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Misfolded Membrane-Bound Cytochrome P450 Activates *KAR2* Induction through Two Distinct Mechanisms[1]

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Using the mRNA differential display technique and Western blot analysis, the present study demonstrates that induction of *KAR2* occurs when misfolded membrane-bound cytochrome P450, mutated in its cytosolically exposed domain, is expressed in *Saccharomyces cerevisiae*. Using various *KAR2* promoter constructs in front of the *Escherichia coli* β-galactosidase reporter gene, we found a fast and strong induction through the heat shock element (HSE), which was enhanced several fold by its adjacent GC-rich region. Additionally, a less pronounced induction was detected for the UPR element (UPRE). As expected, this response was absent in the ire1 disruptant strain. However, the HSE-mediated induction was enhanced upon disruption of *IRE1* suggesting that the HSE pathway can compensate for the lack of a functional UPR pathway. Western blotting confirmed that Kar2p levels were increased to the same extent in the ire1 disruptant and in the non-disruptant strain. Removal of the P450 membrane-spanning region also abolished the UPRE-mediated induction of *KAR2* transcription, but the HSE-mediated response remained. The data show for the first time that the transcription of *KAR2* is significantly induced in response to a misfolded membrane-bound endoplasmic reticulum protein, and identifies the HSE and UPRE regions as *KAR2* promoter elements responding to the misfolded cytosolic P450 domain and to the membrane-integrated mutant P450, respectively.

Key words: cytochrome P450, heat shock element, heat shock protein, *KAR2*, unfolded protein response.

Cytochromes P450 (P450s) are widely distributed heme-thiolate proteins (*1*). They comprise a superfamily of about 900 structurally related variants. In eukaryotes, P450s are an integral component of the ER. As shown for a number of different forms (*2-6*), P450s are anchored in the ER membrane by a short hydrophobic amino-terminal sequence and expose their enzymatically active domain into the cytoplasm. The metabolic function of P450s includes the primary oxidation of a wide variety of endogenous and xenobiotic compounds, which themselves can act as inducing agents stimulating P450 transcription. Such drug-stimulated P450 expression can be accompanied by a massive proliferation of ER membranes, as demonstrated first for phenobarbital-treated rat liver cells (*7*). That the P450 protein itself is sufficient to generate such proliferated ER structures was shown later by overexpressing a *Candida maltosa* P450 form from an artificial multicopy vector (i) heterologously in *Saccharomyces cerevisiae* (*8*) and (ii) homologously in *Candida maltosa* (*9*). In these studies, the P450-containing ER membrane structures demonstrated by immunoelectron microscopy were discussed as the amplification of preexisting ER membranes, a process that might be necessary to accomodate the newly synthesized P450 molecules.

Based on these results, recent studies took advantage of heterologous P450 expression in *S. cerevisiae* to investigate ER membrane biogenesis (*10, 11*) and the intracellular localization of different P450 isoforms (*12*). Interestingly, the morphology of the proliferated membrane structures depends strictly on the quality of the P450 folding state (*12*). When wild-type P450Cm2 was overexpressed in *S. cerevisiae*, a tubular network of ER membranes was found distributed throughout the whole cytoplasm. In contrast, all P450Cm2 variants mutated in their cytosolic domains were generally found to be less stable and to generate nuclear-associated membrane stacks that strongly resemble "karmellae" structures found after the overexpression in yeast of a HMG-CoA reductase isoform (*13*) or karmellae-like ER induced by the overexpression of cytochrome b_5 (*14*). Since even different point mutations introduced into the

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Abbreviations: P450, cytochrome P450; P450Cm2, cytochrome P450Cm2 (CYP52A4); HMG-CoA reductase, 3-hydroxy-3-methylglutaryl coenzyme A reductase; HSE, heat shock element; *lacZ*, *E. coli* β-galactosidase reporter gene; RT-PCR, reverse transcriptase-polymerase chain reaction; UPR, unfolded protein response; UPRE, UPR element.

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cytosolic P450 domain that do not influence the *in vitro* P450 activity were detected in this way by the host cells, a sensitive quality control mechanism was assumed to regulate intracellular P450 sorting and transport (*12*).

