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Role of the Yeast VAP Homolog, Scs2p, in *INO1* Expression and Phospholipid Metabolism

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In the yeast *Saccharomyces cerevisiae*, the expression of phospholipid biosynthetic genes, including the *INO1* gene (encoding inositol-1-phosphate synthase), is coordinately regulated by a *cis*-acting transcriptional element, UAS_{INO} (inositol-sensitive upstream activating sequence). For this paper we studied the effect of *SCS2* disruption on *INO1* expression. *SCS2* encodes a type II membrane protein and its deletion leads to inositol auxotrophy at temperatures above 34°C. We found that the expression of the *INO1* gene was reduced in the *scs2*Δ strain even when the cells were cultured under derepressing conditions for *INO1* expression. However, the β-galactosidase gene fused with the *INO1* promoter region was expressed normally in the *scs2*Δ strain. The phospholipid composition of *scs2*Δ cells was not dramatically changed compared with wild-type cells at 28°C, but the phosphatidylinositol level was reduced in *scs2*Δ cells cultured at 34°C. In addition, elevated phosphatidylcholine synthesis through the CDP-choline pathway was observed in the scs2Δ strain, and the disruption of genes involved in the CDP-choline pathway rescued the *INO1* expression defect of the *scs2*Δ strain. These results indicate that Scs2p can contribute to coordinated phospholipid metabolism including *INO1* expression by regulating phosphatidylcholine synthesis through the CDP-choline pathway.

Key words: CDP-choline pathway, *INO1*, phospholipid, *SCS2*, yeast VAP family.

Abbreviations: PA, phosphatidic acid; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PI, phosphatidylinositol, UAS_{INO}, inositol-sensitive upstream activating sequence; UPR, unfolded protein response; VAP, vesicle-associated membrane protein (VAMP) associated protein.

In the yeast *Saccharomyces cerevisiae*, the transcription of structural genes, which encode many phospholipid biosynthetic enzymes (Fig. 1), is coordinately regulated by a *cis*-acting promoter element named UAS_{INO} (inositol-sensitive upstream activating sequence), also known as ICRE (inositol/choline-responsive element) (*1*–*3*). These genes are repressed in the presence of the soluble phospholipid precursors, inositol and choline, and derepressed in their absence. The *INO1* gene, which encodes inositol-1-phosphate synthase, has two UAS_{INO} in its promoter region, and is highly regulated in response to inositol and choline. Since the *INO1* gene product catalyzes a rate-limiting step in inositol production, defects in *INO1* expression cause inositol auxotrophy (Ino^-). This strong indicative phenotype was used as a marker for the isolation of mutants showing defects in the regulation of *INO1* and other UAS_{INO}-containing genes.

Many genes required for *INO1* expression have been isolated on the basis of their ability to suppress the inositol auxotrophy of several mutant strains. The *INO2* and *INO4* gene products (Ino2p and Ino4p, respectively) have a basic helix-loop-helix motif and form heterodimers with each other. The heterodimer binds to UAS_{INO} and facilitates the expression of genes under UAS_{INO} control (*4*–*7*). The *OPI1* gene product (Opi1p) has a basic leucine zipper motif, and *OPI1* disruption causes elevated *INO1* expression even in the presence of inositol (*8*). Although there is no evidence for the binding of Opi1p to UAS_{INO} (*9*, *10*), Opi1p is thought to repress *INO1* expression by interacting with Ino2p and the pleiotropic repressor Sin3p (*11*).

In contrast to the *INO1* expression apparatus, signal molecules that lead to *INO1* expression in response to the cellular levels of inositol and choline have not been clearly elucidated. Since misregulation of *INO1* expression has been observed in many strains showing altered phospholipid metabolism, some phospholipids, such as phosphatidylcholine (PC) and phosphatidic acid (PA), are thought to regulate *INO1* expression in response to cellular inositol and choline levels (*3*, *12*, *13*).

The *SCS2* gene was originally identified as a suppressor of Ino^- in *CSE1* mutants (*14*). The disruption of *SCS2* causes inositol auxotrophy at temperatures above 34°C (*15*). *SCS2* overproduction also suppresses telomeric silencing defects caused by mutations in the *MEC1* gene, which encodes a protein kinase (*16*), or by a dominant-negative mutation in the *SIR2* gene, which ecodes an NAD^+-dependent deacetylase (*17*). Immunochemical analysis has shown that Scs2p is a type-II membrane protein located on the ER/nuclear membrane with a molecular mass of 35 kDa (*15*). A homology search revealed that Scs2p is a member of a protein family found in various species including human (*18*), rat (*19*), mouse (*20*), *Drosophila melanogaster* (*21*), and *Arabidopsis thaliana* (*22*). This protein family is called the VAP [vesicle-

