Alteration of complex sphingolipid composition and its physiological significance in yeast *Saccharomyces cerevisiae* lacking vacuolar ATPase

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In the yeast *Saccharomyces cerevisiae*, complex sphingolipids have three types of polar head group and five types of ceramide; however, the physiological significance of the structural diversity is not fully understood. Here, we report that deletion of vacuolar H\(^+\)-ATPase (V-ATPase) in yeast causes dramatic alteration of the complex sphingolipid composition, which includes decreases in hydroxylation at the C-4 position of long-chain bases and the C-2 position of fatty acids in the ceramide moiety, decreases in inositol phosphorylceramide (IPC) levels, and increases in mannosylinositol phosphorylceramide (MIPC) and mannosylidinositol phosphorylceramide [M(IP)\(_2\)C] levels. V-ATPase-deleted cells exhibited slow growth at pH 7.2, whereas the increase in MIPC levels was significantly enhanced when V-ATPase-deleted cells were incubated at pH 7.2. The protein expression levels of MIPC and M(IP)\(_2\)C synthases were significantly increased in V-ATPase-deleted cells incubated at pH 7.2. Loss of IPC synthesis or an increase in the hydroxylation level of the ceramide moiety of sphingolipids on overexpression of Scs7 and Sur2 sphingolipid hydroxylases enhanced the growth defect of V-ATPase-deleted cells at pH 7.2. On the contrary, the growth rate of V-ATPase-deleted cells was moderately increased on the deletion of *SCS7* and *SUR2*. In addition, supersensitivities to Ca\(^{2+}\), Zn\(^{2+}\) and H\(_2\)O\(_2\), which are typical phenotypes of V-ATPase-deleted cells, were enhanced by the loss of MIPC synthesis. These results indicate the possibility that alteration of the complex sphingolipid composition is an adaptation mechanism for a defect of V-ATPase.

INTRODUCTION

Complex sphingolipids are major components of the eukaryotic plasma membrane. They consist of a hydrophobic segment, ceramide, with a polar head group. The ceramide moiety comprises a long-chain base (LCB) attached to a fatty acid via an amide bond. Mammalian complex sphingolipids can carry phosphocholine or carbohydrate chains as polar head groups, whereas complex sphingolipids in the yeast *Saccharomyces cerevisiae* contain phosphoinositol. Recent studies have demonstrated that complex sphingolipids, together with sterols, form lipid microdomains, and play important roles in signal transduction, membrane trafficking and stress adaptation (Dickson *et al.*, 2006; Simons & Sampaio, 2011).

According to the hydroxylation state, ceramide in *S. cerevisiae* can be classified into five types (A, B, B’, C and D) (Fig. 1). The A-type contains dihydrosphingosine (DHS) and very long-chain fatty acids, which are mostly of 26-carbon chain length. The hydroxylation patterns of the B-, B’- and C-types are determined by two hydroxylases, Ssr2 and Ssc7, which catalyse hydroxylation at the C-4 position of the DHS and the C-2 position of the very long-chain fatty acids, respectively (Haak *et al.*, 1997). The D-type is generated on further hydroxylation at an unknown position of the fatty acid moiety of the C type, and CCC2 encoding a copper transporter is required for the synthesis (Beeler *et al.*, 1997). *S. cerevisiae* complex sphingolipids have three types of polar head group. Therefore, complex sphingolipids in yeast can be divided into IPC (inositol phosphorylceramide), MIPC (mannosylinositol phosphorylceramide) and M(IP)\(_2\)C (mannosylidinositol phosphorylceramide), all of which include phosphoinositol (Dickson *et al.*, 2006) (Fig. 1). Because each of IPC, MIPC and M(IP)\(_2\)C has five types of
Fig. 1. Synthesis and structure of sphingolipids in the yeast S. cerevisiae. (a) The pathway and genes responsible for the synthesis of yeast sphingolipids are shown. Because of the different hydroxylation states of ceramide (ceramide-A, -B', -B, -C and -D), there are five subtypes each for IPC, MIPC and M(IP)2C. (b) Structure of yeast complex sphingolipids. Sites labelled 1, 2 and 3 in the ceramide moiety are hydroxylated by Sur2, Scs7 and as-yet-unidentified hydroxylase(s), respectively. Sites 1 and 2 are on the C-4 position of the LCBs and the C-2 position of the very long-chain fatty acids, respectively. Site 3 is also on the very long-chain fatty acids, but the position has not been determined.
ceramide, the complex sphingolipids in *S. cerevisiae* can be classified into 15 subtypes in total. IPC is formed by IPC synthase (Aur1), an enzyme catalysing the transfer of the head group of phosphatidylinositol to ceramides (Nagiec et al., 1997) (Fig. 1). MIPC is formed through the addition of mannose to IPC, which is catalysed by two homologous IPC mannosyltransferases, Sur1 and Csh1, and Csg2 functions as a regulatory subunit for both Sur1 and Csh1 (Beeler et al., 1997; Uemura et al., 2003; Zhao et al., 1994) (Fig. 1). MIPC is converted to M(IP)2C through the addition of another phosphoinositol, which is catalysed by Ipt1 (Dickson et al., 1997) (Fig. 1).

