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Deletion of *OSH3* gene confers resistance against ISP-1 in *Saccharomyces cerevisiae*

Tatsuya Yano, Masatoshi Inukai, and Fujio Isono*

Lead Discovery Research Laboratories, SANKYO CO., LTD., 2-58, Hiromachi 1, Shinagawa-Ku, Tokyo, Japan

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Abstract

Sphingolipids have been reported to regulate the growth and death of mammalian and yeast cells, but their precise mechanisms are unknown. In this paper, it was shown that the deletion of the oxysterol binding protein homologue 3 (OSH3) gene confers hyper resistance against ISP-1, an inhibitor of sphingolipid biosynthesis, in the yeast *Saccharomyces cerevisiae*. Furthermore, the overexpression of the *ROK1* gene, which directly binds to Osh3p, conferred resistance against ISP-1, and the deletion of the *KEM1* gene, which regulates microtubule functions, exhibited ISP-1 hypersensitivity. And yet, an ISP-1 treatment caused an abnormal mitotic spindle formation, and the ISP-1-induced cell cycle arrest was rescued by the deletion of the *OSH3* gene. Taken together, it is suggested that the expression levels of the *OSH3* gene influence the ISP-1 sensitivity of *S. cerevisiae*, and the sphingolipids are necessary for normal mitotic spindle formation in which the Osh3p may play a pivotal role.

Keywords: Sphingolipid; ISP-1; OSH3; ROK1; KEM1; Mitotic spindle formation; Cell cycle

Recently, many papers showed that sphingolipids act as second messengers that mediate cell growth and death in mammalian and yeast cells [1–4]. In yeast, long chain bases or their phosphorylated derivatives transiently accumulate in response to heat-shock stress [5–8] and regulate the cell cycle progression through unknown pathways in the yeast *Saccharomyces cerevisiae* [9,10], suggesting that sphingolipids not only exist as the constituents of the membrane, but may also have important roles as a regulator of yeast cell growth.

ISP-1 is a strong inhibitor of serine palmitoyl transferase (SPT), which catalyzes the first step of sphingolipid biosynthesis [11]. ISP-1 induces cell death in mouse cytotoxic T cells and rat Purkinje cells, and also inhibits the growth and synthesis of the yeast *S. cerevisiae* by sphingolipid depletion [12–14]. However, their precise mechanism is still obscure.

OSH3 is one of the seven yeast homologues of the oxysterol binding protein (OSBP), which was originally discovered as a cytosolic oxysterol binding protein and regulator of cholesterol biosynthesis in mammals [15–19]. These genes have a conserved oxysterol binding domain at the carboxy-terminal end. Among them, *OSH1*, 2, and 3 have the pleckstrin homology domains at their amino-terminal end and form a long protein group in the *OSH* gene family [20,21]. Recently, it was shown that Osh3p directly binds to the ATP-dependent DEAD-box protein Rok1 and that an overexpression of the *OSH3* gene enhances *kem1* mutation [22]. The *ROK1* gene was discovered as a high-copy number plasmid suppressor of the *kem1* mutation that enhanced *kar1* mutation [22–24]. The *kar1* mutation was reported to induce a spindle pole body (SPB) duplication defect and chromosome disjunction in mitosis [25–27]. The Kem1p also associated with microtubule functions such as chromosome transmission, nuclear migration, and SPB duplication [24,28]. These results suggest that *OSH3* may participate in microtubule-related functions together with the functions of *KAR1, KEM1*, and *ROK1* genes.

In this report, we showed that the expression level of the *OSH3* gene affected the ISP-1 sensitivity of budding yeast and the sphingolipid depletion by ISP-1 disturbed the mitotic spindle formation and nuclear division. Our data suggest that Osh3p may participate in the

* Corresponding author. Fax: +81-3-5436-8561.
E-mail address: isonof@shina.sankyo.co.jp (F. Isono).

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regulation of downstream events of the sphingolipid depletion by ISP-1, such as the mitotic spindle formation.

