Multiple functions of ergosterol in the fission yeast *Schizosaccharomyces pombe*

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Sterols are a major class of membrane lipids in eukaryotes. In *Schizosaccharomyces pombe*, sterol 24-C-methyltransferase (Erg6p), C-8 sterol isomerase (Erg2p), C-5 sterol desaturase (Erg31p, Erg32p), C-22 sterol desaturase (Erg5p) and C-24 (28) sterol reductase (Sts1p/Erg4p) have been predicted, but not yet determined, to catalyse a sequence of reactions from zymosterol to ergosterol. Disruption mutants of these genes were unable to synthesize ergosterol, and most were tolerant to the polyene drugs amphotericin B and nystatin. Disruption of *erg31*⁺ or *erg32*⁺ did not cause ergosterol deficiency or tolerance to polyene drugs, indicating that the two C-5 sterol desaturases have overlapping functions. GFP-tagged DRM (detergent-resistant membrane)-associated protein Pma1p localized to the plasma membrane in *ergΔ* mutants. DRM fractionation revealed that the association between Pma1-GFP and DRM was weakened in *erg6Δ* but not in other *erg* mutants. Several GFP-tagged plasma membrane proteins were tested, and an amino acid permease homologue, SPBC359.03c, was found to mislocalize to intracellular punctate structures in the *ergΔ* mutants. These results indicate that these proteins are responsible for ergosterol biosynthesis in fission yeast, similar to the situation in *Saccharomyces cerevisiae*. Furthermore, in fission yeast, ergosterol is important for plasma membrane structure and function and for localization of plasma membrane proteins.

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INTRODUCTION

Sterols are essential structural and regulatory components of eukaryotic cell membranes. Mammals, plants and fungi produce similar sterols, which differ in the number and location of double bonds and methyl side chains. Ergosterol is the end product of the sterol biosynthetic pathway and is the major sterol in yeasts (Fig. 1a). Like cholesterol in mammalian cells, it is responsible for membrane fluidity and permeability (Parks *et al.*, 1995).

Ergosterol synthesis and metabolism have been well defined in the budding yeast *Saccharomyces cerevisiae* (Daum *et al.*, 1998). Multiple genetic and biochemical studies have culminated in the virtually complete elucidation of the

pathway leading to ergosterol (Lees *et al.*, 1995; Parks *et al.*, 1995). The enzymic reactions have been largely defined by identification of *erg* mutants defective in ergosterol biosynthesis, and by their complementation based on sterol auxotrophy or altered sterol composition. Most genes involved in the early part of the pathway to lanosterol are essential for growth, because yeasts require sterols and no sterol molecule is synthesized up to this point. In contrast, mutations in the steps from zymosterol to ergosterol (Fig. 1b) are not essential for growth because the intermediates produced can partially substitute for ergosterol. The latter part of the ergosterol biosynthetic pathway is not linear in the sense of consecutive reactions, because the enzymes converting lanosterol to ergosterol do not show a strict substrate preference. Thus, null mutants impaired in the steps from zymosterol to ergosterol accumulate characteristic sterols, which are not defined substrates of the respective enzymes.

Recent studies have identified a membrane microdomain rich in sterols and sphingolipids (Simons & van Meer,

Abbreviations: CHX, cycloheximide; CPY, carboxypeptidase Y; DRM, detergent-resistant membrane; GFP, green fluorescent protein; LY, Lucifer Yellow CH; TX-100, Triton X-100.

Three supplementary figures are available with the online version of this paper.

Fig. 1. Late pathway of ergosterol biosynthesis in *Schiz. pombe* deduced from *Sacch. cerevisiae*. (a) Ergosterol is shown with specific Erg proteins and their functions indicated. (b) Schematic representation of the late stages of the ergosterol biosynthetic pathway.

1988; Simons & Ikonen, 1997; Sturley, 2000). This membrane microdomain is called the lipid raft, which corresponds to a related structure, the caveola, in mammalian membranes. Many functions have been attributed to these structures, including cholesterol transport, endocytosis and signal transduction (Alonso & Millán, 2001; Pelkmans, 2005; Maguy *et al.*, 2006; Wachtler & Balasubramanian, 2006). The most widely used assay for rafts is based on the observation that a subset of associated plasma membrane components is resistant to nonionic detergents, such as Triton X-100, at 4 °C (Bagnat *et al.*, 2000). Lipid rafts are also called detergent-resistant membranes (DRMs) because of their detergent resistance. However, lipid rafts and DRMs can theoretically be separated, as detergent solubilization may involve the formation of nonphysiological structures (Munro, 2003; Lichtenberg *et al.*, 2005).

