Mannosylinositol phosphorylceramide is a major sphingolipid component and is required for proper localization of plasma-membrane proteins in *Schizosaccharomyces pombe*

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Summary

In *Saccharomyces cerevisiae*, three classes of sphingolipids contain *myo*-inositol – inositol phosphorylceramide (IPC), mannosylinositol phosphorylceramide (MIPC) and mannosyldiinositol phosphorylceramide [$M(IP)_2C$]. No fission yeast equivalent of Ipt1p, the inositolphosphotransferase that synthesizes $M(IP)_2C$ from MIPC, has been found in the *Schizosaccharomyces pombe* genome. Analysis of the sphingolipid composition of wild-type cells confirmed that MIPC is the terminal and most abundant complex sphingolipid in *S. pombe*. Three proteins (Sur1p, Csg2p and Csh1p) have been shown to be involved in the synthesis of MIPC from IPC in *S. cerevisiae*. The *S. pombe* genome has three genes (SPAC2F3.01, SPCC4F11.04c and SPAC17G8.11c) that are homologues of *SUR1*, termed *imt1*$^+$, *imt2*$^+$ and *imt3*$^+$, respectively. To determine whether these genes function in MIPC synthesis in *S. pombe*, single and multiple gene disruptants were constructed. Single *imt* disruptants were found to be viable. MIPC was not detected and IPC levels were increased in the triple disruptant, indicating that the three *SUR1* homologues are involved in the synthesis of MIPC. GFP-tagged Imt1p, Imt2p and Imt3p localized to Golgi apparatus membranes. The MIPC-deficient mutant exhibited pleiotropic phenotypes, including defects in cellular and vacuolar morphology, and in localization of ergosterols. MIPC seemed to be required for endocytosis of a plasma-membrane-localized amino acid transporter, because sorting of the transporter from the plasma membrane to the vacuole was severely impaired in the MIPC-deficient mutant grown under nitrogen-limiting conditions. These results suggest that MIPC has multiple functions not only in the maintenance of cell and vacuole morphology but also in vesicular trafficking in fission yeast.

Key words: *Schizosaccharomyces pombe*, Sphingolipid, Inositol phosphorylceramide, Lipid rafts, Mannosyltransferase

Introduction

As in higher eukaryotic cells, phospholipids, sterols and sphingolipids are the major membrane lipid components in yeasts. Sphingolipids are abundant components of eukaryotic plasma membranes, with important functions in bilayer stability, stress adaptation, signaling and possibly the formation of lipid microdomains (Dickson, 1998; Holthuis et al., 2001; Edidin, 2003). Sphingolipids in microorganisms contain a long-chain amino alcohol, phytosphingosine, amide linked to a very-long-chain fatty acid, with inositol phosphate or mannosylated inositolphosphate as the polar head group. The budding yeast *Saccharomyces cerevisiae* has served as a unique model to uncover sphingolipid metabolic pathways and has emerged as the framework from which sphingolipid metabolism and function can be elucidated (Cowart and Obeid, 2007; Dickson et al., 2006). The three classes of yeast sphingolipids – inositol phosphorylceramide (IPC), mannosylinositol phosphorylceramide (MIPC) and mannosyldiinositol phosphorylceramide [$M(IP)_2C$] – are intermediates and products of a common biosynthetic pathway (Cowart and Obeid, 2007; Dickson et al., 2006). Sphingolipid biosynthesis begins with the condensation of palmitoyl-CoA and serine via the serine palmitoyltransferase complex in the endoplasmic reticulum (ER). Synthesis occurs in the ER until formation of ceramide (Ko et al., 1994). After transport to the Golgi apparatus, ceramide is converted to IPC. Next, IPC is converted to MIPC, with GDP-mannose as the carbohydrate donor (Abeijon et al., 1989). Finally, introduction of the second inositolphosphate group results in the formation of $M(IP)_2C$ in the Golgi apparatus (Puoti et al., 1991). This final step of the complex sphingolipid biosynthetic pathway was found previously (Dickson et al., 1997) to require *IPT1* (encoding an inositolphosphotransferase) for synthesis of $M(IP)_2C$, the most abundant complex sphingolipid in budding yeast, which is found primarily in the plasma membrane.

Correct sorting of membrane proteins and lipids is essential for establishing and maintaining the identity and function of cellular organelles. Much progress has been made in uncovering the transport machinery for delivering endosomal and lysosomal proteins (Mellman, 1996; Burd et al., 1998), and the mechanisms for cargo sorting to the cell surface by multiple pathways in most, if not all, eukaryotic cells (Keller and Simons, 1997). It was reported that the organization of the various post-Golgi delivery pathways

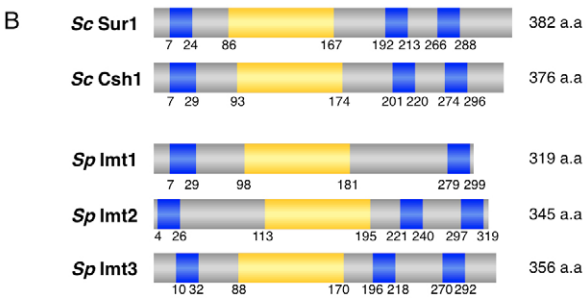
in *S. cerevisiae* does not depend on the production of mannosylated sphingolipids (Lisman et al., 2004).

Many studies have described a membrane microdomain rich in sterols and sphingolipids, known as the 'lipid raft' (Van Meer and Simons, 1988; Simons and Ikonen, 1997; Sturley, 2000; Gaigg et al., 2006). This membrane microdomain corresponds to a related structure, the caveolae, in mammalian membranes. Various functions have been attributed to these structures, including cholesterol transport, endocytosis and signal transduction (Millán et al., 2001; Pelkmans, 2005; Maguy et al., 2006; Wachtler and Balasubramanian, 2006). The most widely used assay for lipid rafts is based on the observation that a subset of associated plasma membrane components is resistant to nonionic detergents, such as Triton X-100 (TX-100) at 4°C (Bagnat et al., 2000). Lipid rafts are also called detergent-resistant membranes (DRMs), because of their detergent resistance. Theoretically, however, lipid rafts and DRMs can be separated as detergent solubilisation might involve formation of nonphysiological structures (Munro, 2003; Lichtenberg et al., 2005). In *S. cerevisiae*, an association between many plasma membrane proteins and DRMs has been studied; some DRM-associated proteins have been identified (Dupré and Haguenauer-Tsapis, 2003; Bagnat et al., 2000; Bagnat et al., 2001; Umebayashi and Nakano, 2003; Malinska et al., 2004; Grossmann et al., 2006; Lauwers and André, 2006).

A

Sp \ Sc	SUR1	CSH1
SPAC2F3.01 *imt1+*	37%	35%
SPCC4F11.04c *imt2+*	50%	48%
SPAC17G8.11c *imt3+*	42%	42%

B



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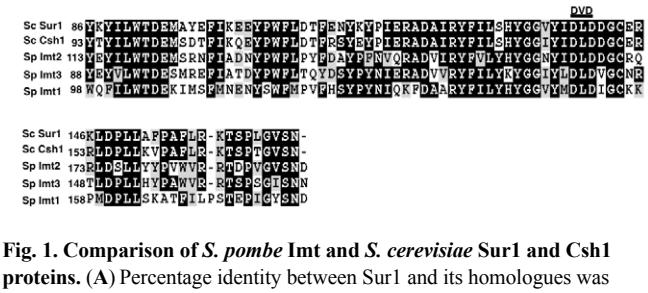

Fig. 1. Comparison of *S. pombe* Imt and *S. cerevisiae* Sur1 and Csh1 proteins. (**A**) Percentage identity between Sur1 and its homologues was determined by Sanger BLAST analysis. (**B**) Domain structure of *S. cerevisiae* Sur1 and Csh1, and the *S. pombe* Imt proteins. Yellow, DXD motif; blue, transmembrane domain. (**C**) Sequence alignments of the lumenal catalytic domain of *S. cerevisiae* Sur1 and Csh1, and the *S. pombe* Imt proteins are shown. Protein names and numbered amino acid residues are given. Solid black boxes indicate identity and shaded boxes indicate similarity between most aligned amino acids. *Sc*, *S. cerevisiae*; *Sp*, *S. pombe*.

