Expression of budding yeast IPT1 produces mannosyldiinositol phosphorylceramide in fission yeast and inhibits cell growth

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In *Saccharomyces* (Sacc.) *cerevisiae*, the final step of the complex sphingolipid biosynthetic pathway requires Lpt1p for synthesis of mannosyldiinositol phosphorylceramide [M(IP)2C]. No fission yeast equivalent to Lpt1p has been found in the *Schizosaccharomyces* (Schiz.) *pombe* genome, and the most abundant complex sphingolipid is mannosyldiinositol phosphorylceramide. To examine the effect of expressing *Sacc. cerevisiae* IPT1 (ScIPT1) in *Schiz. pombe*, the ScIPT1 gene was cloned into an inducible fission yeast integrative vector and expressed in wild-type *Schiz. pombe*. In the *Schiz. pombe* ScIPT1-expressing cells, M(IP)2C was detected, indicating that Scpt1p functions in M(IP)2C synthesis in *Schiz. pombe*. Expression of ScIPT1 caused pleiotropic phenotypes, including aberrant morphology and mislocalization of ergosterols in the plasma membrane. Furthermore, growth of *Schiz. pombe* was severely impaired. We analysed the sphingolipid composition of ScIPT1-expressing cells following a prolonged lag phase, and found that M(IP)2C was not synthesized, indicating that Lpt1p had been inactivated. GFP-tagged Scpt1 localized primarily in the Golgi apparatus in wild-type *Schiz. pombe*. Over time, Scpt1p was eventually transported to the vacuolar lumen through the multivesicular body pathway. These results indicate that M(IP)2C is toxic to *Schiz. pombe* and that fission yeast possesses an unknown mechanism to effectively extrude toxic sphingolipids from cells.

INTRODUCTION

In eukaryotic cells, sphingolipids are essential constituents of the plasma and intracellular membranes, playing important roles in bilayer stability, stress adaptation, signalling and possibly the formation of lipid microdomains (Dickson, 1998; Holthuis et al., 2001; Edidin, 2003; van Meer et al., 2008). The membrane lipid microdomains called lipid rafts are formed by the lateral association of sphingolipids and cholesterol (ergosterol in yeasts). Lipid rafts are involved in endocytosis, exocytosis and, more generally, in lipid-mediated membrane trafficking as well as in establishing platforms for vesicle-linked actin polymerization (Ikonen, 2001; Rozelle et al., 2000). The typical phosphophingolipid in mammalian cells is sphingomyelin with a phosphocholine head group, whereas the phosphosphingolipids of plants and fungi have head groups based on phosphoinositol. The simplest compound of the latter class is inositol phosphorylceramide (IPC), but often sugar residues and additional phosphoinositol units are added to form glycosylated IPC (Warnecke & Heinz, 2003; Pata et al., 2010; Zäuner et al., 2010).

*Saccharomyces* (Sacc.) *cerevisiae* cells contain three major classes of inositol-containing sphingolipids: IPC, mannosylinositol phosphorylceramide (MIPC), and mannosyldiinositol phosphorylceramide [M(IP)2C; Cowart & Obeid, 2007; Dickson et al., 2006]. These lipids comprise 30 % of the total phospholipids present in the yeast plasma membrane, with M(IP)2C accounting for 75 % of these yeast sphingolipids (Smith & Lester, 1974). These complex sphingolipids are synthesized in the Golgi apparatus (Puoti et al., 1991). The final step of the complex sphingolipid biosynthetic pathway in *Sacc. cerevisiae* was found previously to require IPT1 (encoding an inositolphosphotransferase) for synthesis of M(IP)2C (Dickson et al., 1997). Recently, SKN1, a plant defensin-sensitivity gene, was also found to be required for M(IP)2C biosynthesis in *Sacc. cerevisiae* (Thevissen et al., 2005, 2010). When grown in nutrient-rich media, ipt1Δ and skn1Δ single and double deletion mutants are characterized by membranes devoid of M(IP)2C (Dickson et al., 1997; Thevissen et al., 2005). However, when grown under nutrient-limiting conditions in half strength potato...
dextrose broth (PDB), the single deletion mutants ipt1Δ and skn1Δ reacquire M(IP)2C in their membranes, whereas M(IP)2C is completely absent in the membranes of the double ipt1Δskn1Δ deletion mutant (Im et al., 2003; Thevissen et al., 2005). Hence, while nutrient conditions influence biosynthesis of M(IP)2C in Sacc. cerevisiae, the direct role of Skn1p in sphingolipid biosynthesis is still uncertain.

