

***SLI1 (YGR212W)* is a major gene conferring resistance to the sphingolipid biosynthesis inhibitor ISP-1, and encodes an ISP-1 N-acetyltransferase in yeast**

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ISP-1 (myriocin) is a potent inhibitor of serine palmitoyltransferase, the primary enzyme of sphingolipid biosynthesis, and is a useful tool for studying the biological functions of sphingolipids in both mammals and yeast (*Saccharomyces cerevisiae*). In a previous study, we cloned yeast multicopy suppressor genes for ISP-1, and one of these, *YPK1/SLI2*, was shown to encode a serine/threonine kinase which is a yeast homologue of mammalian SGK1 (serum/glucocorticoid-regulated kinase 1). In the present study, another gene, termed *SLI1 (YGR212W)*; GenBank accession number CAA97239.1), was characterized. Sli1p has weak similarity to Atf1p and Atf2p, which are alcohol acetyltransferases. Although a *sli1*-null strain grew normally, the IC₅₀ of ISP-1 for the growth of this strain was markedly decreased compared with that for the parental strain, indicating that Sli1p is a major contributor

to ISP-1 resistance in yeast. On a *sli1*-null background, the increase in resistance to ISP-1 induced by *YPK1* gene transfection was almost abolished. These data indicate that Sli1p co-operates with Ypk1p in mediating resistance to ISP-1 in yeast. Sli1p was found to convert ISP-1 into *N*-acetyl-ISP-1 *in vitro*. Furthermore, *N*-acetyl-ISP-1 did not share the ability of ISP-1 to inhibit the growth of yeast cells, and the serine palmitoyltransferase inhibitory activity of *N*-acetyl-ISP-1 was much lower than that of ISP-1. These data suggest that Sli1p inactivates ISP-1 due to its *N*-acetyltransferase activity towards ISP-1.

Key words: N-acetyltransferase, condensation domain, drug-resistance gene, ISP-1, myriocin, serine palmitoyltransferase.

INTRODUCTION

Sphingolipids are sphingoid-base-containing lipids that are found in both mammals and the yeast *Saccharomyces cerevisiae*. More than 300 derivatives of sphingolipids occur in mammals, and play crucial roles in adhesion, differentiation, growth and apoptosis. Although the molecular species of yeast sphingolipids are much simpler than in those of mammals, sphingolipids are known to be involved in several biological functions in yeast. Ceramide was shown to induce G1 arrest in yeast via a ceramide-activated protein phosphatase consisting of three protein phosphatase 2A components [1,2]. Dihydrosphingosine and phytosphingosine were shown to be transiently elevated and involved in cell cycle arrest during heat stress [3,4]. Phytosphingosine is postulated to activate ubiquitin-dependent proteolysis upon heat stress [5]. These sphingoid bases have also been shown to inhibit nutrient import [6,7]. The sphingolipid synthesis pathway was reported to be necessary for intracellular trafficking of glycosylphosphatidylinositol-anchored proteins [8,9] and for the internalization step of endocytosis [10]. However, most of the downstream pathway of sphingolipid-related signalling has not been well clarified in mammals and yeast.

Yeast is genetically tractable and is therefore a useful system for the study of sphingolipids. Indeed, most genes involved in the early stage of the sphingolipid biosynthetic pathway, which is shared by both mammals and yeast, were originally cloned in yeast and then their mammalian counterparts were studied. In a pion-

ering study using a sphingolipid auxotrophy yeast mutant, genes for the subunits of SPT (serine palmitoyltransferase; *LCB1/2*), which is the enzyme catalysing the first step in sphingolipid biosynthesis, were cloned [11,12]. Later, genes for the sphingosine kinases, sphingosine phosphate phosphatases and sphingosine phosphate lyase were also cloned in yeast before the corresponding mammalian genes [13–16]. More recently *LAG1* and *LAC1*, which were known to be longevity genes in yeast, were identified as encoding ceramide synthases [17].

ISP-1 (myriocin) was originally isolated as an immunosuppressant from a fungus. Structural studies revealed that ISP-1 was identical to myriocin and thermozyomicin, which had been isolated previously as antibiotics [18,19]. ISP-1 exhibited an immunosuppressive potency 10–100-fold greater than that of cyclosporin A, a widely used immunosuppressant, when the activity was measured in a mouse allogenic mixed lymphocyte reaction [20]. Unlike cyclosporin A and FK506 (another commonly used immunosuppressant), ISP-1 did not interfere with interleukin-2 production in a mixed lymphocyte reaction [20], but instead suppressed alloreactive cytotoxic T lymphocyte generation *in vivo* and the interleukin-2-dependent growth of the mouse cytotoxic T cell line CTLL-2 [21]. FTY720 was developed as an analogue of ISP-1 and has little apparent toxicity *in vivo* [22], and therefore it has been tested in clinical immunosuppressive regimens for humans [23]. Both immunosuppressants are involved in sphingolipid function, but their direct targets are different, despite the structural similarity between the two. ISP-1 inhibits SPT in mammals, and

Abbreviations used: EGFP, enhanced green fluorescent protein; MBP, maltose-binding protein; SGK1, serum/glucocorticoid-regulated kinase 1; SPT, serine palmitoyltransferase.