Although the complete genome sequence of *S. pombe* has been reported (Wood et al., 2002), there have been few studies on the biosynthetic pathway and role of sphingolipids. Feoktistova et al. isolated fission yeast mutants that display defects in cell-wall formation (Feoktistova et al., 2001). One of these mutants, *css1,* accumulates significant amounts of α- and β-glucans. Css1p shares sequence similarity with mammalian neutral sphingomyelinases (Feoktistova et al., 2001). Interestingly, the *css1*$^{+}$ gene is essential, unlike the *ISC1* gene in *S. cerevisiae*, and therefore the synthesis of ceramides from sphingolipids through Css1p-mediated hydrolysis might play essential roles in *S. pombe*. We have analyzed the complete genome sequence of *S. pombe* to identify homologues of genes required for the biosynthesis of sphingolipids in *S. cerevisiae*. Through a BLAST search of protein databases, we found that *S. pombe* contains many genes that are homologous to proteins required for sphingolipid biosynthesis in *S. cerevisiae*. Interestingly, no fission yeast equivalents of the inositolphosphotransferase Ipt1p were found. In addition, *S. pombe* has three *SUR1* homologues but no genes homologous to *CSG2* of *S. cerevisiae*. Therefore, we presumed that the composition of sphingolipids and the enzyme complexes required for the mannosylation of IPC in *S. pombe* would differ from those in *S. cerevisiae.* In an effort to better understand the role of MIPC in fission yeast, we constructed strains harbouring disruptions in *SUR1* homologues and subjected them to phenotypic characterization.

We found that the most abundant complex sphingolipid in *S. pombe* cells is MIPC and that three *SUR1* homologues are required for MIPC synthesis. *S. pombe* strains disrupted for all three genes failed to produce MIPC and exhibited pleiotropic phenotypes, including defects in cell morphology, localization of ergosterols, endocytosis and localization of plasma-membrane transporters. This is the first report demonstrating that sphingolipid is essential for lipid-raft-dependent endocytosis of plasma-membrane transporters in *S. pombe* cells.

Results

Three *SUR1* homologous genes encode the putative IPC mannosyltransferase

In *S. cerevisiae*, Sur1p, Csg2p and Csh1p are known to be involved in MIPC synthesis from IPC (Uemura et al., 2003). We analyzed the complete genome sequence of *S. pombe* to identify genes required for MIPC synthesis. Three genes homologous to *S. cerevisiae SUR1* and *CSH1* (which encode IPC mannosyltransferases required for MIPC synthesis) were found. We designated SPAC2F3.01, SPCC4F11.04 and SPAC17G8.11c as *imt* (*S. pombe* IPC mannosyltransferase homologue) *1*$^{+}$, *2*$^{+}$ and *3*$^{+}$, respectively. Comparative sequence analysis revealed that Imt1p, Imt2p and Imt3p share 37, 50 and 42% identify with Sur1p, respectively, and contain two or three potential transmembrane segments (Fig. 1A,B). The fission yeast Imt proteins contain a conserved DXD motif that occurs in a wide range of glycosyltransferase families and probably forms part of a catalytic site (Wiggins and Munro, 1998). Comparative sequence analysis suggested that the catalytic sites of Imt1p, Imt2p and Imt3p closely resemble that of Sur1p (Fig. 1C). Interestingly, no fission yeast equivalent of Csg2p has been found in the *S. pombe* genome.

Phospholipid composition of wild-type and mutant *S. pombe* cells

To determine whether the *imt* genes function in MIPC synthesis, single, double and triple disruptant mutants were constructed (*imt1*Δ, *imt2*Δ, *imt3*Δ, *imt1*Δ*imt2*Δ, *imt1*Δ*imt3*Δ, *imt2*Δ*imt3*Δ and