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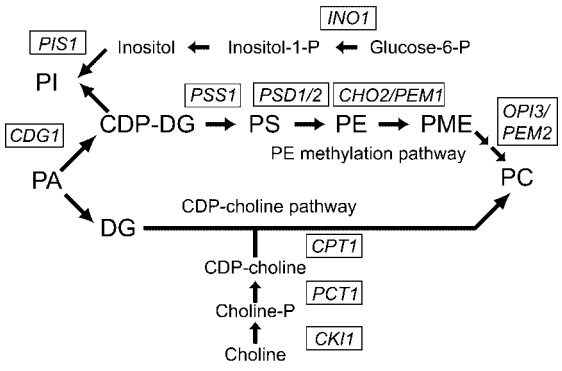

Fig. 1. **Pathways for phospholipid synthesis in *S. cerevisiae*.** Metabolic steps are indicated by the arrows, and the names of genes involved in each step are indicated in boxes. DG, diacylglycerol: PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PME, phosphatidylmonomethylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine.

associated membrane protein (VAMP)-associated protein] family, and is named after VAP-33 found in *Aplysia californica* (*23*). The VAP family proteins associate with various intracellular components such as ER (*20*, *24*), the ER/Golgi intermediate compartment (*25*), the Golgi complex (*24*), COPI vesicles (*19*), GLUT-4 containing vesicles (*26*), microtubules (*20*), and tight junctions (*27*), suggesting conserved membrane-related functions of this family. However, the physiological roles of this protein family are not yet fully understood.

In this report we have studied the role of Scs2p by characterizing the Ino$^-$ phenotype of the *scs2*Δ strains. We find that Scs2p can affect PC biosynthesis through the CDP-choline pathway (Fig. 1), and that the disruption of *SCS2* causes elevated PC synthesis, which leads to a reduction in *INO*1 expression under conditions of inositol depletion. We conclude that the Ino$^-$ of *scs2*Δ cells is significant because this mutant does not express enough *INO1* to produce sufficient amounts of phosphatidylinositol (PI) to meet the elevated PI requirement at 34°C. Therefore Scs2p is required for regulated PC biosynthesis through the CDP-choline pathway, which coordinates phospholipid metabolism including *INO1* expression in yeast cells.

MATERIALS AND METHODS

Strains—Escherichia coli XL1-Blue MRF' was used for routine bacterial transformations and the maintenance of plasmids. The genotypes of the yeast strains used in this study are listed in Table 1.

Media—YPD (1% yeast extract, 2% peptone, 2% glucose) and inositol-free medium were prepared as described by Burke *et al*. (*28*) and Klig *et al*. (*29*). The following amino acids and supplements were added to the inositol-free medium: adenine (20 mg/liter), histidine (20 mg/liter), leucine (100 mg/liter), lysine (30 mg/liter), tryptophan (20 mg/liter) and uracil (20 mg/liter). The inositol-containing medium was prepared by supplementing inositol-free medium with *myo*-inositol to 100 μM. In

Table 1. **Yeast strains used in this study.**

Name	Genotype	Source
KY354	*MAT*α *ade2-101 leu2-*Δ*1 lys2-801 his3-*Δ*200 trp1-*Δ*63 ura3-52*	(*15*)
KY360	*MAT*α *scs2*Δ*::TRP1 ade2-101 leu2-*Δ*1 lys2-801 his3-*Δ*200 trp1-*Δ*63 ura3-52*	(*15*)
KY427	KY354/YEp*lac*195	(*15*)
KY429	KY360/YEp(*SCS2*)	(*15*)
KY430	KY360/YCp(*SCS2*)	(*15*)
KY431	KY360/YEp*lac*195	(*15*)
KY503	KY360/YCp*INO1*Z	This study
KY504	KY354/YCp*INO1*Z	This study
KY506	*MAT*α *scs2*Δ*::TRP1 cki1*Δ*::URA3 ade2-101 leu2-*Δ*1 lys2-801 his3-*Δ*200 trp1-*Δ*63 ura3-52*	This study
KY507	*MAT*α *scs2*Δ*::TRP1 pct1*Δ*::LEU2 ade2-101 leu2-*Δ*1 lys2-801 his3-*Δ*200 trp1-*Δ*63 ura3-52*	This study
KY508	*MAT*α *scs2*Δ*::TRP1 cpt1*Δ*::LEU2 ade2-101 leu2-*Δ*1 lys2-801 his3-*Δ*200 trp1-*Δ*63 ura3-52*	This study
KY509	*MAT*α *scs2*Δ*::TRP1 opi3D::LEU2 ade2-101 leu2-*Δ*1 lys2-801 his3-*Δ*200 trp1-*Δ*63 ura3-52*	This study