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therefore ISP-1 treatment caused sphingolipid depletion that led to apoptosis of the cytotoxic T cell line. On the other hand, FTY720 was phosphorylated *in vivo*, and the phosphorylated form of FTY720 bound agonistically to the sphingosine 1-phosphate receptors on the lymphocyte cell membrane, and the resulting signal induced the recruitment of lymphocytes from blood to secondary lymphocyte tissues [24,25].

In contrast with mammals, yeast does not have sphingosine 1-phosphate receptors. However, similarly to mammals, the initial step of sphingolipid synthesis in yeast is catalysed by SPT. In a previous study [28], we showed that ISP-1 inhibited SPT and induced a growth defect in yeast. ISP-1 is therefore a useful tool for studying sphingolipid function in yeast. *YPK1/SLI2* was identified as a multicopy suppressor gene for ISP-1-induced growth defects in yeast. *YPK1* encodes a serine/threonine protein kinase that is a yeast counterpart of SGK1 (serum/glucocorticoid-regulated kinase 1) in mammals. SGK1 was isolated as a kinase regulated by serum and glucocorticoid [26]. Previously, SGK1 has been reported to be a downstream kinase of PDK1 (phosphoinositide-dependent kinase 1) and to have similar function to protein kinase B/Akt [27]. In yeast, Ypk1p is thought to be involved in the pathway of sphingolipid synthesis [28]. Indeed, the *in vitro* kinase activity of Pkh1/2p, upstream kinases of Ypk1p, was shown to be regulated by sphingoid bases, and the overexpression of Pkh1/2p suppressed the sphingoid base synthesis requirement [29]. However, the pathway downstream of Ypk1p is not well understood.

In the present study, another multicopy suppressor gene for ISP-1, *SLI1*, was characterized, and Sli1p was shown to have N-acetyltransferase activity towards ISP-1.

EXPERIMENTAL

Strains and reagents

S. cerevisiae strains KMY1006a (*MATa leu2-3 ura3-52 his3-200 trp1-901 lys2-802 ADE2*), $\Delta ypk1$ (*MATa leu2-3 ura3-52 his3-200 trp1-901 lys2-802 ADE2 ypk1::KanMX*), BY4741 (*MATa his3 Δ 1 leu2 Δ 0 met15 Δ 0 ura3 Δ 0*) and $\Delta sli1$ (*MATa his3 Δ 1 leu2 Δ 0 met15 Δ 0 ura3 Δ 0 sli1::KanMX*) were used. BY4741 and $\Delta sli1$ were obtained from the Yeast Genome Deletion Project (via Research Genetics Inc.). $\Delta ypk1$ was obtained by disruption of the *YPK1* locus.

N-Acetyl-ISP-1 lactone was synthesized as described previously [30]. N-Acetyl-ISP-1 was obtained by cleavage of N-acetyl-ISP-1 lactone with 0.1 M KOH at 60 °C for 1 h. N-acetyl-ISP-1 was then purified by HPLC on a C₂₂ reverse-phase column (250 mm × 4.6 mm internal diam.) with a 15-min gradient from water containing 25 % (v/v) methanol to 100 % (v/v) methanol. N-Acetyl-ISP-1 was identified by MS.

Disruption of the *YPK1* locus

A replacement cassette with long flanking homology regions was used to disrupt the *YPK1* gene in strain KMY1006. PCR amplification was performed with KOD-Plus-polymerase (TOYOBO) using pFA6a-kanMX6 containing the geneticin resistance gene with the oligonucleotide primers P1 (GTAATCAACTATC-GTCATACCAACAACAGTTGAGTTATTGCCAGCTGAAGC-TTCGTAC) and P2 (CGCCCCATCGACACGGATCTCGGAA-GGGTAAGAGAGGAAAGCCACTAGTGGATCTGATAT), and generated a DNA product corresponding to the kanMX6 marker gene, with extensions of 20 or 22 bp identical to *YPK1*. The fragment was used to transform KMY1006 cells. Correct integration at the *YPK1* locus in geneticin-resistant cells was confirmed by whole-cell PCR as described previously [31].

Molecular cloning of *SLI1*

SLI1 was cloned as described previously [28]. The 2.3 kb *Bam*HI–*Pvu*II fragment containing the wild-type *SLI1* gene from pSLI1 (a plasmid which carries the *SLI* gene) was subcloned into YEp351 (an episomal, multicopy yeast vector containing the *LEU2* selectable marker).

Yeast culture conditions

SD medium (used for SD agar plates) consisted of yeast nitrogen base, essential amino acids and glucose. Colonies selected on agar plates were inoculated into SD medium and then incubated overnight at 30 °C. Exponentially growing cultures were diluted to the desired concentrations, aliquoted into sterile culture tubes, and then treated with ISP-1 or methanol as a control. Proliferation was determined as the *A*₆₀₀.

Construction of plasmids

To construct a Sli1p–EGFP (enhanced green fluorescent protein) fusion protein, the stop codon of *SLI1* was replaced with a leucine codon, and *Xho*I, *Not*I and *Hind*III sites were introduced after the leucine codon by long, accurate PCR using primers 5'-TGT-CACGGATCCGCAAGAATGAATCTTAAACTT-3' and 5'-TG-GTAAGCTTGC GGCCGCAGCTCGAGGTATAAATTTAAGT-AATCTT-3'. The *Bam*HI–*Hind*III fragment of the PCR product was subcloned into YEp351 with the *ADHI* promoter, yielding YEp351*ADHI*-Sli1deltaSTOP. The EGFP DNA fragment was isolated from pEGFP-N1 by *Xho*I and *Not*I digestion and then ligated into YEp351*ADHI*-Sli1deltaSTOP, yielding YEp351-*ADHI*-Sli1p-EGFP. To generate YEp351-Sli1p-EGFP, the *Stu*I–*Hind*III fragment of YEp351*ADHI*-Sli1p-EGFP was swapped into wild-type *SLI1*. The *Bam*HI–*Stu*I fragment of YEp351*ADHI*-Sli1deltaSTOP was isolated and ligated into wild-type *SLI1*, yielding YEp351*ADHI*-Sli1. YEp351-Ypk1 was obtained as described by Sun et al. [28].