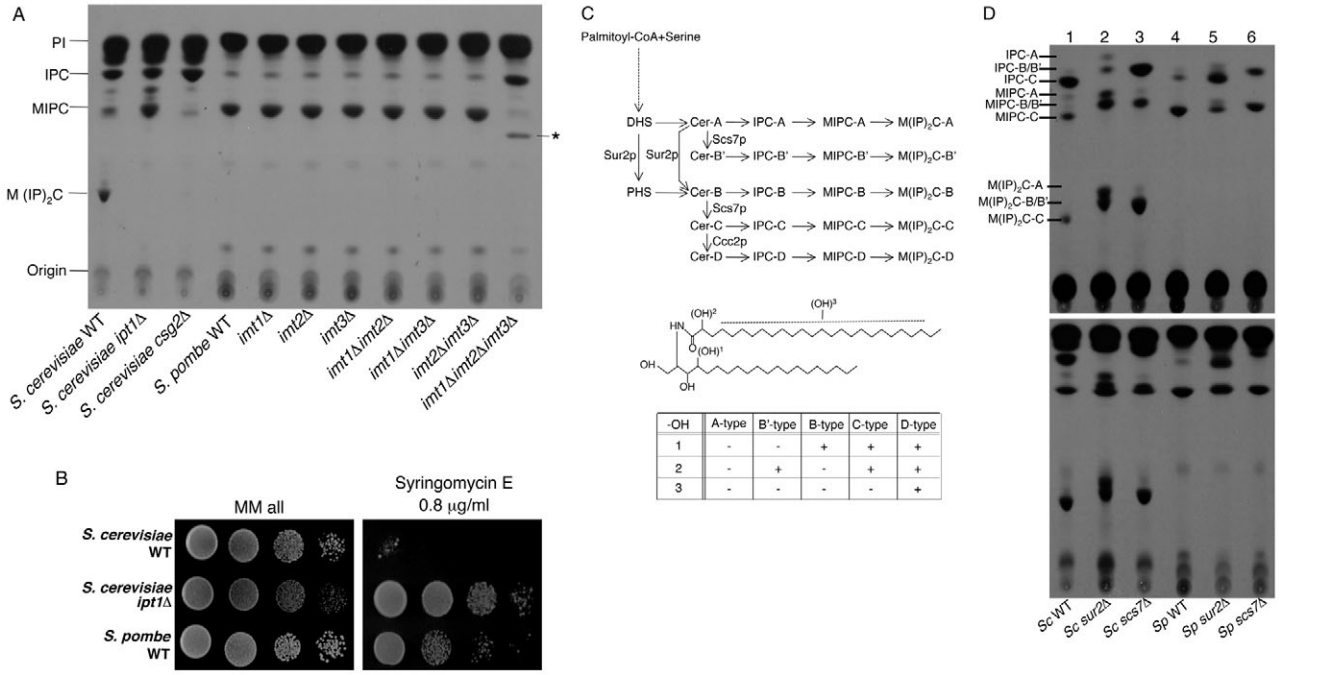

Fig. 2. TLC analysis of sphingolipids from wild-type yeast and various mutants. (**A**) *S. cerevisiae* wild-type, *ipt1*Δ and csg2Δ, and *S. pombe* wild-type, *imt1*Δ, *imt2*Δ, *imt3*Δ, *imt1*Δ*imt2*Δ, *imt1*Δ*imt3*Δ, *imt2*Δ*imt3*Δ and *imt1*Δ*imt2*Δ*imt3*Δ cells were labelled with [^{3}H] *myo*-inositol for 1 hour at 30°C. Lipids labelled with [^{3}H] *myo*-inositol were extracted and separated by TLC and visualized by autoradiography. The asterisk indicates an unidentified band. (**B**) Wild-type fission yeast is resistant to syringomycin E. An overnight culture in YES medium was diluted to an OD_{600} of 1.0 and then serially diluted 1:10. Aliquots (5 μl) were plated on YES medium containing 0.8 μg/ml syringomycin E. Plates were photographed after 3 days at 30°C. (**C**) Structure and sphingolipid biosynthesis pathways in *S. cerevisiae.* Sphingolipid biosynthesis begins with the condensation of palmitoyl-CoA and serine in the ER. Synthesis occurs in the ER until formation of ceramide (middle structure). Ceramide is converted to IPC, MIPC and $M(IP)_2C$. Because of the different hydroxylation states of ceramide (see table), there are five species (ceramides A, B′, B, C and D) each of IPC. The sphingolipid long-chain base moieties of ceramides A and B′ are dihydrosphingosine (DHS), whereas those of ceramides B, C and D are phytosphingosine (PHS). Sur2p is sphingosine hydroxylase, which can convert DHS to PHS or dihydroceramide to phytoceramide. Scs7p is ceramide hydroxylase, which can convert ceramide A to ceramide B′ and ceramide B to ceramide C. (**D**) TLC analysis of sphingolipids from *sur2*Δ and *scs7*Δ mutants. *S. cerevisiae* wild-type, *sur2*Δ and *scs7*Δ, and *S. pombe* wild-type, *sur2*Δ and *scs7*Δ cells were labelled with [^{3}H] *myo*-inositol for 1 hour at 30°C. The lipids were extracted and treated with methanol or alkali treated with 40% methylamine methanol solution (methanol-treated samples: top panel; alkali-treated samples: bottom panel).

*imt1*Δ*imt2*Δ*imt3*Δ). None of the *imt* genes was found to be essential for viability, as the disruptants grew well under normal conditions (data not shown). The growth of *imt1*Δ*imt2*Δ*imt3*Δ cells was slower than that of wild-type cells. In liquid YES medium at 27°C, the cells had a doubling time of 4 hours, in contrast to 2 hours 30 minutes for wild-type cells.

S. pombe wild-type and mutant cells, and *S. cerevisiae* wild-type, *ipt1*Δ and *csg2*Δ cells were cultured in YES or YPD medium, respectively. These cells were incubated with 0.5 μCi/ml [^{3}H] *myo*-inositol for 1 hour at 30°C, and lipids were then extracted and separated by thin layer chromatography (TLC). In *S. cerevisiae* wild-type cells, IPC, MIPC and $M(IP)_2C$ were detected, whereas $M(IP)_2C$ was not detected in *S. cerevisiae ipt1*Δ cells. In *S. pombe* wild-type cells, on the other hand, two major sphingolipid spots (IPC and MIPC) were detected, but $M(IP)_2C$ was not observed (Fig. 2A). Thus, we found that *S. cerevisiae* and *S. pombe* differ in sphingolipid composition, and that the major sphingolipid in wild-type *S. pombe* is MIPC (Fig. 2A). Whereas similar types of sphingolipids were found in most of the *imt* single and double mutants, a distinct sphingolipid composition was found only in *imt1*Δ*imt2*Δ*imt3*Δ cells (Fig. 2A): MIPC was not detected, IPC had accumulated and one unidentified sphingolipid spot was observed (Fig. 2A). These results indicate that all three *imt* genes are required for MIPC synthesis and that MIPC was not produced in *imt1*Δ*imt2*Δ*imt3*Δ disruptant mutants.

In *S. 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). *Kluyveromyces lactis* zymocin inhibits proliferation of *S. cerevisiae* wild-type cells, and its zymocicity is dependent on sphingolipid biosynthesis and the presence of $M(IP)_2C$ (Zink et al., 2005). Syringomycin E is a membrane-disrupting fungicide and studies with *S. cerevisiae* mutants have revealed that $M(IP)_2C$ is required for syringomycin E action (Stock et al., 2000). Because *S. pombe* does not produce $M(IP)_2C$ (Fig. 2A), we expected that *S. pombe* and an $M(IP)_2C$-deficient mutant of *S. cerevisiae* would survive exposure to zymocin and syringomycin E. Toxin tolerance was determined by a visual spotting assay on YES medium. Wild-type *S. cerevisiae* was sensitive to zymocin, whereas the $M(IP)_2C$-deficient mutant (*ipt1*Δ) was tolerant of both toxins, as previously reported (Zink et al., 2005). *S. pombe* wild type was found to be tolerant of both syringomycin E (Fig. 2B) and zymocin (data not shown).

S. cerevisiae sphingolipids [IPC, MIPC and $M(IP)_2C$] contain one of five ceramide backbones (A, B, B′, C and D) that differ in extent of hydroxylation (Fig. 2C) (Uemura et al., 2003; Haak et al., 1997; Dunn et al., 1998). Dihydroceramide contains a dihydrosphingosine

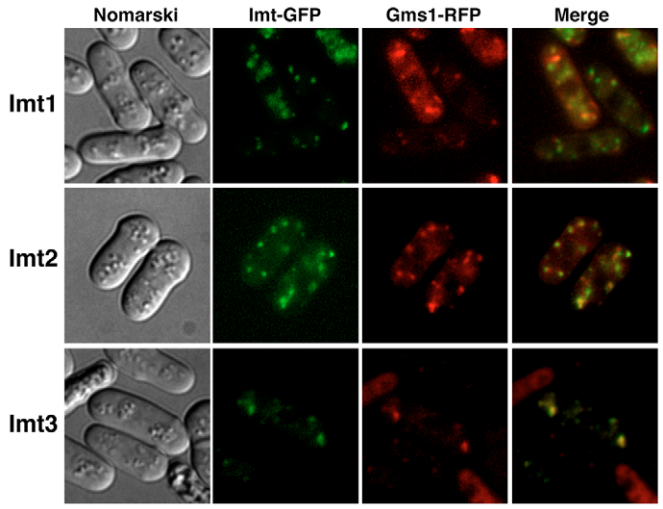

Fig. 3. Intracellular localization of Imt proteins. Cells carrying pTN197-Imt1, Imt2 or Imt3 and pAU-Gms1-RFP were cultured in MM medium without leucine and uracil for 18 hours.

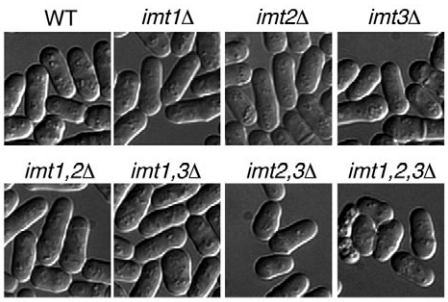

Fig. 4. MIPC-deficient mutant cells have an aberrant morphology. Wild-type, *imt1*Δ, *imt2*Δ, *imt3*Δ, *imt1*Δ*imt2*Δ, *imt1*Δ*imt3*Δ, *imt2*Δ*imt3*Δ and *imt1*Δ*imt2*Δ*imt3*Δ cells were grown in YES medium at 30°C.

(DHS)-type long-chain base and a C26 fatty acid. Sur2p, sphingosine hydroxylase, can convert DHS to phytosphingosine (PHS) or dihydroceramide to phytoceramide. Hydroxylation of the C26 fatty acid of ceramide A and ceramide B at the C-2 position by Scs7p (ceramide hydroxylase) yields ceramide B′ and ceramide C, respectively. Ceramide D is generated by further hydroxylation, at an unknown position, of the fatty acid moiety of ceramide C, a reaction requiring Ccc2p, a possible Cu^{2+} transporter (Uemura et al., 2003). IPC-A, -B, -B′, -C and -D are converted to MIPC-A, -B, B′, -C and -D, respectively, by the mannosyltransferase complexes Sur1p-Csg2p or Csh1p-Csg2p. The Csh1p-Csg2p complex exhibits activity towards IPC-A and -B, whereas the Sur1p-Csg2p complex exhibits activity towards IPC-B and -C. Thus, both Sur1p and Csh1p are involved in MIPC synthesis and have different substrate specificities (Fig. 2C) (Uemura et al., 2003).

