Disruption of the sphingolipid Δ^8 -desaturase gene causes a delay in morphological changes in *Candida albicans*

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Ceramides and glycosylceramides, including desaturated long-chain bases, are present in most fungi as well as animals and plants. However, as the budding yeast *Saccharomyces cerevisiae* is not capable of desaturating long-chain bases, little is known about the physiological roles of these compounds in fungi. To investigate the necessity of desaturation of long-chain backbones in ceramides and glycosylceramides in fungal cells, we have identified and characterized a sphingolipid Δ^8 -desaturase (SLD) gene from the pathogenic yeast *Candida albicans*. Gene disruption of the *C. albicans* SLD homologue led to the accumulation of (E)-sphing-4-enine, a main substrate for the SLD enzyme. Introducing the *Candida* SLD gene homologue into these mutant cells resulted in the recovery of synthesis of (4E, 8E)-sphinga-4,8-dienine and this gene homologue was therefore identified as a *Ca*-SLD gene. Additionally, the *sld* disruptant of *C. albicans* had a decreased hyphal growth rate compared with the wild-type strain. These results suggest that Δ^8 -desaturation of long-chain bases in ceramides plays a role in the morphogenesis of *C. albicans*.

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INTRODUCTION

Sphingolipids are abundant compounds in the cells of eukaryotic organisms, some of which are essential for growth. These lipids contain a ceramide which consists of a sphingobase bound to a long-chain fatty acid. The outer leaflet of the plasma membrane is highly enriched with sphingolipids and, recently, in many organisms, they have been proposed to serve as intra- and intercellular second messengers which play an important role in various cellular events, such as proliferation and differentiation (reviewed by Dickson & Lester, 2002). Glucosylceramide (GluCer), which contains a directly linked glucose, is a typical sphingolipid found in most eukaryotes. On the other hand, glycosylinositolphosphorylceramides, which are linked with inositol via a phosphodiester bond, are present only in fungi and plants. Galactosylceramides, which are linked with galactose, are found in animals and fungi (reviewed by Warnecke & Heinz, 2003).

In animals, glycosylceramides (GlcCers) not only are essential for growth but also are believed to play a crucial role in cell differentiation and cell–cell interactions (reviewed by Merrill *et al.*, 1997; Ternes *et al.*, 2002). Plant GlcCer and its derivatives such as ceramide and long-chain bases (LCBs) have also been suggested to function in

signal transduction and host–pathogen interactions (reviewed by Sperling & Heinz, 2003; Thevisen *et al.*, 2004; Ramamoorthy *et al.*, 2007). In fungi, two yeast species, *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, have been used as models for studying higher eukaryotic cells with regard to the metabolism and function of sphingolipids as well as other intracellular components. However, as these yeasts are not capable of synthesizing GlcCer and are not able to desaturate and methylate LCBs, knowledge of the molecular mechanisms responsible for GlcCer function and the role of modified LCBs in fungi remains limited.

Recently, a number of other yeast species and fungi have been reported to contain GluCers which are thought to play important roles in growth and differentiation. For example, the addition of antibodies against a purified *Cryptococcus* GluCer led to the inhibition of cell budding and growth in *Cryptococcus neoformans*, and antibodies raised against a purified *Pseudallescheria* GluCer prohibited the formation of mycelium in *Pseudallescheria boydii* and germ-tube formation in *C. albicans* (Rodrigues *et al.*, 2000; Pinto *et al.*, 2002). Additionally, inhibition of UDP-glucose:ceramide glucosyltransferase (GCS), which catalyses the final step of GluCer synthesis, influences the growth and differentiation of *Aspergillus nidulans* and *A. fumigatus* (Levery *et al.*, 2002). However, little is known about which structure of GluCer causes these biological phenomena in fungal cells.

Abbreviations: GCS, UDP-glucose, ceramide glucosyltransferase; GlcCer, glycosylceramide; GluCer, glucosylceramide; LCB, long-chain base; SLD, sphingolipid Δ^8 -desaturase.

