microtubules.

To test if the shortening and increase in microtubule number per cell were due to altered dynamic properties of the microtubules, we investigated the microtubule dynamics in living wild-type and cta4Δ cells using strains expressing α-tubulin fused to GFP (Ding et al., 1998; Pidoux et al., 2000). The
analysis of time-lapse movies of α-tubulin–GFP fusion con-
firm ed the alterations observed in fixed cells, namely that the
number of microtubules per cell was markedly increased in
cells lacking Cta4p (Fig. 7, A and B). In addition, cells lacking
cta4Δ exhibited changes in the microtubule growth rate and in the
occurrence of microtubule catastrophe events. Length
measurements of individual microtubules revealed that, al-
though the microtubules of wild-type cells commonly reached
a length of >5 μm before they suddenly started to shrink,
those of cta4Δ cells underwent catastrophe events already at
lengths of ~3 μm (Fig. 7 C). Moreover, the microtubule cata-
strophe events in cta4Δ cells were not limited to a cortical
region near the cell tip, whereas those in wild-type cells in
89% of the cases occurred in the cell tip region (Fig. 7 D).
Thus, the time-lapse analysis revealed that the dynamic fea-
tures of microtubules were strongly altered in cta4Δ cells,
leading to a failure in guiding the microtubules toward the cell
tip so that the cell growth axis could not be maintained.

ctaa4 controls the nuclear Ca2+ levels
Considering that the cta4+ deletion mutant displayed a Ca2+-
sensitive phenotype, it was tempting to speculate that Cta4
ATPase would play a role in controlling cytosolic-free calcium
levels. To test this possibility, we explored the FRET-based
method using "cameleon" reporter constructs expressed in liv-
ing cells to measure Ca2+ levels by time-lapse yellow fluores-
cent protein (YFP)/cyan fluorescent protein (CFP) ratio imaging
(Miyawaki et al., 1997). The cameleon reporter consists of a
CFP-M13-CaM-YFP protein fusion, which gives a relative in-
crease in YFP emission (at 535 nm) when Ca2+ levels are high.
This is due to FRET from CFP to YFP caused by proximity of
CFP and YFP when M13 interacts with Ca2+-bound CaM.
The yellow cameleon YC2 was cloned into pREP3x plasmid
(see Materials and methods), and the resulting plasmid was in-
roduced into wild-type S. pombe cells and cells lacking cta4+ by
transformation. To increase the signal for accurate determina-
tion of the FRET signal, a nuclear localization signal was added
to the YC2 protein fusion and strong expression (thiamine) was
used from the pREP3x nmt1+ promoter. The ratio imaging
measurements indicated that the ratio of 535:480 fluorescence
increased significantly (t = 4.125, P < 0.001) in untreated
cta4Δ cells compared with wild-type cells and in cta4Δ cells
treated with Ca2+ compared with wild-type cells treated with
Ca2+ (t = 3.154, P = 0.003). The 535:480 ratio in Ca2+-
treated cta4Δ cells was on average 1.59 (n = 21), whereas Ca2+-
treated wild-type cells gave 535:480 ratios on average of
1.39 (n = 18). The 535:480 ratios were generally stable over
time. It was noticed that all aberrantly shaped or round cta4Δ
cells examined gave particularly high FRET 535:480 ratios on
average of 1.78 (n = 15); for example, the cell nucleus indicated
in red in Fig. 8 C. Thus, the cell shape defects caused by loss of
cta4Δ are associated with elevated nuclear Ca2+ level.