To obtain more insight into the underlying mechanism of this protein sorting phenomenon we initiated a study to identify genes that are specifically regulated upon the expression of mutant cytochromes P450. As shown first in this study, we found an increase in the intracellular amounts of chaperones, including Kar2p (Bip), the major stress-70 protein in the ER lumen of yeast (*15, 16*). Kar2p, which is known to cooperate in protein translocation through the ER membrane (*17-19*) and in the folding and assembly of polypeptides in the ER lumen (reviewed in Ref. *20*), has previously been shown to be strongly induced when unfolded proteins accumulate in this compartment. This UPR pathway is an important mechanism for protecting the ER lumen from protein aggregates and for mediating correct folding after stress-induced protein conformational changes (*21*). The signal for enhanced Kar2p production is transferred through the ER membrane by dimerization and autophosphorylation of the transmembrane kinase Ire1p/Ern1p (*22-25*). Then, the phosphorylated Ire1p specifically interacts with a transcriptional coactivator complex (*26*) and is responsible for the generation of an active form of the transcription factor Hac1p (*27, 28*), which recognizes the so-called UPR element, a 22 bp sequence in the *KAR2* promoter (*29, 30*). Interestingly, a recent study demonstrated that phosphatase Ptc2p downregulates the UPR pathway by dephosphorylating Ire1p (*31*). Besides this pathway, the induction of *KAR2* has been described to occur in response to heat shock treatment mediated by its HSE (*15, 16, 30*). This induction pathway is also assumed to be responsible for the relatively high basal expression of *KAR2* (*29*).

The present paper shows for the first time that a strong *KAR2* induction occurs in response to the expression of a misfolded ER membrane protein. Using *lacZ* as a reporter gene, the study further identifies the responsible promoter elements, investigates the time course of *KAR2* induction and provides insight into the role of the large cytosolic P450 domain and the short amino-terminal membrane anchor for *KAR2* induction. Since the folding state of cytochromes P450 can be roughly assessed by comparing the results of enzyme activity assays as well as by spectroscopic (CO difference spectra) and immunological (Western blotting) methods, these enzymes provide a suitable model to show protein quality-dependent and selective *KAR2* induction.

EXPERIMENTAL PROCEDURES

Strains and Culture Conditions—The strain *S. cerevisiae* YS18 (*MATα, his 3-11, his 3-15, leu 2-3, leu 2-112, ura3-52, can*^{r}; kindly provided by Ch. Sengstag, ETH Zürich/Switzerland) was used as the host for P450 expression experiments. The wild-type strain and its ire1 disruptant were transformed according to the lithium thiocyanate method of Keszenman-Pereyra and Hieda (*32*). The transformants obtained were cultivated in yeast minimal medium (1.34% yeast nitrogen base without amino acids), 100 mg/liter L-histidine, 1 mg/liter $FeCl_3$, and 2% raffinose as the carbon source to a cell density of 0.5 to 0.7×10^8 cells per ml before adding 2% galactose to induce cytochrome P450 expression controlled by the *GAL10* promoter. The pH of the cultures was adjusted periodically to pH 4.5-5.0 by adding small aliquots of NaOH (10 M stock solution). *Escherichia coli* strain SURE™ (Stratagene) was used for DNA recombination work.

Differential Display Technique—Total RNA was isolated by a phenol extraction procedure (*10*). RT-PCR reactions, product labeling, separation and detection, as well as isolation of labeled DNA fragments from polyacrylamide gels and their reamplification were performed essentially according to Ref. *33*. Briefly, each of the anchored oligo-dTAN primers (*e.g.*, dTAT corresponds to 5′-TTTTTTTTTTTTTTAT-3′) was used to generate a specific cDNA library using M-MLV Reverse Transcriptase (Gibco BRL). These cDNAs were then used for the following PCR reactions by combining the respective oligo-dTAN primer in combination with each of 21 arbitrary 10mer. Labeling was done with [α-^{33}P]dATP (Amersham). PCR reactions were separated in 6% polyacrylamide gels and the fragment patterns were visualized on X-ray film (Fuji). To isolate fragments of interest from the dried gel, the X-ray film was aligned specifically using luminescent peel-off stickers (Glogos™ markers purchased from Stratagene), and bands corresponding to up- or downregulated genes were marked with a needle. The respective gel slices were cut out and subjected to electroelution. Precipitated DNA fragments were then reamplified and analyzed directly by the dideoxy sequencing method using an LI-COR® 4000 automated DNA sequencer (LI-COR) and a SequiTherm™Long-Read™Cycle Sequencing Kit (Epicentre Technologies Corporation). As the sequencing primer, the respective arbitrary 10mers were used at the usual concentration. IR_{770}-9-dATP was used for the labeling reaction according to the protocol of the supplier (Boehringer Mannheim). As a control, yeast cultivation, RNA and cDNA preparations, as well as the resulting primary PCR reactions were repeated at least once, and, generally, only reproducible differences were considered for further analysis. Database search was done using the BLAST software at the National Center for Biotechnology Information provided *via* the world wide web (http://www.ncbi.nlm.nih.gov/BLAST/). The identity of the DNA sequences was proved by analyzing the size and signal intensity of the labeled DNA fragments after specific restriction endonuclease treatment of the original RT-PCR reaction mixture.