Preparation of antibodies specific for Sli1p

The 1.4 kb *Bam*HI–*Hind*III fragment was introduced by long, accurate PCR using primers 5'-TGTCACGGATCCGCAAGAAT-GAATCTTAAACTT-3' and 5'-TCAGAAGCTTCTAGTATAAA-TTTAAGTAAT-3', and ligated in-frame to the 3' end of the MBP (maltose-binding protein) gene in the cytoplasmic expression vector pMalc2 (New England BioLabs). The Sli1–MBP fusion protein was expressed in *Escherichia coli* BL21 following induction with isopropyl β -D-thiogalactopyranoside. After sonication of the cells, the fusion protein was recovered in the soluble fraction, purified using an amylose resin (New England BioLabs) column, and then eluted from an SDS/polyacrylamide gel. Immunization and IgG purification were carried out by Veritas Corp. (Tokyo, Japan).

Analysis of sphingolipid biosynthesis

Cultures of 2 ml were grown to 2×10^6 cells/ml in SD medium at 30 °C and then labelled with [³H]serine (20 μ Ci/ml) for the indicated times. The cultures were chilled on ice after the addition of 0.5 ml of unlabelled stationary-phase cells and then subjected to centrifugation at 2800 g for 10 min at 4 °C. The cells were washed twice with 5 ml of cold water and then treated with 5 % (v/v) trichloroacetic acid at 4 °C for 20 min. Lipids were extracted twice with 1 ml of ethanol/water/diethyl ether/pyridine/NH₄OH (15:15:5:1:0.018, by vol.) at 60 °C, as described elsewhere [32]. The [³H]serine-labelled extract was subjected to mild alkaline

methanolysis with 0.1 M KOH. The lipid products were extracted with 3 ml of chloroform, 1 ml of methanol, and 4.5 ml of 0.5 M NH_4OH , and the chloroform layer was washed three times with 5 ml of water. The lipid products were dried under N_2 , resuspended in 0.04 ml of chloroform/methanol/water (16:16:5, by vol.), applied to a silica gel TLC plate, and then resolved with chloroform/methanol/4.2 M NH_4OH (9:7:2, by vol.). Radioactive bands were visualized with a BAS 3000 image analyser (Fuji Film Co.).

Preparation of yeast extracts and immunoprecipitation

Total protein extracts were prepared from cells grown to mid-exponential phase. The cells were harvested, resuspended in lysis buffer [20 mM Tris/HCl, pH 7.5, containing 1 mM EDTA, 5 mM MgCl_2 , 50 mM KCl, 5% (v/v) glycerol, 1 mM PMSF, 0.5 $\mu\text{g/ml}$ leupeptin, 1 $\mu\text{g/ml}$ pepstatin A, 50 mM NaF and 3 mM dithiothreitol] and vortexed for 10 min with 0.5 vol. of 0.3–0.5-mm-diam. glass beads to lyse the cells, as described by Kohno et al. [33]. Unbroken cells and debris were removed by centrifugation of the homogenates at 12 000 g for 10 min. For immunoprecipitation, Triton X-100 was added to the resulting supernatant to make a 1% solution. Immunoprecipitates were prepared using anti-Sli1p antibodies (10 μl) preadsorbed to a 50:50 (w/v) slurry of Protein G–Sepharose (10 μl). The beads were washed three times with lysis buffer and then incubated with the lysate at 4 °C overnight. Immune complexes were washed three times with lysis buffer and then separated by SDS/PAGE. Sli1p–EGFP was detected using anti-GFP antibodies, and Ypk1p was detected using anti-Ypk1p antibodies. Blots were examined using a chemiluminescent reagent (Pierce) for the detection of overexpressed Sli1 protein. Protein concentrations were measured with a Bio-Rad protein assay kit.

Assay for growth inhibition by ISP-1 and N-acetyl-ISP-1

Cells were grown in SD medium. The culture was diluted with SD medium to $A_{600} = 0.002$. Aliquots of 100 μl of cells were diluted into a series of wells in a microtitre plate. Each well containing 100 μl of SD medium was supplemented with ISP-1 or N-acetyl-ISP-1. Final concentrations of ISP-1 from 10 $\mu\text{g/ml}$ to 1.22 ng/ml and of N-acetyl-ISP-1 from 3.9 $\mu\text{g/ml}$ to 1.9 ng/ml were prepared by serial 2-fold dilutions. The plates were incubated at 30 °C for 42–48 h. The contents of each well were mixed, and A_{595} was measured.