In *Sacch. cerevisiae* and *Candida* species, many experiments have demonstrated a role for ergosterol in physiological functions, such as membrane permeability, resistance to drugs, protein transport to the plasma membrane, sporulation and endocytosis (Geber *et al.*, 1995; Parks *et al.*, 1999; Young *et al.*, 2003; Sanglard *et al.*, 2003; Pasrija

et al., 2005a, b; Mukhopadhyay *et al.*, 2002; Proszynski *et al.*, 2005; Enyenihi & Saunders, 2003; Munn *et al.*, 1999; Heese-Peck *et al.*, 2002; Kishimoto *et al.*, 2005). In addition, an association between many plasma membrane proteins and DRMs has been studied and some DRM-associated proteins have been identified (Dupré & Haguenaer-Tsapis, 2003; Bagnat *et al.*, 2000, 2001; Umabayashi & Nakano, 2003; Malinska *et al.*, 2004; Grossmann *et al.*, 2006; Lauwers & André, 2006). While there is no doubt that DRMs are rich in ergosterol, it is not at all clear if ergosterol is essential for the association between DRMs and plasma membrane proteins (Eisenkolb *et al.*, 2002; Gaigg *et al.*, 2005).

In *Schizosaccharomyces pombe*, a physiological change caused by a genetic defect in ergosterol metabolism is known only in the *sts1* mutant, encoding an Erg4 homologue (Shimanuki *et al.*, 1992). Nonetheless, genes encoding the latter part of the ergosterol biosynthetic pathway have been predicted through studies of transcriptional activation under anaerobic conditions (see Fig. 1b and Table 1 of Todd *et al.*, 2006). It is not known whether these genes are truly involved in the ergosterol biosynthetic pathway, nor is it clear what specific defects are caused by loss of ergosterol in fission yeast. To address these questions, disruption mutants of six sterol biosynthesis genes were constructed. These mutants were found to be deficient in ergosterol and to be resistant to polyene drugs, although no striking defects in endocytosis were observed.

METHODS

Strains, media, and genetic methods. Wild-type *Schiz. pombe* strains ARC039 (*h⁻ ura4-C190T leu1-32*), ARC010 (*h⁻ leu1-32*) and KJ100-7B (*h⁹⁰ leu1-32 ura4-D18*) were used. *vps34Δ* (Takegawa *et al.*, 1995) and MTD2 (*cpy1Δ*; Tabuchi *et al.*, 1997) were used as positive and negative controls, respectively, for the colony blot assay. Strains were grown in standard rich medium (YES), and in synthetic minimal medium (MM). Sporulation medium (ME) was used as described by Moreno *et al.* (1991). *Schiz. pombe* cells were transformed by the lithium acetate method or by electroporation (Suga & Hatakeyama, 2001; Morita & Takegawa, 2004). Standard genetic methods have been described previously (Alfa *et al.*, 1993).

Gene disruptions. Genes encoding ergosterol biosynthesis proteins were cloned into pGEM T-EASY (*erg31⁺*, *erg32⁺* and *erg5⁺*) or pGEM T (*erg6⁺* and *sts1⁺*) vectors (Promega) following PCR amplification using the primers listed in Table 1. The resulting plasmids were digested with restriction enzymes, followed by insertion of a *ura4⁺* gene cassette to generate gene disruption constructs. For *erg2⁺*, 0.6 kb sequences of promoter and terminator regions were amplified by PCR, and sequentially cloned into *KpnI*–*XhoI* sites (promoter) and *EcoRI*–*BamHI* sites (terminator) of pBluescript II-KS (Stratagene) carrying a *ura4⁺* cassette at the *Clal* site. Wild-type strain ARC039 was transformed with the PCR products amplified from these constructs. Gene disruptions were confirmed by PCR using appropriate primers.

Sterol analysis. Cells were grown to stationary phase in MM at 30 °C. Extraction of total sterols and alkaline hydrolysis were carried out as described by Munn *et al.* (1999). Harvested cells were washed twice with distilled water and then resuspended in 3 ml KOH/

Table 1. *Schiz. pombe* ergosterol biosynthetic genes deduced from *Sacch. cerevisiae*

Gene name (systematic name)	P-value*	Oligonucleotides†	Restriction enzymes‡
<i>erg6</i> ⁺ (SPBC16E9.05)	1.5 × 10 ⁻¹¹²	5'-GAGCTTAGCGCATTCCTATCAAAGCGG-3' 5'-TACATGACAGATTGAATGTGCTTGTCTCG-3'	<i>EcoRI</i> <i>HpaI</i>
<i>erg2</i> ⁺ (SPAC20G8.07c)	1.5 × 10 ⁻⁵⁰	NN; 5'-GTTTGGTACCGTCAAATACCCATAATCCG-3' NC; 5'-GTTTTCTCGAGATATCAACAAGCTGGTAGG-3' CN; 5'-GTTTTGAATTTCGCATGCCGTGTGGTGAACC-3' CC; 5'-GTTTGGATCCATAGTAAGGGATATATACGC-3'	
<i>erg31</i> ⁺ (SPAC1687.16c)	9.8 × 10 ⁻⁸⁸	5'-CATGGGCATGTGGTGTAGATGGTAATCACG-3' 5'-CCTTTATAGCAACTGAAGGTACACTGGG-3'	<i>BglII</i>
<i>erg32</i> ⁺ (SPBC27B12.03c)	6.4 × 10 ⁻⁷⁴	5'-GCAAGCAACATGATGGAATACATCCCGG-3' 5'-ATTAGTTATCCTTCTTGCTGGC-3'	<i>HindIII</i>
<i>erg5</i> ⁺ (SPAC19A8.04)	2.2 × 10 ⁻¹³⁶	5'-GCGAGAGACTATGAACGCATGAAGAGGACG-3' 5'-TTATCGACAATGCATGTCTGTTTACGATCC-3'	<i>HindIII</i>
<i>sts1</i> ⁺ (SPAC20G4.07c)	3.0 × 10 ⁻¹³⁹	5'-GTAGACAAGTCGTTTTATTGAGAATGGCC-3' 5'-GTAGATGGGATTTCAGATAGCCGATGAGG-3'	<i>EcoRI</i>