Discussion
In the present work, we undertook a genetic approach to gain
insight into the role of a putative P-type ATPase in fission yeast.
Cta4 ATPase belongs to a P4 subfamily of the P-type ATPases
with an unknown substrate specificity and subcellular localiza-
tion (Catty et al., 1997). This recently identified subfamily
comprises, in addition to cta4+, SFP1 and Yor291 of S. cerevi-
siae and several homologous genes found in the genomes of
Plasmodium, Caenorhabditis, Tetrahymena, and Arabidopsis.
The cell growth was reduced at 25°C and arrested at 36°C. Loss of cta4Δ resulted in multiseptated hyphae-like structures in which cells did not separated and were often branched and aggregated. The microtubule structure was destabilized. Adaptation to cation stress was also impaired. Similarly to spf1 null, cta4Δ cells were resistant to SMKT killer toxin. However, whether the spf1 and cta4 genes are functionally interchangeable remains to be determined. It is of note that contrary to cta4Δ cells, lack of Spf1 ATPase did not cause visible changes in ion homeostasis, since spf1 growth was independent of calcium and manganese (Suzuki and Shimma, 1999). On the other hand, the addition of high CaCl₂ (200 mM) to extracellular medium could restore a defect in hydroxymethylglutaryl-CoA reductase (Cronin et al., 2000). Gene disruption experiments indicated that the cta4Δ gene was nonessential for viability. Yet the cta4-null mutant displayed pleiotropic cellular phenotypes.

Several lines of evidence suggest that cta4Δ is involved in Ca²⁺ homeostasis. First, cta4Δ was unable to grow when calcium was supplied to medium and when it was chelated by EGTA (unpublished data). Second, calcium measurements in cta4Δ cells using fluorescent indicator yellow cameleon showed that nuclear calcium levels were increased in cta4-null cells in comparison with wild-type cells. It is likely that nuclear Ca²⁺ levels are indicative of the cytoplasmic Ca²⁺ levels, since Ca²⁺ ions can diffuse through the nuclear pores (Lipp et al., 1997). Thus, loss of cta4Δ function reduces the ability to sequester Ca²⁺ to internal stores, presumably the ER (see below). The consequence of this is an increase in intracellular Ca²⁺.

The cta4 mutant cells were sensitive to inhibition of calcineurin, Ca²⁺/CaM-dependent protein phosphatase type 2B, by CsA. The ppb1Δ gene of S. pombe encodes a catalytic subunit of calcineurin. In this respect, it is noteworthy that pleiotropic phenotypes of cta4Δ were reminiscent of those reported previously for ppb1Δ mutants regarding growth characteristics, septation, cell shape defects, and microtubule integrity (Yoshida et al., 1994). Moreover, S. pombe mutants lacking cta4Δ were, like S. cerevisiae calcineurin mutants, sensitive to Ca²⁺ and Mn²⁺ cation stress. Thus, it is possible that calcineurin function is defective in cta4-null cells. It remains to be determined if the cta4Δ and ppb1Δ genes interact and if these two genes products share an essential overlapping function. Finally, cta4Δ overexpression was toxic to wild-type cells, and analysis at 30°C. The y axis shows the ratio of the YFP to CFP signal in the nuclei of the cells, which indicates [Ca²⁺]nuc. The x axis shows the real time in seconds.
this could be overcome by addition of \( \text{Ca}^{2+} \) (unpublished data). Therefore, both elevated and lowered \( \text{Ca}^{2+} \) cytosolic levels may be deleterious to \( S. \text{pombe} \). Since \( \text{cta}4^+ \) deletion is not lethal to \( S. \text{pombe} \) cells, it could be concluded that other \( \text{Ca}^{2+} \) transporters deplete cytosolic \( \text{Ca}^{2+} \) in this genetic background, although to a lesser extent than \( \text{cta}4^+ \) normally does. Therefore, the precise regulation of \( \text{Ca}^{2+} \) homeostasis fails and, consequently, \( \text{Ca}^{2+} \) signals.