Enzymic acetylation of ISP-1

Enzymic acetylation of ISP-1 was carried out using a modification of the protocol for the enzymic acetylation of 12-D,L-hydroxystearic acid described by Malcorps and Dufour [34]. The reaction was carried out in a final volume of 250 μl , containing 100 mM KH_2PO_4 , 30 mM n-octyl glucoside, 0.1 mM ISP-1, 200 μM [^3H]acetyl-CoA (25 Ci/mol) and 125 μl of yeast cell lysate prepared with breaking buffer (20 mM Tris/HCl, 10 mM EDTA, 5 mM dithiothreitol and 1 mM PMSF) as enzyme source. After the indicated period of incubation at 30 °C, the reaction was stopped by chilling on ice. The reaction product was extracted two times with 125 μl of n-butanol. The butanol was dried under N_2 and the residue resuspended in 50 μl of methanol, applied to a silica gel TLC plate, and then resolved with chloroform/methanol/4.2 M NH_4OH (9:7:2, by vol.; solvent A) or chloroform/methanol/4 M acetic acid (9:7:2, by vol.; solvent B). Radioactive bands were visualized with a BAS 2000 image analyser (Fuji Film Co.). For alkaline treatment, the labelled compound(s) was dis-

solved in 0.1 M KOH in methanol and incubated for 1 h at 60 °C. After incubation, the sample was neutralized and extracted with n-butanol.

SPT assay

SPT assays were carried out by a modification of the method of Buede et al. [11]. For each assay, the following components were used, in a final volume of 0.2 ml: 0.1 M Hepes (pH 8.3), 5 mM dithiothreitol, 2.5 mM EDTA (pH 8.0), 50 μM pyridoxal phosphate, 200 μM palmitoyl-CoA, 5 mM L-serine, 5 μCi of L-[^3H]serine, 100 nM ISP-1 or N-acetyl-ISP-1, and 0.5 mg of yeast total protein extract prepared with phosphate buffer (50 mM sodium phosphate, pH 7.0, containing 5 mM dithiothreitol and 1 mM PMSF). After incubation for 20 min at 30 °C, 0.5 ml of 0.5 M NH_4OH containing 10 mM L-serine was added, and then the lipid products were extracted with chloroform. The chloroform layer was transferred to a scintillation vial and then dried down completely before the radioactivity was measured with a Beckman LS6000 liquid scintillation counter. The result for background without palmitoyl-CoA was subtracted from all the measurements to calculate the specific activity.

Fluorescence microscopy

For indirect immunofluorescence microscopy, cells were fixed with 3.7% (v/v) formaldehyde in 0.1 M potassium phosphate buffer (pH 6.5), collected by centrifugation, and washed with SP buffer (0.1 M potassium phosphate, pH 7.5, and 1 M sorbitol). The cells were then converted into spheroplasts by adding 1:500 vol. of 2-mercaptoethanol and 15 $\mu\text{g/ml}$ Zymolyase 100T (Seikagaku Co.), and incubated for 30 min at 30 °C. The spheroplasts were centrifuged and permeabilized by adding 0.5 ml of SP with 0.1% Triton X-100 for 10 min, and blocked with PBT (1% BSA and 0.1% Triton X-100 in PBS) for 10 min. The cells were incubated with primary antibodies diluted in PBT overnight. Anti-Kar2p antiserum (a gift from Dr. K. Kohno, NAIST, Ikoma, Japan) was used at 1:500 dilution. After washing, the cells were incubated with secondary antibodies diluted in PBS for 2 h. For secondary staining, Alexa Fluor 546 goat anti-rabbit IgG (H + L) conjugate (10 $\mu\text{g/ml}$; Molecular Probes) was used. The cells were observed under a fluorescence confocal microscope (LSM510; Zeiss).

RESULTS

Sli1p is a major contributor to ISP-1 resistance in yeast cells

In our previous study, we showed that ISP-1 inhibited yeast cell growth, and characterized one of the suppressor genes for ISP-1 resistance, *YPK1/SLI2* [28]. Another gene, termed *SLII* for sphingosine-like immunosuppressant resistant gene 1 (*YGR212W*; GenBank designation CAA97239.1), encodes a putative polypeptide (Sli1p) of 468 amino acids with a deduced molecular mass of 54.5 kDa (Figure 1). Sli1p-overexpressing yeast cells were much more resistant to ISP-1 than were Ypk1p-overexpressing cells (Figures 2A and 2B). To determine the phenotypic consequences of a complete loss of *SLII* function, we analysed the *sli1*-null mutant. The *sli1*-null mutant grew normally (results not shown). However, when the strain was cultured in the presence of ISP-1, cell growth was markedly reduced. The IC_{50} of ISP-1 for the growth decreased to approx. $0.01 \times$ that of the parental strain, suggesting that Sli1p is a major contributor to ISP-1 resistance in yeast (Figure 2C).

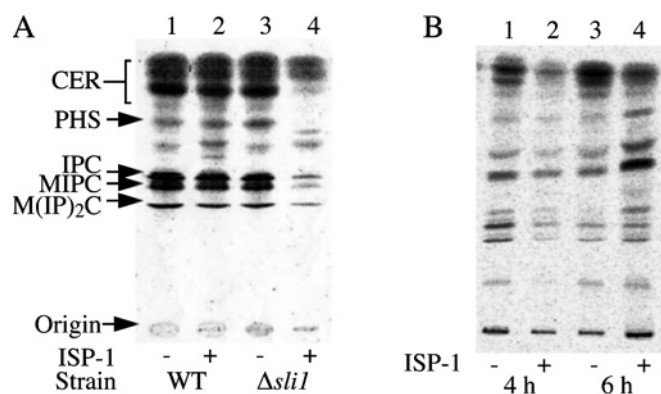


Figure 3 Effects of the *sli1* deletion and of Sli1p overexpression on sphingolipid biosynthesis in yeast cells

