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Null Mutation in *IRE1* Gene Inhibits Overproduction of Microsomal Cytochrome P450Alk1 (*CYP 52A3*) and Proliferation of the Endoplasmic Reticulum in *Saccharomyces cerevisiae*[1]

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Overproduction of microsomal cytochrome P450Alk1 (P450Alk1) of *Candida maltosa* in *Saccharomyces cerevisiae* resulted in an extensive proliferation of endoplasmic reticulum (ER) and induction of Kar2p and Pdi1p. The *ire1* null mutation severely suppressed ER proliferation, reduced the level of functional P450Alk1, and showed no induction of these ER chaperones, suggesting that the function of Ire1p is required for ER proliferation upon the overproduction of P450Alk1. Cerulenin, a potent inhibitor of lipid biosynthesis, also induced these chaperones in an Ire1p-dependent manner and limited the production of functional P450Alk1. These results imply that Ire1p may function to restore the balance between membrane proteins and lipids of the ER when the ER is relatively overcrowded by membrane proteins.

Key words: cerulenin, cytochrome P450, ER chaperones, ER proliferation, *IRE1*, yeast.

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The endoplasmic reticulum (ER) is a central organelle for the biosynthesis of secretory and membrane proteins and lipids in eukaryotic cells. Although its function, especially in the synthesis of secretory proteins, has been well characterized during the last two decades, the mechanisms regulating ER biosynthesis and turnover are only poorly understood, which possibly because of the essential nature of the ER for cellular activity and the difficulty in isolating it as an entity. The ER spreads in the cytosol as a network of fragile and sealed membranous structures and has continuity to the outer nuclear membrane. It is also thought to connect with cytoskeletal structures or other organelles (*1*), and its functional and morphological specialization to substructures, such as smooth and rough ERs, further complicates investigations. However, the recent finding that the ER proliferates upon overproduction of certain ER membrane proteins is potentially useful in a system to analyze how ER is generated and controlled (*2*). In *Saccharomyces cerevisiae*, overproduction of Hmg1p resulted in a multi-layered membranous structure surrounding the nucleus (*3*), which is different from the "crystalloid ER" in HMG-CoA reductase-overproducing Chinese hamster cells (*4*). The former structure, comprising karmellae, also resembled the stacked ER membranes of yeast cells overexpressing mammalian cytochrome *b*5 (*5*) or the mammalian 180 kDa ribosome receptor (*6*). The proliferated ER-derived structures in yeast are not limited to such karmella-like membranes but include tubular structures, Bip bodies, and Russel body-like structures, which emerged when *Candida maltosa* cytochromes P450 (*7, 8*), Sec12p (*9*), or a mutant form of fungal secretory aspartic proteinase (*10*), respectively, were overproduced. In case of cytochrome P450, overproduction of mutant variants preferentially induced stacked membrane layers similar to the "karmellae" that are induced by other ER membrane proteins. Since the mutant cytochromes P450 had shorter half lives than the wild-type forms, it is speculated that karmella-like structures may represent a specialized degradative organelle for overproduced ER membrane proteins (*11*). Although similar phenomena are known in mammalian and yeast cells, the yeast system is more promising for examining the underlying molecular mechanism of ER proliferation, because of its well-developed molecular and genetic manipulation systems.

In this article, we report that, upon overproduction of cytochrome P450Alk1 (CYP52A3) of *C. maltosa*, tubular ER proliferated in *S. cerevisiae*, and this process was accompanied by enhanced expression of ER chaperone genes. P450Alk1 overproduction, ER proliferation, and the induction of ER chaperones were blocked by the disruption of the *IRE1* gene, suggesting the essential role of the Ire1p in ER proliferation. Moreover, cerulenin, a potent inhibitor to lipid *de novo* biosynthesis, induced these chaperones in an Ire1p-dependent manner and limited the production of functional P450Alk1. This implies the importance of

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balanced synthesis of ER membrane proteins and lipids, in which Ire1p may be implicated.

EXPERIMENTAL PROCEDURES

Strains and Culture Conditions—*Escherichia coli* MV1190 [Δ(*srl-recA*)*306::Tn*10 Δ(*lac-pro*) *thi supE* F′ (*proAB lacI*^{q} *lacZ*ΔM15 *traD36*)] was used as a host for construction and preparation of plasmid DNA.

All haploid *S. cerevisiae* strains used in this study are listed in Table I. Strain YPH499ΔIRE1-2 transformed with plasmid YEp51Cm1 (*14*) and strain YS18ΔIRE1-2 were crossed by cultivating them together in YPD medium [2% Bacto Tryptone (Difco), 2% glucose, 1% yeast extract] for 6 h at 30°C. Cells were then plated onto SD medium supplemented with 25 μg/ml uracil. SD medium for yeast growth was prepared as previously described (*10*). Single colonies were used for further P450 induction experiments as well as for spore formation to confirm the ploidy of the diploid strain. The medium for culture before the induction of cytochrome P450 contained 0.5% NH_4SO_4, 2.0% Casamino Acids (Technical, Difco), 0.17% Yeast Nitrogen Base w/o amino acids and ammonium sulfate (Difco), and 2% raffinose. The latter two ingredients were separately sterilized as 10-fold concentrated stock solutions. Induction of the cytochrome P450 was started by adding 100 ml of 20% galactose and 1 ml of 1 mg/ml $FeCl_3$ to 1 liter of culture. *myo*-Inositol in SD was fortified to 100 μg/ml, except for a medium to test inositol auxotrophy of *ire1* mutants.