*The fission yeast proteins were identified by screening the *Schiz. pombe* genome database for new homologues of the known *Sacch. cerevisiae* proteins using BLAST for sequence alignments (<http://www.genedb.org/genedb/pombe/blast.jsp>). Probability scores are shown as P-values.

†Primers used for gene disruptions. For *erg2*⁺, NN and NC, CN and CC were used to amplify promoter and terminator sequences, respectively.

‡Cloned PCR products were digested with the indicated restriction enzymes in order to construct gene disruptions by insertion of *ura4*⁺ as a selectable marker.

methanol solution (20 % KOH in 100 % methanol) with a small amount of pyrogallol. After heating for 2 h at 85 °C, total sterols in the reaction mixture were extracted twice with 2 ml petroleum ether. The organic phases were combined, dried under nitrogen atmosphere, and stored at -20 °C before use. Before separation by reverse-phase HPLC, dried sterols were resuspended in 0.5 ml methanol. HPLC analysis was performed using a reverse-phase HPLC column (TSKgel ODS80Ts; Tosoh) with methanol as eluent at 40 °C, 1 ml min⁻¹ solvent flow, and detection at 280 nm.

Resistance assays. Cells cultured overnight in 5 ml YES medium were diluted with water to an OD₆₀₀ of 0.5, corresponding to about 10⁷ cells ml⁻¹ 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.

Analyses of carboxypeptidase Y (CPY). CPY missorted to the medium was detected by colony blot assay as described by Cheng *et al.* (2002). Briefly, yeast strains were replica-plated on a YES plate in contact with a nitrocellulose membrane and then incubated at 30 °C for 2 days. The nitrocellulose membranes were washed several times with water and then subjected to immunodetection with rabbit antiserum against CPY (Tabuchi *et al.*, 1997) and anti-rabbit IgG-horseradish peroxidase (GE Healthcare). Visualization was enhanced by the ECL system (GE Healthcare).

Pulse-chase analysis and immunoprecipitation of vacuolar CPY from *Schiz. pombe* were carried out as described by Tabuchi *et al.* (1997). Antibody incubations were carried out using rabbit polyclonal antibody against *Schiz. pombe* Cpy1p (Tabuchi *et al.*, 1997).

Visualization of sterol-rich plasma membrane domain. Staining with filipin was carried out as described by Wachtler *et al.* (2003). Filipin was added to the medium at a final concentration of 5 µg ml⁻¹ and cells were observed immediately using a fluorescence microscope (model BX-60; Olympus).

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 min at 30 °C. Cells were then centrifuged at 13 000 g for 1 min, washed by resuspending in YES to remove free FM4-64, and collected by centrifugation at 13 000 g for 1 min. The cells were resuspended in YES and incubated for 90 min at 30 °C before microscopic observation. Stained cells were observed using a fluorescence microscope. For measurement of vacuoles after fusion in response to hypotonic stress, stained cells were incubated in distilled water for 6 h 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).

Analysis of fluid-phase endocytosis. Fluid-phase endocytosis was observed microscopically after cells were treated with Lucifer Yellow CH (LY, Sigma). Staining with LY was performed as described by Murray & 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 LY ml⁻¹. Cells were incubated at 30 °C for 60 min with shaking and then washed three times with fresh medium. Labelled cells were then examined by microscopy.

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.

Plasmid constructs. To tag the C terminus of Pma1p with green fluorescent protein (GFP), the *pma1*⁺ ORF 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.