Biosynthesis of GluCer starts with condensation of palmitoyl-CoA and serine to synthesize 3-ketosphinganine by serine palmitoyltransferase (reviewed by Warnecke & Heinz, 2003). 3-Ketosphinganine reductase catalyses the conversion of 3-ketosphinganine to sphinganine. Ceramide synthase catalyses the condensation of sphinganine to saturated acyl-CoA for synthesis of ceramides. Next, sphingolipid Δ^4 -desaturase (DES) catalyses the Δ^4 -desaturation of LCBs in ceramides (Ternes *et al.*, 2002). Moreover, in plants and many fungi, sphingolipid Δ^8 -desaturase (SLD) catalyses the Δ^8 -desaturation of LCBs in ceramides, while sphingolipid 9-methyltransferase (SLM) introduces a methyl group in carbon-9 of LCBs in ceramides (Sperling *et al.*, 1998; Takakuwa *et al.*, 2002; Ternes *et al.*, 2006). The desaturation of fatty acyl groups in ceramides has also been detected in some organisms. Finally, GCS catalyses the reaction of these ceramides with UDP-glucose, resulting in the synthesis of GluCer (Leipelt *et al.*, 2001). Since it has been suggested that most of the genes that encode enzymes for GluCer synthesis are absent in *Sac. cerevisiae* and *Sch. pombe*, little is known at present about fungal genes involved in GluCer synthesis. It was recently reported that, in *Cryptococcus neoformans*, a pathogenic fungus, a strain lacking the GCS gene and thus not able to synthesize GluCer is incapable of growing in neutral and alkaline solutions *in vitro*, which reduces its pathogenicity in its host (Rittershaus *et al.*, 2006). In contrast, disrupting the GCS gene in *C. albicans* does not affect the growth of the yeast in its unicellular or filamentous forms (Leipelt *et al.*, 2001).

In this study, we have focused on the gene modifying an LCB moiety of GlcCer, namely the gene encoding the SLD enzyme. We identified a *C. albicans* SLD gene from the *Candida* genome database and constructed an *sld* null mutant. We then investigated the effect of the SLD disruption on the morphogenesis of fungal cells. Interestingly, the *C. albicans* *sld* mutant appeared to have an obvious delay in filamentous growth compared with isogenic wild-type strains.

METHODS

Media, growth conditions and basic techniques. The *C. albicans* strains used and constructed in this study are shown in Table 1. Cells were typically grown at 30 °C in YPD medium [1% Bacto yeast

extract, 2% Bacto peptone, 2% glucose (pH 5.6)] or complete minimal (CM) medium (Ausubel *et al.*, 1992) without uracil and/or arginine. The speed of growth was measured as OD₆₀₀ by using an Ultrospec 3000 spectrophotometer (Pharmacia Biotech). The yeast-to-hypha transition was induced at 37 °C in both solid and liquid media. For the solid transition medium, we used either Spider medium (Liu *et al.*, 1994) or 10% fetal bovine serum medium (Hanaoka *et al.*, 2005). For the liquid transition medium, we used 10% fetal bovine serum in YPD, adjusted to pH 7.2. Filamentation in the liquid medium was induced by inoculating 2×10^6 to 5×10^6 cells ml⁻¹ and incubating at 37 °C. For spot tests, cells were grown overnight at 30 °C in YPD medium and tenfold serial dilutions were then spotted onto the indicated YPD agar plate and grown for 31 h at 30 °C. Antifungals used in this study were amphotericin B (Wako Pure Chemical Industries), fluconazole (LKT Laboratories), micafungin (Astellas Pharma) and terbinafine (LKT Laboratories).

Escherichia coli DH5 α and cloning vector pBluescript II SK+ (Stratagene) were used for DNA manipulation. General recombinant DNA procedures were performed as described by Sambrook *et al.* (1989). *C. albicans* was transformed by the method described by Umeyama *et al.* (2005).

Plasmid construction. The primers used in this study are listed in Table 2. The *Ca*-SLD DNA fragment was amplified by PCR using primers *sld*-clo5 and *sld*-clo3, with TUA4 chromosomal DNA as a template, and cloned into the *EcoRV* site of pBluescript II SK+ to generate pBS-SLD. Nucleotide sequencing confirmed that there were no PCR errors. The 1.8 kb *Bgl*II-*Xho*I DNA fragment from pBS-SLD was inserted into the *Bam*HI/*Xho*I sites of pFLAG-Act1 (Umeyama *et al.*, 2002) to generate pFLAG-Act1-SLD.

A plasmid, pBS-hph200-URA3, was constructed for gene replacement. The 2.0 kb *Pvu*II DNA fragment from pUC19-hph200-URA3 (Umeyama *et al.*, 2005) was inserted into the *EcoRV* site of pBluescript II SK+ to generate pBS-hph200-URA3.