Plasmids and DNA Manipulation—Plasmid pYPR-3831X is a YEp-type high-copy number expression vector that carries *TRP1* as a selective marker, the *GAL1* promoter, a downstream multicloning site including an *Sal*I site, and a transcriptional terminator from the *GAPDH* gene (*10*). Plasmid pYPR-ALK1, a derivative of pYPR-3831X, was constructed as follows. Briefly, the coding region of P450Alk1 [*CYP52A3* (*15, 16*)] was excised with *Eco*T22I and inserted into a *Pst*I site of pUC119 to give pUC119-ALK1. An *Sal*I site was introduced just before the translation initiation codon ATG of P450Alk1 on the plasmid pUC119-ALK1 by *in vitro* mutagenesis. A DNA fragment containing P450Alk1-coding region was excised with *Sal*I and inserted into the *Sal*I site of the multiple cloning site of pYPR3831X to obtain pYPR-ALK1. To form plasmid YCp-IRE1, a 5-kb *Hind*III-*Xho*I fragment from the plasmid pIRE1 (*17*) containing the entire *IRE1* gene was inserted between the *Hind*III and *Sal*I sites of YCp50. The same 5-kb fragment was inserted into pUCdKpnI, of which the *Kpn*I site was destroyed by trimming the *Kpn*I ends with T4 DNA polymerase and subsequent re-ligation. The resultant plasmid was digested with *Kpn*I, blunt-ended, and combined with a blunt-ended 2.2-kb *LEU2* DNA from the plasmid YEp13. Finally, this plasmid, named pUC-LEU2ΔIRE1, was digested with *Pvu*II and used for transformation of *S. cerevisiae* to obtain the strain YPH500ΔIRE1, in which 65% of the *IRE1* gene was replaced by *LEU2*. To disrupt *IRE1* in strains YS18, YPH500, and YPH499 according to Menzel *et al.* (*14*), the *HIS3* marker gene was directly inserted into the *Eco*RV site of the *IRE1* coding region in vector pIRE1. The resulting plasmid pUC-HIS3ΔIRE1 was digested with *Pvu*II and further used to transform the respective *S. cerevisiae* strains to obtain YS18ΔIRE1-2, YPH500-ΔIRE1-2, and YPH499ΔIRE1-2 (Table I). Transformants were checked for inositol auxotrophy, and the gene disruptions were confirmed by Southern blotting.

TABLE I. ***Saccharomyces cerevisiae* strains used in this study.**

Strain	Genotype	Origin
YPH500	*MATα ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1*	(*12*)
YPH499	*MATa ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1*	(*12*)
YPH500ΔIRE1[a]	Isogenic to YPH500, *ire1::LEU2*	This work
YPH500ΔIRE1-2[b]	Isogenic to YPH500, *ire1::HIS3*	This work
YPH499ΔIRE1-2[b]	Isogenic to YPH499, *ire1::HIS3*	This work
YS18	*MATα his3-11 his3-15 leu2-3 leu2-112 ura3-52 can*^{r}	(*13*)
YS18ΔIRE1-2[b]	Isogenic to YS18, *ire1::HIS3*	This work

[a] *IRE1* was disrupted by inserting the *LEU2* marker gene between the two *Kpn*I sites, thus replacing 65% of the *IRE1* coding region. [b] *IRE1* was simply disrupted by inserting the *HIS3* marker gene into the unique *Eco*RV site.

Plasmid pSVYB1 bearing *KAR2* was kindly provided by K. Kohno (Research and Education Center for Genetic Information, Nara Institute of Science and Technology, Nara). pUC-PDI1 bearing *PDI1* was from H. Tachikawa (Faculty of Applied Biological Science, Tokyo University of Agriculture and Technology, Tokyo) and T. Mizunaga (Department of Horticulture, Keisen Junior College, Kanagawa).

DNA manipulation was done according the protocols of Sambrook *et al.* (*18*). Restriction enzymes, T4 DNA ligase, and T4 DNA polymerase were the products of Takara and Nippon Gene and used according to the suppliers' protocols. Yeast transformation was done by the lithium acetate method of Ito *et al.* (*19*).