DRM isolation and immunoblotting. DRM was isolated as described by Bagnat *et al.* (2000). One hundred OD₆₀₀ units of cells was collected, washed twice with distilled water, and stored at -80°C . The cell pellet was then lysed in 0.5 ml TNE buffer [50 mM Tris/HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA] containing a protease inhibitor mix (Nakarai tesque) by vortexing with glass beads five times for 1 min with 1 min intervals on ice. The lysate was cleared by centrifugation at 500 g for 5 min. A 0.75 ml aliquot of TNE buffer was added to 0.25 ml of the lysate, and then incubated with Triton X-100 (TX-100; 1% final) for 30 min on ice. After extraction of TX-100, the lysate (1 ml) was adjusted to 40% Optiprep by addition of 2 ml Optiprep solution (Nycomed) and overlaid with 4.8 ml 30% Optiprep in TXNE (TNE with 0.1% TX-100) and 0.8 ml TXNE. The samples were centrifuged at 200 000 g for 2 h, and six fractions of equal volume were collected from the top. The top fraction was subjected to a second incubation with TX-100, loaded onto a second Optiprep gradient, and centrifuged again. Fractions from gradients were precipitated with 10% (w/v) TCA, dissolved in appropriate volumes of cracking buffer [8 M urea, 5% (w/v) SDS, 1 mM EDTA, 50 mM Tris/HCl (pH 6.8), 5% (v/v) 2-mercaptoethanol], and incubated at 65°C for 20 min. Samples (10 μl) were separated by SDS-PAGE and transferred to PVDF filters. Rabbit polyclonal antibody against GFP, a generous gift from Dr R. Sugiura (Kinki University), was used at a 1:10 000 dilution. Protein-antibody complexes were visualized by chemiluminescence using the Amersham ECL plus system (GE Healthcare).

RESULTS

Disruption of *erg* genes leads to alterations in sterol profiles

An examination of the *Schiz. pombe* genome database revealed several genes predicted to encode proteins homologous to the *Sacch. cerevisiae* Erg proteins. A single *ERG* gene homologue was identified per gene, except for *ERG3* homologues (Table 1, see also Supplementary Fig. S1, available with the online version of this paper). In order to determine whether the products of these genes catalysed conversion of zymosterol to ergosterol, gene disruption mutants were constructed. Total sterols extracted from stationary-phase cells of wild-type and the gene disruption mutants were compared by reversed-phase HPLC analyses. These analyses revealed that the single disruption mutants, *erg31* Δ and *erg32* Δ , could synthesize ergosterol (Supplementary Fig. S2), indicating that these proteins were functionally redundant. Therefore, a double disruption mutant, *erg31* Δ *erg32* Δ was also constructed.

HPLC elution patterns are shown in Fig. 2. Ergosterol was not detected in the *erg6* Δ , *erg5* Δ or *sts1* Δ mutants. No clear peaks were detected in the *erg2* Δ and *erg31* Δ *erg32* Δ mutants by absorption at 280 nm (data not shown). The *sts1* Δ mutant accumulated ergosta-5,7,22,24(28)-tetraenol, while the other mutants accumulated other sterols.

In order to examine the cellular distribution of sterols, mutant cells were treated with the fluorescent probe filipin, a polyene antibiotic that forms specific complexes with free 3β -hydroxysterols. It has been reported that *Sacch. cerevisiae* *erg6* Δ cells could not be stained with filipin (Malathi *et al.*, 2004). In *Schiz. pombe* *erg* Δ cells,

Fig. 2. HPLC separation of sterols from ARC039 (wild-type, WT), *erg6* Δ , *erg5* Δ and *sts1* Δ mutants. The dashed line and the small arrow indicate retention times for ergosterol and ergosta-5,7,22,24(28)-tetraenol, respectively.

fluorescence was detected at the plasma membrane and sterols were enriched at the growing cell tips, as observed in the wild-type (Fig. 3). Although the intensity of fluorescence was lower in the *erg* mutants, except for *sts1* Δ , the staining patterns were similar to wild-type (Fig. 3). These results indicated that ergosterol derivatives partly substitute for ergosterol and were transported to the plasma membrane. On the other hand, *erg6* Δ cells had an abnormal cellular morphology, and more than one septum was found in some of the *erg2* Δ cells.

Drug tolerance of *erg* Δ mutants

Tolerance to drugs, other reagents and high temperature was determined by a visual spotting assay on YES medium. The *sts1* Δ mutant exhibited sensitivity to 15 μg cycloheximide (CHX) ml^{-1} (Fig. 4), 100 mM CaCl_2 and 0.01% (w/v) SDS (data not shown), as previously reported (Shimanuki *et al.*, 1992). All the ergosterol-deficient mutants were found to be sensitive to CHX and to 1 μg staurosporine ml^{-1} , and tolerant to 10 μg nystatin ml^{-1} and to 1 μg amphotericin B ml^{-1} (Fig. 4). The *erg31* Δ single mutant grew almost as well as wild-type, while the *erg32* Δ single mutant exhibited slight sensitivity to staurosporine and tolerance to nystatin and amphotericin

Fig. 3. Localization of sterol-rich plasma membrane domains in *ergΔ* mutants. Cells from exponentially growing cultures were briefly incubated with filipin ($5 \mu\text{g ml}^{-1}$) and observed by microscopy.

B. These results indicated that Erg31p and Erg32p have redundant functions and that Erg32p might be a major C-5 sterol desaturase. The polyene drugs nystatin and amphotericin B bind ergosterol, creating pores and causing cell lysis. Therefore, the tolerance to these drugs reflects the ergosterol deficiency of *ergΔ* mutants. However, the *sts1Δ* mutant was not resistant to nystatin, suggesting that nystatin binds ergosta-5,7,22,24(28)-tetraenol.