Strain construction. To disrupt *Ca*-SLD, two different markers were used for two different alleles (see Fig. 2). The 0.6 kb DNA fragment corresponding to the 3' end of *Ca*-SLD was amplified using primers *sld*-mut-Not5 and *sld*-mut-Sac3, digested with *Not*I and *Sac*I, and then cloned into the *Not*I-*Sac*I site of pBS-hph200-URA3 and pUC19-ARG4 (Hanaoka *et al.*, 2005) to generate pBS-hph200-URA3-NS and pUC-ARG4-NS, respectively. The 0.6 kb DNA fragment corresponding to the 5' end of *Ca*-SLD was amplified using primers *sld*-mut-Kpn5 and *sld*-mut-Xho3, digested with *Kpn*I and *Xho*I and then cloned into the *Kpn*I/*Xho*I site of pBS-hph200-URA3-NS to generate pBS-hph200-URA3-disSLD. The 0.6 kb DNA fragment corresponding to the 5' end of *Ca*-SLD was amplified using primers *sld*-mut-Sph5 and *sld*-mut-Kpn3, digested with *Sph*I and *Kpn*I and then

Table 1. Yeast strains used and constructed in this study

Strains TUA4 and TUA6 were obtained from the National Institute of Infectious Diseases, Japan. The remaining strains were constructed in this study.

Strain	Genotype
TUA4	<i>ura3Δ::imm434/ura3Δ::imm434 arg4Δ::hisG200/arg4Δ::hisG200</i>
TUA6	<i>ura3Δ::imm434/ura3Δ::imm434 arg4Δ::hisG200/arg4Δ::hisG200 RP10::p3HA-ACT1</i>
SLD101	<i>ura3Δ::imm434/ura3Δ::imm434 arg4Δ::hisG200/arg4Δ::hisG200 sldΔ::hph200-URA3-hph200/SLD</i>
SLD102	<i>ura3Δ::imm434/ura3Δ::imm434 arg4Δ::hisG200/arg4Δ::hisG200 sldΔ::hph200-URA3-hph200/sldΔ::ARG4</i>
SLD103	<i>ura3Δ::imm434/ura3Δ::imm434 arg4Δ::hisG200/arg4Δ::hisG200 sldΔ::hph200/sldΔ::ARG4</i>
SLD104	<i>ura3Δ::imm434/ura3Δ::imm434 arg4Δ::hisG200/arg4Δ::hisG200 sldΔ::hph200/sldΔ::ARG4 RP10::pFLAG-ACT1</i>
SLD105	<i>ura3Δ::imm434/ura3Δ::imm434 arg4Δ::hisG200/arg4Δ::hisG200 sldΔ::hph200/sldΔ::ARG4 RP10::pFLAG-ACT1-SLD</i>

Table 2. Oligonucleotide primers used in this study

Restriction sites are underlined.

Oligonucleotide	Sequence (5'–3')
sld-clo5	AAAGATCTAGGATGGATGTGAAAAGACCTAAGAAAATCTTTAC
sld-clo3	AACTCGAGCTAATATAAAATCCCCTTCATAGTGCTTTGTTG
sld-mut-Sph5	AAGCATGCTATACTTTTGGCAGGATTCTTGGAAGG
sld-mut-Kpn3	AAGGTACCGGTTTATCTCATAAAGGTTTGTTAACGAAG
sld-mut-Not5	AAGCGGCCGCACAGTATCCTGTTTCTTAACCTTTTATGC
sld-mut-Sac3	TTGAGCTCAAAAGGCAACAAAAACAAAAACAGG
sld-mut-Kpn5	AAGGTACCTATACTTTTGGCAGGATTCTTGGAAGG
sld-mut-Xho3	AACTCGAGGTTTATCTCATAAAGGTTTGTTAACGAAG

cloned into the *SphI/KpnI* site of pUC-ARG4-NS to generate pUC-ARG4-disSLD.

The 3.3 kb *KpnI-SacI* fragment of pBS-hph200-URA3-disSLD was used to transform the *C. albicans* Arg[−] Ura[−] strain TUA4 to generate a Ura⁺ transformant (SLD101). The 3.2 kb *SphI-SacI* fragment of pUC-ARG4-disSLD was then used to transform Arg[−] strain SLD101 to generate SLD102. The resulting Ura⁺ Arg⁺ transformants of SLD102 were plated on medium containing 5-fluoroorotic acid to isolate the Ura[−] segregant (SLD103). To confirm the gene disruption, genomic DNA was isolated from each strain, digested with *HpaI*, run in a 1 % agarose gel and then transferred onto a Hybond-N+ nylon membrane (Amersham Biosciences). Southern hybridization was performed using a ³²P-labelled probe in a solution containing 5 × SSC, 6 × Denhardt's solution and 0.1 mg herring sperm DNA ml^{−1} at 65 °C according to the methods of Sambrook *et al.* (1989). The *SLD* gene was reintroduced into the *Ca-RP10* locus of the null mutant using *AatI*-digested pFLAG-Act1-SLD to transform strain SLD103 to generate SLD105. The *AatI*-digested empty vector pFLAG-Act1 was integrated into strain SLD103 to generate SLD104 as a control.