Southern and Northern Blot Analyses—Yeast genomic DNA was prepared by the spheroplast-proteinase K method of Struhl *et al.* (*20*). Southern blotting and hybridization were done according to the protocol of Sambrook *et al.* (*18*), using Hybond-N membrane (Amersham). A 1.6-kb *Nde*I fragment of the plasmid pUC-LEU2ΔIRE1 was labeled with [α-^{32}P]dCTP (Amersham) by use of a Random Primer DNA Labeling kit (Takara) according to the supplier's protocol and used as a probe.

Yeast total RNA was prepared by the hot-phenol method of Schmitt *et al.* (*21*). Blotting and hybridization were performed following the protocol of Sambrook *et al.* (*18*). Probes were ^{32}P-labeled as described above, and signals were detected by use of BAS2000 Imaging plates (Fuji Film). A 2.0-kb *Sal*I fragment from the plasmid pYPR-ALK1, a 0.8-kb *Eco*RV-*Sal*I fragment from the plasmid pUC-PDI1, a 1.0-kb *Eco*RI fragment from the plasmid pSVYB1, and a 0.5-kb *Cla*I fragment of yeast actin-coding DNA were used to prepare specific probes to *ALK1, PDI1, KAR2*, and *ACT1* mRNAs, respectively.

Western Blot Analysis—Yeast cellular proteins were extracted as previously described (*10*). Briefly, cells collected in a microcentrifuge tube were suspended in 200 μl of 0.2 N NaOH and kept on ice for 10 min. Then 100% (w/v) trichloroacetic acid was added to a final concentration of 10% and the mixture was chilled on ice for 10 min. Precipitates by microfuge centrifugation (15,000 rpm for 5 min at 4°C) were washed twice with ice-chilled acetone and

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dissolved in SDS-sample buffer [125 mM Tris-HCl (pH 6.8), 8% sodium dodecyl sulfate (SDS), 10% glycerol, 0.2% β-mercaptoethanol, 0.01% bromophenol blue (BPB)], being dispersed by use of toothpicks. The resultant turbid solution was neutralized by adding 1 M Tris-HCl (pH 6.8) until the bluish color of BPB was restored, then simmered in boiling water for 5 min. After microfuge centrifugation (15,000 rpm for 5 min at room temperature), the resultant clear supernatant was used as a protein sample.

SDS-polyacrylamide gel electrophoresis was done using a gel of 12.5% acrylamide concentration according to the method of Laemmli (*22*). The separated proteins were transferred to Hybond C membrane (Amersham). The immuno-detection of proteins was done as previously described (*10*). Antiserum against Pdi1p was used at a 5,000-fold dilution. Antibodies against P450Alk1 were used at 1,000-fold dilution (*7*). Peroxidase-conjugated anti-rabbit IgG goat antibody (Organon Technica) was used as the second antibody. The signal intensity was quantified by use of a Laser Scanning Densitometer (Biomed Instruments, USA).

Analysis of Cytochrome P450—The amount of cytochrome P450 in *S. cerevisiae* cell suspension was quantified from the 450 nm peak height of CO-difference spectrum as described (*15*) using a molar extinction coefficient of 91 $mM^{-1}\cdot cm^{-1}$ (*23*).

Extraction and Quantitation of Phospholipids—Cells from 10 ml of culture were collected, washed once, and suspended in 2.0 ml of 0.1 M potassium phosphate buffer (pH 7.2). The suspension was vortexed briefly with glass beads to disrupt cells, and phospholipids were extracted from the smashed cell suspension with chloroform. Extracted lipids were finally dissolved in chloroform, ashed, and their phosphorus content was measured as described previously (*24*).

Electron Microscopy—Yeast cells were collected by brief microfuge centrifugation, fixed with OsO_4 using the rapid freeze-substitution method, stained, embedded in resin, and thin-sectioned as previously described (*10*).

RESULTS

Overproduction of Cytochrome P450Alk1 Induces ER Proliferation in S. cerevisiae—To determine whether the overproduction of cytochrome P450Alk1 (P450Alk1) can induce proliferation of ER structures, and to establish a model system for investigation of ER biogenesis, the *ALK1* gene coding for P450Alk1 of *C. maltosa* was expressed in *S. cerevisiae* under the control of *GAL1* promoter using a multicopy plasmid, pYPR-ALK1. Transformants were first grown on raffinose, then shifted to galactose-containing medium to induce P450Alk1 production. After 12 h of induction, the amount of spectrally active P450Alk1 reached 0.25 nmol/10^8 cells, even higher than that in the *n*-alkane grown *C. maltosa*, judging from the peak at 450 nm in the CO-difference spectra (Fig. 1A). This overproduction of P450Alk1 resulted in extensive proliferation of tubular ER in the host cells as shown in Fig. 2 (panels A and B). The proliferation of ER was reflected in the higher cellular content of lipid phosphorus, which was nearly 150% of the control that carried only vector plasmid (Table II, compare the 1st and 2nd rows), and also in the increase of the fluorescence intensity of staining by DiOC6, which mostly binds to ER (data not shown).