***erg* disruptions do not affect sorting of CPY, vacuolar fusion or endocytosis**

In *Sacch. cerevisiae*, certain *erg* mutations cause defects in vacuolar morphology and endocytosis, but do not affect maturation of vacuolar carboxypeptidase Y (CPY) (Kato & Wickner, 2001; Munn *et al.*, 1999; Heese-Peck *et al.*, 2002),

even though vacuolar morphology and CPY maturation are known to be closely related (Raymond *et al.*, 1992). Therefore, we determined if these vesicular trafficking events had a sterol requirement. Missorting of CPY was determined by colony blot assay, because some mutants defective in CPY transport to the vacuole mis-sort significant amounts of CPY to the cell surface. A fraction of CPY was detected in *erg6Δ* and *erg2Δ* cells, although CPY secretion was not detected in *erg31Δerg32Δ*, *erg5Δ* or *sts1Δ* cells (Fig. 5a). In order to determine whether CPY secretion in *erg6Δ* and *erg2Δ* cells was caused by lytic release, pulse-chase analysis was performed. During the initial 15 min of labelling, the endoplasmic reticulum- and Golgi-specific precursor form (proCPY) and a small amount of the vacuole-specific mature form (mCPY) were produced in the wild-type. After a 30 min chase, proCPY was almost completely converted to the mature form (Fig. 5b). Processing defects were not found in *erg6Δ* and *erg2Δ* cells. These results indicate that CPY secretion was due to lytic release, because it has been suggested that CPY is processed to a mature form by unidentified proteases in the vacuole (Tabuchi *et al.*, 1997).

To investigate vacuolar morphology and fusion, yeast vacuoles were stained with a lipophilic styryl dye, FM4-64. Wild-type cells had numerous small vacuoles while *ergΔ* cells other than *erg5Δ* had slightly smaller vacuoles (Fig. 6a, c). Vacuoles in wild-type cells had a mean diameter of $0.80 \pm 0.14 \mu\text{m}$, while the means in *ergΔ* cells were as follows: *erg6Δ*, $0.70 \pm 0.18 \mu\text{m}$; *erg2Δ*, $0.52 \pm 0.09 \mu\text{m}$; *erg31Δerg32Δ*, $0.60 \pm 0.11 \mu\text{m}$; *erg5Δ*, $0.7 \pm 0.15 \mu\text{m}$; *erg4Δ*, $0.60 \pm 0.09 \mu\text{m}$. When cells were transferred to water, a smaller number of much larger vacuoles were observed as a result of vacuolar fusion (Bone *et al.*, 1998). All of the ergosterol-deficient mutants were able to undergo normal vacuolar fusion in response to hypotonic stress (Fig. 6b, c), in contradiction with a previous study reporting an ergosterol requirement for homotypic vacuolar fusion (Kato & Wickner, 2001). Vacuoles in wild-type cells had a mean diameter of $1.24 \pm 0.43 \mu\text{m}$, while the means in *ergΔ* cells were as follows: *erg31Δerg32Δ*, $1.07 \pm 0.28 \mu\text{m}$; *sts1Δ*, $1.08 \pm 0.34 \mu\text{m}$. Vacuolar fusion in *erg5Δ* occurred to the same extent as in wild-type cells (Fig. 6a, b), so measurement of *erg5Δ* vacuoles was omitted. Vacuoles of *erg6Δ* and *erg2Δ* could not be measured because some were not circular in shape. The difference in mean diameter between pre-fusion and post-fusion was 0.44 (wild-type) to 0.48 μm (*sts1Δ*). Therefore, we conclude that homotypic vacuolar fusion was not affected by loss of ergosterol.

In *Sacch. cerevisiae*, ergosterol is required for endocytosis (Munn *et al.*, 1999; Heese-Peck *et al.*, 2002; Pichler & Riezman, 2004). In order to determine whether *ergΔ* mutants are impaired in endocytosis, vacuolar accumulation of the soluble fluorescent dye Lucifer Yellow CH (LY) was assayed. In assays for fluid-phase endocytosis, cells were incubated with LY at 30°C for 1 h and observed by fluorescence microscopy. Endocytic uptake of dye resulted in LY accumulation in vacuoles. Vacuolar accumulation of

Fig. 4. Phenotypic characterization of fission yeast strains harbouring disrupted *erg* alleles. An overnight culture in YES medium was diluted to an OD₆₀₀ of 0.5, and then serially diluted 1 : 10. Aliquots (5 μ l) were plated on YES containing 15 μ g cycloheximide (CHX) ml⁻¹, 1 μ g staurosporine (Sta) ml⁻¹, 1 μ g amphotericin B (AmB) ml⁻¹ or 10 μ g nystatin (Nys) ml⁻¹. Plates were photographed after 3 days at 30 °C.

LY in all the *erg* Δ mutants was similar to that observed in wild-type cells (Fig. 7).