To determine the substrate specificities of the fission yeast Imt mannosyltransferases, we constructed deletion strains of *sur2* and *scs7* homologues and analyzed sphingolipid composition. In *S. pombe*, to date no information is available on whether ceramides and complex sphingolipids contain ceramide backbones that differ in hydroxylation state. We found genes homologous to *S. cerevisiae SUR2* and *SCS7*, designated SPBC887.15c and SPAC19G12.08, which we named *sur2*$^{+}$ and *scs7*$^{+}$, respectively. Extracted lipids from *sur2*Δ and *scs7*Δ mutants were alkali treated to remove phosphatidylinositol and separated by TLC (Fig. 2D). The sphingolipid composition of the *S. pombe sur2*Δ and *scs7*Δ mutants was different to that of both wild-type *S. cerevisiae* and *S. pombe*, suggesting that wild-type *S. cerevisiae* and *S. pombe* might contain different types of ceramides (Fig. 2D). Interestingly, a significant amount of IPC accumulated and MIPC decreased in *sur2*Δ cells. The *scs7*Δ mutation also caused a reduction in MIPC content. These results suggest that Imt proteins exhibit weak activity towards dihydroceramide-containing IPC, such that a reduction in MIPC was observed in *sur2*Δ and *scs7*Δ mutants.

Intracellular localization of the fission yeast Imt proteins

In *S. cerevisiae*, it has been reported that Sur1p localizes to the medial Golgi and is required for mannosylation of IPC (Lisman et al., 2004). To determine the localization of the fission yeast Imt proteins, we constructed C-terminal GFP fusions to Imt1p, Imt2p and Imt3p. The Imt-GFP fusion proteins were found to be functional, as assessed by their ability to complement the Ca^{2+}-sensitive growth defect of the *imt1*Δ *imt2*Δ *imt3*Δ disruptant (data not shown). Cells expressing Imt1-GFP, Imt2-GFP and Imt3-GFP exhibited punctate fluorescence, suggesting that Imt1-GFP, Imt2-GFP and Imt3-GFP localized to the Golgi apparatus (Fig. 3). The GFP fusion proteins were coexpressed with the Golgi marker protein Gms1-RFP. Gms1p is a UDP-galactose transporter that localizes to the Golgi membrane (Tabuchi et al., 1997; Tanaka et al., 2001). Imt1-GFP, Imt2-GFP and Imt3-GFP fluorescence largely colocalized with Gms1-RFP or was observed as adjacent spots of fluorescence (Fig. 3). These results indicate that all three Imt-GFP fusion proteins primarily localize to the Golgi and *trans*-Golgi network, similar to *S. cerevisiae* Sur1p (Lisman et al., 2004).

Phenotypic characterization of *imt* mutants

To elucidate the role of MIPC in *S. pombe* cells, microscopic observations were made of single and multiple *imt* deletion mutants. Single and double disruptants had a normal cell shape, but the *imt1*Δ*imt2*Δ*imt3*Δ disruptants were round or pear shaped under normal growth conditions (Fig. 4). Such abnormal shapes were characteristic phenotypes of *S. pombe* mutants with defective cell walls and glycosylation (Tanaka et al., 2001). In the glycosylation modification defective strains, the abnormal cell shape was suppressed by the addition of 1 M sorbitol to the growth medium. However, the morphological defect of the *imt1*Δ*imt2*Δ*imt3*Δ strain was not rescued in 1 M sorbitol-YES medium (data not shown). To determine the localization of actin patches in the *imt1*Δ*imt2*Δ*imt3*Δ mutant, cells were stained with Alexa488-phalloidin. The *imt1*Δ*imt2*Δ*imt3*Δ cells displayed comparatively normal actin structures under normal growth conditions (data not shown).

In *S. cerevisiae*, Sur1p and Csg2p play important roles in Ca^{2+} tolerance; *sur1* and *csg2* mutants were originally identified as Ca^{2+} sensitive (Beeler et al., 1994). Therefore, we investigated the Ca^{2+} sensitivity of *imt* mutants. Sensitivity to Ca^{2+} was determined by a visual spot assay on YES medium (Fig. 5). Single *imt* mutants did not exhibit obvious Ca^{2+} sensitivity, whereas the *imt1*Δ*imt2*Δ*imt3*Δ mutant did. Sensitivity to drugs, other reagents and heavy metals was also tested. *imt1*Δ*imt2*Δ*imt3*Δ cells exhibited sensitivity to 20 μg/μl hygromycin B and several heavy metal ions, including 0.7 mM $CdCl_2$ and 3 mM $ZnCl_2$ (data not shown).

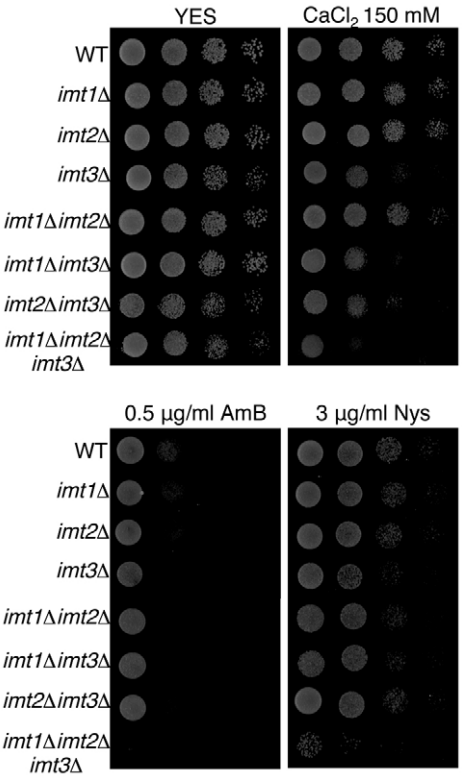

Fig. 5. Phenotypic characterization of *imt* disruptants. An overnight culture in YES medium was diluted to an OD_{600} of 1.0 and then serially diluted 1:10. Aliquots (5 μl) were plated on YES medium containing 150 mM $CaCl_2$, 0.5 μg/ml amphotericin B (AmB) or 3 μg/ml nystatin (Nys). Plates were photographed after 3 days at 30°C.

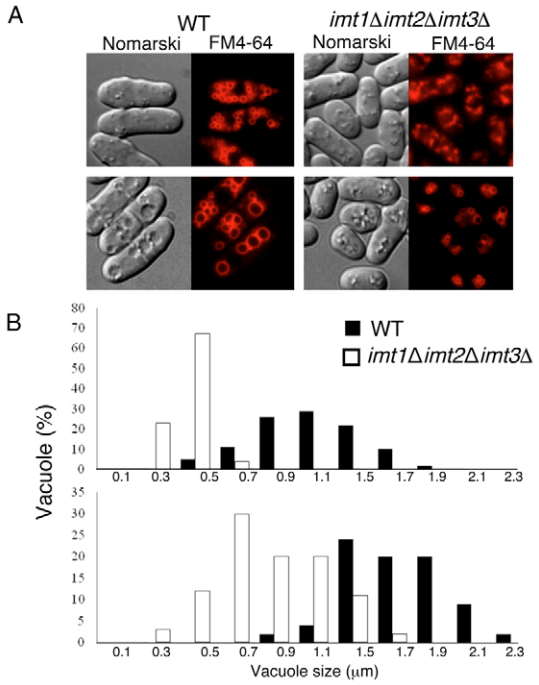

Fig. 6. Vacuole morphology of MIPC-deficient mutants. (**A**) Wild-type and MIPC-deficient mutant (*imt1*Δ*imt2*Δ*imt3*Δ) cells were grown in YES at 30°C and stained with FM4-64. Wild-type cells have numerous small vacuoles. The vacuoles of MIPC-deficient cells seem to be slightly smaller than those of wild-type cells (top panels). Cells were shifted to distilled water for 5 hours to induce homotypic vacuolar fusion. A small number of large vacuoles were observed in wild-type cells. The vacuoles of MIPC-deficient cells failed to fuse normally (bottom panels). (**B**) Vacuole size distribution before (top panel) and after (bottom panel) hypotonic stress. Black bars: wild type. White bars: MIPC-deficient mutant.