Materials and methods

Strains, media, and growth conditions. Yeast Deletion Clones and wild type strain BY4741 (MATa *his*Δ*1 leu2*Δ*0 met15*Δ*0 ura3*Δ*0*) were purchased from Invitrogen. All yeast transformants were constructed using wild type strain YPH250 (MATa *ura3-52 lys2-801 ade2-101 trp1-*Δ*1 his3-*Δ*200 leu2-*Δ1), Minimal (SD), and synthetic complete (SC) liquid media and agar plates were prepared as previously described [29]. Selections of yeast transformants were carried out using the SC plates containing appropriate supplements (Clonetech). Transformants carrying expression vectors with *GAL* promoters were cultured in the SC liquid or agar media containing 2% galactose/raffinose and supplements to induce the expression of genes. To obtain a synchronized culture, exponentially growing cells were further cultivated for 4–6 h in 4 μg/ml of α-mating factor (Wako Chemical) containing media. The cells were released by washing with fresh media. For the growth phenotype assays, 5 μl of the 5-fold serial dilutions of each strain was spotted on to the SC or SD plates containing different concentrations of ISP-1 (BioMol) and aureobasidin A (TaKaRa).

Cloning and plasmid constructions. Genetic manipulations were done as previously described [30]. PCRs were carried out for 20 cycles by using Plutinum *Taq* High Fidelity Polymerase (Invitrogen). Osh3p and Rok1p were expressed using the 2 μm-based vectors with the *GAL* promoters, pYES2.1TOPO, pYES3 (Invitrogen), and pESC-HIS (Novagen), or the CEN6/ARSH4-based vector pYC2 (Invitrogen). To generate a high-copy number plasmid containing an *OSH3* gene, *OSH3* was cloned by PCR with the yeast genomic DNA from the YPH250 strain using primers OSH3-F (5′-ATGGAAACAATTG ATATACAAAATCGATCA-3′) and OSH3-R (5′-TAACCAGAGT TGAGAAATATCAGACCAATC-3′). The PCR fragment was introduced into the pYES2.1TOPO to yield pYES2OSH3. To construct the high-copy number plasmid containing the *ROK1* gene, the *ROK1* gene was amplified by PCR with primers 5′-GGGGTACCCCATGGAT ATTTTTAGAGTATTAACTAGAGGA-3′ and 5′-CCGCTCGAG CGGTAATTTCGAG AAATGTTTTTTTGAAAGTTC-3′. The PCR product was digested with *Kpn*I and *Xho*I and introduced into the multicloning site of pYES3 to obtain pYES3ROK1. The high-copy number plasmid pESCROK1 was constructed by PCR amplification of the *ROK1* gene with primers 5′-GGAATTCCATGGATATTTTT AGAGTATTAACTAGAGGA-3′ and 5′-GACTAGTCTCGAGAAA TGTTTTTTTGAAGTTC-3′, and insertion of the PCR product into the multicloning site of pESC-HIS after digestion with *Eco*RI and *Spe*I.

Analysis of sphingolipid biosynthesis. The wild type strain YPH250 was grown to saturation in the SD medium containing supplements and diluted to an A_{660} of 1.0 with fresh medium. Then, 5 ml aliquots were cultured for 2 h to bring the cells to an exponentially growing phase. Next, various concentrations of drugs were added. After incubation with the drugs for 30 min, the cultures were labeled with 10 μCi *myo*-[2-^{3}H]inositol (20 Ci/mmol, DuPont NEN) for 1 h. Cell pellets were obtained by centrifugation at 3000*g* for 10 min and washed with 2 ml ice-cold water. Total lipids were extracted from the pellets as previously reported [31]. The lipid extracts were dried under a N_2 gas flow and dissolved in 100 μl of diethylmethane:methanol (1:1, v/v). Then, aliquots of 20 μl were spotted on the silica-gel thin-layer chromatography plates (20 cm Whatman LK5) and developed with diethylmethane:methanol:acetic acid:water (16:6:4:1.6, v/v). Visualization and densitometric analysis of ^{3}H-labeled lipids were performed with BAS-2000 imaging analyzer (FUJI film). The respective sphingolipids were detected by comparing the TLC profiles of the wild type, *sur2*Δ, *cgs2*Δ, and *ccc2*Δ strain.