Sphingoid base analysis. Analysis of the sphingoid base composition was performed as described by Tanji *et al.* (2004) with optimizations. Lyophilized cells were treated in chloroform-methanol (2:1, v/v) for 10 min with a subsonic homogenizer (HOM-100; Asahi Glass). Total lipids were then extracted with 4 vols chloroform-methanol (2:1, v/v) and chloroform-methanol (1:2, v/v) and hydrolysed in 0.4 M KOH-methanol at 37 °C for 2 h. After washing, the organic phase was dried on a rotary evaporator to yield the alkali-stable lipids. GluCers were isolated from the other lipids on preparative TLC developed with chloroform-methanol (95:12, v/v). GluCers obtained were degraded with 10 % Ba(OH)₂-dioxane (1:1, v/v) and the sphingoid bases released were extracted with diethyl ether and reacted with 0.2 M sodium periodate for 2 h to obtain fatty aldehydes. The fatty aldehydes were extracted in dichloromethane and analysed by GLC (GC-18A; Shimadzu) on a 0.25 mm × 50 m CP-Sil 88 capillary column (Varian). The initial column temperature of 150 °C was maintained for 2 min, increased by 2 °C min^{−1} to 190 °C and then maintained for 6 min.

RESULTS

Identification of a *C. albicans* *SLD* gene homologue and its sequence analysis

From the *Candida* genome database website (<http://www.candidagenome.org/>), we detected one sequence having high similarity with fungal *SLD* proteins from

Saccharomyces kluyveri (*Sk*-SLDp), *Kluyveromyces lactis* (*Kl*-SLDp) and *Pichia pastoris* (*Pp*-SLDp). The resulting amino acid sequence, *Ca*-SLDp (orf19.260), was predicted to be a 584 amino acid polypeptide. The deduced amino acid sequence of *Ca*-SLDp had high identities with *Sk*-SLDp (58.5 %), *Kl*-SLDp (56.5 %) and *Pp*-SLDp (53.7 %). The haem-binding motif (HPGG) in cytochrome *b₅* and three histidine motifs (HXXXH, HXXHH, QXXHH), considered essential for desaturase activity and as potential ligands for iron atoms, are found in *Ca*-SLDp (Fig. 1).

Construction of *Ca-sld* mutants

To investigate the functions of *Ca*-SLD in *C. albicans*, we constructed *Ca-sld* mutants as described above. For the first allele, *Ca*-SLD was replaced with an *hph200-URA3-hph200* cassette (SLD101) and the remaining allele was replaced by *ARG4*. The resulting strain was termed as SLD102. The *URA3* gene was excised from SLD102, generating the Ura-auxotrophic derivative SLD103. Southern blot analysis was performed to confirm that the obtained mutants were disrupted at the *SLD* locus (Fig. 2). To confirm that loss of *SLD* function was responsible for the phenotypes observed, the *SLD* expression plasmid pFLAG-Act1-SLD and the pFLAG-ACT1 vector alone were reintroduced into the *Ca-RP10* locus (Murad *et al.*, 2000) of *sld* mutant strain SLD103, yielding strains SLD105 and SLD104, respectively.

Sphingoid base analysis of *Ca-sld* mutants

To investigate the profile of sphingoid base residues in *C. albicans* transformants, GluCers were isolated from SLD104 (*sld* mutant), SLD105 (*SLD* transformant) and the corresponding wild-type strain, TUA6. Their sphingoid base compositions were compared (Fig. 3). The GluCers of the wild-type strain contained the three sphingoid bases (*E*)-sphing-4-enine, (*E,E*)-sphinga-4,8-dienine and (*E,E*)-9-methylsphinga-4,8-dienine. In contrast, the *sld* mutant contained only one sphingoid base, (*E*)-sphing-4-enine. The sphingoid base profile of the *SLD* transformant strain was similar to that of the wild-type. These results show that *Ca*-SLDp is the only enzyme that introduces a double bond at the Δ⁸ position of the LCB in ceramides.