Only a few budding yeast strains have IPT1 homologues, including Candida glabrata, Candida albicans and Kluyveromyces lactis (Prasad et al., 2005). We previously reported that no fusion yeast genes equivalent to Lpt1p have been found in the Schizosaccharomyces (Schiz.) pombe genome (Nakase et al., 2010). Sacc. cerevisiae and Schiz. pombe differ in sphingolipid composition, with MIPC being the major type in wild-type Schiz. pombe. Schiz. pombe cells deficient in MIPC have been found to exhibit pleiotropic phenotypes, including defects in cell morphology and endocytosis, and in localization of ergosterols and plasma membrane-associated transporters (Nakase et al., 2010).

In this paper, we report the effects of ScIPT1 expression on Schiz. pombe cells, and show that ScIpt1p synthesizes M(IP)2C. M(IP)2C-producing Schiz. pombe cells exhibited pleiotropic phenotypes, including defects in cell morphology and in localization of ergosterols. Although formation of M(IP)2C inhibited cell growth, fission yeasts possess an unknown mechanism that effectively extrudes toxic sphingolipids from cells.

**METHODS**

**Strains, media and genetic methods.** Escherichia coli strain XL-1-blue (Stratagene) was used for all cloning procedures. Wild-type Schiz. pombe ARC039 (h+ ura4-C190T leu1-32) and Sacc. cerevisiae BY4742 (MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ1) were used. Strains were grown in standard rich medium [Sacc. cerevisiae, YPD (1 % yeast extract, 2 % peptone, 2 % glucose); Schiz. pombe, YES (0.5 % yeast extract, 3 % glucose, and adenine, histidine, leucine, uracil and/or lysine hydrochloride; all at 225 mg l⁻¹) or in synthetic minimal medium [Sacc. cerevisiae, SD (2 % glucose, 0.67 % yeast histogen base without amino acids); Schiz. pombe, MM (0.3 % potassium hydrogen phthalate, 0.22 % sodium phosphate, 0.5 % ammonium chloride, and 2 % glucose, vitamins, minerals and salts)]. Schiz. pombe cells were transformed by the lithium acetate method (Morita & Takegawa, 2004). Standard genetic methods have been described by Alfa et al. (1993).

**Plasmids.** To construct the ScIPT1 expression vectors (nmt1-IPT1-pJK148), the IPT1 coding region was first amplified from Sacc. cerevisiae chromosomal DNA using Taq polymerase and the sense primer 5'-GTTCCTCATGATGCTGATTTTCTTGGCC-3', containing an NdeI restriction site and the anti-sense primer 5'-GTTTTGATCCTGCTGAACGGATC-3', containing a BamHI restriction site. The PCR fragment was digested with NdeI and BamHI and introduced into NdeI and BamHI sites present in the Rep1 plasmid. The Rep1-IPT1 plasmid was then digested with PstI and SacI to produce an nmt1 promoter-IPT1 ORF-nmt1 terminator fragment. The digested DNA fragment was subsequently introduced into PstI and SacI sites present in plasmid pJK148. The leu1Δ-containing pJK148 plasmid used for the homologous integration at the leu1-32 locus has been described (Keeney & Boeke, 1994).

Aat1-pTN197 (ura4+) was constructed as follows. The Aat1-pTN197 (LEU2 marker) (Nakase et al., 2010) was digested with Xhol and NotI, and cloned into Xhol- and NotI-digested pTN197 (ura4+) derived from a thiamine-repressible expression vector pRep41 (Nakamura et al., 2001).

To construct the GFP-ScIPT1 expression vectors (nmt1-GFP-IPT1-pJK148), the IPT1 coding region was first amplified from Sacc. cerevisiae chromosomal DNA using Taq polymerase and the sense primer 5'-GTTCCTCATGATGCTGATTTTCTTGGCC-3', containing a BamHI restriction site and the anti-sense primer 5'-GTTCCTCATGATGCTGATTTTCTTGGCC-3', containing a NotI restriction site. The PCR fragment was digested with BamHI and NotI and introduced into BamHI and NotI sites present in the pJK148/pREP1-KS C-EGFP plasmid. The leu1Δ-containing pJK148 plasmid used for the homologous integration at the leu1-32 locus has been described (Keeney & Boeke, 1994).