Sphingolipids were labelled with [³H]serine and extracted, then separated on TLC plates as described in the Experimental section. The positions of standard lipids are indicated by arrows: CER, ceramide; PHS, phytosphingosine; IPC, inositol phosphoceramide; MIPC, mannose inositol phosphoceramide; M(IP)₂C, mannose di-inositol diphosphoceramide. (A) Sphingolipid biosynthesis of *sli1*-null mutant cells (lanes 3 and 4) and wild-type (WT) cells (lanes 1 and 2) in the presence (lanes 2 and 4) or absence (lanes 1 and 3) of 10 ng/ml ISP-1 for 2.5 h. [³H]Serine was added 0.5 h after the addition of ISP-1. (B) Sphingolipid biosynthesis of yeast cells (KMY1006a) containing YEp351-Sli1 (*SLI1*). Cells were treated with (lanes 2 and 4) or without (lanes 1 and 3) 0.5 μg/ml ISP-1 for 4 h (lanes 1 and 2) or 6 h (lanes 3 and 4). [³H]Serine was added 2 h (lanes 1 and 2) or 4 h (lanes 3 and 4) after the addition of ISP-1.

of SPT to ISP-1. To test these possibilities, sphingolipid biosynthesis was monitored during ISP-1 treatment in Sli1p-overexpressing cells. At the beginning of ISP-1 treatment (Figure 3B, lanes 1 and 2), sphingolipid biosynthesis was decreased. However, during further incubation (Figure 3B, lanes 3 and 4), biosynthesis recovered to the level in untreated cells. These data indicate that Sli1p overexpression blocks the ISP-1-induced inhibition of sphingolipid biosynthesis. This is most probably due to a decrease in the ISP-1 concentration inside the cell, since the inhibition of biosynthesis was observed at the beginning of ISP-1 treatment.

Sli1p acetylates ISP-1

Sli1p has weak similarity to Atf1p and Atf2p, which are yeast alcohol acetyltransferases [35,36] (Figure 1). Atf1p and Atf2p catalyse the synthesis of isoamyl acetate from acetyl-CoA and isoamyl alcohol. Based on the sequence similarity between Sli1p and Atf1/2p, we predicted that Sli1p may acetylate some hydroxy groups of ISP-1 (Figure 4A). We investigated whether Sli1p acetylates ISP-1 using [³H]-labelled acetyl-CoA. One major band of putative acetylated ISP-1 was detected when ISP-1 and [³H]acetyl-CoA were incubated with yeast cell lysate containing Sli1p (Figure 4B). The band was not detected when the reaction was performed either with cell lysate that lacked Sli1p, or without ISP-1. Thus it is very likely that the labelled band represents acetyl-ISP-1 produced due to the acetyltransferase activity of Sli1p. Surprisingly, the radioactive compound co-migrated with *N*-acetyl-ISP-1 on a TLC plate in two different solvent systems (Figures 4B and 4C). Furthermore, the compound was stable after alkaline treatment (Figure 4C). These data suggest that the compound is not *O*-acetyl-ISP-1, but rather *N*-acetyl-ISP-1, because *O*-acetyl groups are known to be labile under alkaline treatment conditions. The acetyltransferase activity towards ISP-1 was dependent on the protein concentration of the enzyme source (results not shown). The specific activity of the crude cell lysate was calculated to be 7.69 nmol/min per mg of protein.

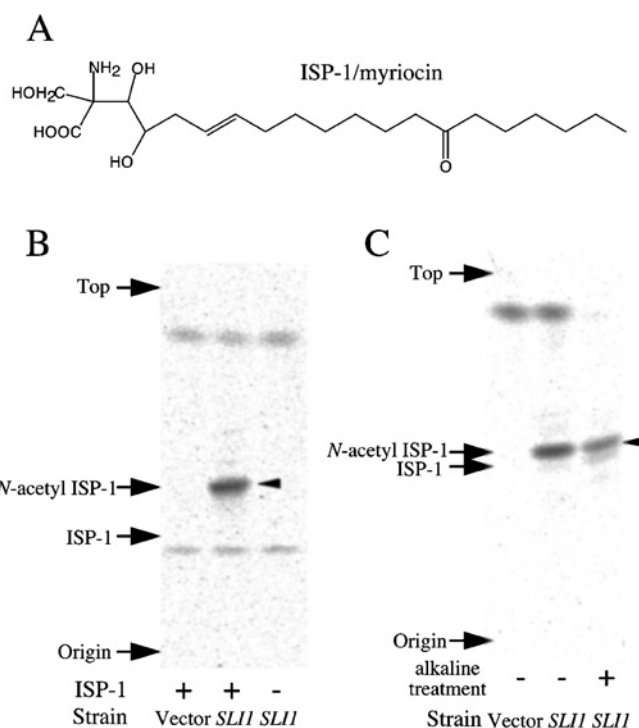


Figure 4 Acetylation activity towards ISP-1 of Sli1p-overexpressing cell lysate

(A) Chemical structure of ISP-1. (B) ISP-1 acetyltransferase activity of Sli1p-overexpressing cells. Cell lysates prepared from *sli1*-null cells containing YEp351 (Vector) or YEp351*ADH1*-Sli1 (*SLI1*) were used as enzyme sources (0.5 μg/μl). Sli1p was overexpressed under the *ADH1* promoter. Samples were incubated for 5 min at 30 °C with (left and middle lanes) or without (right lane) ISP-1. Products were extracted and separated on a TLC plate as described in the Experimental section using solvent A. A major putative band of acetylated ISP-1 is indicated by an arrowhead. Authentic standards are also indicated. (C) The radioactive acetyl-ISP-1 was treated under alkaline conditions as described in the Experimental section and separated on a TLC plate using solvent B. A major putative band of acetylated ISP-1 is indicated by an arrowhead. Authentic standards are also indicated.