*imt1*Δ*imt2*Δ*imt3*Δ cells were also found to be sensitive to 3 μg/ml nystatin and to 0.5 μg/ml amphotericin B (Fig. 5). These results indicate that Imt1p, Imt2p and Imt3p share redundant functions, and that MIPC-deficient *imt1*Δ*imt2*Δ*imt3*Δ cells are sensitive to various metal ions and drugs.

Vacuolar function of the *imt1*Δ*imt2*Δ*imt3*Δ mutant

To investigate vacuolar morphology and fusion, yeast vacuoles were stained with a lipophilic styryl dye, FM4-64 (Vida and Emr, 1995). Wild-type and *imt1*Δ*imt2*Δ*imt3*Δ cells were grown in YES media, stained with FM4-64 and shifted to water for 5 hours to observe the vacuolar morphology (Fig. 6). Wild-type cells contained numerous vacuoles of small size, with hypotonic stress seeming to cause transitory fusion of vacuoles. By contrast, *imt1*Δ*imt2*Δ*imt3*Δ cells had slightly smaller vacuoles compared with those of wild-type cells under normal conditions (Fig. 6). When *imt1*Δ*imt2*Δ*imt3*Δ cells were transferred to hypotonic stress conditions, the vacuoles failed to fuse normally. This result suggests that MIPC is required for normal vacuole morphology and for the fusion process.

Vacuolar morphology and vacuolar protein sorting are known to be closely related (Raymond et al., 1992). Because defects in vacuolar morphology were observed in *imt1*Δ*imt2*Δ*imt3*Δ cells, we investigated vacuolar protein transport in the mutant cells. Mis-sorting of CPY was not detected in the *imt1*Δ*imt2*Δ*imt3*Δ cells, suggesting that vacuolar protein transport was normal in MIPC-deficient cells (data not shown).

Cell-surface distribution of ergosterol in MIPC-deficient cells

Sterol-rich membrane domains (or lipid rafts) are primarily composed of complex sphingolipids and sterols (Harmouch et al., 1995; Xu et al., 2001). As described above, fission yeast MIPC-deficient cells are sensitive to nystatin and amphotericin B (Fig. 5). Nystatin and amphotericin interact with membrane sterols and cause damage to the cell membranes (Kerridge et al., 1986). Recently, we reported that fission yeast ergosterol-deficient mutants were tolerant to nystatin and amphotericin B (Iwaki et al., 2008). Therefore, we examined the localization of sterols in *S. pombe* cells using the fluorescent probe filipin, a polyene antibiotic that forms specific complexes with free 3-β-hydroxysterols. Most *S. pombe* wild-type cells exhibited intense staining at the tips of the cell, as previously reported (Takeda et al., 2004; Wachtler et al., 2003). Whereas *imt1*Δ*imt2*Δ*imt3*Δ cells exhibited enhanced levels of fluorescence, sterols were detected throughout the plasma membrane (Fig. 7). These results suggest that aberrant localization or enrichment of sterols in the plasma membrane caused sensitivity to polyene antibiotics in MIPC-deficient cells.

MIPC is required for lipid-raft-mediated endocytosis of plasma-membrane proteins

The intracellular trafficking of general amino acid permease Gap1p has been extensively studied in *S. cerevisiae* (Grenson et al., 1970).

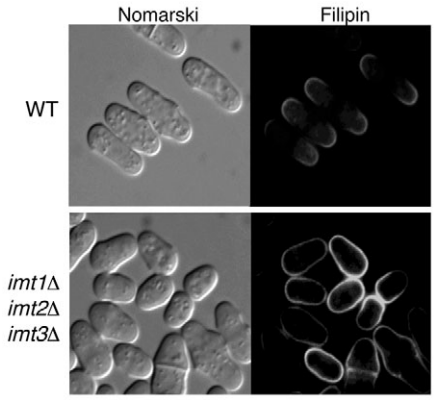

Fig. 7. Localization of sterol-rich plasma-membrane domains in MIPC-deficient mutant cells. Cells from exponentially growing cultures were briefly incubated with filipin (5 μg/ml) and observed by microscopy.

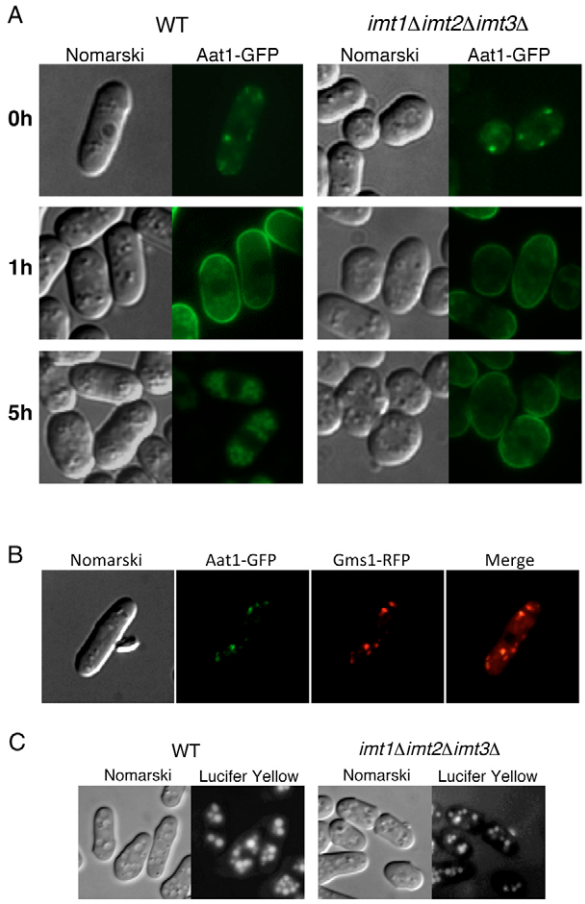

Fig. 8. Localization of an amino acid permease and fluid-phase endocytosis in MIPC-deficient cells. (**A**) Wild-type and MIPC-deficient mutant (*imt1*Δ*imt2*Δ*imt3*Δ) cells carrying pTN197-Aat1 were cultured in MM without leucine for 18 hours (designated time 0 hours), and shifted to nitrogen-free medium after 1 hour or 5 hours. (**B**) Cells carrying pTN197-Aat1 and pAU-Gms1-RFP were cultured in MM medium without leucine and uracil for 18 hours. (**C**) Wild-type and MIPC-deficient mutant cells were grown in YES at 30°C and stained with LY for 60 minutes, after which cells were visualized using Nomarski optics and fluorescence microscopy.

The fate of the Gap1 permease is regulated according to medium nitrogen source and abundance (Magasanik and Kaiser, 2002). Endocytosis of Gap1p is triggered when a good nitrogen source, such as ammonium or an excess of amino acids, is added to the medium (Hein et al., 1995; Stanbrough et al., 1995; Springael and Andre, 1998). The Gap1 permease, when present at the plasma membrane, fractionates with DRMs enriched in sphingolipids and ergosterol (Lauwers and Andre, 2006).