ER Chaperones Kar2p and Pdi1p Are Up-Regulated upon the Induced ER Proliferation—It was of great interest to investigate whether the synthesis of ER chaperones is further induced beyond the normal level, along with the proliferation of ER. Figure 3 shows that *KAR2* and *PDI1* mRNA levels in P450Alk1-overproducing cells are both more abundant than in the control cells. At 9 h after the carbon source shift, the difference was more than twofold in both cases (Fig. 3, A and B). These results indicate that the transcription of both genes is accelerated upon P450Alk1 overproduction and ER proliferation. By using the antibody-directed method, a threefold increase of Pdi1p was detected (Fig. 3C), suggesting that the Kar2p was also at higher level in the induced cells.

Disruption of IRE1 Blocked P450Alk1 Overproduction and ER Proliferation—The increased transcription of the ER chaperones led us to examine the involvement of the *IRE1* gene, whose product activates their transcription in response to misfolded proteins in the ER lumen. The *IRE1* gene was disrupted by replacing its carboxy terminal two-thirds, surrounded by two *Kpn*I sites (*17*), with the *LEU2* marker DNA. The replaced region of *IRE1* included the putative membrane-spanning and protein kinase domains (*17, 25, 26*). The resultant *ire1* disruptant YPH500ΔIRE1 required *myo*-inositol for its growth, as previously described (*17*). When P450Alk1 was overproduced in the disruptant in the same way as done in the wild-type strain, enhanced transcription of *KAR2* and *PDI1* genes were not observed (Fig. 4). These results show that their induction upon the overproduction of P450Alk1 was obviously

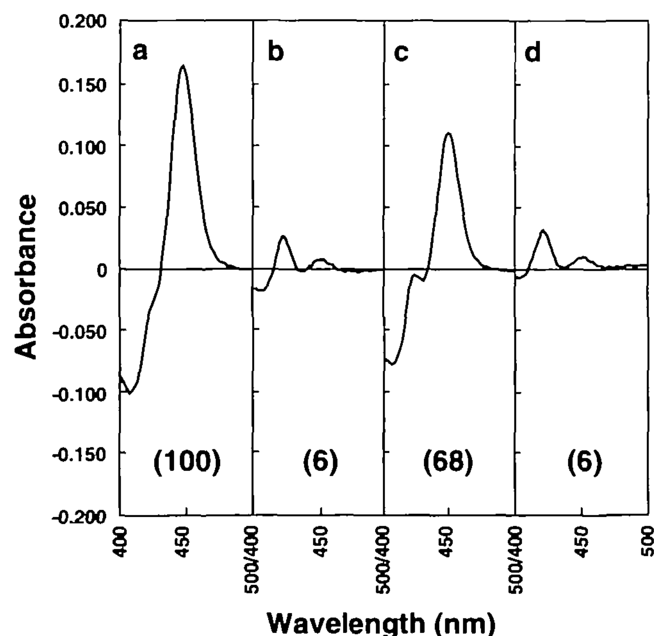

Fig. 1. **Production of P450Alk1 in *S. cerevisiae* under various conditions.** The CO-difference spectra of *S. cerevisiae* cells with the plasmid pYPR-ALK1 at 12 h after addition of galactose are shown. Strains and conditions are: A, YPH500; B, YPH500ΔIRE1; C, YPH500ΔIRE1/YCpIRE1; D, YPH500+cerulenin (15 μg/ml). Relative amounts of P450Alk1 were indicated in parentheses. The amount of P450Alk1 in the wild-type strain YPH500 was 0.25 nmol/10^8 cells (referred to as 100%).