Acetylation activity towards ISP-1 was only detected when Sli1p was expressed under the *ADH1* promoter, but not when expressed under its own promoter. The difficulty of detecting acetylation activity of the cell lysate may be due to a very low level of expression of Sli1p under its own promoter, even when overexpressed using a multicopy plasmid. Moreover, immunoprecipitated Sli1p did not show detectable acetyltransferase activity towards ISP-1. It is possible that Sli1p is a component of a multi-component complex enzyme. When wild-type yeast cells were treated with chemically synthesized *N*-acetyl-ISP-1, growth inhibition was not detected under the conditions used (Figure 5A). The *sli1*-null mutant was hypersensitive to ISP-1 (Figure 2C). However, the strain grew normally in the presence of *N*-acetyl-ISP-1. ISP-1 (100 nM) almost completely inhibited SPT activity *in vitro*, but *N*-acetyl-ISP-1 did not show any inhibitory activity against SPT at this concentration (Figure 5B). Furthermore, approx. 40% of wild-type SPT activity was still detected in the presence of a >20-fold molar excess of *N*-acetyl-ISP-1 (2.26 μM). These data suggest that Sli1p inactivates ISP-1 due to its N-acetyltransferase activity.

The ISP-1-resistance activity of Ypk1p is markedly decreased on a *sli1*-null background

YPK1/SLI2 has been reported to be a multicopy suppressor of ISP-1, and to encode a serine/threonine kinase [28]. Ypk1p is a

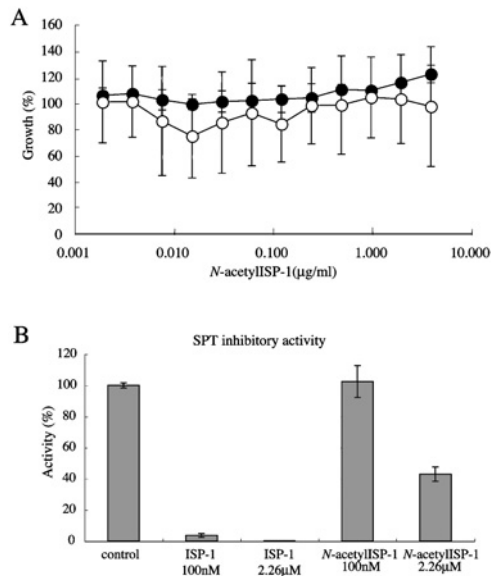


Figure 5 Effects of *N*-acetyl-ISP-1 on yeast cell growth and SPT activity

(A) IC_{50} of *N*-acetyl-ISP-1 for the wild-type (●) and *sli1*-null (○) strains was measured as described in the Experimental section. A_{595} was measured after a 42 h incubation at 30 °C. Results are expressed as a percentage of the cell density achieved by the culture in the absence of ISP-1. Data are the means \pm S.D. from one experiment done in triplicate. (B) The effect of *N*-acetyl-ISP-1 on SPT activity was studied. SPT activity was measured using the wild-type cells as described in the Experimental section. Results are expressed as a percentage of control activity in the absence of reagents. Data are the means \pm S.D. from one experiment done in triplicate.

functional homologue of the mammalian protein kinase SGK1, which was originally reported as a serine/threonine protein kinase that is transcriptionally regulated by serum and glucocorticoids in rat mammary tumour cells [37]. To clarify the relationship between Sli1p and Ypk1p, the effect of overexpression of Ypk1p on resistance to ISP-1 was examined on the *sli1*-null background. Comparison between Ypk1p-overexpressing cells and vector control cells on the wild-type background demonstrated that the IC_{50} of ISP-1 was increased by approx. 0.27 μ g/ml in the Ypk1p-overexpressing cells (Figure 6A). Strikingly, the increase in IC_{50} due to Ypk1p expression was only 0.006 μ g/ml on the *sli1*-null background. Under these conditions, the expression of Ypk1p was not significantly affected (Figure 6B), indicating that Sli1p is necessary for the ISP-1-resistance activity of Ypk1p. On the other hand, Sli1p-overexpressing *ypk1*-null cells showed ISP-1 resistance (Figure 6C). It has been shown that *ypk1*-null strains have a slow growth phenotype [38]. However, Sli1p overexpression did not recover the slow growth phenotype of the *ypk1*-null mutant in the absence of ISP-1 (Figure 6C).

Localization of Sli1p-EGFP fusion protein

The intracellular localization of Sli1p was visualized by monitoring a Sli1p-EGFP fusion protein. The Sli1p-EGFP fusion protein was distributed in the area around the nucleus and co-localized with Kar2p, which was localized in both the endoplasmic reticulum and the plasma membrane (Figure 7), suggesting that the Sli1p-EGFP fusion protein localizes in the endoplasmic reticulum.