The most abundant species of complex sphingolipid in *S. cerevisiae* is the C-type, which is hydroxylated at both LCs and fatty acids, and the levels of IPCs and M(IP)2Cs are much higher than those of MIPC (Uemura et al., 2014). Although loss of all sphingolipids in *S. cerevisiae* is lethal, the addition of hydroxyl groups to the ceramide moiety, and the biosynthesis of MIPC and M(IP)2Cs, are basically non-essential for growth (Dickson et al., 2006). However, several lines of evidence indicated the physiological significance of these modifications in complex sphingolipids. For instance, deletion of *SURI*, *IPT1* or *SUR2* suppresses phenotypic defects associated with the loss of Rvs161 or Rvs167, N-BAR family proteins involved in the regulation of endocytosis and in the actin cytoskeleton (Balguerie et al., 2002; Desforges et al., 1993). The C-4 hydroxylation of DHS is important for formation of lateral diffusion barriers, which compartmentalize the plasma membrane and the endoplasmic reticulum (Clay et al., 2014), and proper lipid microdomain formation (Idkowiak-Baldys et al., 2004). In addition, fluorescence recovery after photobleaching (FRAP) analysis revealed that loss of *SCS7* and/or *SUR2* affects the lateral diffusion of membrane proteins (Uemura et al., 2014). Loss of MIPC synthesis causes supersensitivity to Ca2+ (Uemura et al., 2003; Zhao et al., 1994), rapid cell death under nitrogen starvation (Yamagata et al., 2003; Zhao et al., 2013), and impairment of a specific endosomal trafficking pathway and cell integrity (Tani & Kuge, 2010, 2012; Morimoto & Tani, 2015). In addition, MIPC regulates the activity of aminophospholipid flippases via Fpk1 and Fpk2 kinases, and phospholipid asymmetry in plasma membranes (Roelants et al., 2010). Loss of M(IP)2C synthesis affects multidrug sensitivity (Hallstrom et al., 2001). Thus, elucidation of the relationship between the physiological functions of complex sphingolipids and their detailed structural properties has become essential.

Proton-translocating vacuolar +ATPase (V-ATPase) functions in the acidification of cellular components in eukaryotic cells (Li & Kane, 2009). It consists of approximately 14 subunits arranged into two subcomplexes, V(0) and V(I) (Li & Kane, 2009). Complete loss of V-ATPase activity in mouse and *Drosophila* is lethal, often at very early stages of development (Davies et al., 1996; Sun-Wada et al., 2000). In contrast, *S. cerevisiae* mutants that lack V-ATPase are viable; however, deletion of any subunits of V-ATPase in *S. cerevisiae* causes a defect of vacuolar acidification and various abnormal phenotypes, such as a growth defect under alkaline conditions, supersensitivity to metal ions and oxidative stress (Li & Kane, 2009). In this study, we found that the composition of complex sphingolipids is dramatically altered in V-ATPase-deleted mutants; that is, the MIPC and M(IP)2C levels are increased, and the hydroxylation level of the ceramide moiety is decreased. Furthermore, it was found that loss of MIPC synthesis or an increase in the hydroxylation of the ceramide moiety in V-ATPase-deleted mutants greatly enhanced the growth defect of V-ATPase-deleted mutants cells at neutral pH. These results indicate that an increase in MIPC and M(IP)2C and a decrease in the hydroxylation level of the ceramide moiety as observed in V-ATPase-deleted mutants may compensate for the loss of V-ATPase. This is indicative of the physiological importance of the detailed structural properties of complex sphingolipids in the absence of V-ATPase.

**METHODS**

**Yeast strains and media.** The *S. cerevisiae* strains used are listed in Table 1. Disruption of VMA2, VMA3, VMA21, VPH1, STV1, SCS7, SUR2, SURI, CSH1 and IPT1 was performed by replacing their ORFs with the *kanMX4* marker from a genome from a yeast knockout library or the pFA6a-*kanMX4* vector (Wach et al., 1994), the *natMX4* marker from the p4339 vector (pCRII-TOPO::natMX4) (Tong & Boone, 2006), the *natNT2* marker from the pFA6a-*natNT2* vector (Wach et al., 1994), the *hphNT1* marker from the pFA6a-*hphNT1* vector (Wach et al., 1994), the *ura3* marker from the pRS406 vector, or the LEU2 marker from the pRS405 vector (Sikorski & Hieter, 1989). Occasionally, *kanMX4* and *natMX4* were replaced with the hygromycin B-resistance gene (from the pFA6a-*hphNT1* vector; Janke et al., 2004) to create *hphMX4*. For tagging of the C-terminus of Aur1 or Sur1 with six copies of the HA epitope (6 × HA), a 6 × HA fusion cassette with the *hphNT1* marker from the pYM16 vector was introduced immediately upstream of the stop codon of chromosomal *AUR1* or *SUR1* as described elsewhere (Janke et al., 2004). To tag the N-terminus of Scc7, Sur2 or Ipt1 with three copies of the FLAG epitope (3 × FLAG), a 3 × FLAG tag was introduced immediately downstream of the initiator ATG of chromosomal *SCS7*, *SUR2* or *IPT1* without changing the potential promoter region according to the method described previously (Tani & Kuge, 2014). The sequences of the oligonucleotide primers described below are listed in Table S1 (available in the online Supplementary Material). A DNA fragment of the *SCS7*, *SUR2* or *IPT1* ORF without the initiator ATG was amplified by PCR using *SCS7*-3 × FLAG-HindIII-F and *SCS7*-3 × FLAG-BamHI-R (for *SCS7*), *SUR2*-3 × FLAG-NorF and *SUR2*-3 × FLAG-BamHI-R (for *SUR2*), or *IPT1*-3 × FLAG-HindIII-F and *IPT1*-3 × FLAG-BamHI-R (for *IPT1*), and yeast genomic DNA as a template. The PCR products were inserted into the HindIII and BamHI, or NorI and BamHI sites of the p3 × FLAG-CMV-7.1 vector (Sigma). A DNA fragment of 3 × FLAG-SCS7, 3 × FLAG-SUR2 or 3 × FLAG-IPT1 was amplified by PCR using 3 × FLAG-SCS7-F1 and SCS7-Hyg-R (for SCS7), 3 × FLAG-SUR2-F1 and SUR2-Hyg-R (for SUR2), or 3 × FLAG-IPT1-F1 and IPT1-Hyg-R (for IPT1), and the p3 × FLAG-CMV-7.1 vector containing the *SCS7*, *SUR2* or *IPT1* ORF as a template. A DNA fragment containing the *hphNT1* marker was amplified by PCR using SCS7-Hyg-F and SCS7-S2 (for SCS7), SUR2-Hyg-F and SUR2-S2 (for SUR2), or IPT1-Hyg-F and IPT1-S2 (for IPT1), and pYM16 (Janke et al., 2004) as a template. These two DNA fragments were combined by PCR, and the resultant DNA
fragment (3 × FLAG-SCS7::hphNT1, 3 × FLAG-SUR2::hphNT1 or 3 × FLAG-IPT1::hphNT1) was used to transform the cells. The cells were cultured in YPD medium (1% yeast extract, 2% peptone and 2% glucose). Buffered medium was prepared by the addition of 2% glucose. Buffered medium was prepared by the addition of