**myo-[3H]inositol labelling assay.** Sacc. cerevisiae strains were grown in YPD and Schiz. pombe strains were grown in YES or MM-leucine medium at 30 °C to OD600 1.0. Cells were then incubated with 1 μCi myo-[3H]inositol ml⁻¹ in 0.5 ml SC-inositol, MM all-inositol or MM-leucine and inositol medium for 1 h at 30 °C. The cells were washed with water and suspended in 150 μl lipid extraction solvent [ethanol:water:diethyl ether:pyridine:15 M ammonium (15:15:5:1:0.018, by volume)] and incubated at 60 °C for 15 min. After centrifugation at 13 000 g for 3 min, the resulting supernatant was transferred to fresh tubes, and the pellet was re-extracted. The supernatant was dried and suspended in 20 μl chloroform:methanol:water (5:4:1, by volume). Lipids of equal radioactivity were resolved by TLC on silica gel 60 using chloroform:methanol:4.2 M ammonium (9:7:2, by volume) as the solvent system (Uemura et al., 2003).

**Northern blotting.** RNA was prepared using a Qiagen RNeasy kit according to the manufacturer’s instructions for isolating total RNA from yeast. RNA was run on a formaldehyde gel, followed by blotting to a Pall Biodyne Transfer Membrane (Pall). Probes were sequences of ~300 bp that had been amplified by PCR, consisting of the 5' terminus of ScIPT1 gene: 5'- AATGTCATATTCTTGTGGGATTTCGTC-3' and 5'- AAAAGGAATACATATCAGAAATACTAA-3'. Pre-hybridization and hybridization reactions were performed with an AlkPhos direct kit module (GE Healthcare). Transcripts were visualized using a CDP-Star detection reagent (GE Healthcare) and an LAS4000 Imaging System (Fuji Film).

**Western blot analysis.** Cells were grown in 5 ml MM medium at 30 °C to OD600 1.0. The cells were collected by centrifugation, washed once with water and washed in lysis buffer A [50 mM Tris/HCl, 5 mM EDTA, 1 mM PMSF, containing a tablet of EDTA-free complete protease inhibitor cocktail (Roche) per 50 ml (pH 7.5)]. Cells were lysed with glass beads. Unbroken cells and debris were removed by centrifugation at 300 g for 2 min. An aliquot (50 μl) of the cleared lysates was mixed with 2 x sample buffer (100 mM Tris/HCl, 4 mM EDTA, 4 % SDS, 20 % glycerol pH 6.8, 5 % 2-mercaptoethanol) at 37 °C for 20 min to denature proteins. Samples were resolved by 8 % SDS-PAGE. Antibodies used for immunoblotting included mouse monoclonal anti-GFP antibody (Roche) at a dilution of 1:5 000 and rabbit anti-mouse IgG (H+L) HRP conjugate (Invitrogen) as the second antibody at a dilution of 1:10 000. Signals were detected with an LAS4000 imaging system (Fuji Film).

**Fluorescence microscopy.** To visualize the sterol-rich plasma membrane domain, Filipin staining was used. Filipin staining was performed as described by Wachter et al. (2003). Briefly, Filipin was added to the medium at a final concentration of 5 μg ml⁻¹, after which cells were observed immediately using a fluorescence microscope.
To stain F-actin, cells were fixed with 2% formaldehyde and stained with rhodamine-conjugated phalloidin (Molecular Probes) at 100 ng ml\(^{-1}\) (Alfa et al., 1993). Stained cells were observed under a Nikon Y-FL fluorescence microscope using appropriate filter sets (Nikon). Images were captured with a SenSys Cooled CCD camera using MetaMorph (Roper Scientific) software, and were saved as Adobe Photoshop files.

**RESULTS**

**Expression of the Sacc. cerevisiae IPT1 gene in Schiz. pombe cells**

In Sacc. cerevisiae, Ipt1p is known to be involved in M(IP)_2C synthesis from MIPC (Fig. 1a) (Uemura et al., 2003). In previous work, we analysed the complete genome sequence of Schiz. pombe in order to identify homologues of genes required for the biosynthesis of sphingolipids in Sacc. cerevisiae. Through a BLAST search of protein databases, we found that Schiz. pombe contains many homologues of proteins required for sphingolipid biosynthesis in Sacc. cerevisiae. However, no fission yeast equivalents of the inositolphosphotransferase Ipt1p were found (Nakase et al., 2010). In wild-type Sacc. cerevisiae, IPC, MIPC and M(IP)_2C were detected, whereas in wild-type Schiz. pombe, two major sphingolipid spots (IPC and MIPC) were detected, but M(IP)_2C was not observed. Thus, Sacc. cerevisiae and Schiz. pombe were found to differ in sphingolipid composition, with MIPC being the major Schiz. pombe sphingolipid.