Localization of plasma membrane proteins and their association with DRM in *erg* Δ mutants

Ergosterol interacts closely with sphingolipids, providing the membrane microdomain required for membrane

sorting and trafficking. This membrane microdomain is also called the lipid raft or detergent-resistant membrane (DRM). In order to determine the localization of plasma membrane proteins and their association with DRM, Pma1-GFP was expressed in the *erg* Δ mutants. Pma1p is a P-type H⁺-ATPase that localizes to the plasma membrane and has been reported to associate with DRM (Takeda *et al.*, 2004). Pma1-GFP was found to localize to the cell surface in all *erg* Δ cells (Fig. 8a). Crude membrane was solubilized with 1% Triton X-100 and fractionated after discontinuous gradient centrifugation using Optiprep. Each fraction was analysed by Western blotting using rabbit anti-GFP serum. In wild-type cells, Pma1-GFP was detected in the TX-100-insoluble fraction even after the second gradient centrifugation, in agreement with a previous report (Takeda *et al.*, 2004). In most of the *erg* Δ mutants, the distribution of Pma1-GFP was similar to that in wild-type cells, although it was found in a broad range of fractions in *erg6* Δ cells (Fig. 8b). These results indicated that association of Pma1-GFP to DRM was weakened in *erg6* Δ cells.

Localization of other plasma membrane proteins was also determined in the *erg* Δ mutants. GFP-tagged ATP-binding cassette (ABC) transporters Bfr1-GFP and Pmd1-GFP were found to localize normally in the plasma membrane (Iwaki *et al.*, 2006) (Supplementary Fig. S3). One of the amino acid permease homologues, SPBC359.03c, has been reported to localize to the plasma membrane when expressed as a C-terminal GFP-tagged allele (Matsumoto *et al.*, 2002). GFP-tagged SPBC359.03c (SPBC359.03c-GFP) localized mainly to the plasma membrane in wild-type cells (Fig. 9). In *erg* Δ mutants, it localized to numerous tiny dots within the cytoplasm and could not be found in the plasma membrane in stationary-phase cells (Fig. 9). These observations indicate that mutations in the *erg* genes have a modest affect on membrane structure and may alter localization of some, but not all, plasma membrane proteins. Moreover, these observations indicate that plasma membrane proteins can be divided into two classes, i.e. sensitive or resistant to changes in sterol composition, although the cause of these differences is currently unknown.

Fig. 5. Analyses of the vacuolar protease CPY. (a) CPY secretion in *erg* Δ mutants. Membranes were immunoblotted with rabbit anti-CPY at a 1 : 500 dilution. Wild-type, *vps34* Δ (positive control) and *cpy1* Δ (negative control) strains were included for comparison. (b) Analysis of CPY processing. Wild-type, *erg6* Δ and *erg2* Δ strains were pulse-labelled with Express-³⁵S (NEN-Perkin Elmer) for 15 min and chased for the indicated times. Immunoprecipitations were performed from the lysates using antibody against CPY. The positions of precursor (proCPY) and mature (mCPY) forms are indicated.

Fig. 6. Vacuole morphology of *ergΔ* cells. (a) Exponentially growing cells in YES medium have numerous small vacuoles. Vacuoles of *ergΔ* cells seem to be slightly smaller than in wild-type cells, while *erg5Δ* has normal vacuoles. (b) Cells were shifted to distilled water to induce homotypic vacuolar fusion. A smaller number of large vacuoles were apparent in wild-type cells. Vacuoles of *ergΔ* cells were able to undergo fusion in response to hypotonic stress. (c) Vacuole size distribution before (white bars) and after (black bars) hypotonic stress. Vacuole size increased in all *ergΔ* cells after fusion in response to hypotonic stress.

Fig. 7. Vacuolar accumulation of LY. All *ergΔ* mutants accumulated the dye. Background fluorescence in *erg6Δ* and *erg2Δ* could not be removed by additional washing of cells.

Sporulation in *ergΔ* mutants

Sacch. cerevisiae erg4Δ cells sporulate inefficiently (Enyenihi & Saunders, 2003). In order to determine the sporulation efficiency in *Schiz. pombe ergΔ* mutants, some of the *ergΔ* mutants were crossed to the wild-type homothallic strain KJ100-7B, and homothallic *ergΔ* mutants were obtained. The strains were then streaked onto ME medium, and observed microscopically after incubation for 3 days, after which cells were counted. As can be seen from the morphology of spores under these conditions (Fig. 10), the asci of *erg5Δ* cells resembled those of wild-type cells, while the asci of *erg2Δ* cells appeared aberrant. The *erg2Δ* cells formed zygotes but did not differentiate into mature spores (asci:zygotes:vegetative cells 6:77:122). In *sts1Δ* cells, the numbers of asci and zygotes were quite low: asci:zygotes:vegetative cells 49:11:609. These ratios in wild-type and *erg5Δ* cells were 105:8:200 and 121:20:183, respectively. When *erg6Δ* cells were crossed to wild-type, viable Ura autotrophic mutants were not obtained. Thus, this observation indicates that ergosterol is involved in

Fig. 8. Analyses of Pma1-GFP. (a) Localization of Pma1-GFP. Cells carrying pTN197/pma1 were cultured in MM without leucine and thiamine for 20 h. (b) Detergent solubilization. Solubilization of Pma1-GFP from wild-type, *erg6Δ*, *erg2Δ* and *sts1Δ* was tested in Optiprep gradients. Fractions 1–6 (top to bottom of the gradient) were analysed by Western blotting.

sporulation or zygote formation and that some sterol derivatives produced in *erg5Δ* cells can completely substitute for ergosterol with respect to sporulation function.