Cell cycle analysis. Exponentially growing BY4741 cells were arrested in the G1 phase with α-mating factor as described above. After releasing the cells from G1 arrest by washing 3 times with the drug in fresh SD medium, the cells were further cultured with various concentrations of drugs for 4 h. The cell pellets were obtained by centrifugation of 4 ml of cell culture. The cell pellets were immediately washed with ice-cold PBS and fixed with ethanol at 4 °C overnight. The fixed cells were rehydrated and washed with ice-cold PBS. The cells were treated with lyticase (Sigma) and 2 mg/ml RNase A overnight. The cell pellets were washed with PBS and stained with propidium iodide as previously described [32]. DNA content analysis was carried out on CytoAce-300 (JASCO).

Fluorescence microscopy. The exponentially growing BY4741 cells were arrested in the G1 phase and released as described above. Then, the cells were cultured with drugs in the SD medium for 4 h and processed for immunofluorescence as described by Guthrie and Fink [30]. Microtubules were observed using rat monoclonal anti-α-tubulin YOL1/34 (Halan-Sera Laboratory) as a primary antibody, and AlexaFluor 594-conjugated goat anti-rat IgG as a secondary antibody (Molecular Probes). The nuclei were stained with 10 μg/ml of 4′,6-diamidino-2-phenylindole (DAPI). All images were viewed with a 100× oil immersion or 40× objective lens on a Nikon Eclipse E800 microscope. Images were taken with a Hamamatsu C5810 3CCD camera and Image-Pro Plus software.

Cell viability assay. Dead cells were observed by staining the cells with 10 μM of SYTOX-GREEN (Molecular Probes) for 10 min at room temperature. Cell viabilities were calculated by dividing the counts of SYTOX-GREEN stained cells by the total cell number.

Results and discussion

*ISP-1 resistance of the S. cerevisiae osh3*Δ *strain*

ISP-1 completely inhibited the cell growth of budding yeast *S. cerevisiae*, with cell cycle arrest and cytokinesis defect [14]. In order to elucidate the inhibition mechanism of growth, we screened ISP-1-resistant strains using a Yeast Deletion Clone set. Among the mutants, *osh3*Δ mutant strain was found to grow at a concentration of 1.0 μg/ml of ISP-1, although the wild type yeast BY4741 was completely inviable at that concentration. As shown in Fig. 1A, the strains with deletion mutations in the *OSH* genes did not show any resistance against ISP-1 except the *osh3*Δ mutant strain. There was no difference between the wild type and *osh3*Δ strain in the sensitivity against 2.5 μg/ml aureobasidin A (AbA), an inhibitor of IPC synthase that catalyzes sphingolipid biosynthesis at a site downstream of SPT [33,34]. Even with 1.0 μg/ml AbA, significant differences were not observed between both strains, although the growth of the strains was partially inhibited (data not shown). As in Fig. 2, although 87% of the wild type cells were killed by a treatment with 1.0 μg/ml ISP-1 for 16 h, only 30% of the *osh3*Δ cells were killed. Furthermore, *osh3*Δ cells transfected with the *OSH3* gene cloned in a low-copy number plasmid (*osh3*Δ [pYCOSH3]) restored ISP-1 sensitivity, and the *OSH3*-overexpressing YPH250 strain (pYES2OSH3) showed hypersensitivity to ISP-1 (Fig. 1B). These results indicated that the ISP-1 sensitivity was influenced by the expression levels of *OSH3* gene, and this effect may be specific to the *OSH3* gene