Ca-SLDp	MDVKRPKHTFTRSCIDLTADCKVIVIKNNVNLTSWIKKHPGGDKAAHYHVGKDATDEMAYHCHDETVDTRFKIGE	80
Sk-SLDp	MD-----NIIISRGEDRIARQQAIVIEGLVLNIEKWKIFHPGGDKAIHHMICRATDEMAYHCHDETVEIRKWHIGR	75
Kl-SLDp	MS-----RVLRRRDADRIAKQOTIVIVEDSVLNIDKWKIFHPGGDKSIYHMICRATDEMAYHCHDETITKIKWIGR	75
Pp-SLDp	M-----VVSREIEVREIISRGNAIVIEDHLNLNGLERHPGGDKAIHHMICRATDEMAYHCHDETPTVKTKKWSIGR	73
Ca-SLDp	IEGFRWENLLPPIQGGIVAKGGHYGSLNKNKTSNKKTDLSKLDNDSSNSTSDLECLTTTNSCDSAISETEIANYKIGFV	140
Sk-SLDp	IDQF--WENELPPIQGGVFRF-----LHQHDSID--GLSNKWIAPNTSDQFKFK-----NEKHMCGEPDVKIY	137
Kl-SLDp	IDYP--WENMLPPIQGGREFSK-----IDERRDIGDYDINLTSKWSVDESNOYTKIP-----KNDRLAGAEVKIY	139
Pp-SLDp	VKLE--WONLVPPIQGGNYFSF-----DKVDQQRVIY-----KKLGIFPQVKIE	113
Ca-SLDp	ELVKFVVPQNQLVFKSNMDIVFEIIDEETKKKVIINPKILLNNYDNKLSQEEVMSLPALDYDSQVLRDKYNELHOTIID	210
Sk-SLDp	EKIPQGVIPSLNLKEAYEKKV-----VTFDFIVADNYDNELVROLETLPDLDPKTCEWLSKBYNKKMHETIE	205
Kl-SLDp	EKIPQGVIPSLDLKEAYEKKI-----VVDFIVVSENYDNERYVEDLTNFBSDLVKNCEWIASERYKIHGETTA	207
Pp-SLDp	EKVQENIV---LIEKSASKLLVGG-----VRDFKIIIDFDNKLYVEDIKQIPESLDHETORNLSQYNELHOTIIN	182
Ca-SLDp	YGLYBCDNDVYRVVKITGSLFL--VSTSLKINQLF--LSAVFMGMANHOGTFAIHDAGHICITHNYQIDNIFGMLTAD	276
Sk-SLDp	AGLYQCNVFRVVKELTRIGLLHALSYLLYHRDQKFWAHSFGC--AAQOLVFIADHAGHISITHNYQIDNIFGMLTAS	282
Kl-SLDp	AGLYQCNVFRVRLRTRIGLLGSGISFYLLSLV--WFAISICLFAMQOLVFIADHAGHISITHNYQIDNIFGMLTAS	283
Pp-SLDp	RGYQCQVWQVFKFCRITSSGFL--FVLFIRSK--WYTLSTAISTCLMQLVFIADHAGHISITHNYQIDNIFGMLTAN	258
Ca-SLDp	WFGGLSLGWWKRNHNVHLITNDPVHDPDIQHLPPFAVSVRLFQNVYSTYYDKILPFDKFSQFLIPLQKLYLYPILCFGR	346
Sk-SLDp	WFGGLSLGWWKRNHNVHLITNDPVHDPDIQHLPPFAVSTRLEFDMYSTYYDKFLWDFPAKVVWVWONLYYPMIAFGR	362
Kl-SLDp	WFGGLSLGWWKRNHNVHLITNDPVHDPDIQHLPPFAVSTRLEFHNVYSTYYDKFLWDFKFAQKVVVIOHLYLYPILCFGR	363
Pp-SLDp	FIGGLSLGWWKRNHNVHLITNDPVHDPDIQHLPPFAVSSRLGNVYSTYYEKYLWDFKIAQKMLQIQHLYLYPILSFGR	338
Ca-SLDp	FNLYRLSWTHVLGCGPREGKAAPFRYFEFFGLSFFFWFFYLLVFKTEGGWNRFNVMVSHITTMLVHVOITLSHFAM	416
Sk-SLDp	FNLYRLSWMHVLLGLGPRRGKAAPFRYFELCGLIFENYWFYLLVGLKLOTGWDRQYIMVSHITTMLVHVOITLSHFAM	442
Kl-SLDp	FNLYRLSWMHVLLGCGPREGKAAPFRYFELABELSFENYWFYLLIYKOMPNAERKVMISHIATMIVHVOITLSHFAM	443
Pp-SLDp	FNLYRLSWSHLIMGLGPRRGKAAPFRYLELIGLCPESYWFYKTMYSY--PTKTLRFWFLISHWTMIVHVOITLSHFAM	417
Ca-SLDp	STADLGVSSESFPSCVVRTMDVDCPEWDLFLHGGLOFOATHHLFPRLPRHNLRAAOPVVIKCFCEVGLSYSYGFGGNE	486
Sk-SLDp	STSDLGVCCEFPMPRLRTSMVDVDCPRWDLDFHGGLOFOVTHHLFPRLPRHNLRAAOPVVIDFCEKVGIKYSYIGFSKNG	522
Kl-SLDp	STSDLGVISEFPMPRLRTSMVDVDCPRWDLDFHGGLOFOVTHHLFPRLPRHNLRAAOPVVIKFDKVGIKYSYIGFAAGND	523
Pp-SLDp	STSDLGSTSESFVRQLRTMDVDCPEWDLFHHGGLOFOATHHLFPRLPRHNLRAAOPVVIDFCKNTGLHYSYIGFVDNG	497
Ca-SLDp	IVISRLADIGKCCSFLL-----ATKHYEGDLY-----	584
Sk-SLDp	VVLKQLQIAVCAKTMLECASAMKKEATGEREADKTYRTKSI-----KNA	568
Kl-SLDp	VVISHLQIAQAHTMLECAKTMKKEATDEFEITNKHVLAANVNEKRKQE	573
Pp-SLDp	KVNVKMAVASQVVLNCLHSIHLENT-----TGKNHYEAKVESVSIGK	542