Table 2. **Oligonucleotides used in this study.**

Name	Sequence (5′ to 3′)
ACT1F	TGGTGAACGATAGATGGACC
ACT1R	TCTGGTATGTGTAAAGCCGG
CKI1F	AACTGCAGTCTTCTTCTAAGCGA
CKI1R	CCCAAGCTTACTACAGTATATTGATC
CPT1ΔF	TATCGATTAAAGTGCATGTGAAAATTCGCGGGAAGTGACGTGTTAATTTCAGAGGTCGCC
CPT1ΔR	CCATGGATAGCAAGTGTCAAGCCGAGTCCCATCCAAACCAGTTTCGTCTACCCTATGAAC
INO1F	TAACTCTCTGTTGCCCATGG
INO1R	CTTCTTTAACTGGGTCCACC
OPI3ΔF	AGAACATTTCGAAGGGTAAACCACCAGGGCAAGTTGAGGCTGTTAATTTCAGAGGTCGCC
OPI3ΔR	GGGAAGAACCTTTTACTGCCATGATCTACGCTAACCGTGAGTTTCGTCTACCCTATGAAC
PCT1F	CCGCATGCATGGCAAACCCAACAACAGG
PCT1R	CCTCTAGATAACCTGTCGTTGTCGTCGT

some experiments, uracil was removed for plasmid maintenance.

Yeast Transformation—Yeast transformation was performed by the modified lithium acetate method (*28*).

Gene Disruption—Genes were disrupted by the one-step gene disruption method (*28*). For *CKI1* disruption, the *CKI1* coding region was amplified by PCR [primers: CKIF and CKIR (Table 2); template: genomic DNA]. The PCR product was digested with *Pst*I and *Hin*dIII and subcloned into the *Pst*I and *Hin*dIII sites of pBluescriptII to yield pKY208. A 1.1 kb *Eco*RI region of pKY208 was replaced with *URA3* to yield pKY210. A 2.4 kb *Pst*I–*Hin*dIII fragment of pKY210 was used for *CKI1* disruption. For *PCT1* disruption, the *PCT1* coding region was amplified by PCR [primers: PCT1F: and PCT1R (Table 2); template: genomic DNA]. The PCR product was digested with *Sph*I and *Xba*I and subcloned into the same sites of pUC18 to yield pRZE4. A 1.8 kb *LEU2* fragment was inserted into the *Pst*I site of pRZE4 to yield pRZE5. A 3.1 kb *Pst*I–*Hin*dIII fragment of pRZE5 was used for *PCT1* disruption. The *CPT1* and *OPI3* genes were replaced by *LEU2* using a DNA fragment generated by PCR. The primers used for the fragment generation were: CPT1ΔF and CPT1ΔR for *CPT1*, OPI3ΔF, and OPI3ΔR for *OPI3* (Table 2). The template used was pACT2 (Clontech). The recombinant genes were identified by positive PCR diagnosis with the use of one primer hybridizing to sequences within the marker gene coding region and another primer hybridizing to sequences outside the deleted region of the targeted gene.

β-Galactosidase Assays—Yeast strains (KY503 and KY504; Table 1) containing YCp*INO1*Z were used for the β-galactosidase assays. YCp*INO1*Z is a low-copy plasmid containing *URA3* and *INO1*-lacZ (*30*). The yeast cells were grown in inositol-containing medium at 28°C to an OD_{600} of 1.0. The cells were washed three times with inositol-free medium and incubated at 28°C or 37°C for 3 h in inositol-containing or inositol-free medium. The cells were disrupted by $CHCl_3$ and SDS, and the β-galactosidase activities were measured using *o*-nitrophenyl-β-D-galactopyranoside as a substrate. The β-galactosidase units were calculated as described by Burke *et al*. (*28*).

Northern Analysis—The appropriate yeast strains were grown overnight in inositol-containing medium at 28°C. The cells were washed three times with fresh inositol-free medium and incubated in inositol-free medium for 3 h at 28°C or 34°C. Total RNA extraction was performed by the method of Cross and Tinkelenberg (*31*). Total RNA (15 μg) was separated in a 0.66 M formaldehyde-containing agarose gel and transferred to Hybond N+ (Amersham Pharmacia Biotech). DNA probes were generated by PCR using primer pairs (INO1F and INO1R for *INO1*; ACT1F and ACT1R for *ACT1*; Table 2) and yeast genomic DNA as a template. DNA labeling with digoxigenin (DIG) and Northern hybridization were performed using a DIG-High Prime DNA Labeling and Detection kit (Roche Molecular Biochemicals) according to the manufacturer's instructions. The hybridization signals were visualized by autoradiography using RX-U X-ray film (Fuji Film).