Lipids were extracted from S. cerevisiae as described by Hanson & Lester (1980) with minor modification. Briefly, the cells (3 OD_{600} units) were suspended in 350 μl ethanol/water/diethyl ether/pyridine/15 M ammonia (15 : 15 : 5 : 1 : 0.018, by vol.), and then incubated at 65 °C for 15 min. The lipid extract was centrifuged at 10,000 g for 1 min and then extracted once more in the same manner. The resulting supernatants were discarded and subjected to mild alkaline treatment using monomethylamine (MMA). For this reason, the lipid extracts were dissolved in 130 μM MMA [40% methanol solution/water (10 : 3, v/v)], incubated for 1 h at 53 °C and then discarded. The lipids were suspended in 50 μl of chloroform/methanol/water (5 : 4 : 1, by vol.), and then separated on silica gel 60 TLC plates (Merck) with chloroform/methanol/4.2 M ammonia (9 : 7 : 2, by vol.) as the solvent system. The TLC plates were sprayed with 10% copper sulphate in 8% orthophosphoric acid and then heated at 180 °C to visualize complex sphingolipids. Identification of each complex sphingolipid band was performed as described in a previous study (Uemura et al., 2014).

Table 1. Strains used in this study

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Lipid extraction and TLC analysis. Lipids were extracted from S. cerevisiae as described by Hanson & Lester (1980) with minor modification. Briefly, the cells (3 OD_{600} units) were suspended in 350 μl ethanol/water/diethyl ether/pyridine/15 M ammonia (15 : 15 : 5 : 1 : 0.018, by vol.), and then incubated at 65 °C for 15 min. The lipid extract was centrifuged at 10,000 g for 1 min and then extracted once more in the same manner. The resulting supernatants were dried and subjected to mild alkaline treatment using monomethylamine (MMA). For this reason, the lipid extracts were dissolved in 130 μM MMA [40% methanol solution/water (10 : 3, v/v)], incubated for 1 h at 53 °C and then discarded. The lipids were suspended in 50 μl of chloroform/methanol/water (5 : 4 : 1, by vol.), and then separated on silica gel 60 TLC plates (Merck) with chloroform/methanol/4.2 M ammonia (9 : 7 : 2, by vol.) as the solvent system. The TLC plates were sprayed with 10% copper sulphate in 8% orthophosphoric acid and then heated at 180 °C to visualize complex sphingolipids. Identification of each complex sphingolipid band was performed as described in a previous study (Uemura et al., 2014).

Quantification of sphingolipids by HPLC analysis. HPLC analysis of sphingolipids was performed as described elsewhere (Jenkins et al., 1997; Tani et al., 2006; Toume & Tani, 2014) with some modifications. Yeast cells (2 OD_{600} units) were collected by centrifugation, and then washed with distilled water. After the addition of 1 nmol sphingosine (d18 : 1) (Biomol) as an internal standard, lipids were extracted as described above. For acid hydrolysis, the lipids were dissolved in 500 μl of methanol/water (82 : 18, v/v) containing 1 M HCl, and then heated at 80 °C for 18 h. After the addition of 500 μl of 3 M NH_{4}OH, the hydrolysed LCBs were extracted twice with 500 μl of chloroform. The combined chloroform extracts were washed with 300 μl of 3 M NH_{4}OH three times, dried and then dissolved in 120 μl
of ethanol by heating at 67 °C for 25 min. The lipid solution was mixed with 15 μl of o-phthalaldehyde (OPA) reagent (1 mg of OPA, 20 μl of ethanol, 2 μl of 2-mercaptoethanol, and 1 ml of a 3 %, w/v, boric acid solution adjusted to pH 10.5), followed by incubation at room temperature for 30 min. Samples were centrifuged at 10 000 g for 5 min, and the resulting supernatants were resolved by HPLC on a pre-packed C18 reversed-phase column (Cosmosil 5C18-AR-II; Nacalai Tesque) using an isocratic eluent composition of acetonitrile/distilled water (90 : 10, v/v) and a flow rate of 1 ml min \(^{-1}\). The OPA derivatives were monitored at an excitation wavelength of 340 nm and an emission wavelength of 435 nm. The areas of peaks of LCBs [phytosphingosine (PHS) (t18 : 0 and t20 : 0) and DHS (d18 : 0)] were determined using sphingosine as an internal standard.

**Yeast protein extraction, SDS-PAGE and Western blotting.**

Protein extraction, SDS-PAGE and Western blotting were performed as described previously (Tani & Kuge, 2010). Anti-HA (Sigma), anti-FLAG (Stratagene) and anti-Pgk1 (Molecular Probes) were used as primary antibodies. Horseradish peroxidase-conjugated anti-mouse IgG (Molecular Probes) was used as the secondary antibody.