Fig. 1. Amino acid sequence alignment of Ca-SLDp and closely related sphingolipid Δ^8 -desaturases. *Sk-SLD*, *Sac. kluyveri* SLD (Takakuwa *et al.*, 2002); *Kl-SLD*, *K. lactis* SLD (Takakuwa *et al.*, 2002); *Pp-SLD*, *P. pastoris* SLD (Ternes *et al.*, 2006). Sequences were aligned using the FASTA algorithm. Conserved amino acids are in white on black. Positions of amino acid residues relative to the start codon are given to the right. The haem-binding motif (HPGG) in cytochrome b_5 and three histidine motifs (HXXXH, HXXXH, QXXXH), considered essential for desaturase activity and as potential ligands for iron atoms, are boxed.

Fig. 2. Gene disruption of Ca-SLD. (a) Disruption strategy by homologous recombination. (b) Restriction maps for each modified allele. WT, Wild-type. (c) Confirmation of disruption constructs by Southern hybridization using the probe indicated in (b). Lanes: 1, *SLD/SLD*, *ura3*, *arg4*; 2, *sld/SLD*, *URA3*, *arg4*; 3, *sld/sld*, *URA3*, *ARG4*; 4, *sld/sld*, *ura3*, *ARG4*.

Fig. 3. GLC analysis of fatty aldehydes derived from sphingoid bases. Cells from strains TUA6 (*SLD/SLD*) (a), SLD104 (*sld/sld*) (b) and SLD105 (*sld/sld SLD*) (c) were grown overnight at 30 °C. Sphingoid base fractions were subjected to periodate oxidation to obtain fatty aldehydes and were identified by comparison of retention times to those of known standards prepared from mushroom sphingoid bases. Abbreviations: d18:1 Δ^4 , 4-*trans*-sphingenine; d18:2 $\Delta^{4,8}$, 4-*trans*-, 8-*trans*-sphingadienine; d18:2 $\Delta^{4,8}$ -9m, 9-methyl-, 4-*trans*-, 8-*trans*-sphingadienine.

Phenotype analysis of the *Ca-sld* disruptant

At first, to investigate the necessity of Δ^8 -desaturation of the LCB in ceramides and GluCers, we examined the growth rate of the *sld* disruptant and the control strains at 30 and 10 °C in liquid YPD medium (Fig. 4). There was no significant difference in the growth rate among these strains at 30 °C. However, the growth rate of the *sld* disruptant was lower than that of the wild-type at 10 °C. These results indicate that the fungal ceramide and GluCer containing a double bond at the Δ^8 -position in the LCB play some role in growth of *C. albicans* at 10 °C.