DISCUSSION

Ergosterol is the major sterol component in fungal membranes and contributes to a variety of functions, including permeability and integrity of the membranes and proper function of membrane-bound enzymes. The ergosterol biosynthetic pathway has been a target for clinical antifungal agents because it is specific to fungi. The pathway has been well characterized and the implicated genes have been identified in many yeasts and filamentous fungi, including pathogenic *Candida* species and *Sacch. cerevisiae*. The observed conservation of the *erg* genes suggests that *Schiz. pombe* synthesizes ergosterol in a similar manner (Todd *et al.*, 2006). However, there is no evidence to date that these conserved genes are truly required for ergosterol synthesis in fission yeast. In the present study, it was shown that the *Schiz. pombe* *ergΔ* mutants, with the exception of the single mutants *erg31* and *erg32*, are deficient in ergosterol synthesis and are resistant to polyene drugs, indicating that the proteins encoded by the *erg* genes are responsible for ergosterol biosynthesis.

As shown in Fig. 2, ergosterol was not detected in all *ergΔ* mutants, while other sterol derivatives were. These detected peaks were identified as cholesta-5,7,22,24-tetraenol (main peak of *erg6Δ*), cholesta-5,7,24-trienol (a small peak at 10.6 min in *erg6Δ*), and ergosta-5,7-dienol (main peak of *erg5Δ*), inferred from studies of *Sacch. cerevisiae* (Munn *et al.*, 1999; Skaggs *et al.*, 1996; Shobayashi *et al.*, 2005). Although none of the sterols in *erg2Δ* or *erg31Δerg32Δ* could be detected by HPLC with a UV-detector, several unidentified sterols were detected by gas chromatography, and the molecular masses of some of these sterols were estimated to be 398 and 396 by gas chromatography-mass spectrometry (data not shown). These molecular masses contain multiple candidates. Identification of the sterols produced in these *ergΔ* mutants is the subject of ongoing work.

Two *ERG3* homologues are specific to *Schiz. pombe*. These proteins share 57 % identity and 71 % similarity. A single *ERG3* gene is found in *Sacch. cerevisiae*, *Candida albicans* and *Candida glabrata*, while *Aspergillus fumigatus* has three *ERG3* homologues (Arthington *et al.*, 1991; Geber *et al.*, 1995; Miyazaki *et al.*, 1999; Alcazar-Fuoli *et al.*, 2006). While ergosterol was not detected in the double disruption mutant *erg31Δerg32Δ*, it was present in the single mutants. *Schiz. pombe* Erg31p and Erg32p were found to be functional during aerobic growth, similar to the situation for *A. fumigatus*, in which all three *ERG3*-encoded proteins

Fig. 9. Localization of SPBC359.03c-GFP. Cells carrying pTN197/ SPBC359.03c were cultured in MM without thiamine and supplements including amino acids for 20 h. ARC001 was used as the wild-type strain.

have been suggested to function as C-5 sterol desaturases (Alcazar-Fuoli *et al.*, 2006). Although fission yeast Erg31p and Erg32p share redundant functions, these two proteins seem to have distinct roles in ergosterol synthesis. *erg31*⁺ is induced about fourfold under anaerobic conditions, while transcription of *erg32*⁺ decreases (Todd *et al.*, 2006). Tolerance of *ergΔ* mutants to nystatin and amphotericin B (Fig. 4) suggests that Erg32p is the major C-5 sterol desaturase under aerobic conditions. A shift from aerobic to anaerobic conditions might result in a change in induction of the major C-5 sterol desaturase from Erg32p to Erg31p. In order to understand why the major C-5 sterol desaturase changes due to transcriptional regulation, the enzymic properties of these proteins need to be determined.

Interestingly, *Schiz. pombe ergΔ* cells were not found to be deficient in endocytosis or vacuolar fusion processes (Figs 6 and 7). In *Sacch. cerevisiae*, *erg3Δ* and *erg2Δ* have been reported to be impaired in endocytosis (Munn *et al.*, 1999; Heese-Peck *et al.*, 2002). It has also been reported that vacuoles isolated from these *ergΔ* mutants are incapable of fusion *in vitro*, and that vacuolar fusion is partially restored by addition of sterol (Kato & Wickner, 2001). These reports suggest that ergosterol may be transported from the plasma membrane to vacuoles via endocytosis, and provide a binding site for certain vacuolar peripheral membrane proteins required for vacuolar fusion. If ergosterol has a similar function in *Schiz. pombe*, normal vacuolar fusion is then consistent with the normal endocytosis in *ergΔ* cells, and the sterols in these mutants can apparently substitute for ergosterol in this process.