From our results it could be presumed that \( \text{Cta}4p \) might transport \( \text{Ca}^{2+} \). However, direct biochemical evidence is needed to establish the exact substrate specificity of \( \text{Cta}4p \). Our attempts to measure ATP-dependent \( \text{Ca}^{2+} \) transport in isolated membranes of \( S. \text{pombe} \) 972 have shown that all \( \text{Ca}^{2+} \) uptake was abolished by protonophore FCCP, indicating that \( \text{Ca}^{2+} \) transport was due to \( \text{Ca}^{2+} / \text{H}^+ \) exchange (Okorokov et al., 2001). The similar result was obtained with \( S. \text{pombe} \) strain Hu237 used for determination of subcellular localization of \( \text{Cta}4p \) (unpublished data). Further experiments will be necessary to find the conditions favoring a detection of biochemical activity of \( \text{Cta}4p \) ATPase. The biochemical characterization of \( \text{Cta}4p \) will contribute to our comprehension of the physiological function of \( P4 \) ATPases.

\( \text{Cta}4p \) localizes to the ER in \( S. \text{pombe} \). In animal cells, ER is equipped with SERCA-type \( \text{Ca}^{2+} \)-ATPase and is a main \( \text{Ca}^{2+} \)-store compartment (Mendolesi and Pozzan, 1998; Carafoli and Brini, 2000). The ATPases belonging to SERCA-type were also found in plant, protista, and insect (Liäng et al., 1997; Lockyer et al., 1998; Talla et al., 1998). Interestingly, the gene encoding for SERCA-type \( \text{Ca}^{2+} \)-pump has not been identified in yeast, although \( \text{Ca}^{2+} \)-ATPase activity could be detected in the \( S. \text{cerevisiae} \) membranes derived from the ER (Okorokov and Lehle, 1998; unpublished data). These observations raise a possibility that \( \text{Cta}4p \) could represent a primary ancient pump serving the ER. It is likely that the evolution of the ER as an organelle was driven by a wide range of functions supporting the development of complex signaling networks within the eukaryotic cell. This may have been the driving force leading to the appearance of additional specialized ATPases, such as SERCA, which may sequester calcium ions into the ER.

The pleiotropic defects exhibited by \( \text{cta}4\Delta \) could be interpreted by either a direct or an indirect involvement of \( \text{cta}4^+ \) in microtubule integrity, cell shape, and cytokinetics through regulated changes in \( \text{Ca}^{2+} \) concentrations. Since \( \text{Ca}^{2+} \) is a well-known secondary messenger, any transient elevations in the intracellular \( \text{Ca}^{2+} \) concentration would result in \( \text{Ca}^{2+} \) binding to multiple classes of \( \text{Ca}^{2+} \)-binding proteins, each of which can, in its turn, regulate multiple downstream signaling pathways. On the other hand, \( \text{Ca}^{2+} \) is emerging as the regulatory ion for many ER/Golgi functions. Oscillations in free \( \text{Ca}^{2+} \) concentrations in the ER of animal cells were shown to control diverse processes, including protein synthesis, chaperone function, and glycoprotein processing (Corbett and Michalak, 2000). The budding yeast secretory pathway requires \( \text{Ca}^{2+} \) for proper glycosylation, sorting, and ER-associated protein degradation (Antebi and Fink, 1992; Durr et al., 1998; Okorokov and Lehle, 1998). Further studies are required to identify components of the \( \text{Ca}^{2+} \) signaling machinery, which depend on \( \text{Cta}4p \). However, some speculations about possible downstream targets can be made already.

We showed that loss of \( \text{cta}4^+ \) enables yeast cells to complete cytokinesis. Previously, this process was shown to be dependent, in part, on the \( \text{cps}1^+ \) gene encoding \( \beta-(1,3)-\text{D-glucan synthase} \) (Ishiguro et al., 1997; Liu et al., 2000). Expression of a homologue of \( \text{cps}1^+ \) in budding yeast, \( \text{FKS}2 \), is induced by PKC together with calcineurin in a \( \text{Ca}^{2+} \)-dependent manner (Zhao et al., 1998). \( \text{cps}1^+ \) also appears to be dependent on calcineurin, since the \( \text{cps}10^+ \) mutant is hypersensitive to \( \text{CSA} \) (Ishiguro et al., 1997). In addition, \( \text{cps}10^+ \) mutants are, like \( \text{cta}4\Delta \), multiseptated and branched. Considering our supposition that calcineurin function might be compromised in \( \text{cta}4\Delta \), then defects in cytokinetics could be explained through changes in \( \text{Cps}1 \) activity, and this raises the possibility that \( \text{cta}4^+ \) and \( \text{cps}1^+ \) act in the same pathway.