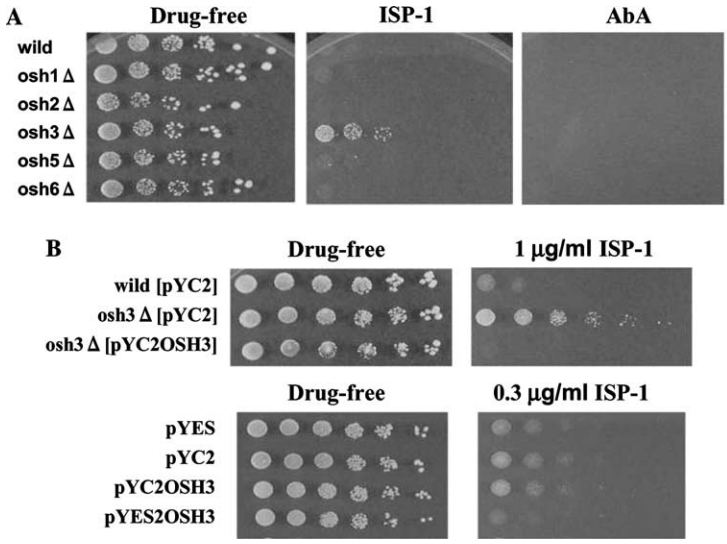

Fig. 1. *OSH3* expression level affects ISP-1 sensitivity of the budding yeast. (A) *osh3Δ* strain is resistant to ISP-1. Cell suspensions of deletion mutants of each *OSH* gene (*osh*1 ~ 3*Δ*, 5*Δ*, 6*Δ*) and the isogenic wild type strain (BY4741) were serially diluted and spotted on SD plated with no drug (drug-free), with 1 μg/ml ISP-1 (ISP-1) and 2.5 μg/ml aureobasidin A (AbA). (B) *OSH3* overexpression confers ISP-1 hypersensitivity. Growth of *osh3Δ* cells transfected with a low-copy number plasmid vector containing the *OSH3* gene (*osh3Δ*[pYC2OSH3]) was compared with that of the empty plasmid vector-transfected *osh3Δ* cells (*osh3Δ*[pYC2]) and BY4741 cells (wild[pYC2]) using SC–ura plates containing 1 μg/ml ISP-1 (upper panels). Cell suspensions of YPH250 strains transfected with the empty plasmid vectors (pYES2, pYC2), and low-copy number *OSH3* gene expression vector (pYC2OSH3) or high-copy number *OSH3* gene expression vector (pYES2OSH3) were serially diluted and spotted on SC–ura plates containing galactose/raffinose as the carbon source without drug (drug-free) or with 0.3 μg/ml ISP-1 (0.3μg/ml ISP-1) (lower panels).

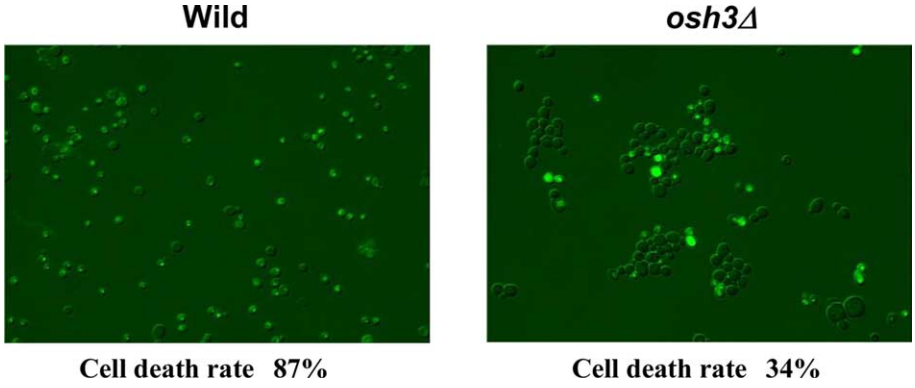

Fig. 2. ISP-1-induced cell death is suppressed by *osh3Δ* mutation. The *osh3Δ* mutant (osh3*Δ*) and isogenic wild type strain BY4741 (wild) were cultured with 0.3 μg/ml ISP-1 in SD medium for 16 h. Dead cells were visualized as fluorescing cells after SYTOX-GREEN staining. Nomarski (DIC) images viewed with a 40× objective lens are shown. Cell death rates are indicated as the mean values of triplicate determinations.

among the *OSH* family. Furthermore, it was also suggested that the inhibition mechanisms of growth by ISP-1 and AbA are different, although both drugs deplete the major types of sphingolipids, such as IPC, MIPC, and $M(IP)_2C$.