Phospholipid Analysis—Steady-state phospholipid labeling with $^{32}PO_4^{3-}$ was performed following the method of McGee *et al*. (*32*) with minor modifications. The appropriate yeast strains were grown in inositol-containing medium overnight and then subcultured in the same medium to an OD_{600} of 0.01. In some experiments, the inositol concentration of the inositol-containing medium was adjusted to 10 μM. The cultures were then presented with $^{32}PO_4^{3-}$ (3 μCi/ml; Perkin Elmer) and incubated at 28°C or 34°C with shaking for 20 h. The yeast cells were disrupted by incubation with 0.3 ml of zymolyase solution [20 μg/ml zymolyase 100T (Seikagaku Kogyo)] at 37°C for 30 min. Total phospholipids were recovered from the cell suspension by the addition of 3 ml of $CHCl_3$/CH_3OH (2:1), followed by incubation at 4°C for 1 h. The extraction mixtures were subsequently centrifuged for 10 min to separate the organic and aqueous phases. The organic phase was removed, dried under N_2 gas, and resuspended in 200 μl $CHCl_3$/CH_3OH (2:1). Radiolabeled phospholipids were resolved by 2-D paper chromatography on SG81 paper (Whatman) or by 1-D chromatography on HPTLC Silica Gel 60 plates (Merck). For the 2-D chromatography, the first dimension solvent was $CHCl_3$/CH_3OH/NH_4OH/H_2O (22:9:1:0.26) and the second dimension solvent was $CHCl_3$/CH_3OH/CH_3COOH/H_2O (8:1:1.25:0.25). For the 1-D chromatography, the solvent was $CHCl_3$/$(CH_3)_2CO$/CH_3OH/CH_3COOH/H_2O (40:15:13:12:8). The radiolabeled lipids were visualized and quantified using a BAS2000 Bioimaging Analyzer (Fuji Film), and identified by comparison with commercial phospholipid standards (Doosan Serdary Research Laboratories) and phospholipids labeled with *myo*-[^{14}C]inositol or [methyl-^{14}C]choline chloride (American Radiolabeled Chemicals).

Incorporation of Radiolabeled Choline into PC—Overnight cultures of appropriate strains were subcultured in inositol-free medium to an OD_{600} of 1.0. After preincubation for 1 h at 28°C, cells were incubated for 30 min in inositol-free medium supplemented with 10 μM [methyl-^{14}C]choline chloride (0.5 μCi/ml) at 28°C or 34°C. Lipid extraction, separation, and quantitation were performed as described above.

RESULTS

scs2Δ Cells Have Defects in INO1 Expression in Response to Inositol Depletion—To see whether *scs2*Δ cells express the *INO1* gene in response to inositol depletion, *INO1* mRNA levels in *scs2*Δ cells grown in inositol-free medium were examined. Total RNA was prepared from cells grown in inositol-containing or inositol-free medium for 3 h at 28°C and subjected to Northern analysis with an *INO1* probe. In the inositol-free medium, the *scs2*Δ strain expressed less *INO1* mRNA compared with the wild-type strain (Fig. 2). Overexpression of the *SCS2* gene from a low-copy plasmid [YCp(*SCS2*)] increased the *INO1* mRNA level to that of the wild-type strain (Fig. 2). The reduced mRNA levels observed in the *scs2*Δ strain might be due to an accelerated degradation of *INO1* mRNA rather than a defect in mRNA production. To exclude this possibility, we examined the *INO1* mRNA levels of an *scs2*Δ strain containing the *INO1* gene under the control of a promoter that is independent of UAS_{INO} control. For this, we introduced the P_{SEC14}::*INO1* construct, which has the *INO1* gene placed under the constitutive transcriptional control of the yeast *SEC14* promoter (*33*), into an *scs2*Δ strain and examined *INO1* mRNA levels by North-

ern analysis. In inositol-free medium the strain containing the YCp(P_{SEC14}::*INO1*) plasmid produced *INO1* mRNA as well as the wild-type strain (data not shown). This result suggests that *scs2*Δ cells can retain *INO1* mRNA levels produced.

In the former study we found that *scs2*Δ inositol auxotrophy became significant at temperatures above 34°C (*15*). As expected, the *INO1* mRNA level in *scs2*Δ cells grown at 34°C in inositol-free medium was reduced more than in cells grown at 28°C (Fig. 2). This result suggests that the elevated inositol auxotrophy at 34°C is partly due to the *INO1* expression defect at this temperature.