An examination of the *S. pombe* genome database revealed several genes predicted to encode proteins homologous to the *S. cerevisiae* Gap1 protein. Among these genes, we chose SPBC359.03c as a *Gap1* homologue. Comparative sequence analysis revealed that SPBC359.03cp is 48% identical to Gap1p and has a 12-transmembrane domain, as does Gap1p. Thus, we named the SPBC359.03c gene *aat1*$^{+}$ (amino acid transporter 1).

To examine the transport mechanism of Aat1p, we constructed a C-terminal GFP-Aat1 fusion allele. Expressed in wild-type *S. pombe* grown in rich medium, Aat1-GFP exhibited punctate fluorescence, suggesting that Aat1-GFP localized to the Golgi apparatus (Fig. 8A,B). When the cells were shifted to a nitrogen-free medium, Aat1-GFP was transported from the Golgi apparatus to the plasma membrane within 30 minutes, followed by endocytosis and transport to the vacuolar lumen. When Aat1-GFP was expressed in *imt1*Δ*imt2*Δ*imt3*Δ cells, the protein localized to the Golgi apparatus in rich medium and then was transported to the plasma membrane upon shift to a nitrogen-free medium, as observed in wild-type cells. Interestingly, endocytosis of Aat1-GFP was severely impaired and Aat1-GFP remained at the plasma membrane after 5 hours of incubation (Fig. 8A). Thus, we conclude that the MIPC-deficient mutant is impaired in its ability to internalize plasma-membrane proteins to the vacuole.

Because *imt1*Δ*imt2*Δ*imt3*Δ cells exhibited an endocytosis defect for plasma-membrane proteins, we examined whether this mutant was also defective in fluid-phase endocytosis, as assayed by vacuolar accumulation of Lucifer yellow CH (LY). Wild-type and *imt1*Δ*imt2*Δ*imt3*Δ cells were incubated in LY for 1 hour at 30°C. Aliquots were washed extensively to remove excess LY and cells were observed under Nomarski and fluorescence optics (Fig. 8C). LY accumulated in the vacuoles of both wild-type and the *imt1*Δ*imt2*Δ*imt3*Δ strain. This result suggests that MIPC is essential for internalization of multispanning membrane proteins from the plasma membrane to the vacuole, but not for fluid-phase endocytosis.

Discussion

The purpose of this study was to analyze sphingolipid composition and to determine the physiological roles of sphingolipids in *S. pombe*. We report five major findings. First, we demonstrated that the most abundant complex sphingolipid in *S. pombe* is MIPC. Second, the *S. pombe* genome has three genes (*imt1*$^{+}$, *imt2*$^{+}$ and *imt3*$^{+}$) that are homologues of *SUR1* and *CSH1*. We constructed all possible corresponding single, double and triple deletion mutants. TLC analysis of sphingolipids in these mutants revealed that MIPC was not detected in the triple mutant, suggesting that all three Imt proteins are required for the mannosylation of IPC. Third, although Imt1p, Imt2p and Imt3p are not essential for growth, the MIPC-deficient mutants exhibited pleiotropic phenotypes, including aberrant morphology, and sensitivity to various drugs. Fourth, we found that MIPC-deficient mutants exhibited aberrant localization

of ergosterols and of multipass membrane proteins localized to the plasma membrane. Fifth, sorting of an amino acid transporter from the plasma membrane to the vacuole was severely impaired in MIPC-deficient mutant cells grown under nitrogen-limiting conditions.

No fission yeast equivalents of Ipt1p (an inositolphosphotransferase) have been found in the *S. pombe* genome. During a search for proteins with strong homology to Ipt1p from *S. cerevisiae*, we found that only a few budding yeast strains have *IPT1* homologues, including *Candida grabrata*, *Candida albicans* and *Kluyveromyces lactis* (Prasad et al., 2005). Therefore, the major sphingolipid of most fungi might not be $M(IP)_2C$, but MIPC as in *S. pombe*. Recently, it has emerged that $M(IP)_2C$ is a target of the toxin zymocin and the antibiotic syringomycin E. The growth of *S. cerevisiae* was strongly inhibited by zymocin and syringomycin E, whereas *S. pombe* was resistant to these compounds (Fig. 2B). This result suggests that the effects of these compounds are limited, and that most fungi are resistant because of the absence of $M(IP)_2C$. In *S. cerevisiae*, MIPC synthesis is catalyzed by either of two homologous IPC mannosyltransferases, Sur1p and Csh1p, and a Ca^{2+}-binding protein, Csg2p, is also required for MIPC synthase activity (Uemura et al., 2003). Csg2p can form a complex with either Sur1p or Csh1p and is considered a regulatory subunit (Uemura et al., 2007). The absence of Csg2p resulted in a dramatic decrease in MIPC levels (Uemura et al., 2003). Interestingly, no fission yeast equivalent of Csg2p has been found in the *S. pombe* genome and all three mannosyltransferase homologues are required for MIPC synthesis (Fig. 2A). Therefore, the fission yeast Imt proteins might have redundant functions and their regulation might differ from that of budding yeast. To determine the expression level of the three *imt* genes, northern blot analysis was performed. Expression of the three *imt* genes was very weak and was not affected by heat stress or nitrogen starvation (data not shown). The predicted amino acid sequences of the Imt proteins have N-terminal signal sequences and transmembrane helices, as does *S. cerevisiae* Sur1p. GFP-fused Imt proteins localized to the Golgi apparatus, indicating that MIPC from IPC is also mannosylated in the Golgi compartment in *S. pombe* cells. It is not clear whether the *S. pombe* Imt proteins form a complex that functions as an MIPC synthase. Co-immunoprecipitation experiments with different epitope-tagged Imt proteins are in progress to determine potential interactions among Imt proteins and to identify Csg2-like protein(s) that might regulate fission yeast IPC mannosyltransferase activity in MIPC synthesis.

In *S. cerevisiae*, ceramide and complex sphingolipids contain one of five ceramide backbones that differ in extent of hydroxylation. Previous work has shown that the substrate specificities of *S. cerevisiae* Sur1p and Csh1p differ. Csh1p exhibits activity similar to that of Sur1p towards IPC-A and IPC-B′, but weaker activity towards IPC-B and IPC-C than Sur1p (Uemura et al., 2003). In this study, we showed that three mannosyltransferases (Imt1p, Imt2p and Imt3p) are required for MIPC synthesis in *S. pombe*. However, their precise functions and interactions in MIPC synthesis are unclear. Therefore, we attempted to determine the substrate specificities. To our knowledge, no reports have appeared concerning fatty-acid length and ceramide type in complex sphingolipids in *S. pombe*. In *S. cerevisiae*, Sur2p and Scs7p are required for hydroxylation of ceramides. Because there are also *SUR2* and *SCS7* homologues in the *S. pombe* genome, we constructed *sur2* and *scs7* deletion strains and analyzed their sphingolipid composition. To determine the effect of the *imt* mutation on the synthesis of MIPC, we attempted to construct multiple disruption mutants, including *imt1-3*Δ*sur2*Δ, and *imt1-3*Δ*scs7*Δ. The sphingolipid composition of the *S. pombe sur2*Δ and *scs7*Δ mutants differs from that of wild-type *S. pombe*. The most prominent differences were an accumulation of IPC and a reduction in MIPC in *sur2*Δ cells (Fig. 2D). This result suggests that fission yeast Imt proteins exhibit weak activity towards dihydroceramide-containing IPC. However, we could not determine the substrate specificity of each Imt protein. Further studies will be required to determine the structure and extent of hydroxylation of ceramide fatty acids in *S. pombe*.