DISCUSSION

In the present study, one of the multicopy suppressor genes for ISP-1 was characterized. This gene, termed *SLI1*, showed a

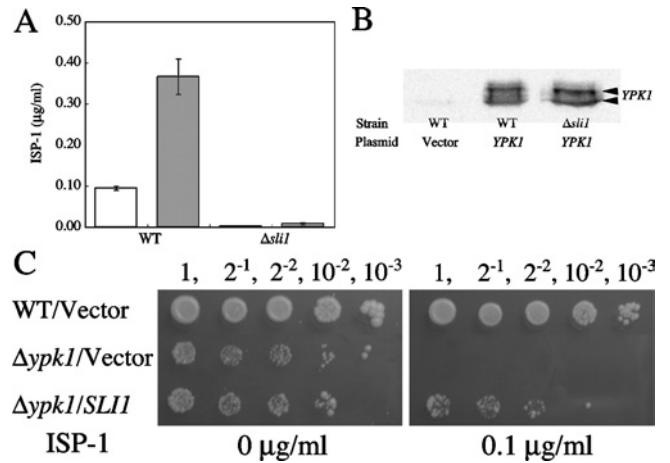


Figure 6 Genetic interaction between *YPK1* and *SLI1* in mediating resistance to ISP-1

(A) IC_{50} of ISP-1 in the wild-type (WT) strain and the *sli1*-null mutant strain. Cells containing YEp351 (empty bars) or YEp351-Ypk1 (shaded bars) were grown for 48 h at 30 °C. The IC_{50} of ISP-1 was measured as described in the Experimental section. Data are the means \pm S.D. from one experiment done in triplicate. (B) The expression level of Ypk1p was detected by immunoblotting. Cell-free extracts were prepared from the wild-type (WT) strain containing YEp351 (Vector) or YEp351-Ypk1 (*YPK1*), and the *sli1*-null mutant containing YEp351-Ypk1. Ypk1p was detected with anti-Ypk1p antibodies. (C) Resistance to ISP-1 of *SLI1*-overexpressing yeast cells on SD plates. Serially diluted wild-type (WT) and *ypk1* null mutant strains containing YEp351 (Vector) or YEp351-Sli1 (*SLI1*) were grown on SD plates containing methanol or 0.1 μ g/ml ISP-1 for 5 days. Serial dilutions of each strain are indicated above the panels.

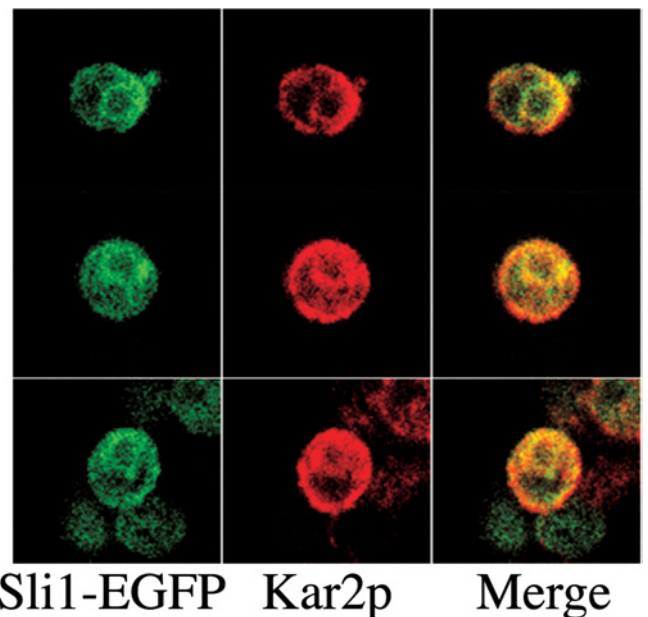


Figure 7 Intracellular localization of Sli1p

Yeast cells containing YEp351*ADH1*-Sli1p-EGFP were fixed with formaldehyde, converted into spheroplasts, and permeabilized with 0.1% Triton X-100. They were then stained with anti-Kar2p antibodies. Green indicates the localization of Sli1p-EGFP (left panel) and red indicates the localization of Kar2p (middle panel). In the merged image (right panel), yellow indicates the co-localization of Sli1p-EGFP and Kar2p.

stronger ISP-1 resistance phenotype than *YPK1/SLI2*, which has already been characterized [28].

Sli1p is a major contributor to ISP-1 resistance in yeast cells. Cell lysates of Sli1p-overexpressing cells showed ISP-1

acetyltransferase activity (Figure 4). ISP-1 has three hydroxy groups and an amino group, which could potentially be acetylated (Figure 4A). Sli1p has weak similarity to the yeast alcohol acetyltransferases Atf1p and Atf2p, which catalyse the synthesis of short-chain and medium-chain aliphatic esters [34–36]. However, the labelled acetyl-ISP-1 was shown to co-migrate with *N*-acetyl-ISP-1 on a TLC plate using two different solvent systems. Furthermore, the labelled compound was stable under alkaline treatment conditions where *O*-acetyl groups are unstable. These data suggest that the amino group of ISP-1 is acetylated by Sli1p. This *N*-acetylation abolished the growth-inhibitory activity of ISP-1 in yeast cells (Figure 5A). In addition, *N*-acetylation of ISP-1 markedly reduced its inhibitory activity against SPT *in vitro* (Figure 5B).