**RESULTS**

**Loss of vacuolar ATPase causes drastic changes of the complex sphingolipid composition**

It was previously reported that deletion of ELO3 or ORM1 and ORM2 causes a reduction in the activity of V-ATPase (Chung et al., 2003; Finnigan et al., 2011). ELO3 is involved in synthesis of very long-chain fatty acids, which are incorporated into sphingolipids (Oh et al., 1997), and ORM1 and ORM2 encode negative regulators of serine palmitoyltransferase, which catalyses the initial step of de novo sphingolipid biosynthesis (Breslow et al., 2010), suggesting a functional connection between sphingolipids and V-ATPase. To investigate the effects of the loss of V-ATPase on the growth defect caused by aberrant metabolism of sphingolipids, a deletion mutant of VMA2 encoding the V1 subunit B of V-ATPase was treated with myriocin and aureobasidin A. Myriocin is an inhibitor of serine palmitoyltransferase. Aureobasidin A is an IPC synthase inhibitor, which causes reductions in complex sphingolipid levels and increases in ceramide levels. As reported elsewhere, the growth of vma2Δ cells was hardly observed on YPD plates buffered to pH 7.6 (Li & Kane, 2009) (Fig. 2a); however, vma2Δ cells exhibited slow growth at pH 7.2 (Fig. 2a). On YPD plates buffered to both pH 5.5 and 7.2, vma2Δ cells exhibited a more severe growth defect with myriocin and aureobasidin A as compared with wild-type cells (Fig. 2b), suggesting that the deletion of VMA2 causes enhancement of the growth defect with aberrant metabolism of sphingolipids. To determine the compositions of complex sphingolipids in wild-type and vma2Δ cells incubated in YPD buffered to pH 5.5, 7.2 and 7.6, lipids were extracted, and complex sphingolipids were visualized with a copper sulphate and orthophosphoric acid reagent (Fig. 3a, b) (Uemura et al., 2014). Interestingly, drastic differences were observed between wild-type and vma2Δ cells. That is, the deletion of VMA2 caused: (i) a reduction in the IPC levels under all pH conditions; (ii) an increase in the MIPC levels, especially at pH 7.2 and 7.6; (iii) an increase in the M(IP)2C levels under all pH conditions; and (iv) increases in A- and B/B'-type complex sphingolipids. It should be

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**Fig. 2.** Effects of sphingolipid biosynthesis inhibitors on the growth of V-ATPase-deleted mutants. (a) Wild-type and vma2Δ cells were cultured overnight in YPD medium, and then spotted onto agar plates containing YPD medium buffered to pH 5.5, 7.2 or 7.6 in 10-fold serial dilutions starting with a density of 0.7 OD\(_{600}\) units ml\(^{-1}\). (b) Cells were spotted onto agar plates containing YPD medium buffered to pH 5.5 or 7.2 with or without the indicated concentrations of myriocin and aureobasidin A as described in (a). All plates were incubated at 30 °C and photographed after 2 days. The details are given in Methods.
Fig. 3. TLC analysis of complex sphingolipids of V-ATPase-deleted mutants. (a) Wild-type and vma2Δ cells were cultured overnight in YPD medium, diluted (0.3 OD600 units ml⁻¹) in fresh YPD medium buffered to pH 5.5, 7.2 or 7.6, and then incubated for 5 h. Lipids (3 OD600 units) were extracted, treated with MMA and then separated by TLC. The lipids were visualized.
with a copper sulphate and orthophosphoric acid reagent. The asterisk indicates unidentified bands. The details are given in Methods. (b) Complex sphingolipids [IPCs, MIPCs (plus IPC-D) and M(IP)2Cs] were quantified with ImageJ software (National Institutes of Health). The amount of IPCs in wild-type cells cultured at pH 5.5 was taken as 1. Data represent means ± SD from at least three independent experiments. (c) Time-course of changes in the complex sphingolipid composition in vma2 Δ cells after replacement of YPD with fresh YPD buffered to pH 7.2. vma2 Δ cells were cultured overnight in YPD medium, diluted (1 OD600 units ml⁻¹) in fresh YPD medium and then incubated for 5 h. The cells were resuspended in fresh YPD medium buffered to pH 7.2 or 5.5 to 0.7 OD600 units ml⁻¹ and then incubated for the indicated times. Lipids (3 OD600 units) were extracted and analysed by TLC as described in (a). (d) Wild-type, ccc2 Δ, vma2 Δ and ccc2 Δ vma2 Δ cells were cultured overnight in YPD medium, diluted (0.3 OD600 units ml⁻¹) in fresh YPD medium buffered to pH 7.2, and then incubated for 5 h. Lipids (3.7 OD600 units) were extracted and analysed by TLC as described in (a). (e) TLC analysis of the complex sphingolipids of wild-type, vma2 Δ, vma3 Δ, vma21 Δ, stv1 Δ and vph1 Δ cells.