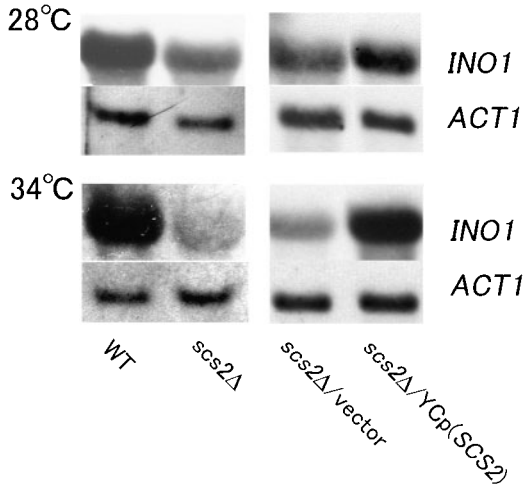

Fig. 2. ***INO1* expression in *scs2*Δ cells.** Cells were grown in inositol-free medium for 3 h at 28°C or 34°C. Total RNA was isolated and used for Northern analysis with the *INO1* probe. The *ACT1* probe was used as a control as an example of a constitutively expressed gene. The strains used were KY354 (WT), KY360 (*scs2*Δ), KY431 (*scs2*Δ/vector), and KY430 [*scs2*Δ/YCp(*SCS2*)].

SCS2 Disruption Does Not Abolish UAS_{INO} Regulation—The overproduction of *INO1* from a low-copy plasmid suppresses *scs2*Δ inositol auxotrophy (*15*). This result is not fully compatible with the finding of this study that *INO1* expression is impaired in the *scs2*Δ strain. To address this question, we examined whether *scs2*Δ cells express a UAS_{INO}-containing gene when the gene is located in a plasmid vector. To this end, we introduced YCp(*INO1Z*), which contains a β-galactosidase gene fused with the *INO1* promoter (*30*), into wild-type and *scs2*Δ strains and measured the β-galactosidase activities of these strains grown in the presence or absence of inositol. In both the wild-type and *scs2*Δ strains, β-galactosidase activity increased in response to inositol depletion (Fig. 3). The tendency did not change when cells were grown at 37°C (Fig. 3), the temperature at which *scs2*Δ cells show a more severe Ino⁻ phenotype (*15*). This result suggests that *scs2*Δ cells can express a gene under the control of the *INO1* promoter, which contains two UAS_{INO} sequences, in response to inositol depletion when the gene is inserted into a plasmid vector. Thus, Scs2p dependent *INO1* expression may be related to structural changes in the chromosomal *INO1* region.

Scs2p Is Required for Normal Steady-State PI and PC Contents—The *INO1* expression defect in *scs2*Δ cells implies that *scs2*Δ cells cannot synthesize PI under derepressing conditions. To examine this possibility, we examined the steady-state PI contents of *scs2*Δ cells grown under derepressing conditions for *INO1* expression. Since *scs2*Δ cells do not grow in inositol-free medium at 34°C, we cultured the cells in medium con-

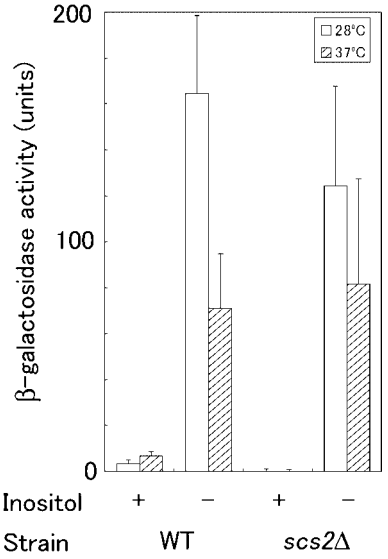

Fig. 3. **Expression of the β-galactosidase gene under the control of UAS_{INO} in *scs2*Δ cells.** Strains [KY504(WT) and KY503(*scs2*Δ)] carrying YCp*INO1Z* were precultured in inositol-containing medium overnight. The cells were washed and incubated in inositol-containing (+) or inositol-free (–) medium for 3 h at 28°C or 37°C. After cell disruption, β-galactosidase activity was measured as described in Materials and Methods. Data presented represent the average ± standard deviation of the results of three experiments.