ISP-1 inhibits sphingolipid biosynthesis in the osh3Δ and wild type strain with the same potency

Next, the inhibitory activities of ISP-1 against sphingolipid biosynthesis were compared between the wild type and *osh3Δ* strain. Exponentially growing cells were cultured with various concentrations of ISP-1 and [^{3}H] inositol for 60 min. In both strains, the synthesis of inositolphosphorylceramide (IPC), mannosylinositol-phosphorylceramide (MIPC), and mannosyldiinositol-phosphorylceramide ($M(IP)_2C$), the final products of sphingolipid biosynthesis, was completely abolished with 0.3 μg/ml ISP-1 (Fig. 3). However, the sphingolipid biosynthesis in the *osh3Δ* strain seemed to be more sensitive to ISP-1 than BY4741, because the biosynthesis of the phosphatidylinositols and IPCs was generally decreased in the *osh3Δ* strain for unknown reasons. These results indicated that the ISP-1 resistance of the *osh3Δ* strain was not due to an impaired inhibition of sphingolipid biosynthesis by ISP-1.

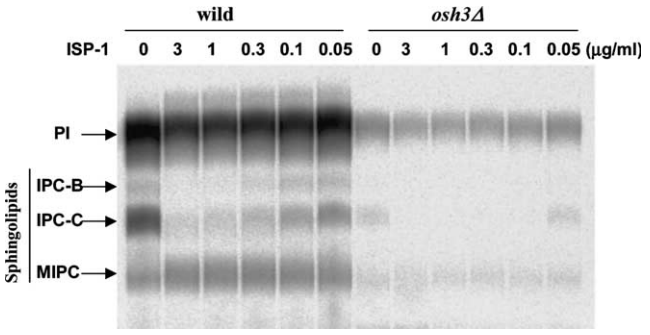

Fig. 3. Sphingolipid biosynthesis in *osh3Δ* strain is normally inhibited by ISP-1. Exponentially growing cells of *osh3Δ* (osh3Δ) and the isogenic wild type BY4741 (wild) strain were cultured with [^{3}H]inositol and various concentrations of ISP-1. Inositol-containing sphingolipids were analyzed as described in Materials and methods. The positions of each sphingolipid are indicated by arrows. PI, phosphatidylinositol; IPC-B, inositolphosphorylceramide B; IPC-C, inositolphosphorylceramide C; and MIPC, mannosylinositolphosphorylceramide.

Contribution of ROK1 and KEM1 genes to ISP-1 sensitivity of S. cerevisiae

Recently, the Osh3 protein was shown to bind directly to the Rok1 protein, a suppressor of *kem1* mutation [22,23]. Based on this evidence, we next examined the ISP-1 sensitivity of *kem1Δ* mutant and *ROK1* overexpressing strain. As in Figs. 4A and B, the *kem1Δ* strain showed hypersensitivity to ISP-1 and the *ROK1* overexpressing strain was viable with 0.1 μg/ml ISP-1. Furthermore, the ISP-1 hypersensitivity of the *OSH3* overexpressing strain was impaired by a co-expression of the *ROK1* gene (Fig. 5). These results indicate that *OSH3* and *ROK1* gene have opposite effects on ISP-1 sensitivity, and the sphingolipid depletion by ISP-1 and the loss of Kem1p function synergistically inhibited the growth of the budding yeast. Previously, *kem1* mutation was reported to enhance the phenotypes of *kar1* mutation, and the *ROK1* and *OSH3* gene overexpression adversely affected the *kem1* mutation [22,24]. These genetic interactions suggest that ISP-1 may inhibit microtubule functions and these genes may participate in the growth inhibition triggered by the ISP-1-induced sphingolipid depletion.