noted that wild-type cells incubated at pH 7.2 and 7.6 also exhibited a decrease in the IPC levels and an increase in the MIPC levels as compared with ones incubated at pH 5.5. However, the changes were not as drastic as compared with the vma2 Δ cells (Fig. 3a, b). Fig. 3(c) shows the time-course dependency of changes in complex sphingolipid composition in vma2 Δ cells after replacement of YPD with fresh YPD buffered to pH 7.2. The increase in the MIPC levels was clearly observed after 5 h but not 1 h after the replacement. The Rf values of IPC-D and MIPC-A on the TLC plate overlapped under these experimental conditions (Uemura et al., 2003). Thus, we examined the complex sphingolipid composition with deletion of CCC2, which is required for the synthesis of D-type complex sphingolipids (Beeler et al., 1997). As shown in Fig. 3(d), the deletion of CCC2 caused disappearance of the band corresponding to MIPC-A/IPC-D for wild-type cells; however, the deletion did not have a significant effect in vma2 Δ cells, indicating that the observed band of MIPC-A/IPC-D for vma2 Δ cells is mostly MIPC-A. To confirm that loss of V-ATPase causes alteration of the complex sphingolipid composition, genes encoding other components of V-ATPase were deleted. VMA3 and VMA21 encode the V₀ subunit c of V-ATPase and a protein required for assembly of the complex of V-ATPase, respectively. As shown in Fig. 3(e), vma3 Δ and vma21 Δ cells exhibited alteration of the complex sphingolipid composition like vma2 Δ ones, supporting the notion that this change is caused by loss of V-ATPase. VPH1 and STV1 encode the V₀ subunit α of V-ATPase localized in vacuoles and Golgi/endosomes, respectively (Kawasaki-Nishi et al., 2001). The alteration of the complex sphingolipid composition in vph1 Δ cells is more drastic than that in stv1 Δ cells (Fig. 3e), indicating that loss of vacuole-localized V-ATPase, but not Golgi/endosome-localized V-ATPase, is a major cause of alteration of the complex sphingolipid composition.

Changes in the hydroxylation level of the ceramide moiety of complex sphingolipids on loss of V-ATPase

Increases in A- and B/B'-type complex sphingolipids in V-ATPase-deleted cells are suggestive of decreases of hydroxylation of both LCBs and fatty acids in the ceramide moiety. To confirm the hydroxylation state of LCBs, cellular sphingolipids were hydrolysed with methanolic HCl, and the resultant free LCBs were derivatized with OPA and then analysed by HPLC. As reported previously (Toume & Tani, 2014), peaks of C18-PHS (PHS18), C20-PHS (PHS20) and C18-DHS (DHS18) were observed on the HPLC analysis (Fig. 4a). Fig. 4(b) shows the quantification results for LCBs in each strain. The PHS18 and PHS20 levels in vma2 Δ cells were significantly reduced as compared with that in wild-type cells. However, the DHS18 level increased in vma2 Δ cells. A significant difference between cells incubated at pH 5.5 and 7.2 was not observed (Fig. 4b). These results indicated that the deletion of VMA2 causes a change in the ratio of DHS- and PHS-based sphingolipids. To examine the hydroxylation of the fatty acid moiety of sphingolipids, SUR2 was deleted in vma2 Δ cells. With the loss of hydroxylation of LCBs caused by the deletion of SUR2, the most predominant species of complex sphingolipid in VMA2-deleted cells were of the A-type, which contains DHS and non-hydroxylated fatty acids (Fig. 4c), indicating a decrease in hydroxylation of fatty acids in complex sphingolipids. Collectively, these results indicated the hydroxylation levels of both LCBs and fatty acids in sphingolipids decreased due to the loss of V-ATPase.

Changes in the expression levels of enzymes involved in the synthesis of complex sphingolipids

The increase in MIPC and M(IP)2C levels, the decrease in IPC levels, and the decrease in hydroxylation of the ceramide moiety of complex sphingolipids in V-ATPase-deleted mutants suggests the possibility of changes in the expression levels of IPC, MIPC and M(IP)2C synthases, and sphingolipid hydroxylases. Thus, we next examined the expression levels of Aur1 (IPC synthase), Sur1 (primary MIPC synthase), Ipt1 ([M(IP)2C synthase], Ssc7 (sphingolipid C-2 hydroxylase) and Sur2 (sphingolipid C-4 hydroxylase) by Western blotting. The chromosomal AUR1 and SUR1 were tagged with 6 × HA at the C-terminus in wild-type and vma2 Δ cells, and the proteins were detected with an anti-HA antibody. The chromosomal IPT1, SUR2 and SCS7 were tagged with 3 × FLAG at the N-terminus without changing the potential promoter region, and the proteins were detected with an anti-FLAG antibody. As shown in Fig. 5, the expression level of

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Fig. 4. Hydroxylation of the ceramide moiety of sphingolipids in V-ATPase-deleted mutants. (a) HPLC analysis of LCBs derived from total cellular sphingolipids. Wild-type and vma2Δ cells were cultured overnight in YPD medium, diluted (0.3 OD600 units ml⁻¹) in fresh YPD medium buffered to pH 5.5 or 7.2, and then incubated for 5 h. Lipids (2 OD600 units) were extracted, hydrolysed with methanol/HCl, derivatized with OPA and then analysed by reversed-phase HPLC. Sphingosine (d18 : 1) was added as an internal standard. (b) The area of each peak of LCB in (a) was determined using sphingosine and (c) Relative amount of sphingolipids. (d) The area of each peak of LCB in (a) was determined using sphingosine and (e) Relative amount of sphingolipids.
Aur1-6 × HA was decreased to approximately 36% in vma2A cells incubated at pH 7.2 compared to pH 5.5. On the contrary, the expression level of Sur1-6 × HA was significantly increased in vma2A cells incubated at pH 7.2 compared to pH 5.5. At pH 7.2, the expression level of Sur1-6 × HA in wild-type cells was increased to approximately 33% as compared with that in cells incubated at pH 5.5. The expression level of 3 × FLAG-Ipt1 in wild-type cells incubated at pH 7.2, and vma2A cells incubated at pH 5.5 and 7.2 was significantly increased as compared with that in wild-type cells incubated at pH 5.5. The results of an increase in Sur1-6 × HA and 3 × FLAG-Ipt1 may explain the increases in the levels of MIPCs and M(IP)2Cs in vma2A cells. The expression levels of 3 × FLAG-Scs7 and 3 × FLAG-Sur2 were not changed under all experimental conditions (Fig. 5).