Plasmid Construction for P450 Overexpression and β-Galactosidase Assay—Plasmid YEp51 (*34*) was used as the basic P450 expression plasmid. This vector contains the *LEU2* marker gene, the 2μ DNA origin, and the strong galactose-inducible *GAL10* promoter. Construction of plasmids YEp51Cm2, YEp51M2, and YEp51M4 has been reported previously (*12, 35*). Plasmid YEp51V1 was constructed using a recombinant PCR technique. First, P450Cm2 DNA regions coding for the short N-terminal part (amino acid positions 1 to 26) and the cytosolic P450 domain (positions 79 to 538), but excluding sequences coding for both the putative membrane anchor segments (positions 27 to 78), were amplified in separate PCR reactions. Primers were designed to allow recombination of both fragments in a second PCR round by adding respective overlapping sequences at the 5′-end. The resulting DNA fragment was finally ligated into the *SalI*/*KpnI* site of plasmid YEp51Cm2, thus replacing the original sequence

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with the shortened variant to obtain YEp51V1. The correctness of the construct was proved by DNA sequencing analysis. Plasmid pJS401 (*36, 37*) was used as the basic plasmid for β-galactosidase expression. This plasmid contains the 2μ DNA origin, the *URA3* marker gene, and a transcriptionally silent *ICL1* promoter in front of *lacZ*, which can be switched on by inserting transcriptionally active promoter sequences (*36, 37*). Plasmids pJS-U, pJS-H, and pJS-HG, which contain the UPRE, HSE, and HSE/GC-rich regions, respectively, were obtained by integrating oligonucleotide pairs into the *Bgl*II/*Sal*I site of pJS401: 5′-GATCGGAACTGGACAGCGTGTCGAAAGATCTCGCGAG-3′ and 5′-TCGACTCGCGAGATCTTTCGACACGCTGTCCAGTTCC-3′ (for pJS-U), 5′-GATCTGAGCTCGAGAGAACCTTCTGGAAATTTCAG-3′ and 5′-TCGACTGAAATTTCCAGAAGGTTCTCTCGAGCTCA-3′ (for pJS-H), and 5′-GATCGAGAACCTTCTGGAAATTTCACCCGGCGCGGCACCCGATCGCGAG-3′, and 5′-TCGACTCGCGATCGGGTGCCGCGCCGGGTGAAATTTCCAGAAGGTTCTC-3′ (for pJS-HG). Plasmid pJS-5HG was constructed by PCR amplification and subcloning of the *KAR2* promoter region (−284 to −170) into the *Bgl*II/*Xba*I site of plasmid pJS401. pJS-5ΔHG was obtained from pJS-5HG by modifying both HSE nucleotide segments (GAANNTTC) by recombinant PCR. The following oligonucleotides were used as the internal primer pair containing the respective modifications: 5′-CCTATTGTTTATTAAACCCGGCGCGGCACCCGA-3′ and CCGGGTTTAATAAACAATAGGCCTTATTAAAATAGAAAGTTGCAGCT-3′. This mutagenesis step replaced the characteristic HSE nucleotide segment (GAACCTTCTGGAAATTTC) with a random sequence (AGGCCTATTGTTTATTAA). Preparation, digestion, and ligation of the DNA were carried out according to established procedures (*38*). The transformation of *E. coli* SURE™ (Stratagene) was performed by electroporation (*39*). The correctness of the DNA constructs was checked by the dideoxy-DNA sequencing method (*40*).