There is a strong link between cell shape/polarity and microtubules in fission yeast (Sawin and Nurse, 1998). Therefore, our data provide evidence that microtubule integrity relies on \( \text{cta}4^+ \) function. From this study, we cannot distinguish direct from indirect effects of \( \text{Ca}^{2+} \) on microtubules. Thus, additional studies will be necessary to gain a deeper comprehension of the involvement of \( \text{cta}4^+ \) in this process. One of the possibilities is that \( \text{cta}4^+ \) would regulate microtubule integrity, controlling the stability and/or deposition of microtubule-associated proteins. A recent study in animal cells has shown that \( \text{Ca}^{2+} \)-binding proteins, such as \( \text{S}100\text{A}1 \) and \( \text{S}100\text{B} \), might have a role in the in vivo regulation of the state of assembly of microtubules in a \( \text{Ca}^{2+} \)-regulated manner (Sorci et al., 2000). Also, in \( S. \text{pombe} \), microtubule-associated factors, such as the CLIP170-like protein Tip1, are involved in microtubule dynamics (Brunner and Nurse, 2000). The phenotypes of \( \text{tip}1 \Delta \) null and \( \text{cta}4\Delta \) are related, since the mutants cells are not rod-shaped like wild-type \( S. \text{pombe} \) cells. Furthermore, both mutants show shorter microtubules and an increase in the frequency of microtubule catastrophe events throughout the cell rather than exclusively in the cell tips, which are the regions of polarized growth (Fig. 7) (Brunner and Nurse, 2000). In both cases, the changes in cell shape are associated with a defective guidance mechanism for microtubules. In this respect, since \( \text{Tip}1p \) is a microtubule-binding protein it would be interesting to investigate if \( \text{Tip}1p \) is directly or indirectly dependent on \( \text{Ca}^{2+} \) and/or \( \text{Cta}4p \).

We provide evidence that the \( \text{Ca}^{2+} \) concentration is crucial for establishing the correct cell polarity by regulating microtubule dynamics. This finding is not without precedence, since studies in plant root tips demonstrate that root hair polarity is dependent on a \( \text{Ca}^{2+} \) gradient that increases in \( \text{Ca}^{2+} \) concentration toward the tip of the root hair (Bibikova et al., 1997; Wymer et al., 1997; Gadella et al., 1999). Furthermore, the polarity marked by this gradient is dependent on microtubules (Bibikova et al., 1999). Thus, the \( \text{Ca}^{2+} \)-dependent mechanisms operating with respect to cell polarity may likely be of general significance in eukaryotes.

Further measurements of cytosolic \( \text{Ca}^{2+} \) are needed to clarify whether a \( \text{Ca}^{2+} \) gradient exists within the yeast cell and if there is a correlation between localized high \( \text{Ca}^{2+} \) and the sites of polarized growth. At the moment, it is tempting to speculate that increased \( \text{Ca}^{2+} \) at the ends of the cells would be a guiding signal for microtubule growth and a factor that induces the occurrence of microtubule catastrophe events.
brane fractions were collected from the bottom and stored at onto a step gradient formed of 56, 52, 48, 45, 42, 39, 36, 33, 30, and 25% rokov and Lehle, 1998). The resuspended total membranes were loaded lysis and isolation of membranes followed published procedures (Oko- lytic enzymes from

Materials and methods
Strains and media
Media used were prepared according to standard methods (Moreno et al., 1991). Strains used in this study are listed in Table III.