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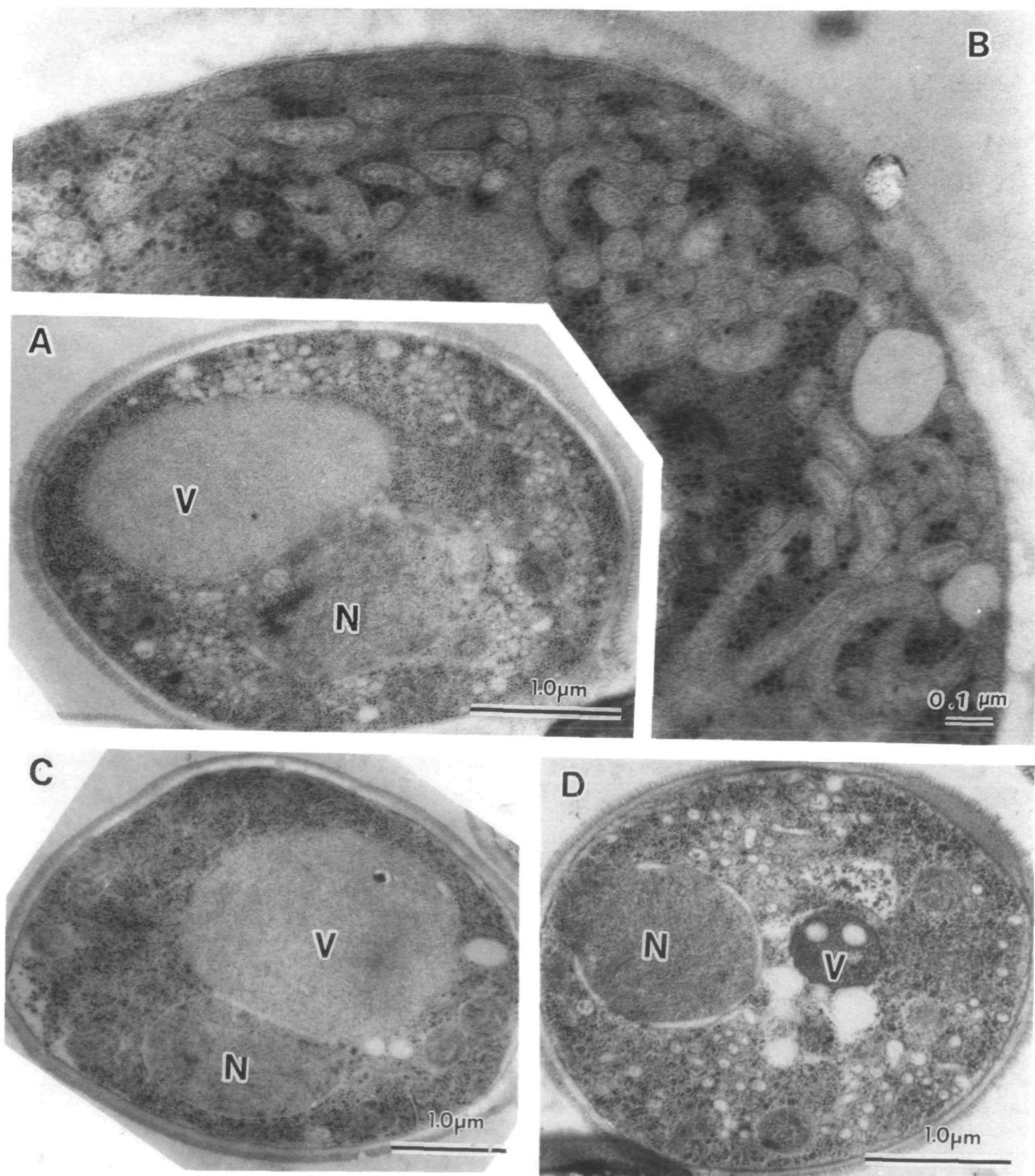

Fig. 2. **Electron micrographs of ultrathin sections of P450Alk1-induced cells after 12 h of induction.** Letters in panels indicate: N, nucleus; V, vacuole. Panels are: A, YPH500/pYPR-ALK1; B, YPH500/pYPR-ALK1, enlarged; C, YPH500⊿IRE1/pYPR-ALK1; D, YPH500 ⊿IRE1/pYPR-ALK1 and YCpIRE1.

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mediated by the *IRE1* gene product. In addition, the *ire1* disruption inhibited ER proliferation, and tubular ER structures were scarcely observed after 12 h of P450Alk1 induction (Fig. 2, panel C). These structures reappeared on introduction of the *IRE1* gene on a YCp vector (Fig. 2, panel D). The ratio of lipid phosphorus to protein was not increased upon induction of P450Alk1 synthesis (Table II, 3rd and 4th rows).

The *ire1* disruption also affected P450Alk1 production. The functional P450Alk1 of the 12-h sample in the null

TABLE II. **Cellular lipid contents after 12 h of induction of cytochrome P450Alk1 under various conditions.**

Strain	Plasmid	Lipid-P[a] (A) (μg)	Protein[b] (B) (mg)	Ratio (A)/(B)×10³
YPH500	pYPR-3831X	32	7.0	4.6
	pYPR-ALK1	44	6.6	6.7
YPH500ΔIRE1	pYPR-3831X	26	6.0	4.3
	pYPR-ALK1	28	6.0	4.7
YPH500[c]	pYPR-3831X	12	2.3	5.2
	pYPR-ALK1	12	2.2	5.5

[a,b]Amounts of lipid phosphorus and protein in 1.0 ml of broken cell suspension, respectively. [c]Cerulenin (2 mg/ml) was added to 15 μg/ml 2 h before cytochrome P450Alk1 induction.