Disruption of IRE1—The *HIS3* marker gene was first ligated into the unique *Eco*RV site of the *IRE1* coding region in plasmid pUC119-IRE1 (*11*). The *Pvu*II fragment of the resulting plasmid, pUC119-ΔIRE1::HIS3, was used to transform *S. cerevisiae* YS18. Transformants obtained on histidine-free agar plates were tested for inositol auxotrophy, and the gene disruption was finally proved by Southern blotting.

Analytical Methods—The β-galactosidase activities were measured in cell-free supernatants obtained after mechanical disruption of yeast cells and centrifugation at $3{,}000 \times g$ for 3 min according to Ref. *41*. An extinction coefficient of $1.91\ mM^{-1} \times cm^{-1}$ at pH 7.0 was used. Protein concentrations were determined using a Bio-Rad Protein Assay kit (Bio-Rad Laboratories GmbH). Cytochrome P450 content was determined by CO difference spectra using an extinction coefficient of $91\ mM^{-1} \times cm^{-1}$ (*42*). For measurements to assess the *in vivo* folding state of the P450 variants after heterologous expression, whole cell measurements were recorded as described previously (*8, 12*). SDS-PAGE and Western blotting were carried out according to Refs. *43* and *44*, respectively. Antibodies against the P450 variants and Kar2p were kindly provided by W.-H. Schunck and E. Kärgel (Max Delbrück Centre Berlin, Germany).

RESULTS

Several Genes Including KAR2 Are Specifically Induced upon Expression of Mutant Cytochromes P450 in Saccharomyces cerevisiae—To identify genes that are subject to transcriptional control after the expression of misfolded P450, we screened for differences in the mRNA population of strains expressing wild-type P450Cm2 or mutant P450-M4 by the mRNA differential display technique. P450M4 is a chimeric P450 (Fig. 1A) that has no enzymatic activity after heterologous expression in *S. cerevisiae*. Despite its high level of expression as detected by Western blotting (see also Fig. 3, lane 4), the carbon monoxide (CO) difference spectra showed no peak at 450 nm, but a significantly increased absorption at 420 nm, compared to cells expressing wild-type P450Cm2. This abnormal CO difference spectra indicates the presence of incorrectly incorporated heme due to disturbed intramolecular interactions in the P450 protein. Because of these features, it is reasonable to conclude that mutant P450M4 accumulates as a misfolded protein in the host cells.

Applying nearly 80 different primer combinations (for details see "EXPERIMENTAL PROCEDURES"), a total of 16 genes were found to be reproducibly up- or downregulated (Table I). Among the upregulated genes, we detected sequences coding for ribosomal protein Rps8ap and its homologue on chromosome V, Sec11p, Sec27p, sequences coding for Ty1bp and for chaperones Mdj1p, Lhs1p, and Kar2p. Furthermore, a considerable number of ORFs were found which have not yet been assigned specific functions. Among them, ORF YLR106c, which codes for the largest hypothetical protein in the *S. cerevisiae* genome (nearly 4,600 amino acids), was detected.

The strong induction of *KAR2* transcription (Fig. 2) was the most surprising result, since Kar2p (Bip) is known to function in the secretory pathway as a lumenal chaperone of the ER that is induced in response to the accumulation of unfolded proteins in the ER lumen. Eukaryotic cytochromes P450, however, are known to be anchored and retained within the ER (*6*). Moreover, the mutation was introduced into the C-terminal part of the cytosolically exposed P450 domain while the short 26 amino acid N-terminal region that is assumed to be exposed to the ER lumen remained unchanged. Therefore, we concentrated our efforts on proving the possible function of Kar2p in the process of quality control of an ER membrane protein.

Induction of KAR2 Transcription Correlates with the P450 Folding State—Since the mRNA differential display technique is a highly sensitive method that may produce false positive data, we first analyzed the Kar2p levels by means of Western blotting to confirm the RT-PCR derived results by an independent method. As shown in Fig. 3A, Kar2p was indeed clearly induced in cells expressing mutant P450M4 (lane 4) compared to cells expressing wild-type P450Cm2 (lane 2) or cells harboring the control plasmid only (lane 1).