IF microscopy
S. pombe cells were prepared for IF microscopy according to the form- aldehyde fixation procedure with some modifications (Hagan and Ay- scough, 2000). Log phase cultures were incubated for 5–30 min in YES plus 1.2 M sorbitol before harvest. In most cases PEMAL (PEM plus 5 or 0.03% milk, 0.1 M l-lysine HCl, cleared by centrifugation during 30 min at 20,000 g was used instead of PEMBAL. As primary antibodies, rabbit anti-GFP (Molecular Probes), mouse anti-myc (Sigma-Aldrich), and rab- bit anti-BiP (Pidoux and Armstrong 1993) were used. FITC- or Texas red–conjugated secondary antibodies were purchased from Jackson Immunomeasurements, K. Cunningham and S. Suzuki for SMKT toxin, K. Gull for the Tat1 antibody, J. Armstrong for BiP antibody, A. Pidoux for the Fy2773 strain, and R. Tsien for cameleon pYC2.

Live analysis of S. pombe cells
We performed GFP and CFPLCFP time-lapse analysis using the ratio imaging module of Openlab software version 2.25; Improvision, was performed using 0.2–0.3-μm sample z spacing and nearest neighbor deconvolution method. An object magnification of 100× and a lens aperture of 1.4 were used.

Membrane fractionation
Yeast cells were grown to late log phase. After incubation in 1.2 M sorbitol and 30 mM mercaptoethanol, pH 8.5, for 10 min at 25°C, they were washed with 1.2 M sorbitol and 50 mM NaH2PO4 adjusted with citric acid to pH 5.8. Spheroplasts were then isolated by incubation of the cells with lytic enzymes from Trichoderma at 30°C in the same buffer. Spheroplasts and isolation of membranes followed published procedures (Oko- rokov and Lehle, 1998). The resuspended total membranes were loaded onto a step gradient formed of 56, 52, 48, 45, 42, 39, 36, 33, 30, and 25% sucrose (w/v/w). After centrifugation at 140,000 g for 2 h 45 min, the mem- hbrane fractions were collected from the bottom and stored at –70°C.

Immunoblotting
Yeast membranes from the sucrose gradient fractions (10 μl) were spotted on nitrocellulose membrane and probed with antibodies. Anti-GFP and anti-myc antibodies were purchased from Molecular Probes and Sigma-Abrich, respectively. Anti-BiP antibodies were provided by Prof. J. Armstrong (University of Sussex, Brighton, UK). The blots were developed with peroxidase-conjugated secondary antibody.

Recombinant DNA
All procedures with recombinant DNA were performed according to stan- dard techniques (Maniatis et al., 1982). The YC2 construct was PCR ampli- fied from the original DNA clone (Miyawaki et al., 1997) using oligonucleo- otides that add a PKKKRKV (SV40) nuclear localization signal fused to the YC2 NH2 terminus and cloned into pREP3× multicopy plasmid digested with Sall. The expression of cta4+ was induced in PMG medium lacking thiamine. The cta4+ gene was tagged at its endogenous site with GFP using the method from Bahler et al. (1998).

We thank A. Mutvei for comments on the article, C. Retamal for help with immunomeasurements, K. Cunningham and S. Suzuki for S. cerevisiae strains, C. Stirling for Sec61-myc, S. Suzuki for SMKT toxin, K. Gull for the Tat1 antibody, J. Armstrong for BiP antibody, A. Pidoux for the Fy2773 strain, and R. Tsien for cameleon pYC2.

This work was supported by the Swedish Medical Research Council VR-M grant no. 12562 to K. Ekwall, Fundação Carlos Chagas Filho de Am- paro à Pesquisa do Estado do Rio de Janeiro grant E-26/171.374/99 to L. Okorokova, a Natural Science Council VR-N open postdoc grant to H. Appelgren, and a Wennergren stipend to M. Tabish.

Submitted: 5 November 2001
Revised: 28 February 2002
Accepted: 3 April 2002

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