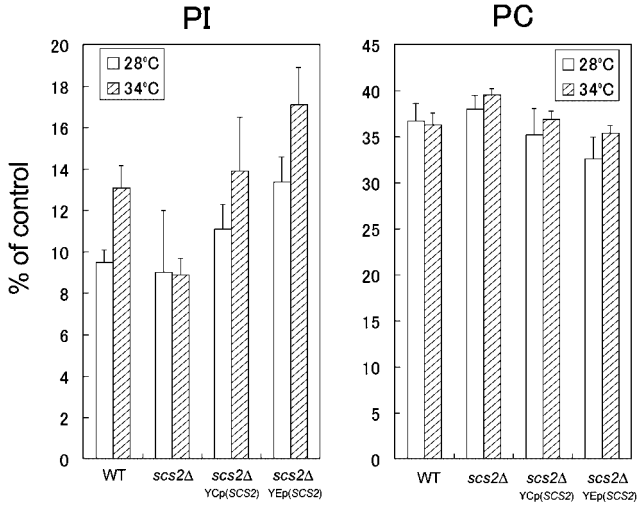

Fig. 4. **Steady-state PI and PC contents of *scs2*Δ cells.** Cells were cultured in inositol-containing (10 μM) medium containing $^{32}PO_4^{3-}$ for 20 h at 28°C (open bars) or 34°C (hatched bars). Phospholipids were extracted and resolved as described in Materials and Methods, and the percentage of total lipid-associated ^{32}P incorporated into PI (left) or PC (right) was determined. Data presented represent the average ± standard deviation of the results of three experiments. Strains used were: KY427 (WT/vector), KY431 (*scs2*Δ/vector), KY430 [*scs2*Δ/YCp(*SCS2*)] and KY429 [*scs2*Δ/YEp(*SCS2*)].

taining limiting amounts of inositol (10 μM). Medium containing 10 μM inositol is used to produce partial derepressing conditions that can induce the expression of *INO1* and other UAS_{INO} containing genes (*34*, *35*). When cells were grown in this medium at 28°C, the PI content of the *scs2*Δ strain was similar to that of the wild-type strain (Fig. 4). At 34°C, however, the *scs2*Δ strain showed a similar PI content (about 9% of total phospholipid) as when grown at 28°C, while the wild-type strain showed a 1.4-fold increase in PI content (Fig. 4). On the other hand, the *scs2*Δ strain showed an approximate 10% increase in PC content at 34°C compared with wild-type cells. There were no significant differences in the contents of other phospholipids between wild-type and *scs2*Δ cells regardless of whether the cells were cultured at 28°C or at 34°C (data not shown). The reduced PI content recovered to the wild-type level in *scs2*Δ cells containing YCp(*SCS2*) (Fig. 4). The *scs2*Δ strain containing YEp(*SCS2*), a high-copy plasmid carrying the *SCS2*, showed about a 1.4-fold increase in PI content at both 28°C and 34°C as compared with the wild-type strain (Fig. 4). The strain also showed a slight decrease in PC content. These results suggest that Scs2p contributes to steady-state PI and PC contents under derepressing conditions.

Disruption of the CDP-Choline Pathway Rescues the Inositol Auxotrophy of scs2Δ Cells—SCS2 was originally isolated as a suppressor gene for inositol auxotrophy of *CSE1* mutants (*14*). Since *CSE1* inositol auxotrophy is rescued by disruption of the genes involved in the CDP-choline pathway for PC biosynthesis (Fig. 1) (*30*), we examined the effect of CDP-choline pathway disruption on the Ino^- phenotype of *scs2*Δ strains. To this end, we prepared *scs2* strains in which each of three CDP-choline pathway genes, including *CKI1*, which encodes choline kinase (*36*), *PCT1*/*CCT1*, which encodes phosphocholine cytidyltransferase (*37*), and *CPT1*, which encodes choline phosphotransferase (*38*), was disrupted. All of these strains (*scs2*Δ *cki1*Δ, *scs2*Δ *pct1*Δ, *scs2*Δ *cpt1*Δ) grew on the plate containing the inositol-free medium (Fig. 5A). On the other hand, disruption of the *OPI3*/*PEM2* gene (*39*, *40*), which is involved in another PC biosynthetic pathway [the phosphatidylethanolamine (PE) methylation pathway, Fig. 1], did not lead to the recovery of the growth defect of the *scs2*Δ strain (Fig. 5A). The growth of the *scs2*Δ *cpt1*Δ strain was slow compared with the other two strains (Fig. 5A). This may be due to incomplete disruption of the CDP-choline pathway because *EPT1*, which encodes ethanolamine phosphotransferase, can partially suppress *cpt1* mutations by contributing the net PC synthesis through the CDP-choline pathway (*41*, *42*). Northern analysis showed that the *INO1* mRNA levels in the strain grown in inositol-free medium at 34°C recovered to the wild-type level (Fig. 5B), indicating that recovery was due to increased *INO1* expression rather than an alteration in inositol metabolism.