To observe the effects of ScIPT1 expression on Schiz. pombe cells, we constructed an integrating construct, ScIPT1-pJK148, with ScIPT1 under the control of an attenuated

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**Fig. 1.** Comparison of sphingolipid biosynthesis in Sacc. cerevisiae and Schiz. pombe and construction of an ScIPT1-expressing Schiz. pombe strain. (a) Sphingolipid biosynthetic pathways in Sacc. cerevisiae and Schiz. pombe. Sphingolipid biosynthesis begins with the condensation of palmitoyl-CoA and serine in the endoplasmic reticulum (ER). Synthesis occurs in the ER until formation of ceramide. Ceramide is converted to IPC, MIPC and M(IP)_2C in the Golgi apparatus. The enzyme which forms M(IP)_2C from MIPC in Sacc. cerevisiae is Ipt1p. Known sphingolipids in Schiz. pombe include IPC and MIPC. (b) Construction of ScIPT1-expressing fission yeast strain. The constructed vector was linearized and integrated at the leu1 locus. (c) Sacc. cerevisiae wild-type and ipt1Δ strains and Schiz. pombe wild-type and ScIPT1-expressing strains were labelled with [3H]-myo-inositol for 1 h at 30 °C. Lipids labelled with myo-[3H]inositol were extracted and separated by TLC and visualized by autoradiography. PI, phosphatidylinositol; PIP, phosphatidylinositol phosphate. (d) mRNA levels of ScIPT1 in Schiz. pombe wild-type and ScIPT1-expressing strains. Cells were cultured at 30 °C, and total RNA was extracted. Each RNA sample was separated on a 1% agarose gel in the presence of formaldehyde.
nt1 promoter (Fig. 1b). In minimal medium supplemented with 0.5 μM thiamine, transcription at the nmt promoter was undetectable. Removal of thiamine resulted in a detectable message after 10 h with maximal steady-state levels reached after 16 h (Maundrell, 1990).

To determine whether Sacc. cerevisiae Ipt1p functioned in M(IP)2C synthesis in Schiz. pombe cells, the composition of intracellular sphingolipids of the transformant was analysed. The Schiz. pombe transformant and wild-type strain were grown in YES and MM (thiamine-free), while the Sacc. cerevisiae ipt1Δ mutant and wild-type strain were grown in YPD. The Schiz. pombe transformant was inoculated from minimal medium supplemented with thiamine, and incubated for 18 h. The cells were incubated with 1 μCi [3H] myo-inositol ml⁻¹ for 1 h at 30 °C, and lipids were then extracted and separated by TLC (Fig. 1c). In wild-type Sacc. cerevisiae cells, IPC, MIPC and M(IP)2C were detected, whereas M(IP)2C was not detected in the Sacc. cerevisiae ipt1Δ mutant or in wild-type Schiz. pombe cells. In the Schiz. pombe ScIPT1 transformant, on the other hand, IPC, MIPC and M(IP)2C were detected. We examined the transcription of ScIPT1 in Schiz. pombe cells. Wild-type and Schiz. pombe ScIPT1 transformant cells were grown in YES and MM (thiamine-free). RNA samples were taken out and subjected to Northern blot analysis using an ScIPT1-specific probe (Fig. 1d). ScIPT1 was highly expressed in Schiz. pombe cells, indicating that Ipt1p functions in M(IP)2C synthesis in Schiz. pombe.

**Cell morphology of ScIPT1-expressing cells**

To elucidate the effects of ScIPT1 expression on Schiz. pombe cell morphology, microscopic observations were made of transformants that were grown in minimal medium (with or without thiamine) for 18 h. ScIPT1-expressing cells were round under normal growth conditions, similar to budding yeast (Fig. 2a). In fission yeast, cells maintain an elongated morphology by growing from both ends, which are marked by F-actin patches. To examine the effect of ScIPT1 expression on Schiz. pombe cell shape and F-actin organization, wild-type and transformant cells were monitored by staining with rhodamine-phalloidin. In wild-type and transformant cells that were grown in thiamine-free minimal medium for ~16 h, the actin cytoskeleton showed two identifiable structures (Fig. 2b). Actin cables run in the longitudinal direction of the cell from one end to the other, while cortical patches are highly polarized and concentrated at both growing ends. In transformant cells grown in thiamine-free minimal medium for 18–20 h, on the other hand, actin cables were not present and actin patches were depolarized. In these cells, the abnormal cell shape correlated with the depolarization of the actin patches.