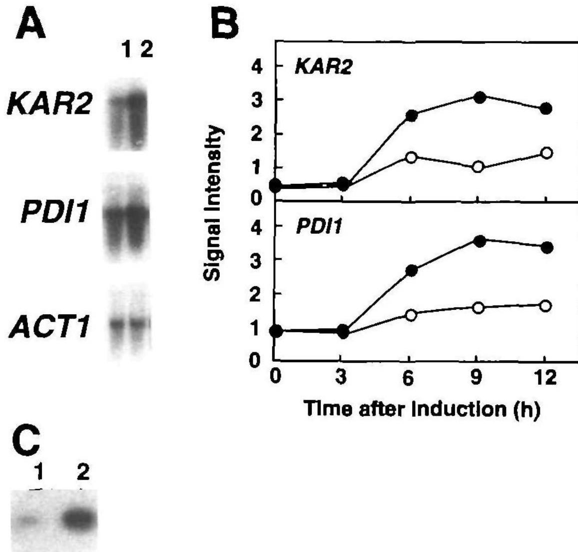

Fig. 3. **Enhanced expression of *KAR2* and *PDI1* genes upon P450Alk1 overproduction.** A and B: A portion of culture was withdrawn after induction of P450Alk1 overproduction, and RNA was analyzed by Northern blot analysis. Results are shown relative to the signal intensity of *ACT1* mRNA. Signals at 9 h are shown in A. Symbols are: open circle, YPH500/pYPR-3831X; closed circle, YPH500/pYPR-ALK1. C: Enhanced production of Pdi1p. Protein samples were prepared after 12 h of induction and analyzed by immuno-blotting as described in "EXPERIMENTAL PROCEDURES." Lanes are: 1, YPH500/pYPR-3831X; 2, YPH500/pYPR-ALK1.

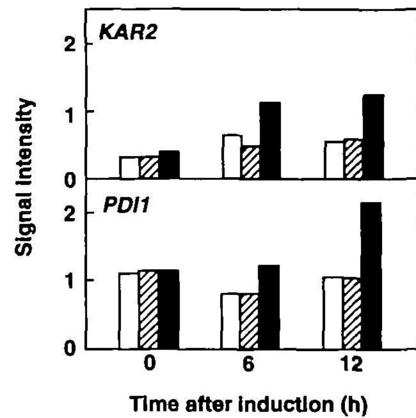

Fig. 4. **Effects of *ire1* disruption on the amounts of *KAR2* and *PDI1* mRNAs.** After addition of galactose, a portion of culture was withdrawn as indicated and analyzed by Northern blotting. Data are expressed relative to the signal intensity of *ACT1* mRNA at each time point. The host strain was YPH500 and the plasmids are indicated in the figure.

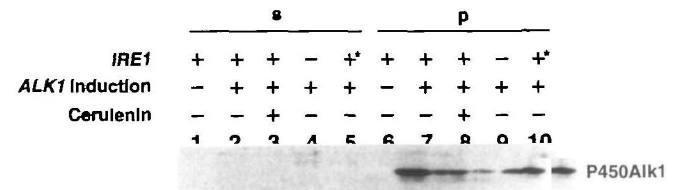

Fig. 5. **The amount of P450Alk1 in the total membrane fraction under various conditions.** After 12 h of induction, cells were collected and disrupted by passing three times through with a French pressure cell (20,000 psi). After removing unbroken cells by brief centrifugation (1,200×*g*, 5 min), membranes were separated by ultracentrifugation (100,000×*g*, 30 min) and analyzed by immuno-blotting. Lanes are: 1-5, 100,000×*g* supernatant; 6-10, 100,000×*g* pellet; 1 and 6, YPH500/pYPR-3831X; 2 and 7, YPH500/pYPR-ALK1; 3 and 8, YPH500/pYPR-ALK1+cerulenin; 4 and 9, YPH500 ΔIRE1/pYPR-ALK1; 5 and 10, YPH500ΔIRE1/pYPR-ALK1, YCp-IRE1.

mutant was about 6% of that of the wild-type strain (Fig. 1B), and the amount of P450Alk1 protein in the total membrane fraction was about 20% of that of P450Alk1-overproducing wild-type cells (Fig. 5, lane 9). The accumulation of *ALK1* mRNA was less influenced by the *ire1* disruption (data not shown).

Inhibition of Fatty Acid De Novo Synthesis Inhibited P450Alk1 Overproduction and ER Proliferation—Cerulenin is a potent inhibitor of fatty acid synthesis. When fatty acid synthesis is inhibited by cerulenin, ER proliferation caused by P450Alk1 overproduction should not occur, because of the limited supply of membrane lipids. Actually, as shown in Table II (5th and 6th rows), the content of lipid phosphorus of the P450Alk1-induced cells was essentially the same as that of non-induced cells. The restricted ER proliferation by cerulenin might give some clue to explain why the overproduction of P450Alk1 did not result in ER proliferation in the *ire1* mutant. The 450 nm peak height of the CO-difference spectrum, which indicates the functional form of P450Alk1, was drastically reduced in the cerulenin-treated P450Alk1 overproduced cells (Fig. 1D), whereas overall P450Alk1 protein content in the membrane fraction was still about 40% of the untreated control (Fig. 5, lane 8), and mRNA level was not reduced (data not shown). These results, which are similar to those observed with the *ire1* disruptant, suggest that lipid supply is necessary for the ER proliferation upon P450Alk1 overproduction and also for the effective assembly of native P450Alk1. Unexpectedly, cerulenin treatment raised the mRNA levels for ER chaperones Kar2p and Pdi1p, whereas induction of P450Alk1 overproduction did not further increase them (Fig. 6, note that probes for the respective genes were the same preparations as those in Fig. 5).