To answer the question of whether the *KAR2* induction level is unspecific for mutated P450s or whether it correlates with the P450 folding state, a second mutant P450 form was included in this study. This P450 form, named P450M2, contains only a single point mutation at position 498 (Fig. 1A). After expression in *S. cerevisiae*, this P450

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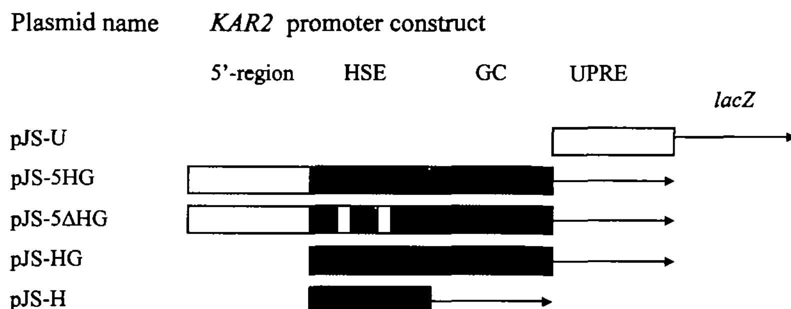
A

Plasmid name	P450 form	Scheme
YEp51Cm2	wild-type P450Cm2	
YEp51M2	mutant P450M2	
YEp51M4	mutant P450M4	
YEp51V1	deletion variant P450V1	

B



C

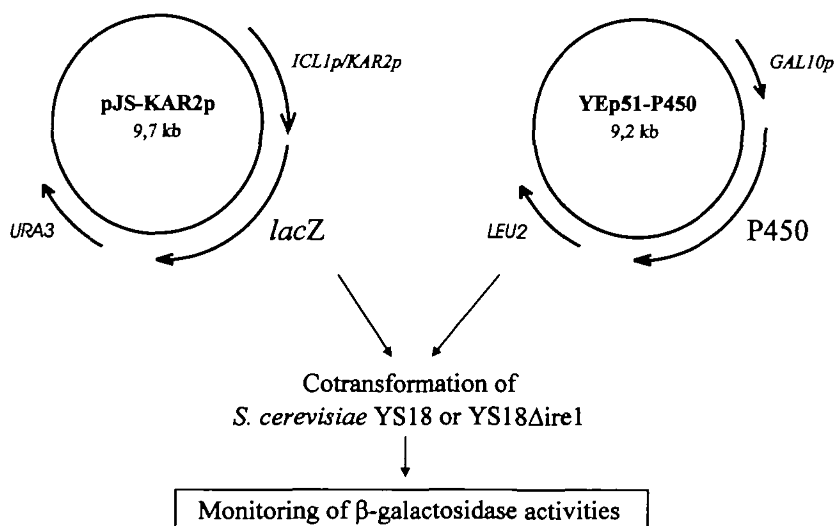


Fig. 1. **Cytochrome P450 forms (A), KAR2 promoter/*lacZ* fusion constructs (B), and experimental strategy (C) used in this study.** (A) P450M2 shows a Val[498]Ala mutant of P450Cm2, P450M4, is a chimeric construct between parental P450Cm2 (1-466) and P450Cm1 (467-523; open box). To obtain P450V1, both of the two putative membrane spanning regions were deleted as described in "EXPERIMENTAL PROCEDURES." (B, C) The different *KAR2* promoter regions were inserted into vector pJS401 which contains a transcriptionally silent deletion variant of the *ICL1* promoter in front of *lacZ* (*36*; see also "EXPERIMENTAL PROCEDURES").

form could be isolated as an enzymatically active P450 form (*35*). Moreover, significant absorption at 450 nm in the CO difference spectra measured with whole yeast cells indicated a spectrally intact protein. In contrast to wild-type P450Cm2, however, this peak was lower at a normal cultivation temperature (28 to 30°C) and an additional absorption at 420 nm was observed. Moreover, the peak at 450 nm shifted rapidly at higher temperatures (>34°C) to a peak at 420 nm. These features indicate that P450M2 represents a partially unstable P450 form, in contrast to P450M4 (for a detailed characterization of P450M2 and M4 see Ref. *12*).

Expression of P450M2 in *S. cerevisiae* indeed led to a weaker *KAR2* induction compared to the signal obtained for P450M4 (see Fig. 3A, lane 3). At the same time, the total amount of P450 produced was nearly the same in all strains investigated, indicating that the differences in the *KAR2* induction levels were not due simply to differences in P450 expression levels. These data show that Kar2p is indeed induced in response to the presence of mutant P450 forms and that its induction level is dependent on the P450 form expressed (P450M4≫P450M2>wild-type P450Cm2 ≥control), which implies a correlation between the P450 folding state and the intensity of *KAR2* induction.