**Cell surface distribution of ergosterol in ScIPT1-expressing cells**

We previously reported that MIPC-deficient cells were round, similar to ScIPT1-expressing cells (Nakase et al., 2010). Sterols and sphingolipids are the major components of membrane microdomains (or lipid rafts) (Harmouch et al., 1995; Xu et al., 2001), and the localization of sterols in the plasma membrane was found to be abnormal in MIPC-deficient cells (Nakase et al., 2010). Therefore, we examined the localization of sterols in ScIPT1-expressing cells using the fluorescent probe Filipin, a polyene antibiotic that forms specific complexes with free 3-β-hydroxysterols. While most wild-type Schiz. pombe cells exhibited intense staining at the cell tips, as previously reported (Takeda et al., 2004; Wachtler et al., 2003), ScIPT1-expressing cells exhibited enhanced levels of fluorescence, with sterols being detected throughout the plasma membrane (Fig. 3a). These results suggest that production of M(IP)2C in Schiz. pombe leads to alterations in sterol localization within the plasma membrane.

The intracellular trafficking of amino acid permease Aat1 has been reported in Schiz. pombe (Nakase et al., 2010, 2012). The fate of the Aat1 permease is regulated according to the nitrogen source in the medium and abundance. Under nitrogen-sufficient conditions, Aat1 is localized at the Golgi apparatus. In contrast, under nitrogen-insufficient concentrations, Aat1 was sorted to the plasma membrane of cell tips. Over time, plasma membrane-localized Aat1 was internalized and sorted to the lumen of the vacuole, where it was degraded (Nakase et al., 2012). When Aat1-GFP was expressed in MIPC-deficient mutant
These results suggest that production of M(IP)_2C in ScIPT1 plasma membrane in (Fig. 3b). However, Aat1-GFP localized throughout the a nitrogen-free medium, as observed in wild-type cells then transported to the plasma membrane upon shift to ScIPT1 pressed Aat1-GFP in lipid raft domains in fission yeast. To examine localization of 2010). Thus, we conclude that the Aat1 is enriched in lipid raft domains in Schiz. pombe (Fig. 3). Localization of sterol-rich plasma-membrane domains and amino acid permease in ScIPT1-expressing cells. (a) The wild-type (+ vector) and transformant were grown for 18 h in MM medium without leucine at 30 °C. Cells from exponentially growing cultures were briefly incubated with Filipin (5 μg ml⁻¹) and observed by microscopy. (b) The wild-type and transformant carrying Aat1-GFP (ura4⁺ marker) were cultured in MM without leucine and/or uracil for 18 h (designated +N), and shifted to nitrogen-free medium after 2 h (designated −N 2 h).

Fig. 3. Localization of sterol-rich plasma-membrane domains and amino acid permease in ScIPT1-expressing cells. (a) The wild-type (+ vector) and transformant were grown for 18 h in MM medium without leucine at 30 °C. Cells from exponentially growing cultures were briefly incubated with Filipin (5 μg ml⁻¹) and observed by microscopy. (b) The wild-type and transformant carrying Aat1-GFP (ura4⁺ marker) were cultured in MM without leucine and/or uracil for 18 h (designated +N), and shifted to nitrogen-free medium after 2 h (designated −N 2 h).

cells, endocytosis of Aat1-GFP was severely impaired and Aat1-GFP remained at the plasma membrane (Nakase et al., 2010). Thus, we conclude that the Aat1 is enriched in lipid raft domains in fission yeast. To examine localization of Schiz. pombe plasma membrane proteins that are enriched in lipid raft domains in ScIPT1-expressing cells, we expressed Aat1-GFP in ScIPT1-expressing cells. The protein localized to the Golgi apparatus in rich medium and was then transported to the plasma membrane upon shift to a nitrogen-free medium, as observed in wild-type cells (Fig. 3b). However, Aat1-GFP localized throughout the plasma membrane in ScIPT1-expressing cells (Fig. 3b). These results suggest that production of M(IP)_2C in Schiz. pombe affected normal localization of Aat1 to the plasma membrane.

Inhibition of cell growth in ScIPT1-expressing cells
To elucidate the influence of ScIPT1 expression on growth, growth rates were compared between wild-type and ScIPT1-expressing Schiz. pombe cells. Cells were pre-incubated in minimal medium without thiamine for 18 h to induce expression of ScIPT1. After 18 h, wild-type and ScIPT1-expressing cells were shifted to fresh thiamine-free minimal medium to measure growth rates. Similarly, cells grown in parallel in minimal medium with thiamine were shifted to fresh minimal medium with thiamine as a control. For wild-type cells, growth began after 20 h with cells reaching stationary phase after 50 h (Fig. 4a). In contrast, in ScIPT1-expressing cells, growth was severely inhibited, as shown by a prolonged lag phase. Interestingly, apparently normal growth began after about a 60 h lag, with cells attaining about the same growth rate and same final cell yield as the wild-type culture. This pattern of growth is consistent with an ScIPT1-inactivating mutation arising in the culture, or some other adaptation that reversed the apparent toxic consequences of ScIPT1 expression.