ISP-1-induced aberrant SPB assembly and nuclear division

The *kar1* mutation shows the SPB duplication defect and influences the length and morphology of microtubules in mitosis and the Kar1 protein is known as a

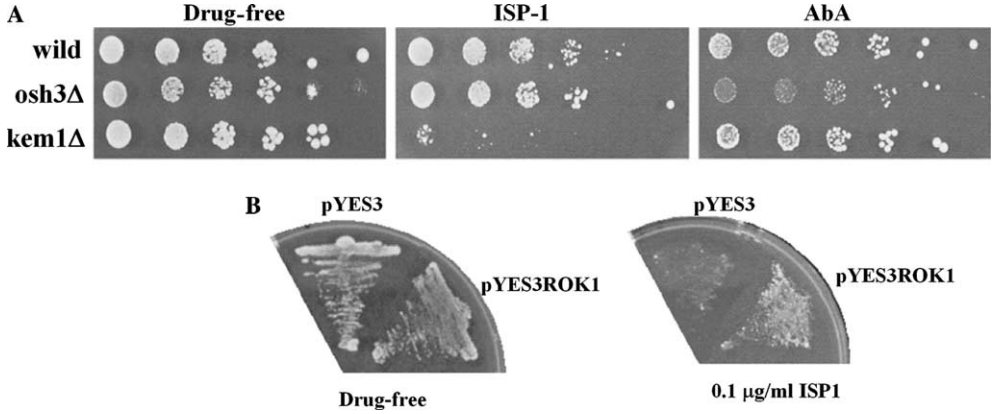

Fig. 4. Effects of *KEM1* and *ROK1* on ISP-1 sensitivity of budding yeast. (A) *kem1Δ* mutation confers hypersensitivity to ISP-1. Cell suspensions of *osh3Δ*, *kem1Δ*, and their isogenic wild type strain BY4741 were serially diluted and spotted on SD plates without drug (drug-free), and with 0.1 μg/ml ISP-1 (ISP-1) or 1 μg/ml aureobasidin A (AbA). (B) ROK1 overexpression confers slight resistance against ISP-1. SANK51893 strain transfected with the high-copy plasmid vector containing ROK1 gene (pYES3ROK1) or empty vector (pYES3) was cultured on SC–trp plates containing galactose/raffinose as the carbon source.

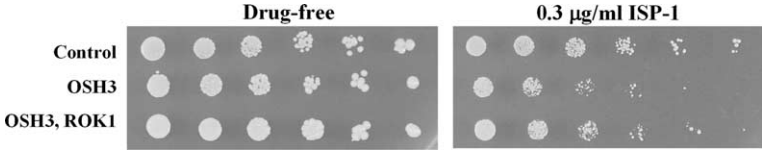

Fig. 5. *ROK1* overexpression-induced suppression of ISP-1 hypersensitivity in *OSH3*-overexpressing cells. Cell suspensions of YHP250 strains transfected with empty vectors pYES2 and pESC2 (Control), high-copy number *OSH3* gene expression vector pYES2OSH3 and empty vector pESC2 (OSH3), and high-copy number vectors pYES2OSH3 and pESCROK1 (OSH3, ROK1) were serially diluted and spotted on SC–ura–his plates containing galactose/raffinose as the carbon source.

component of SPB [26]. In this context, it was examined whether ISP-1 affects the mitotic spindle formation. As shown in Figs. 6A–C, the ISP-1 untreated cells showed extended spindles with the normally separated nuclei in the metaphase. However, the ISP-1 treated cells exhibited inadequately extended spindles and their nuclei