Phenotypic analysis revealed that ergosterol deficiency causes pleiotropic phenotypes. Sensitivity to certain reagents and abnormal localization of SPBC359.03c-GFP might be a consequence of the change in sterol composition, and aberrant sterol composition might alter plasma membrane structures, causing mislocalization and/or changes in activities of membrane pumps. *ERG* genes have

Fig. 10. Mating and sporulation of homothallic strains. Wild-type, *erg2Δ*, *erg5Δ* and *sts1Δ* cells were cultured on ME plates at 30 °C for 3 days, and observed using Nomarski optics.

been shown to be required for correct delivery of a chimeric marker protein to the cell surface in *Sacch. cerevisiae* (Proszynski *et al.*, 2005), and correct localization of tryptophan permease, Tat2p, has also been shown to be dependent on ergosterol (Umebayashi & Nakano, 2003). A recent study in higher eukaryotes indicated that sterol directly binds part of a multiprotein-channel complex and partly regulates the function of a cholesterol-protein supercomplex (Huber *et al.*, 2006). Although changes in localization of plasma membrane proteins (Pma1-GFP, Pmd1-GFP and Bfr1-GFP) were hardly detected, some effects on activity were apparent as tolerance or sensitivity to drugs (Fig. 4). Bfr1p and Pmd1p are involved in CHX tolerance (Nishi *et al.*, 1992; Turi & Rose, 1995; Nagao *et al.*, 1995), and sensitivity to CHX in *ergΔ* mutants might reflect a decrease in activity of these ABC transporters. The multidrug resistance pump Pdr5p has been reported to function with reduced efficiency in *Sacch. cerevisiae erg* mutants (Kaur & Bachhawat, 1999). Among our mutants, only *erg2Δ* showed Li⁺ sensitivity (data not shown); this might be due to decreased activity of the Na⁺/H⁺ exchanger Sod2p, which has been shown to be essential for Na⁺ and Li⁺ tolerance (Jia *et al.*, 1992). Abnormal cell morphology and multiseptum formation in *erg6Δ* and *erg2Δ* might reflect mislocalizations or lowered activities of the cytoskeleton, cell wall synthesizing enzymes, and degrading enzymes, such as glucan synthase, chitin synthase, and glucanases. Inefficient sporulation in *erg2Δ* and *sts1Δ* (Fig. 10) and the failure to isolate homothallic *erg6Δ* mutants might be due to decreased activity of proteins required for formation of zygotes and spores, including mating pheromone transporters and receptors.

These phenotypes might correlate with the membrane microdomain that is rich in sterols and sphingolipids. Protein association with this microdomain is commonly assayed by solubilization with TX-100. Among tested proteins, Pma1-GFP was detected solely in the TX-100-insoluble fraction (DRM), while all other proteins were distributed in a broader range of fractions (data not shown). Loose association of Pma1-GFP was detected in *erg6Δ*, but not in the other *ergΔ* mutants. These results suggest that proteins loosely associated with the DRM may be difficult to detect in *Schiz. pombe*, and that many ergosterol derivatives can substitute functionally for ergosterol in the DRM. Similar to our findings in *Schiz. pombe*, the P-type ATPase Pma1-GFP localized to the plasma membrane in *Sacch. cerevisiae ergΔ* mutants (Gaigg *et al.*, 2005) and Pma1p associated with the DRM in an *erg3Δ* mutant (Kishimoto *et al.*, 2005). Technical difficulties may limit the ability to detect small amounts of solubilized DRM-associated protein and very subtle mislocalization of DRM-associated protein.

Alternatively, changes in sterol composition may cause increased membrane permeability, resulting in increased uptake of drugs. *Sacch. cerevisiae erg* mutants have altered phospholipids as well as sterols, resulting in increased fluidity due to membrane disorder (Sharma, 2006). In

Sacch. cerevisiae, deletion of *ERG6* increases the rate of passive diffusion of small lipophilic drugs (Emter *et al.*, 2002). Budding yeast cells lacking Pdr16p and Pdr17p have altered membrane sterol and lipid composition, display increased rates of passive drug diffusion, and are hypersensitive to many drugs (van den Hazel *et al.*, 1999). The CPY colony blot assay indicated that cell integrity might be reduced in *erg2Δ* and *erg6Δ* cells (Fig. 5), although solubilization of DRM was hardly detected in *erg2Δ* cells (Fig. 8). Cell lysis might be caused by non-DRM sterol function.

Further studies will be required to distinguish between DRM- and non-DRM sterol functions in *Schiz. pombe*. It is possible that once the genes required for sphingolipid biosynthesis are identified, studies using sphingolipid biosynthetic mutants will be informative in relation to DRM function.

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