Expression of Mutant P450s Activates the UPR Pathway—In the UPR pathway known to function in the quality control process of secretory proteins, *KAR2* transcription is activated by Hac1p *via* a short 22bp promoter element, the UPRE (*29*). To test whether the same mechanism is responsible for the enhanced Kar2p production, as induced

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TABLE I. **Genes found to be transcriptionally regulated after the expression of mutant P450M4.**

Accession	ORF name	Gene	Chromosome	ΔIntensity[a]	Found by primer combination (5'-3')
		Genes found to be upregulated			
Z35833	YBL072c	*RPS8A*	II	+	CGCCAGGTCA/dTAT
U18839	YER102w	*RPS8B*	V	+	CGCCAGGTCA/dTAT
Z49309	YJL034w	*KAR2*	X	+++	AGGTCGCTTA/dTAT
Z28336	YFL016c	*MDJ1*	VI	++	CCAAGGAGAG/dTAA
Z72659	YGL137w	*SEC27*	VII	+	CCAAGGAGAG/dTAG
Z28073	YKL073w	*LHS1*	XI	++	GGCTCGGTAA/dTAT
Z73278	YLR106c	unknown	XII	+	GGAATCACGC/dTAC
Z72643	YGL121c	unknown	VII	++	CCAAGGAGAG/dTAT
Z74196	YDL148c	unknown	IV	++	CCAAGGAGAG/dTAG
Z72983	YGR198w	unknown	VII	+	CCAAGGAGAG/dTAC
Z49589	YJR089w	unknown	X	+	CCAAGGAGAG/dTAG
Z28325	YKR100c	unknown	XI	+	CCAAGGAGAG/dTAA
several		*TY1B*	several	++	CGCACTAAGG/dTAT
		Genes found to be downregulated			
X07694	YIR022w	*SEC11*	IX	++	CCTGGACCGT/dTAT
Z73041	YGR256w	unknown	VII	++	CGCACTAAGG/dTAC
U28373	YDR379w	unknown	IV	+	CCTGGACTGA/dTAT

[a]Intensity differences were estimated as follows: +, 2-fold; ++, 2- to 4-fold; +++, >4-fold.

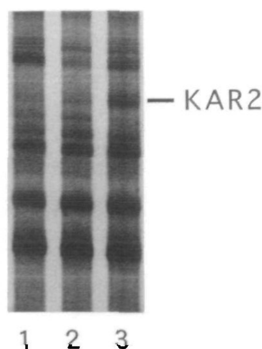

Fig. 2. **Differential display pattern showing upregulation of *KAR2* transcription upon the expression of mutant P450M4.** Respective *S. cerevisiae* strains were cultured in parallel as described in "EXPERIMENTAL PROCEDURES," and P450 expression was induced by adding galactose to 2%. Strains were transformed using the following plasmids: lane 1, control plasmid YEp51; lane 2, P450Cm2 overexpression plasmid YEp51Cm2; lane 3, P450M4 overexpression plasmid YEp51M4. Total RNA was prepared from the cultures 4 h after induction. The growth curves of the three different *S. cerevisiae* cultures were almost identical. The identities of the bands corresponding to *KAR2* were proved for each lane by extraction of the respective gel pieces and sequencing analysis of reamplified DNA fragments, as described in "EXPERIMENTAL PROCEDURES." The respective primer combination is given in Table I. The transcriptional upregulation of *KAR2* was confirmed twice by repeating cultivation, RNA preparation, and RT-PCR reactions.

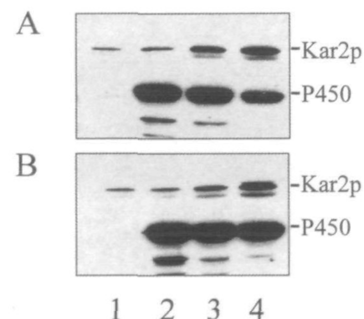

Fig. 3. **Western blot analysis demonstrating elevated levels of Kar2p in *S. cerevisiae* wild-type strain YS18 (A) and in the corresponding ire1 disruptant (B).** Strains were cultured on raffinose (see "EXPERIMENTAL PROCEDURES") to a cell density of 0.5×10^7 cells/ml before adding galactose (final 2%) to induce P450 transcription from the *GAL10* promoter. Cells were harvested after an induction time of 15 h. 20 μg protein from respective cell extracts was used. Lane 1, strain bearing control plasmid YEp51; lane 2, strain overexpressing P450Cm2; lane 3, strain overexpressing P450M2; lane 4, strain overexpressing P450M4. In both cases, the blot was first treated with the Kar2p antibody, and then reprobed with the P450 antibody to get signals for both proteins.