We examined the Ipt1 protein by expressing the GFP-Ipt1 fusion protein (Fig. 4b). Ipt1 protein levels dropped simultaneously with growth recovery and free GFP was detected, suggesting that GFP-Ipt1 protein was degraded by vacuolar proteases (Fig. 4b).

Inactivation of ScIpt1p in Schiz. pombe
We tested the possibility that post-lag-phase growth in the ScIPT1-expressing cells occurred due to inhibition of M(IP)_2C synthesis by analysing the composition of complex sphingolipids. The Schiz. pombe transformant was grown in thiamine-free minimal medium for 18, 20 and 24 h. Cells were then incubated with 0.5 μCi myo-[³H]inositol ml⁻¹ for 1 h at 30 °C, and lipids were extracted and separated by TLC (Fig. 5a). M(IP)_2C was detected in cells harvested at 18 and 20 h, but not at 24 h, suggesting that ScIpt1p activity was inactivated sometime between 20 and 24 h. We showed that Ipt1 protein levels dropped in cells harvested at 24 h (Fig. 5b). Because growth was observed after about 60 h of continuing incubation (Fig. 4a), we speculate that the growing cells not only had some means of inactivating ScIpt1p but also were able to neutralize or eliminate the presumably toxic M(IP)_2C formed prior to cessation of synthesis.

ScIpt1 protein is transported to the vacuole via the multivesicular body (MVB) pathway
In Sacc. cerevisiae, it has been reported that Sur1p and Ipt1 localize in the Golgi apparatus (Lisman et al., 2004). We previously reported that localization of the fission yeast Imt proteins, Sur1p homologues, primarily localized in the Golgi apparatus, similar to Sacc. cerevisiae Sur1p. To determine localization of ScIpt1p in fission yeast, we constructed N-terminal GFP-tagged ScIpt1p. Cells expressing GFP-ScIpt1 incubated for 18 h in thiamine-free minimal medium exhibited punctate fluorescence, suggesting that GFP-ScIpt1 also localized in the Golgi apparatus (Fig. 6a). The GFP fusion proteins were co-expressed with the Golgi marker protein Gms1-RFP. Gms1p is an UDP-galactose transporter that localizes to the Golgi membrane (Tabuchi et al., 1997; Tanaka et al., 2001). GFP-ScIpt1
fluorescence largely colocalized with Gms1-RFP or was observed as adjacent spots of fluorescence (data not shown). These results indicate that the GFP-ScIpt1 fusion protein primarily localized in the Golgi apparatus, similar to \textit{Sacc. cerevisiae} Sur1p and the 3 \textit{Schiz. pombe} Imt proteins (Lisman et al., 2004; Nakase et al., 2010).

When cells expressing GFP-ScIpt1 were incubated for 24 h in thiamine-free minimal medium, GFP-ScIpt1 was sorted from the Golgi apparatus to the vacuolar lumen (Fig. 6a).

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**Fig. 4.** Cell growth of \textit{ScIPT1}-expressing cells. (a) The wild-type (+ vector) and transformant were pre-incubated in MM medium without thiamine to induce expression of \textit{ScIPT1}. After 18 h pre-incubation, cells were shifted to fresh thiamine-free MM without leucine to analyse growth at 30 °C. As a control, cells pre-incubated in MM without leucine supplemented with thiamine were shifted to fresh MM without leucine supplemented with thiamine. (b) After 18 h pre-incubation, the wild-type (+ vector) and GFP-Ipt1 transformant were incubated for 0, 2, 6 and 30 h in MM medium without leucine at 30 °C. After incubation, whole-cell extracts were prepared. These samples were solubilized and analysed by Western blotting using an anti-GFP antibody.