In order to observe hyphal formation of the disruptant, overnight cultures were inoculated into liquid YPD medium (pH 7.2) plus 10 % serum medium. Time-course observation of growth at 37 °C with shaking demonstrated that hyphal growth of the *sld* mutant was similar to that of the wild-type, and there was no significant difference in germ-tube formation (Fig. 5). In contrast, on agar media such as 10 % serum agar at 37 °C or spider medium at

25 °C, filamentation of the *sld* disruptant was slower than that of the wild-type strain (Fig. 6). This phenotype was restored by the reintroduction of *Ca-SLD* to the *sld* mutant.

To investigate other effects of the loss of Δ^8 -desaturation of GluCer in *C. albicans*, growth of the *sld* mutant in various agents or environments was examined by drop assays (Fig. 7). The *sld* disruptant was sensitive to 0.02 % SDS in comparison with the wild-type. Other stresses such as heat shock (42 °C), salt (1 M NaCl or 1 M KCl), 6 % ethanol or 50 μ g Calcofluor white (CFW) ml⁻¹ had no effect. Moreover, the *sld* disruptant was more susceptible than the wild-type to the lanosterol 14 α -demethylase inhibitor fluconazole, while other antifungals such as amphotericin B, micafungin and terbinafine had no effect. These results indicate that Δ^8 -desaturated GluCers may have a role in membrane integrity in *C. albicans*.

DISCUSSION

In this study, we characterized the sphingolipid Δ^8 -desaturase gene from *C. albicans* (*Ca-SLD*) and also constructed a disruptant of this gene. This is the first report on the physiological characterization of an *SLD* gene disruptant in any organism. The deduced amino acid sequence of the gene product encoded by orf19.260 in the *C. albicans* genome had high identities with those of other yeasts, confirming that the ORF encodes a *C. albicans* sphingolipid Δ^8 -desaturase (*Ca-SLDp*).

We constructed the *sld* disruptant by replacing two *Ca-SLD* ORFs with two different marker genes. From the result of the analysis of LCB in GluCers, the *sld* mutant contained only the sphingoid base (*E*)-sphing-4-enine. In contrast, the *SLD* transformant strain contained the three sphingoid bases (*E*)-sphing-4-enine, (*E,E*)-sphinga-4,8-dienine and (*E,E*)-9-methylsphinga-4,8-dienine in GluCers, a result that was similar to the wild-type strain. The amounts of GlcCer from these cells were unlikely to make much of a difference. These results show that *Ca-SLDp* is required for desaturation at the Δ^8 position of *C. albicans* glucosylcer-

Fig. 4. Growth of *C. albicans* strains in liquid YPD medium. Cells from strains TUA6 (*SLD/SLD*; circles), SLD104 (*sld/sld*; squares) and SLD105 (*sld/sld SLD*; triangles) were precultured overnight at 30 °C and then inoculated into 50 ml medium to OD₆₀₀ 0.2. Inoculated cells were grown in a reciprocal shaker (130 r.p.m.) at 30 °C (a) and 10 °C (b). Vertical bars represent the SEM for three replicate experiments.

Fig. 5. Germ-tube formation in liquid YPD (pH 7.2) plus 10% serum at 37 °C. Cells from strains TUA6 (*SLD/SLD*), SLD104 (*sld/sld*) and SLD105 (*sld/sld SLD*) were grown overnight at 30 °C. For induction of filamentation, 2×10^6 to 5×10^6 cells ml^{-1} were inoculated into YPD (pH 7.2) with 10% serum and incubated at 37 °C with shaking.

Fig. 6. Morphology of *C. albicans* strains grown on solid agar medium. Cells from strains TUA6 (*SLD/SLD*), SLD104 (*sld/sld*) and SLD105 (*sld/sld SLD*) were grown overnight at 30 °C and 10^6 cells were then spotted onto the indicated agar plate and grown for 7 days at 37 °C on agar medium containing 10% serum or at 25 °C on Spider medium. Results of a representative experiment are shown; four further independent experiments showed the same results.

Fig. 7. Stress-, temperature- and reagent-related phenotypes of *C. albicans* strains. Cells from strains TUA6 (*SLD/SLD*), SLD104 (*sld/sld*) and SLD105 (*sld/sld SLD*) were grown overnight at 30 °C, and tenfold serial dilutions of cells were then spotted onto the indicated YPD agar plate and grown for 31 h at 30 °C. AMB, Amphotericin B; CFW, Calcofluor white; FCZ, fluconazole; MFG, micafungin; TB, terbinafine.

amides. Additionally, Δ^8 -desaturation by *Ca*-SLDp is necessary for 9-methylation by sphingolipid 9-methyltransferase that occurs in LCBs within ceramides.