**Growth defect of V-ATPase-deleted cells caused by loss of MIPC synthesis**

To investigate the importance of MIPC and M(IP)2C synthesis in V-ATPase-deleted cells, SUR1 and CSH1, or IPT1 were deleted. In both wild-type and vma2A cells, the deletion of SUR1 and CSH1 caused loss of MIPCs and M(IP)2Cs, and accumulation of IPCs (Fig. S1). In both ipt1A and ipt1A vma2A cells, loss of M(IP)2Cs and accumulation of IPCs were observed (Fig. S1). Fig. 6(a) shows the growth of each strain under pH 5.5 and 7.2 conditions. Significant differences in growth pattern between wild-type, sur1A csh1A and ipt1A cells were not observed under these experimental conditions; however, sur1A csh1A vma2A cells did not grow at pH 7.2. The growth of ipt1A vma2A cells at pH 7.2 was only slightly delayed as compared with that of vma2A cells (Fig. 6a). It was previously reported that some abnormal phenotypes found in MIPC synthesis-deficient mutants are caused by accumulation of IPC-C, but not by loss of MIPC itself. For example, the Ca2+ sensitive phenotype of csg2A cells is suppressed by mutation of SUR2, SCS7, LCB1, LCB2, TSC3 or TSC10, all of which suppress the accumulation of IPC-C (Beeler et al., 1998; Zhao et al., 1994). Thus, we investigated whether or not the growth defect of sur1A csh1A vma2A cells at pH 7.2 is suppressed by the deletion of SUR2 or SCS7, either of which causes loss of IPC-C (Haak et al., 1997; Uemura et al., 2014). As shown in Fig. 6(b), sur1A csh1A vma2A sur2A and sur1A csh1A vma2A scs7A cells did not grow at pH 7.2, like sur1A csh1A vma2A cells, indicating that the growth defect caused by loss of MIPC synthesis and V-ATPase is not caused by the accumulation of IPC-C. Overall, it was suggestive that loss of total mannosyl-containing complex sphingolipids caused the strong growth defect of V-ATPase-deleted mutants under neutral pH conditions, whereas depletion of M(IP)2C alone did not.

**Effects of increases and decreases in the hydroxylation levels of sphingolipids on the growth of V-ATPase-deleted cells**

To investigate the effects of changes in the hydroxylation level of sphingolipids on the growth of V-ATPase-deleted cells, Scs7 and Sur2 were overexpressed in vma2A cells. For the overexpression, the promoter regions of chromosomal SCS7 and SUR2 were substituted with a strong constitutive TEF promoter (TEFp-SCS7 and TEFp-SUR2). The overexpression of Scs7 and Sur2 with the TEF promoter was confirmed by tagging with 3 × FLAG at the N-terminus of Scs7 and Sur2 (Fig. S2a). As shown in Fig. S2(b), the overexpression of Scs7 in vma2A cells caused a decrease in B/B’-type and an increase in C-type complex sphingolipids as compared with in vma2A cells. In TEFp-SUR2 vma2A cells, A-type complex sphingolipids were decreased as compared with in vma2A cells. The effects of both the overexpression of Sur2 and Scs7 were observed in TEFp-SCS7 TEFp-SUR2 vma2A cells (Fig. S2b). These results indicated increases in the levels of hydroxylation of the complex sphingolipids in vma2A cells on the overexpression of Scs7 and/or Sur2. The growth of wild-type, TEFp-SCS7, TEFp-SUR2 and TEFp-SCS7 TEFp-SUR2 cells was indistinguishable at pH 5.5 and 7.2 (Fig. 6c). At pH 7.2, the growth of TEFp-SCS7 vma2A cells appeared to be slightly delayed as compared with that of vma2A cells; however, a clear difference was observed between TEFp-SCS7 TEFp-SUR2 vma2A and vma2A cells (Fig. 6c). Thus, the results were indicative that the overexpression of Scs7 and Sur2 causes enhancement of the growth defect of V-ATPase-deleted cells under neutral pH conditions. Next, to examine the effects of loss of hydroxylation of the LCBs and fatty acids in vma2A cells, sur2A vma2A, scs7A vma2A and sur2A scs7A vma2A cells were used. As shown in Fig. S3, in both scs7A vma2A and sur2A vma2A cells, C-type complex sphingolipids were not detected. sur2A scs7A vma2A cells contained only A-type complex sphingolipids (Fig. S3). The growth rates of sur2A vma2A and sur2A scs7A vma2A cells on YPD plates buffered to pH 7.2 seemed to be slightly increased as compared with that of vma2A cells (Fig. S4). In liquid culture, growth rates of sur2A vma2A, scs7A vma2A and sur2A scs7A vma2A cells at pH 7.2 and 7.6 were significantly increased as compared with that of vma2A cells.
Fig. 5. Protein expression levels of sphingolipid-metabolizing enzymes. Cells were cultured overnight in YPD medium, diluted (0.3 OD₆₀₀ units ml⁻¹) in fresh YPD medium buffered to pH 5.5 or 7.2, and then incubated for 5 h. Yeast cell extracts were
immunoblotted using anti-FLAG, anti-HA or anti-Pgk1 antibody. Sur1-6 × HA gave two bands, the upper band being the N-glycosylated form (Uemura et al., 2007). The asterisks indicate unidentified bands. The relative amount of each enzyme was determined with ImageJ software (National Institutes of Health). The amount of Aur1-6 × HA, Sur1-6 × HA, 3 × FLAG-Ipt1, 3 × FLAG-Sur2 or 3 × FLAG-Scs7/Pgk1 in wild-type cells incubated at pH 5.5 was taken as 1. Data represent means ± SD from at least three independent experiments. The details are given in Methods.

(Fig. 6d). The growth rates of sur2Δ vma2Δ and sur2Δ scs7Δ vma2Δ cells at pH 5.5 were higher than that of vma2Δ cells after 10 h culture (Fig. 6d). Taken together, these results indicated that enhancement of the hydroxylation of LCBs and fatty acids in the ceramide moiety causes a decrease in the growth rate of V-ATPase-deleted mutants at neutral pH, whereas loss of the hydroxylation causes an increase in growth rate.