The IC₅₀ of ISP-1 in the *sli1*-null mutant was decreased to about 0.01 times that in the parental strain. The sphingolipid biosynthesis of the *sli1*-null mutant was inhibited at a low concentration of ISP-1 (10 ng/ml), although that of the parental strain was not. The concentration used was closer to the IC₅₀ of ISP-1 for the inhibition of the *in vitro* activity of SPT in wild-type cells [28] than that for the inhibition of cell growth in wild-type cells (Figure 2C). These data suggest that the SPT activity is directly affected by ISP-1 in the *sli1*-null strain, as *in vitro*, but that inhibition of the enzyme activity by ISP-1 in wild-type cells is suppressed by the function of Sli1p to some extent. On the other hand, overexpression of Sli1p restores sphingolipid biosynthesis that was inhibited by ISP-1. Although sphingolipid biosynthesis was inhibited by ISP-1 at the beginning of the treatment period, the biosynthesis recovered to the same level as without ISP-1 during further incubation (Figure 3B). The ISP-1 concentration was probably high enough to inhibit SPT at early time points, but would be decreased due to the activity of Sli1p, and hence biosynthesis recovered at a later time point. This profile shows good accordance with the possibility that Sli1p inactivates ISP-1. Taken together, these data suggest that ISP-1 is converted into *N*-acetyl-ISP-1 and hence inactivated by Sli1p *in vivo*. Sli1p has a domain similar to the condensation domain (pfam 00668) (Figure 1). The condensation domain is usually involved in amide bond formation in non-ribosomal peptide biosynthesis [39]. Thus the condensation-like domain of Sli1p may be involved in amide bond formation in the *N*-acetylation reaction catalysed by Sli1p.

FTY720, an analogue of ISP-1, has been tested in clinical immunosuppressive regimens in humans [23]. In spite of the structural similarity between the two compounds, cell lysates prepared from Sli1p-overexpressing cells did not show any acetylation activity towards FTY720 (results not shown). The structure of ISP-1 is also similar to those of sphingosine and phytosphingosine, which are intermediates in the sphingolipid biosynthesis pathway. When yeast cells were treated with a high concentration of these compounds, a growth defect was also observed. Interestingly, Sli1p overexpression did not suppress the growth defect, despite the structural similarity. In accordance with these data, cell lysates prepared from Sli1p-overexpressing cells did not show any acetylation activity toward these long-chain bases (results not shown). Fumonisin B1 [40] and aureobasidin [41] also inhibit sphingolipid biosynthesis in a different manner to ISP-1: fumonisin B1 inhibits ceramide synthase [17,40] and aureobasidin inhibits inositol phosphoceramide synthase [41]. Treatment with these reagents also induces a growth defect in yeast cells. However, Sli1p did not overcome the growth defect caused by such treatment (results not shown). Taken together, these findings imply that the action of Sli1p seems to be specific for ISP-1.

YPK1/SLI2 is another multicopy suppressor gene for ISP-1, and encodes a serine/threonine kinase [28]. The multicopy suppressor activity of Ypk1p was almost abolished on the *sli1*-null

background. On the other hand, Sli1p-overexpressing *ypk1*-null cells showed ISP-1 resistance. It is very difficult to compare the ISP-1 resistance activity of Sli1p in the *ypk1*-null mutant cells with that in wild-type cells, because *ypk1*-null strains show a slow growth phenotype [38]. These findings suggest that there is a genetic interaction between *SLI1* and *YPK1*. However, we regard it likely that this interaction is not direct, because Sli1p was not phosphorylated *in vitro* by Ypk1p, while a peptide substrate was well phosphorylated by Ypk1p (results not shown), and the putative phosphorylation sequence for Ypk1p (RXRXXS/T) is not detected in Sli1p. These observations suggest that Sli1p is not a direct substrate of Ypk1p. Furthermore, the slow growth phenotype of *ypk1*-null strains was not compensated by the overexpression of Sli1p. Therefore the mechanism of interaction between *SLI1* and *YPK1* remains to be clarified.

To study the intracellular localization of Sli1p, a Sli1p–EGFP fusion protein was expressed. However, the fusion protein was not detected in yeast cells when expressed under its own promoter from a multicopy-base vector. The *ADHI* promoter was therefore used to overcome this problem. The Sli1p–EGFP fusion protein was co-localized with the part of Kar2p known to localize in the endoplasmic reticulum [42], showing that Sli1p localizes in this organelle. SPT is also reported to localize in the endoplasmic reticulum. These data suggest that Sli1p may function in the endoplasmic reticulum and decrease the ISP-1 concentration in the vicinity of SPT.

ISP-1 was originally discovered as an immunosuppressant. This action of ISP-1 in mammalian cells is restricted to a few cell lines: CTLL-2 [21], Purkinje cells [43], and CHO cells [44,45]. However, the mechanism of action has not been clarified. Further studies on the function of Sli1p may facilitate elucidation of the detailed mechanism, although the mammalian homologue of Sli1p has yet to be identified.

This work was supported in part by Grants-in-Aid for Scientific Research (13470488 and 13877373 to Y.K.) from the Japan Society for the Promotion of Science, by a Grant-in-Aid for Scientific Research on Priority Areas (12140202 to Y.K.) from the Ministry of Education, Culture, Sports and Technology, and by RIKEN.

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Received 20 January 2004/9 March 2004; accepted 16 March 2004

Published as BJ Immediate Publication 16 March 2004, DOI 10.1042/BJ20040108