The fission yeast MIPC-deficient mutants exhibited Ca^{2+} sensitivity, as did *S. cerevisiae sur1*Δ and *csg2*Δ strains (Beeler et al., 1997; Beeler et al., 1994; Tanida et al., 1996). Furthermore, MIPC-deficient mutants also exhibited sensitivity to hygromycin B (data not shown). These two phenotypes are characteristic of mutants with defective cell walls and glycosylation (Tanaka et al., 2001; Ikeda et al., 2009). Therefore, Imt proteins might participate in *N*- and *O*-mannosylation of glycoproteins. We determined the glycosylation of acid phosphatase, an indicator protein for *N*-linked glycosylation (Ikeda et al., 2009), by electrophoresis, and the localization of Fus1, an indicator protein for *O*-linked glycosylation in *S. pombe* (Tanaka et al., 2005). However, each single *imt*Δ mutant and the triple disruptant mutant produced acid phosphatase and Fus1 with electrophoretic mobilities similar to those of the wild-type strain (data not shown), indicating that the Imt proteins do not seem to function in mannosylation of secreted glycoproteins. In *S. cerevisiae*, MIPC-deficient *csg2*Δ mutants exhibited no notable phenotypes other than Ca^{2+} sensitivity. It is not clear why the fission yeast MIPC-deficient mutants exhibit these pleiotropic phenotypes upon loss of MIPC or accumulation of IPC. *S. pombe* might have a unique mannose-recognition mechanism for mannosylated sphingolipids.

MIPC-deficient mutants were very sensitive to the polyene antibiotics nystatin and amphotericin B (Fig. 5). For this reason, we presume that aberrant localization of ergosterol, which is one of the main target molecules for polyene antibiotics in yeast cells, might cause changes in the sphingolipid composition of lipid microdomains of the plasma membrane. Whereas ergosterol localized at the cell tips during the logarithmic growth phase in wild-type cells, based on filipin staining (Wachtler et al., 2003), it was distributed throughout the plasma membrane in MIPC-deficient mutants (Fig. 7). This result suggests that loss of the mannose residue of MIPC also affected normal localization of ergosterol to the plasma membrane in *S. pombe*. The intensity of filipin staining in the MIPC-deficient cells increased, as judged visually by fluorescence microscopy (Fig. 7). Recently, filipin staining of *lcb1*^{ts} (temperature-sensitive *lcb1* mutant) cells was reported in *S. cerevisiae* (Jin et al., 2008). Lcb1 protein is a component of serine palmitoyltransferase responsible for the first step in sphingolipid synthesis. The *lcb1*^{ts} mutant was found to have a 50% reduction in sphingolipid synthesis when grown under permissive conditions. Mitotic *lcb1*^{ts} cells had bright uniform plasma-membrane staining, as seen in the fission yeast MIPC-deficient cells. The authors suggested that the bright filipin staining of the *lcb1*^{ts} plasma membrane was a direct consequence of alterations in membrane lipid composition (Jin et al., 2008). We speculate that loss of the mannose residue in MIPC might lead to an increase in ergosterol levels, because of an unknown compensation mechanism. Differences in gene expression between wild-type and MIPC-deficient cells were analyzed using DNA microarrays. However, significant changes in the gene expression pattern of the ergosterol

biosynthetic pathway (Iwaki et al., 2008) were not detected (data not shown). Recently, Guan et al. reported that sterol biosynthesis mutants have altered sphingolipid profiles in *S. cerevisiae* (Guan et al., 2009). These data suggest a functional interaction between sterols and sphingolipids. Quantification of the sterols in the MIPC-deficient mutant will be required to clarify the interaction between sterols and sphingolipids in *S. pombe*.

MIPC-deficient mutants have smaller vacuoles than the wild type (Fig. 6). It has been reported that there is little ergosterol in the vacuolar membrane, but that trace amounts are important for vacuolar fusion in *S. cerevisiae* (Kato and Wickner, 2001). Therefore, ergosterol might be transported from the plasma membrane to vacuoles by means of endocytosis and provide a binding site for certain vacuolar peripheral membrane proteins required for vacuolar fusion. The aberrant vacuolar morphology of MIPC-deficient cells might be caused by mislocalization of ergosterol. We expect that it will be informative to determine whether lipid rafts play a key role in vacuolar fusion or in the localization of vacuolar membrane proteins.

To determine whether the lateral membrane distribution of multispanning proteins was affected in mutants lacking MIPC, we chose one marker protein, Aat1. Aat1p is a fission yeast homologue of the general amino acid permease Gap1p. Intracellular trafficking of Gap1p has been extensively studied and is tightly regulated depending on nitrogen availability (Lauwers and Andre, 2006). Gap1p is also associated with DRMs when present in the plasma membrane (Lauwers and Andre, 2006). Therefore, we examined the localization and membrane trafficking of Aat1p in *S. pombe*. In wild-type cells, an Aat1-GFP fusion localized to the Golgi apparatus when cells were grown in rich medium, but was quickly transported to the plasma membrane upon shift to nitrogen-limiting conditions (Fig. 8A). Aat1-GFP was then endocytosed by sorting into the multivesicular body (MVB) pathway and subsequent delivery to the vacuolar lumen for degradation (Iwaki et al., 2007). Aat1-GFP was also transported normally from the Golgi apparatus to the plasma membrane in MIPC-deficient cells (Fig. 8A). Interestingly, we found that cell-surface Aat1-GFP was not transported normally to the vacuolar lumen after prolonged incubation under nitrogen-limiting conditions. This result suggests that mannose residues in MIPC are required for endocytosis of plasma-membrane proteins and for sorting into the MVB pathway in *S. pombe*. However, fluid-phase endocytosis was not blocked, based on normal internalization of LY and FM4-64 through transport to the vacuole in MIPC-deficient cells (Fig. 8C and Fig. 6A).

To further examine internalization of Aat1p to the vacuole in *S. pombe*, we observed localization of Aat1-GFP over a prolonged period using fluorescence microscopy. Movies of the endocytosis of Aat1p in wild-type or MIPC-deficient cells are available (supplementary material Movies 1 and 2, respectively). Live imaging experiments revealed that some Aat1-GFP was actually internalized in MIPC-deficient cells. Nonetheless, Aat1-GFP was still largely localized to the plasma membrane, even after prolonged incubation under starvation conditions. These results suggest that association of Aat1-GFP with lipid rafts was significantly impaired in the MIPC-deficient cells. Ergosterols are enriched at the growing ends of cells in fission yeast and endocytosis has been observed to occur towards the poles of growing cells (Wachtler et al., 2003; Gachet and Hyams, 2005). Sterol-rich domains (lipid rafts) of MIPC-deficient cells become unstable, and Aat1p dissociates from sterol-rich domains during trafficking along the endocytic pathway. Alternatively, Aat1p might dissociate from sterol-rich domains before its internalization at the plasma membrane. Aat1p might also be endocytosed and subsequently recycled to the plasma membrane under starvation conditions.

Although new observations on the role of sphingolipids in fission yeast have been made in this study, many important issues remain unresolved. For example, how does Aat1p localize to the Golgi apparatus in cells grown in rich medium? In *S. cerevisiae*, Gap1p is transported to the plasma membrane under nitrogen-limiting conditions, with ubiquitin-dependent modification triggering endocytosis (Springael and Andre, 1998). Recently, we have confirmed that fission yeast Aat1p is ubiquitinated and that ubiquitination is important for internalization of Aat1p after it reaches the plasma membrane (M.N. and K.T., unpublished data). Further analysis of Aat1p with regard to cell-surface delivery and internalization is likely to reveal the role of the mannose component of sphingolipids as well as lipid-raft-dependent protein sorting in *S. pombe*.