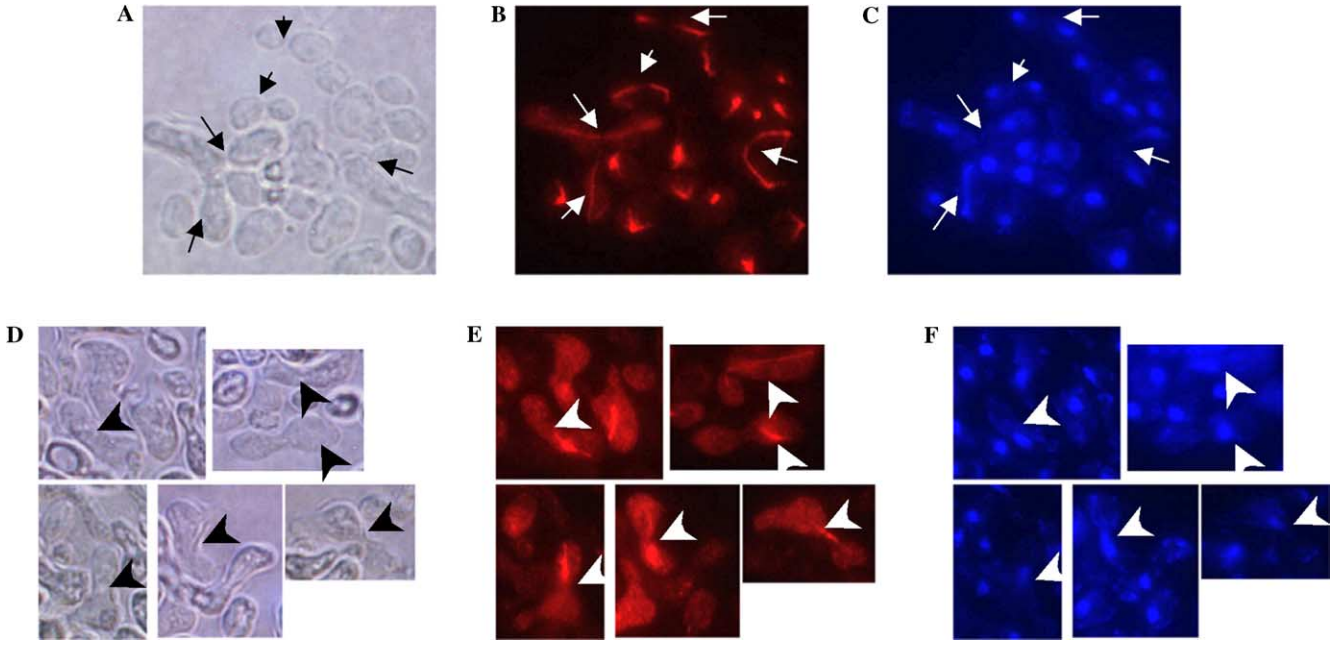

Fig. 6. Effects of ISP-1 on the mitotic spindle formation. BY4741 strain was cultured for 4 h with (D–F) or without (A–C) 1 μg/ml ISP-1 after release from G1 arrest by adding an α-mating factor. The mitotic spindles were visualized with monoclonal anti-α-tubulin (B and E) and the nuclei were stained with DAPI (C and F) as described in Materials and methods. Concomitant Nomarski (DIC) images are also shown (A and D). Arrows point to normally dividing cells with separated nuclei. Arrowheads indicate abnormal metaphase cells with aberrant mitotic spindles and non-separated nuclei.