Effects of mutation of sphingolipid-metabolizing enzymes on typical phenotypes of V-ATPase-deleted cells

The deletion of V-ATPase in S. cerevisiae causes various abnormal phenotypes including supersensitivities to metal ions and oxidative stress, due to a defect of vacuolar proton homeostasis (Eide et al., 1993; Li & Kane, 2009; Milgrom et al., 2007). Thus, we investigated whether or not loss of MIPC synthesis or a change in the hydroxylation levels of sphingolipids affects the typical phenotypes of V-ATPase-deleted cells. As shown in Fig. 7(a), the sensitivities to Zn2+, Ca2+ and H2O2 of vma2Δ cells were enhanced by the deletion of SUR1 and CSH1. sur2Δ scs7Δ vma2Δ cells were relatively resistant to Zn2+ as compared with vma2Δ cells, whereas the sensitivity to Zn2+ of vma2Δ cells was slightly increased by the overexpression of SCS7 and SUR2 (vma2Δ versus TEFp-SCS7 TEFp-SUR2 vma2Δ cells). A clear difference in sensitivities to Zn2+, Ca2+ and H2O2 was observed between sur2Δ scs7Δ vma2Δ and TEFp-SCS7 TEFp-SUR2 vma2Δ cells (Fig. 7a). These results suggested that the loss of MIPC synthesis and the change in the hydroxylation levels in sphingolipids can broadly affect the phenotypes caused by the deletion of V-ATPase. As reported previously, sur1Δ csh1Δ cells exhibited supersensitivity to Ca2+, and the sensitivity was rescued by the deletion of SUR2 or SCS7 (Beeler et al., 1998; Zhao et al., 1994) (Fig. 7a, b). However, the supersensitivity to Ca2+ of sur1Δ csh1Δ vma2Δ cells was not rescued by the deletion of SUR2 or SCS7 (Fig. 7b), suggesting that the cause of the supersensitivity to Ca2+ of sur1Δ csh1Δ vma2Δ cells is different from that of sur1Δ csh1Δ cells.

DISCUSSION

In the present study, we showed that loss of V-ATPase causes dramatic alteration of the complex sphingolipid composition, which includes decreases in the hydroxylation levels in the ceramide moiety, and a decrease in the IPC levels, and increases in the MIPC and M(IP)2C levels (Figs. 3 and 4). In particular, the MIPC levels in V-ATPase-deleted cells were markedly increased when the cells were incubated at pH 7.2 and 7.6 (Fig. 3a, b). It should be noted that wild-type cells incubated at pH 7.2 and 7.6 also exhibited the increase in MIPC levels as compared with ones incubated at pH 5.5; however, the increase rate was lower than that in V-ATPase-deleted mutants (Fig. 3a, b). In V-ATPase-deficient mutants, the vacular pH increases with increasing extracellular pH; that is, the vacular pH is approximately 5.9 when the cells are cultured at pH 5.5, whereas it increases to approximately 7 when the pH of culture medium is switched to 7.5 (Plant et al., 1999). The impaired acidification of vacuoles is thought to be one of the major causes of the growth inhibition of V-ATPase-deficient mutants at neutral pH (Li & Kane, 2009). Even in wild-type cells, the vacular pH can vary slightly depending on the extracellular pH; that is, the vacular pH increases from approximately 5.5 to 5.9 when the pH of the culture medium is switched from 5.5 to 7.5 (Plant et al., 1999). Thus, it is likely that the increase in the MIPC levels is induced by the impaired acidification of vacuoles.

The increase in the MIPC levels was suggested to be important for maintenance of the growth of V-ATPase-deleted mutants, because growth of sur1Δ csh1Δ vma2Δ cells, which lack MIPCs and M(IP)2Cs, was hardly observed when cells were cultured at pH 7.2 (Fig. 6a). It should be noted that IPC levels were increased by the loss of MIPC synthesis (Fig. S1). The Ca2+ supersensitivity of MIPC synthesis-deficient mutants is caused by the accumulation of IPC-C (Beeler et al., 1998; Zhao et al., 1994), and the cell death under nitrogen starvation due to the loss of MIPC synthesis is rescued by the deletion of SUR2 or myriocin, both of which prevent the accumulation of IPC-C (Yamagata et al., 2013). However, the growth defect of sur1Δ csh1Δ vma2Δ cells at pH 7.2 was not suppressed on the deletion of SUR2 or SCS7 (Fig. 6b). Furthermore, vma2Δ cells exhibited supersensitivity to myriocin and aureobasidin A, which causes reductions in all complex sphingolipid levels including that of IPCs and MIPCs (Fig. 2). Thus, it is suggested that the cell-growth defect caused by the loss of MIPC synthesis is caused by loss of MIPC, not by accumulation of IPCs. In contrast, ipt1Δ vma2Δ cells, which lack only M(IP)2C, can grow at pH 7.2 (Fig. 6a), suggesting the importance of MIPC, but not M(IP)2C, in V-ATPase-deleted mutants. In addition, increases in the hydroxylation of LCBs and fatty acids in the ceramide moiety of sphingolipids caused by overexpression of Scs7 and Sur2 enhanced the growth defect of V-ATPase-deleted mutants at pH 7.2 (Fig. 6c). On the contrary, the deletion of SCS7 and/or SUR2 partly rescued the growth of V-ATPase-deleted...
Fig. 6. Effects of deletion and overexpression of sphingolipid-metabolizing enzymes on the growth of V-ATPase-deleted mutants. (a, b, c) Cells were cultured overnight in YPD medium and then spotted onto agar plates containing YPD medium buffered to pH 5.5 or 7.2 in 10-fold serial dilutions starting with a density of 0.7 OD₆₀₀ units ml⁻¹. All plates were incubated at 30 °C and photographed after 2 days. (d) Cell growth of vma2Δ, scs7Δ vma2Δ, sur2Δ vma2Δ and sur2Δ scs7Δ vma2Δ cells in liquid cultures. Cells were cultured overnight in YPD medium and then diluted (0.07 OD₆₀₀ units ml⁻¹) in fresh YPD medium buffered to pH 5.5, 7.2 or 7.6, and aliquots of cell suspensions were subjected to cell density measurement (OD₆₀₀) at the indicated times. Data represent means ± SD from one experiment (triplicate) representative of three independent experiments.
mutants (Fig. 6d). In addition, supersensitivities to Ca\(^{2+}\), Zn\(^{2+}\) and H\(_2\)O\(_2\), which are typical phenotypes of V-ATPase-deleted cells, were affected by the loss of MIPC synthesis and the change in the hydroxylation levels in sphingolipids (Fig. 7a). Collectively, these results strongly suggested the possibility that the increase in the MIPC levels and decreases in the hydroxylation levels in the ceramide moiety, which are observed in V-ATPase-deleted mutants, suppress the phenotypes caused by the deletion of V-ATPase, which is indicative that alteration of the complex sphingolipid composition is one of the adaptation mechanisms for a defect of V-ATPase.