P450 Overproduction Can Be Restored in a Diploid S. cerevisiae Strain Derived from Strain YPH499 and

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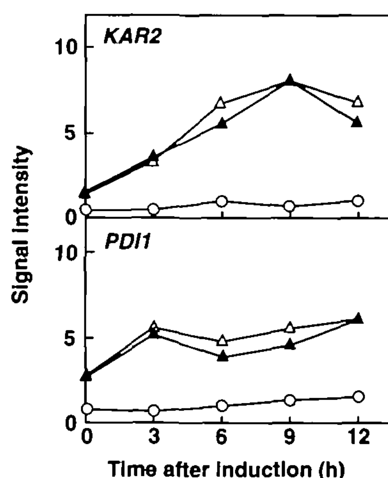

Fig. 6. **Effects of cerulenin on the transcription of *KAR2* and *PDI1* genes.** After addition of galactose, a portion of culture was withdrawn as indicated and analyzed by Northern blotting. Cerulenin (final 15 μg/ml) was added 2 h before the addition of galactose. Data are expressed relative to the signal intensity of *ACT1* mRNA at each time point. Symbols are: open circle, YPH500/pYPR3831X, no addition; open triangle, YPH500/pYPR3831X, plus cerulenin; closed triangle, YPH500/pYPR-ALK1, plus cerulenin.

YS18—Recently, *IRE1* was disrupted by another group of researchers using *S. cerevisiae* strain YS18 and another disruption strategy (*14*). In contrast to our results, however, the *ire1* mutant was still able to overproduce the P450 form to the same extent as the wild-type strain, and ER proliferation still occurred after induction of P450 expression. To confirm the data obtained in our study, we investigated whether the differences in the *IRE1* gene disruption strategy or possibly a difference in genetic background of the two yeast strains might be responsible for the contrary results obtained.

In the study mentioned above, *IRE1* was simply disrupted by insertion of the *URA3* marker gene in the unique *Eco*RV site, which is located downstream of the sequence coding for the putative membrane anchor. Therefore, we speculated that the remaining protein part of Ire1p in the mutant YS18 strain might retain some key functions for ER biogenesis, thus allowing P450 overproduction. We disrupted *IRE1* in strain YPH500 accordingly (insertion of the *HIS3* marker gene into the unique *Eco*RV site of *IRE1*) and tried to overproduce P450Cm1, which represents the CYP52A3 form used in the study of Menzel *et al.* (*14*) and which is different in only 3 amino acids from P450Alk1. As shown in Table III, however, this *ire1* disruptant was not able to overproduce the P450 form, indicating that the striking difference between our data and those reported in the other study (*14*) was not due to the *IRE1* disruption method.

Therefore, we had to assume that a difference in genetic background of strains YPH500 and YS18 was responsible for the clear differences in P450 expression and ER proliferation when *IRE1* was deleted. To prove this hypothesis, we constructed a suitable diploid strain and tested its ability to induce P450 overproduction. As the first step, we disrupted *IRE1* in strains YS18 (*MATα, ura3, leu2, his3*) and YPH499 (isogenic to YPH500; *MAT***a**, *ura3, leu2, his3, lys2, ade2, trp1*) according to Ref. *14* by insertion of the *HIS3* gene into the *Eco*RV site of the *IRE1* coding region. The resulting *ire1* disruptant strain (YPH499-ΔIRE1-2) was then transformed with the P450Cm1 overexpression plasmid YEp51Cm1 which had *LEU2* as a selective marker and was used for CYP52A3 overproduction in the study of Menzel *et al.* (*14*). Finally, both haploid strains (YS18ΔIRE1-2 and YPH499ΔIRE1-2 transformed with YEp51Cm1) were crossed to produce a diploid strain carrying the P450 overexpression plasmid. As shown in Table III, this strain overexpressed P450Cm1 up to 85 pmol/10^8 cells, which corresponds to nearly 50% of the expression level obtained in the strain YS18ΔIRE1-2 or in the YPH499 wild-type strain.

TABLE III. **Influence of the genetic background of *S. cerevisiae* strains YPH499 and YS18 on P450 overproduction in *ire1* disruptants.**

Strain	Plasmid	P450 expression level (pmol/10^8 cells)	(%)
YPH500ΔIRE1-2	YEp51Cm1	<10	<5
YPH499ΔIRE1-2	YEp51Cm1	<10	<5
YS18ΔIRE1-2	YEp51Cm1	180	100
YPH499ΔIRE1-2/ YS18ΔIRE1-2	YEp51Cm1	85	47

This result clearly shows that the two strains differ in their genetic background, which itself determines the ability to overproduce P450 and to proliferate the ER when *IRE1* is deleted. Moreover, the ability to overproduce the P450 form was transferred from strain YS18 to the diploid strain, indicating the dominant nature of the gene(s) involved.