**Fig. 5.** Synthesis of M(IP)$_2$C in \textit{ScIPT1}-expressing cells. (a) The wild-type (+ vector) and transformant were grown for 18, 20 and 24 h in MM medium without leucine, and in MM without leucine supplemented with thiamine as a control at 30 °C. Cells were labelled with \textit{myo-}$[^3$H]inositol for 1 h at 30 °C. Lipids labelled with \textit{myo-}$[^3$H]inositol were extracted and separated by TLC and visualized by autoradiography. (b) The wild-type (+ vector) and GFP-Ipt1 transformant were grown for 18, 20 and 24 h in MM medium with/without leucine at 30 °C. After incubation, whole-cell extracts were prepared. These samples were solubilized and analysed by Western blotting using an anti-GFP antibody.
investigate ScIpt1p sorting to the vacuole in more detail, ScIpt1p was monitored in several intracellular-trafficking-deficient mutants. Most membrane proteins sorting to the vacuolar lumen are sorted from the Golgi apparatus to the endosome. After reaching the endosome, membrane proteins are sorted into vesicles that bud into the lumen of this compartment as it matures into an MVB. Upon fusion of the MVB with the vacuole, internal vesicles are released into the vacuolar lumen where the proteins are ultimately degraded. Sorting of membrane proteins into the vacuole in class E vps mutants was assessed, as we have previously shown that ESCRT machinery is present in Schiz. pombe (Nikko et al., 2003; Babst, 2005; Iwaki et al., 2007). In the class E vps mutants tested, ScIpt1p was not properly transported to the vacuolar lumen (Fig. 6b), indicating that ScIpt1p was transported from the Golgi apparatus to the vacuolar lumen through the MVB pathway.

**DISCUSSION**

Previously, we reported that the most abundant complex sphingolipid in Schiz. pombe was MIPC, and that a Sacc. cerevisiae IPT1 homologue was not present in the Schiz. pombe genome (Nakase et al., 2010). Only a few budding yeast strains have IPT1 homologues, including C. glabrata, C. albicans and K. lactis (Prasad et al., 2005). In Sacc. cerevisiae, it has been reported that M(IP)2C is a potential target for toxins and antibiotics, such as zymocin and syringomycin E (Zink et al., 2005; Stock et al., 2000). Growth of Sacc. cerevisiae was reported to be strongly inhibited by zymocin and syringomycin E, whereas Schiz. pombe was resistant to these compounds (Nakase et al., 2010). These observations suggest that the effects of these compounds are limited and that most fungi are resistant because of the absence of M(IP)2C.

In the present report on the effect of ScIPT1 expression on Schiz. pombe cells, the ScIPT1 gene was cloned into an inducible and integrative fission yeast vector and expressed in wild-type Schiz. pombe. Significant amounts of M(IP)2C were detected in the ScIPT1 transformant (Fig. 1c), indicating that M(IP)2C was synthesized by ScIpt1p from MIPC. We previously reported that MIPC-deficient cells also exhibited pleiotropic phenotypes, including defects in cellular and vacuolar morphology, and in localization of ergosterols (Nakase et al., 2010). Therefore, our initial presumption was that the pleiotropic phenotypes of the ScIPT1-expressing transformant were caused by reduced levels of MIPC. However, we determined that levels of MIPC were not reduced in the ScIPT1-expressing transformant (Fig. 1c), suggesting that production of M(IP)2C was the likely cause.

In Sacc. cerevisiae, M(IP)2C normally accounts for about 75% of the sphingolipids in wild-type cells (Smith & Lester, 1974). Although the ipt1 deletion mutant completely lacked M(IP)2C, it grew normally and did not exhibit any apparent defects in the localization of membrane proteins. In contrast, expression of ScIPT1 in Schiz. pombe
caused abnormal localization of sterols within the plasma membrane. In *Sacc. cerevisiae*, sterol biosynthesis mutants have altered sphingolipid profiles (Guan et al., 2009), which suggests a functional interaction between sterols and sphingolipids. We reported that ergosterol was also distributed throughout the plasma membrane in MIPC-deficient cells (Nakase et al., 2010) and, therefore, the polar head group of sphingolipids may play important roles in lipid raft formation in *Schiz. pombe*.

Actin patches assemble at sites of endocytosis, accompanying polarized cell growth at the tips of interphase cells and at the centre of dividing cells (Gachet & Hyams, 2005; Arellano et al., 1997; Balasubramanian et al., 1998; Kovar et al., 2011). Their distribution changes throughout the cell cycle. Early in the cycle, actin patches are localized at one cell tip (the old end) and then, in G2 phase, appear at both cell tips. The patches are delocalized during mitosis and are finally concentrated around the medial septum during cytokinesis (Gachet & Hyams, 2005; Arellano et al., 1997). Their distribution changes throughout the cell cycle. Early in the cycle, actin patches are localized at one cell tip (the old end) and then, in G2 phase, appear at both cell tips. The patches are delocalized during mitosis and are finally concentrated around the medial septum during cytokinesis in *Schiz. pombe* (Marks et al., 1986). In *ScIPT1*-expressing cells incubated in thiamine-free minimal medium for 18–20 h, actin cables were not present while actin patches were depolarized (Fig. 2). This suggests that M(IP)2C destabilized the lipid raft and that M(IP)2C-containing cells were unable to maintain actin-associated protein complexes at the cell tip (Martin et al., 2005, 2007; Perez & Rincón, 2010). It is also reported that the polarized localization of the protein complex depends on sterol-rich membrane domains in *Aspergillus nidulans* (Takehita et al., 2008). As a result of defective actin-filament formation, *ScIPT1*-expressing cells acquire a round shape.