The effects of CDP-choline pathway disruption described above suggest that the CDP-choline pathway is upregulated in *scs2*Δ strains. To test this possibility, we examined the efficiency of PC synthesis through the CDP-choline pathway by estimating the amounts of radiolabeled PC in cells cultured in the presence of [methyl-^{14}C]choline. As expected, the *scs2*Δ strain incorporated

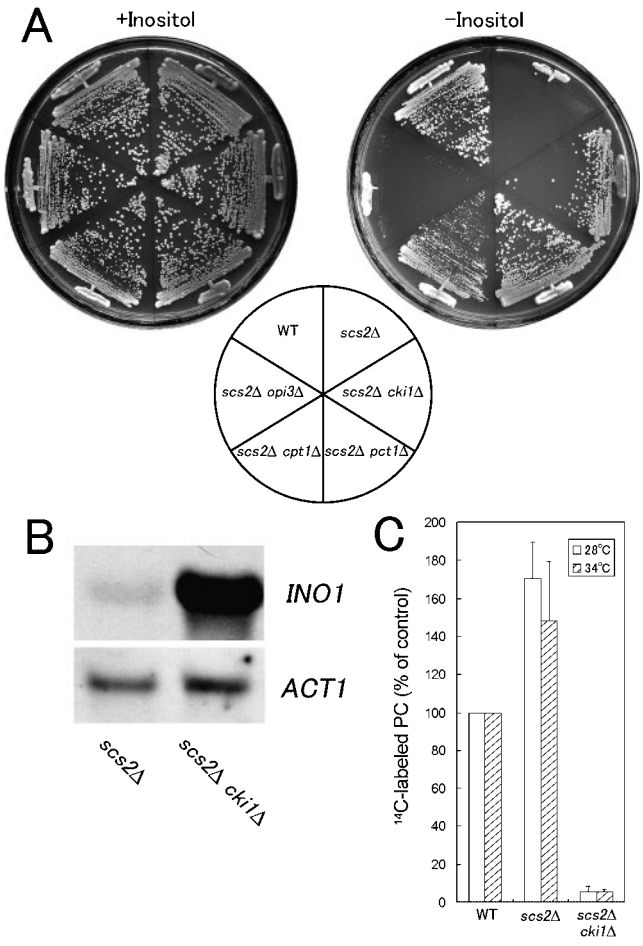

Fig. 5. **Effect of the CDP-choline pathway on the inositol auxotrophy of *scs2*Δ cells.** (A) Indicated strains were grown in inositol-containing (+Inositol) or inositol-free (–Inositol) medium for 2 days at 34°C. The strains used were KY354(WT), KY360(*scs2*Δ), KY506(*scs2*Δ *cki1*Δ), KY507(*scs2*Δ *pct1*Δ), KY508(*scs2*Δ *cpt1*Δ), and KY509(*scs2*Δ *opi3*Δ). (B) Northern analysis. Total RNA was isolated and used for Northern analysis with the *INO1* probe. The *ACT1* probe was used as a control as an example of a constitutively expressed gene. The strains used were KY360 (*scs2*Δ) and KY506 (*scs2*Δ *cki1*Δ). (C) Incorporation of choline into PC. Indicated strains were pulse radiolabeled for 30 min with [methyl-^{14}C]choline (0.5 μCi/ml) in inositol-free medium at 28°C (open bars) or 34°C (hatched bars). Total phospholipids were extracted and resolved by one-dimensional chromatography. Radiolabeled PC was visualized and quantified as described in Materials and Methods. Data are the means ± standard deviations of the results of four independent experiments expressed as the percentage of wild-type cells (WT). The strains used were KY354(WT), KY360(*scs2*Δ), KY506(*scs2*Δ *cki1*Δ).

about 1.5-fold more radioactive choline into PC than the wild-type strain when incubated in inositol-free medium for 30 min at either 28°C or 34°C (Fig. 5C). The slight decrease in choline incorporation efficiency at 34°C compared with 28°C may be due to the growth defect of the *scs2*Δ strain in inositol-free medium at 34°C. *CKI1* disruption reduced choline incorporation to about 5% the level of incorporation observed in the wild-type strain (Fig. 5C), indicating that the elevated PC synthesis observed in the *scs2*Δ strain requires the CDP-choline pathway. The residual PC biosynthesis activity in the

*scs2*Δ *cki1*Δ strain was similar to activities reported previously (*43–45*).

DISCUSSION

In this study we found that *scs2*Δ cells do not fully induce *INO1* expression in response to inositol depletion (Fig. 2). Although the Ino⁻ phenotype of *scs2*Δ cells is significant at 34°C (*15*), the *INO1* expression defect and the accelerated incorporation of radioactive choline into PC were observed in cells cultured at 28°C, indicating that Scs2p is not a protein that only acts at higher temperatures (Figs. 2 and 5). The phospholipid analysis data suggest that the severe Ino⁻ phenotype at 34°C is due to the inability of *scs2*Δ cells to produce sufficient amounts of PI to meet the elevated PI requirement observed in wild-type cells at this temperature (Fig. 4). Other than the PI content at 34°C, *scs2*Δ cells showed no significant differences in phospholipid composition under derepressing conditions at 28°C (unpublished data). These results support our former conclusion that Scs2p does not act as a general transcriptional activator for UAS_{INO}-containing genes (*15*), since misregulation of UAS_{INO}-containing genes leads to significant abnormalities in phospholipid composition (*1–3*).