DISCUSSION

The results presented here describe, (i) the enhanced expressions of *KAR2* and *PDI1* genes and simultaneous ER proliferation upon the overproduction of P450Alk1, and (ii) the inhibitory effects of *ire1* null mutation on these processes. Since mRNA levels of these ER chaperone genes are not elevated at all in the *ire1*-disrupted mutant, the enhanced transcription of these genes could be explained as a result of an unfolded protein-triggered, Ire1p-mediated signal transduction (*25, 26*).

One possible explanation for the function of Ire1p in the ER proliferation is that Ire1p might influence the integration of membrane proteins into the ER by controlling the ER chaperone level. Many membrane proteins are integrated first into the ER membrane through the secretory process (*27, 28*), and so should be P450Alk1. Recently, direct involvement of some of the yeast ER chaperones in translocation of proteins across the ER membrane was reported (*29, 30*). Kar2p works together with Sec63p for co-translational and post-translational protein translocation across the ER membrane (*30*). Lhs1p, another Hsp70 family protein in the ER, is also involved in translocation of certain proteins (*31*). The increased production of *KAR2* mRNA and its protein product upon overproduction of cytochrome P450Cm1 in *S. cerevisiae* was recently reported by Menzel *et al.* (*14*). In contrast to strain YS18 that was used by Menzel *et al.* (*14*), the basal level of ER chaperones in strain YPH500 might be lower, and without Ire1p-mediated enhanced production, the effective concentration of chaperones in the ER could not be maintained upon the

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overproduction of P450Alk1, which would limit the integration of P450Alk1 and other membrane proteins into the ER and would not result in the ER proliferation.

Another explanation for the function of the Ire1p in the ER proliferation is that the Ire1p-mediated signaling system might control ER lipid supply. Cox *et al.* (*32*) reported that the Ire1p-mediated regulatory pathway was necessary for maintaining the expression level of the *INO1* gene and that the unfolded protein response through Ire1p resulted in enhanced expression of this gene. Furthermore, a growth inhibitory effect of overproduction of Hmg1p, an ER-resident membrane protein, in an *ire1*-disrupted mutant was overcome by disruption of the *OPI1* gene, which encodes a transcriptional repressor of *INO1* and of many other genes of yeast lipid biosynthesis (*33*). Although their report lacks conclusive data linking the ER unfolded protein response and the function of the *OPI1* gene, the absence of Ire1p-mediated suppression of the *OPI1* gene function, as they hypothesized, might have limited the ER lipid supply and the ER proliferation upon the induction of P450Alk1. It is plausible that the genetic background of the YPH500 *ire1*-disrupted mutant is different from that of the mutant YS18 strain, in which the repressive function of the *OPI1* gene on the lipid biosynthetic genes is less strict than that in the YPH500 *ire1* disruptant.

How the Ire1p was activated by the overproduction of P450Alk1 is a yet unsolved question. The N-terminus of overproduced P450Alk1 molecules protruding into the ER lumen might have been partly recognized as unfolded proteins, which somehow activated Ire1p. Alternatively, ER proliferation caused by the integration of overproduced P450Alk1 into the ER membrane might have enlarged the ER luminal space and reduced the concentrations of ER chaperones. Ire1p may sense the reduced chaperone concentrations or the resultant possible increase in unfolded proteins and generate a signal to enhance the transcription of ER chaperone genes. As an example of the effect of reduced chaperone function, the strains with mutant Kar2p had higher level of transcription of ER chaperone genes including *KAR2* itself (*34*). Also, disruption of the *LHS1* gene resulted in enhanced transcriptions of other ER chaperone genes (*31*).

The fact that the cerulenin treatment caused enhanced transcriptions of chaperone genes in an Ire1p-dependent manner may suggest a new hypothesis to explain Ire1p activation. This result suggests that Ire1p could sense other conditions of the ER, such as over-crowding of ER membrane proteins or an increase in improperly folded ER membrane proteins. Under these conditions, ER membranes of could be produced either by the limitation of lipid supply by cerulenin treatment or by the insertion of the overproduced P450Alk1 into the ER. The finding of Cox *et al.* (*32*) that the inositol deprivation from media resulted in the increased production of Hac1p, which indicates activation of the Ire1p-signaling pathway, could be explained in a similar context. An abrupt reduction of inositol supply will reduce PI synthesis and then PC synthesis through the Sec14p-mediated regulation (*35*), meaning that the inositol deprivation will also result in the limitation of ER phospholipid supply. As a membrane-spanning protein that functions as a protein kinase in a dimeric or multimeric complex (*36*), the Ire1p might be able to sense the overcrowding or mal-folding of the ER membrane proteins.

Finally, the difference between our results and those of Menzel *et al.* (*14*) offers a clue to the function of Ire1p in ER proliferation upon overproduction of P450Alk1. Our strategy of using the physiological strain difference between *S. cerevisiae* YPH500 and YS18 by crossing the two strains and analyzing the resulting spores could reveal the function of novel genes involved in the complicated process of ER biogenesis.

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