The presence of M(IP)2C in the plasma membrane is toxic to fission yeast and, therefore, growth does not occur unless M(IP)2C can be degraded. When enzymically active ScIpt1p is eliminated from the Golgi apparatus to the vacuolar lumen, plasma membrane containing M(IP)2C is also degraded or extruded. We presume that the time needed for degradation of the enzyme and elimination of M(IP)2C accounted for the prolonged lag phase (~60 h) observed in *ScIPT1*-expressing cells (Fig. 4). At present, we have not determined how M(IP)2C in the plasma membrane of *ScIPT1*-expressing *Schiz. pombe* cells is extruded. Because an M(IP)2C-specific antibody and/or fluorescent dyes that specifically bind to M(IP)2C are not currently available, it is not possible to monitor the precise pattern of degradation or intracellular transport of M(IP)2C in *Schiz. pombe*. In *Sacc. cerevisiae*, the enzyme that hydrolyses complex sphingolipids was identified as Isc1p (Sawai et al., 2000). In *Schiz. pombe*, Css1p is an Isc1p homologue and is able to hydrolyse inositolphingolipids such as IPC (Feoktistova et al., 2001). Although it is not known if the fission yeast Css1p can hydrolyse M(IP)2C, Css1p may be important for the extrusion system of toxic M(IP)2C in *ScIPT1*-expressing cells. Recently, we examined the intracellular localization of Css1p, and found that a Css1-GFP fusion protein was mainly localized at the cell tips where lipid rafts are found in *Schiz. pombe* (M. Nakase & K. Takegawa, unpublished results). We are currently analysing expression of *ScIPT1* in a css1 temperature-sensitive mutant.

In the present study, we found that Scpt1 was eliminated from the Golgi apparatus via transport to the vacuolar lumen through the MVB pathway. In the fission yeast class E vps mutants, Scpt1p was not properly transported to the vacuolar lumen (Fig. 6b). If this is the case, then cell viability should be heavily compromised in the class E vps mutants compared with control cells. However, the cell viability did not decrease in class E vps mutants (data not shown). Therefore, we speculate that Scpt1 was eliminated from Golgi apparatus to endosome, and M(IP)2C was not synthesized in the fission yeast class E vps mutants. Because we found that Scpt1 was localized at the vacuolar lumen in most *ScIPT1*-expressing cells, it is highly unlikely that the observed growth recovery after 60 h was due to a spontaneous mutation in a single cell. However, many important issues remain unresolved. For example, how is Scpt1 selectively recognized as ‘toxic’ in the Golgi apparatus, and what kinds of signals are required for Golgi-to-vacuole transport of Scpt1? In *Sacc. cerevisiae*, several membrane-spanning proteins are transported from the Golgi apparatus to the vacuolar lumen via the MVB pathway, including Cps1p, Phm5p and Sna3p (Reggiori & Pelham, 2001; Odorizzi et al., 1998). Cps1p and Phm5p are ubiquitylated, and non-ubiquitylatable variants of these proteins fail to reach the vacuolar lumen (Odorizzi et al., 1998). In contrast, Sna3p enters internal vesicles in a ubiquitin-dependent manner (Reggiori & Pelham, 2001). In eukaryotic cells, receptor downregulation is a key regulatory step in ensuring the correct duration and magnitude of cell signalling, and ubiquitin modification of these receptors at the plasma membrane is necessary for rapid receptor internalization and downregulation. However, both intracellular sorting and ubiquitination of cell surface membrane-associated transporters are not well understood in *Schiz. pombe*. Recently, we confirmed that the fission yeast amino acid permease Aat1p is ubiquitinated and that ubiquitination is important for internalization of Aat1p (M. Nakase & K. Takegawa, unpublished results). We are currently analysing ubiquitination of Scpt1p in *Schiz. pombe*. Further analysis of Golgi-to-vacuole Scpt1p transport is likely to reveal the mechanism(s) by which cells are able to selectively eliminate ‘toxic’ proteins (Wang et al., 2011) in *Schiz. pombe*.

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REFERENCES


Expression of Sacch. cerevisiae IPT1 in fission yeast


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