The growth rate of the *sld* disruptant at 30 °C in liquid YPD medium was similar to that of the wild-type. However, the *sld* mutant showed slower growth than the wild-type strain at 10 °C. These results indicate that Δ^8 -desaturation of LCBs in ceramide and GluCer is necessary for growth of *C. albicans* at 10 °C. (*E,E*)-9-Methylsphing-4,8-dienine in GluCers is assumed to be essential in *Kluyveromyces lactis* to maintain sufficient membrane fluidity at low temperatures (Tanji *et al.*, 2004). Therefore, the loss of this molecule in the cell membrane may result in the sensitivity of the *sld* mutant to low temperatures.

When grown in liquid media to induce the hyphal form, the *sld* disruptant was able to elongate and its germ tubes evaginated normally. However, when grown on hyphal-inducing solid medium, the *C. albicans sld* disruptant showed slower elongation than the wild-type. These results suggest that Δ^8 -desaturation of LCBs in ceramide and GluCer is closely related to hyphal elongation on solid medium. Sphingolipids, a major component of the lipid raft, are known to be important for normal hyphal growth in *C. albicans* (Martin & Konopka, 2004). In this latter report, myriocin, a specific inhibitor of serine palmitoyltransferase, which catalyses the first step of sphingolipid biosynthesis, affected hyphal elongation in *C. albicans*.

Therefore, GluCer containing Δ^8 -desaturated LCB, among many kinds of sphingolipids, may play an important role in hyphal elongation in *C. albicans*. Moreover, some GluCers of fungi and plants are thought to be highly active in inducing fruiting body formation in the fungus *Schizophyllum commune* (Kawai & Ikeda, 1985; Kawai *et al.*, 1986). Structural analysis of these GluCers has revealed that a *cis*- or *trans*- Δ^8 double bond in the sphingoid base moiety is essential, while the sugar moiety has no effect. However, a *C. albicans gcs* mutant that is unable to condense ceramides with glucose grew in both yeast and filamentous forms (Leipelt *et al.*, 2001). These results and our finding suggest that Δ^8 -desaturated and 9-methylated ceramides may be active in inducing hyphal morphogenesis of *C. albicans*.

The *C. albicans sld* disruptant was sensitive to SDS in comparison with the wild-type but CFW, a cell-wall stress inducer, had no effect on this mutant. Moreover, the *C. albicans sld* disruptant was more susceptible than the wild-type to the lanosterol 14 α -demethylase inhibitor fluconazole. We surmise that the defect of Δ^8 -desaturation may have led to a disturbance in the membrane integrity of *C. albicans*. Sphingolipids, including GluCer, are enriched in the detergent-resistant membrane microdomains known as lipid rafts. Lipid rafts have been implicated in numerous cellular processes, including signal transduction, protein and lipid sorting, cellular entry by toxins and viruses and viral budding (reviewed by Chazal & Gerlier, 2003; Bollinger *et al.*, 2005). In addition, antimicrobial peptide

inhibitors of fungal plasma membrane ATPase (Pmalp), which is known to be associated with the lipid raft, block the azole resistance of *C. albicans* (Monk *et al.*, 2005). Therefore, the sensitivity to SDS and susceptibility to fluconazole of the *sld* disruptant may result from some defect of lipid rafts in *C. albicans*. In future experiments, the detergent-resistant microdomain in the membrane of the *C. albicans sld* mutant will be analysed in detail.

The *sld* disruptant did not contain (*E,E*)-9-methylsphing-4,8-dienine or (*E,E*)-sphing-4,8-dienine. (*E,E*)-9-Methylsphing-4,8-dienine is the most abundant sphingoid base in ceramide and GluCer of the *C. albicans* wild-type strain. Therefore, it may be that 9-methylation of the LCB and its relation with filamentous growth of *C. albicans* may be more important than the Δ^8 -desaturation. A *C. albicans* ORF (orf19.4831) encoding a homologue of sphingolipid C9-methyltransferase of *Pichia pastoris* has been found in the *C. albicans* genome (Ternes *et al.*, 2006). Analysis of this gene disruptant will further clarify the most important sphingoid base in *C. albicans*.

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