Several lines of evidence indicated sphingolipids have important roles in the maintenance of activity of V-ATPase. For example, in *S. cerevisiae*, the proton-pump activity of V-ATPase decreases on deletion of *ELO3* or *ORM1* and *ORM2*, all of which are involved in sphingolipid biosynthesis (Chung et al., 2003; Finnigan et al., 2011). In addition, impaired biosynthesis of ergosterol also causes a reduction in V-ATPase activity (Zhang et al., 2010). Since ergosterol together with complex sphingolipids is involved in the formation of lipid microdomains (Simons & Sampaio, 2011), it is likely that proper formation of lipid microdomains is required for the maintenance of V-ATPase activity. Indeed, it was reported that complex sphingolipids are distributed in vacuoles, as well as plasma membranes and the Golgi, in *S. cerevisiae* (Hechtberger et al., 1994), and V-ATPase is associated with lipid microdomains in baby hamster kidney cells and *Arabidopsis* (Lafourcade et al., 2008; Yoshida et al., 2013), supporting the notion of the importance of lipid microdomains in the maintenance of V-ATPase activity. In *Schizosaccharomyces pombe*, overexpression of ceramide synthase rescues the defect in vacuolar acidification in the absence of a regulator protein of V-ATPase, in which V-ATPase activity is not completely abolished (Dawson et al., 2008). However, the overexpression of ceramide synthase does not restore the defect of vacuolar acidification in V-ATPase-deleted mutants, which indicates that the enhancement of ceramide synthesis cannot rescue the phenotypic defect when the activity of V-ATPase is completely abolished (Dawson et al., 2008). In this study, we found that deletion of *VMA2*, *VMA3* or *VMA21*, which causes complete loss of V-ATPase activity (Li & Kane, 2009), causes a dramatic alteration of the complex sphingolipid composition (Fig. 3e). This implies that complex sphingolipids play some important roles even under circumstances in which V-ATPase is absent. It should be noted that a recognizable difference in ergosterol levels was not observed between wild-type and vma2A cells when...
ergosterol was separated by TLC and visualized with a copper sulphate and orthophosphoric acid reagent (data not shown).

Very recently, it was reported that the loss of hydroxylation of both the C-4 position of LCBs and the C-2 position of fatty acids of the ceramide moiety affects membrane fluidity and lateral diffusion of membrane-bound proteins in *S. cerevisiae* (Uemura et al., 2014). Furthermore, loss of the C-4 hydroxylation influences the physical and structural properties of lipid microdomains in *S. cerevisiae* (Idkowiak-Baldys et al., 2004). In addition, studies involving ceramide monolayers revealed that both the C-4 and C-2 hydroxylations promote condensation of the lipid lateral packing, probably due to enhancement of hydrogen-bonding interaction among ceramides, which indicates that these hydroxylations affect the structural integrity of membranes (Lofgren & Pascher, 1977). Thus, collectively, it is suggested that hydroxylation of the ceramide moiety significantly affects the properties of membranes, especially lipid microdomains. In addition, in human erythrocytes, the membrane fluidity is significantly affected by a change in extracellular pH (Yamaguchi et al., 1982). Although it remains unclear why decreases and increases in the hydroxylation levels influence the phenotypes of V-ATPase-deleted mutants, it would be interesting to know the relationship between membrane fluidity and the growth defect due to impaired intracellular proton homeostasis. Thus, investigations as to effects of the alteration of the complex sphingolipid composition on the properties of membranes, especially lipid microdomains, in V-ATPase-deleted mutants are required in the future. In addition to the effects of complex sphingolipids on the membrane properties, numerous studies have also demonstrated the roles of sphingolipids as signalling molecules (Dickson et al., 2006; Dickson, 2008; Montefusco et al., 2014). For example, MIPC stimulates Fpk1 and Fpk2 kinases, and subsequently regulates aminophospholipid flippases (Roelants et al., 2010). Thus, it is also possible that certain species of complex sphingolipid act on specific signalling proteins and compensate for the loss of V-ATPase.

In summary, the present study indicated that loss of V-ATPase causes dramatic alteration of the complex sphingolipid composition. In addition, our data also were suggestive of the possibility that the alteration suppresses the phenotypes caused by loss of V-ATPase. These results indicate the importance of the detailed structural properties of complex sphingolipids in the absence of V-ATPase. Further detailed investigation of this molecular mechanism will provide new insights into the physiological significance of the structural complexity of complex sphingolipids.

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