Materials and Methods

Strains, media and genetic methods

Escherichia coli strain XL1-blue (Stratagene) was used for all cloning procedures. Wild-type *S. pombe* ARC039 (*h⁻ ura4-C190T leu1-32*) and ARC001 (*h⁻ leu1-32*), and wild-type *S. cerevisiae* BY4741 were used. Strains were grown in standard rich medium (YES) and in synthetic minimal medium (MM). Nitrogen-free derivative MM-N medium was modified slightly so that it contained only 1% glucose (Isshiki et al., 1992). *S. pombe* cells were transformed by the lithium acetate method (Morita and Takegawa, 2004). Standard genetic methods have been described previously (Alfa et al., 1993).

Gene disruptions

The *imt1*$^+$-*imt3*$^+$, *sur2*$^+$ and *scs7*$^+$ genes were disrupted using *ura4*$^+$ as a selective marker (Grimm et al., 1988). Genes encoding MIPC biosynthesis proteins were cloned into pGEM T-EASY (*imt1*$^+$, *sur2*$^+$) or pGEM T (*imt2*$^+$, *scs7*$^+$) vectors (Promega). To disrupt the *imt1*$^+$ locus, a DNA fragment carrying the *imt1*$^+$ gene was amplified by PCR, using the following oligonucleotides: sense 5′-TCTCTACTG-CATTTCATCCAGCATCAAGGC-3′ and antisense 5′-GAAGCCGCAATTC-CACGTTTCCTTCTCCG-3′. The *Hin*dIII-*Bam*HI sites within the cloned *imt1*$^+$ open reading frame (ORF) were digested and a 1.6 kb *ura4*$^+$ cassette was inserted. To disrupt the *imt2*$^+$ gene, the following oligonucleotides were used: sense 5′-GCAACAGTTGAAATCGATTAATGCGCCGC-3′ and antisense 5′-TCGAAG-TATGGATGATGAGGAACACTTCCC-3′. The *Hin*dIII-*Eco*RI sites within the cloned *imt2*$^+$ ORF were digested and *ura4*$^+$ was inserted. To disrupt the *imt3*$^+$ locus, upstream fragments were amplified by PCR using a sense primer carrying a *Kpn*I site and an antisense primer carrying a *Xho*I site. Downstream fragments were amplified by PCR using a sense primer carrying a *Hin*dIII site and an antisense primer carrying a *Bam*HI site. The primers had the following sequences: upstream, sense 5′-GGATGC-CGTTAACGTTGGCTTTGCGATG-3′ and antisense 5′-GTTTTCTCGAGCTT-GGTAGATTCAGCACAG-3′; downstream, sense 5′-TCTTGTCGATAATATG-GAGTGCTTGGCGG-3′ and antisense 5′-TCACCATACAGATGGACTTA-AGCAACACCG-3′. Amplified fragments were digested with the corresponding restriction enzymes and fragments were inserted into the pBS *ura4* cassettes. Wild-type strain ARC039 was transformed with the PCR products amplified from these constructs. Gene disruptions were confirmed by PCR using appropriate primers. To disrupt the *sur2*$^+$ locus, a DNA fragment carrying the *sur2*$^+$ gene was amplified by PCR, using the following oligonucleotides: sense 5′-CAGATTTGCCTACAAT-GAGATGATACG-3′ and antisense 5′-CACAAATACCTAGAGTCATAGGAGGG-3′. The *Hin*dIII-*Hpa*I sites within the cloned *sur2*$^+$ ORF were digested and a 1.6 kb *ura4*$^+$ cassette was inserted. To disrupt the *scs7*$^+$ locus, a DNA fragment carrying the *scs7*$^+$ gene was amplified by PCR, using the following oligonucleotides: sense 5′-TCCACGCCACAACATCGTGCCATTAACGC-3′ and antisense 5′-CCGA-TTACAAGACGGTACTTGTCAGCAGG-3′. The *Hin*dIII-*Hpa*I sites within the cloned *sur2*$^+$ ORF were digested and a 1.6 kb *ura4*$^+$ cassette was inserted.

[^{3}H] *myo*-inositol labelling assay

S. cerevisiae and *S. pombe* strains were grown in YPD or YES medium at 30°C to 1.0 A_{600} units. The cells were then incubated with 2 μCi/ml [^{3}H] *myo*-inositol in 0.5 ml SC-inositol or MM all-inositol medium for 1 hour at 30°C. The cells were washed with water, suspended in 150 μl of lipid extraction solvent [ethanol:water:diethyl ether:pyridine:15 N ammonia (15:15:5:1:0.018, volume basis)] and incubated at 60°C for 15 minutes. After centrifugation at 13,000 ***g*** for 3 minutes at 4°C, the resulting supernatant was transferred to fresh tubes and the pellet was re-extracted. The supernatant was dried and suspended in 20 μl of chloroform:methanol:water (5:4:1, volume basis). Lipids of equal radioactivity were resolved by TLC on silica gel 60 using chloroform:methanol:4.2 N ammonia (9:7:2,

volume basis) (Uemura et al., 2003) as the solvent system. To remove phosphatidylinositol, extracted lipids were treated with methanol or alkali treated with 40% methylamine methanol solution. These samples were dried and suspended in 20 µl of chloroform:methanol:water (5:4:1, volume basis).

Resistance assays

Cells cultured overnight in 5 ml YES medium were diluted with water to an optical density (OD_{600}) of 0.5, corresponding to about 10^7 cells/ml, and used as inoculum. Cell suspensions were serially diluted 1:10 and 1:100, and 5 µl aliquots were spotted onto plates containing potential inhibitors at the indicated concentrations. Growth was scored after 3 days.

Fluorescence microscopy

Cells were observed with an Olympus BX-60 fluorescence microscope using appropriate filter sets (Olympus). Images were captured with a SenSys cooled CCD camera using MetaMorph (Roper Scientific) and were saved as Adobe Photoshop files on a Macintosh G4 computer.

Visualization of sterol-rich plasma membrane domain

Filipin staining was performed as described (Wachtler et al., 2003). Briefly, filipin was added to the medium at a final concentration of 5 µg/ml and cells were observed immediately using a fluorescence microscope.

Analysis of fluid-phase endocytosis

Fluid-phase endocytosis was observed microscopically after cells were treated with LY (Sigma). Staining with LY was performed as described (Murray and Johnson, 2001). Briefly, 1 ml of exponentially growing cells in YES medium was collected by centrifugation, washed twice with fresh medium and resuspended in 0.5 ml YES medium containing 5 mg/ml LY. Cells were incubated at 30°C for 60 minutes with shaking and then washed three times with fresh medium. Labelled cells were then observed microscopically.

Vacuole staining

Vacuolar membranes were labelled with FM4-64 (Iwaki et al., 2003). Cells were grown to exponential phase in YES medium at 30°C and 500 µl of cells was then incubated in medium containing 8 µM FM4-64 for 30 minutes at 30°C. Cells were then centrifuged at 13,000 ***g*** for 1 minute, washed by resuspending in YES to remove free FM4-64, and collected by centrifugation at 6000 ***g*** for 1 minute. Cells were then resuspended in YES and incubated for 90 minutes at 30°C before analysis by fluorescence microscopy. For measurement of vacuoles after fusion in response to hypotonic stress, stained cells were incubated in distilled water for 5 hours to ensure full fusion. The diameter of every vacuole visible in one focal plane per cell was measured using NIH-image software and downloaded to Microsoft Excel for analysis (more than 100 vacuoles were counted).

Plasmid constructs

To tag the C terminus of Pma1p and Hmt1p with GFP, the *pma1*$^+$ ORF or *hmt1*$^+$ was amplified by PCR and cloned into pTN197, a derivative of the thiamine-repressible expression vector pREP41 (Nakamura et al., 2001). To tag SPBC359.03c with GFP, the SPBC359.03c ORF was amplified by PCR and subcloned into pTN197, resulting in plasmid pTN197-SPBC359.03c.

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Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/123/9/1578/DC1

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