The Ino⁻ phenotype of *scs2*Δ cells was suppressed by disruption of the CDP-choline pathway (Fig. 5). The relationship between PC biosynthesis and *INO1* expression has been studied substantially (*3*, *13*). Inactivation of genes involved in the PE methylation pathway causes an over-production of the inositol phenotype (*40*, *43*, *46*). Henry and co-workers have proposed that ongoing PC turnover, not metabolites in PC synthesis, is required for the transcriptional regulation of *INO1* and other UAS_{INO}-containing genes (*43*). This hypothesis is supported by detailed studies on *CKI1*-dependent *INO1* expression using the *cki1 sec14*^{ts} double mutant (*44*, *46*). *SEC14* is a structural gene for a phospholipid transfer protein (*47*), and *CKI1* disruption can bypass a normal essential function of *SEC14* (*48*). It is found that *sec14*^{ts} *cki1* cells overexpress the *INO1* gene even in the presence of inositol at the restrictive temperature (37°C) (*44*). The misregulated *INO1* expression in *sec14*^{ts} *cki1* cells is recovered when the *SPO14*/*PLD1* gene, which encodes a phospholipase D, is disrupted (*46*). Thus, elevated *INO1* expression is thought to be caused by increased PA production catalyzed by phospholipase D (*3*, *46*). In this context, our results (Fig. 5) can be explained by the idea that PC synthesized through the CDP-choline pathway may accumulate in *scs2*Δ cells, since, in contrast to the elevated PC turnover that leads to *INO1* derepression, PC accumulation can inhibit *INO1* derepression. The PC-dependent *INO1* repression is consistent with the result that *SCS2* overexpression can suppress the CDP-choline pathway dependent Ino⁻ phenotype of the *CSE1* mutant (*14*, *49*).

The effects of CDP-choline pathway disruption were observed when *scs2*Δ cells were cultured in inositol-free medium not supplemented with choline. This result is consistent with recent findings that the CDP-choline pathway can contribute to PC biosynthesis in the absence of exogenous choline (*32*, *44*, *50*, *51*). This means that the effect of the PE methylation pathway cannot be estimated by disrupting the genes involved in that pathway even when cells are cultured in choline-free medium, because PC can be synthesized by the CDP-choline pathway. Therefore, although *OPI3* disruption did not rescue the Ino⁻ phenotype of the *scs2* strain (Fig. 5A), we cannot exclude the possibility that PC synthesized through the PE methylation pathway is responsible for the repression of the *INO1* gene as well as that synthesized through the CDP-choline pathway.

A large-scale survey of yeast protein-protein interactions (*52*) has shown that Scs2p associates with the yeast oxysterol binding protein (OSBP) homologs, Osh1p and Osh2p (*53*, *54*). On the other hand, the mammalian Scs2p homolog, VAP-A, has been shown to be associated with OSBP (*56*), indicating that Scs2p and VAP-A have conserved functional roles. The constitutive interaction between VAP-A and OSBP induced by a mutant form of the OSBP causes the accumulation of unfolded proteins and ceramide in the ER (*55*). This result suggests a potential requirement for VAP-A for the unfolded protein response (UPR) regulating the synthesis of ER luminal and phospholipid biosynthetic enzymes (*56–58*). In yeast, at least two genes, *IRE1* and *HAC1,* mediate the UPR pathway. *IRE1* encodes a transmembrane kinase/endoribonuclease and *HAC1* encodes a transcriptional factor activated by *IRE1*. The disruption of either *IRE1* or *HAC1* causes inositol auxotrophy (*59*, *60*). Interestingly, *SCS2* overproduction can suppress the Ino⁻ phenotype of *hac1* cells (*14*). The potential involvement of VAP family proteins in the UPR pathway and the relationship between PC biosynthesis and *INO1* expression observed in *scs2*Δ cells (Fig. 5) suggest that Scs2p resides in a signal transduction pathway that induces derepression of the *INO1* gene in response to the hyperactivation of PC biosynthesis.

A protein–protein interaction study (*52*) also revealed the interaction of Scs2p with Opi1p, a negative regulator of *INO1* expression (*8*), and Stt4p, a PI-4-kinase (*61*), in addition to Osh1p and Osh2p. This potential interaction with proteins involved in lipid metabolism and the results obtained in this study suggest that Scs2p is involved in the coordinated regulation of both lipid metabolism in the ER and specific gene expression. Further studies on proteins that interact with Scs2p will help to elucidate the fundamental functions of Scs2p and other VAP family proteins.

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