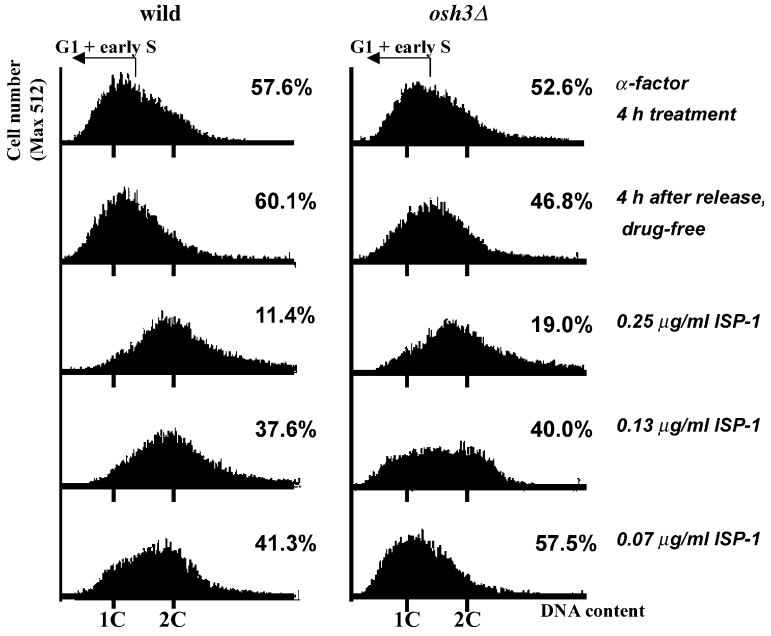

Fig. 7. *osh3Δ* strain shows resistance against G2/M arrest by ISP-1 treatment. BY4741 (wild) and *osh3Δ* cells (osh3Δ) were arrested at the G1 phase by adding an α-factor (α-factor 4 h treatment) as described in Materials and methods. After release from G1 arrest, the cells were cultured for 4 h without (4 h after release, drug-free) or with ISP-1 (0.07, 0.13, and 0.25 μg/ml ISP-1). The cell populations at G1 and early S phase were indicated as a percentage of total cells.

could not migrate into their daughter cells (Figs. 6D–F). Thus, the sphingolipid depletion by ISP-1 disrupts proper mitotic spindle formation and nuclear division.

Release from ISP-1-induced G2/M arrest by the deletion of OSH3 gene

In order to investigate the effects of *OSH3* gene on mitosis, the G2/M phase transition states were compared between the wild type and *osh3Δ* strain. Both cells were released from the G1 arrest and proceeded through the cell cycle for 4 h with or without ISP-1 (Fig. 7). In the wild type strain, the cells passed the M phase and reached the G1 and early S phase 4 h after release from G1 arrest without drugs, but most cells were arrested at the G2/M phase in the presence of 0.07–0.25 μg/ml ISP-1. These results were consistent with the effect of ISP-1 on the mitotic spindle formation shown in Fig. 6. However, the *osh3Δ* strain proceeded to the G1 phase through the G2/M phase even in the presence of 0.07 or 0.13 μg/ml of ISP-1, suggesting that *osh3Δ* strain can overcome the G2/M arrest by ISP-1.

In this paper, we showed that deletion of the *OSH3* gene conferred resistance against ISP-1 in the budding yeast *S. cerevisiae*. And yet, it was indicated that Osh3p might regulate mitotic spindle formation together with Kem1p and Rok1p, in which sphingolipids may play indispensable roles. From these experiments, two hypotheses were speculated as to the sphingolipid function in mitotic spindle formation. First, sphingolipids directly regulate the Osh3p functions in the mitotic spindle formation. As *OSH3* has a highly conserved oxysterol binding domain, it is supposed that this protein likely senses the changes in the amounts of certain lipids by the ISP-1 treatment. There is also the possibility that Osh3p recognizes non-steroidal lipids as its ligand because the amino acid sequence of the highly conserved region of the oxysterol binding domain of Osh3p (EKVSHRPP, 741–748) is slightly different from those of other homologues Osh1p and Osh2p (EQVSHHPP) [35]. A next subject for future investigation will be to determine which lipids are sensed by Osh3p. Second, sphingolipids may play essential roles in the mitotic spindle formation indirectly through the Osh3p functions. In this case, sphingolipids may be indispensable as constituents of the nuclear membrane where SPBs are organized or in the regulation of protein functions that are essential for mitotic spindle formation. Recently, a reduction of the sphingolipid amount by *acc1* (acetyl-CoA carboxylase) mutation in the budding yeast was reported to cause considerable morphological change in the nuclear membrane and cell cycle arrest at the G2 phase [36]. Whether the nuclear membrane morphologies are disturbed without the sphingolipids in the *osh3Δ* strain should be further examined.

Elucidation of the physiological roles and mechanisms of action of sphingolipids will shed some light on the new aspects that the lipids composing the cellular membrane directly regulate cell proliferation. Such investigations will bring about new molecular targets of novel antifungal and anticancer agents.

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