by the presence of misfolded P450, we inserted the 22bp UPRE into a ΔICL1-*lacZ* fusion construct (*36*) and monitored β-galactosidase activities in the respective co-expression strains (see Fig. 1, B and C). As shown in Fig. 4A, the presence of UPRE led to clearly higher β-galactosidase activities when a mutant P450 protein was expressed, whereas no significant increase was observed for the wild-type protein. These results demonstrate that the UPR pathway is activated in response to the presence of misfolded membrane protein, cytochrome P450M2, and M4. Interestingly, the intensity of *KAR2* induction *via* this pathway, was not different for either mutant P450 variant.

KAR2 Remains Induced When the UPR Pathway Is Blocked—Although the β-galactosidase system is useful to demonstrate the significance of the UPR pathway, it can not explain the difference in the Kar2p level between strains expressing P450M2 and M4, since both mutant enzymes activate *lacZ* transcription to the same extent (compare Fig. 3A and Fig. 4A). This suggests the existence of a second additional mechanism for the induction of *KAR2* transcription when mutant cytochromes P450 are expressed. To prove this hypothesis, we blocked the UPR pathway by disrupting the *IRE1* locus in the *S. cerevisiae* genome according to Ref. *10*. This modification has been shown to eliminate the functional UPR pathway without decreasing the ability of the yeast strain to overproduce cytochrome P450 or to proliferate its ER (*10*). After integrative transformation, a strain which strictly requires inositol for growth (typical phenotype of an ire1 disruptant strain) was selected, and the gene disruption was confirmed by South-

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ern blotting. Then, the different P450 forms were expressed in the presence of the UPRE-*lacZ* fusion (see vector pJS-U in Fig. 1B) and β-galactosidase activities were monitored. As shown in Fig. 4B, no increases in the β-galactosidase activities were found when P450M4 was overproduced, demonstrating a complete block of the UPR pathway. At the same time, however, the Kar2p levels were almost identical in the wild-type and ire1 disruptant

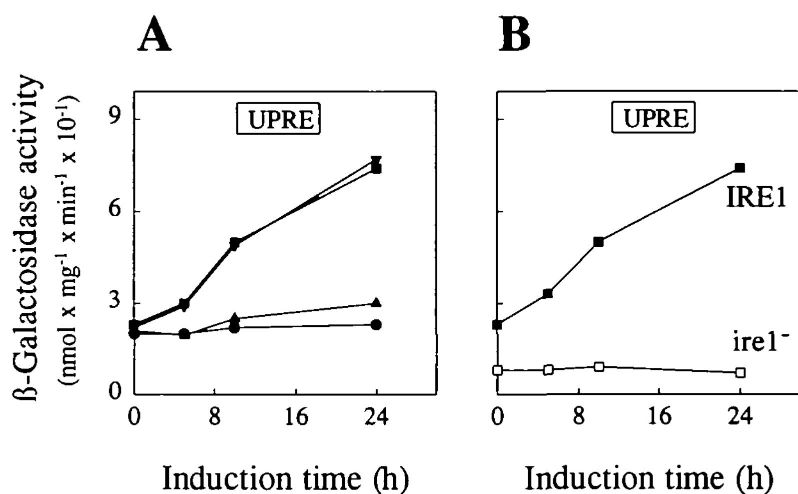

Fig. 4. UPRE-dependent β-galactosidase activities in wild-type strain YS18 after the expression of different P450 variants (A), and the effect of *IRE1* disruption on β-galactosidase activities upon the expression of mutant P450M4 (B). *S. cerevisiae* was co-transformed according to the strategy given in Fig. 1C. Then, the transformants obtained were cultivated in 100 ml yeast minimal medium containing raffinose (2%) at 30°C to a cell density of about 0.5×10^7 cells/ml. After adding galactose to 2%, 10 ml aliquots were removed at the time points indicated and used to determine β-galactosidase activities (see "EXPERIMENTAL PROCEDURES"). ●, control plasmid, ▲, P450Cm2, ▼, P450M2; ■, □, P450M4.

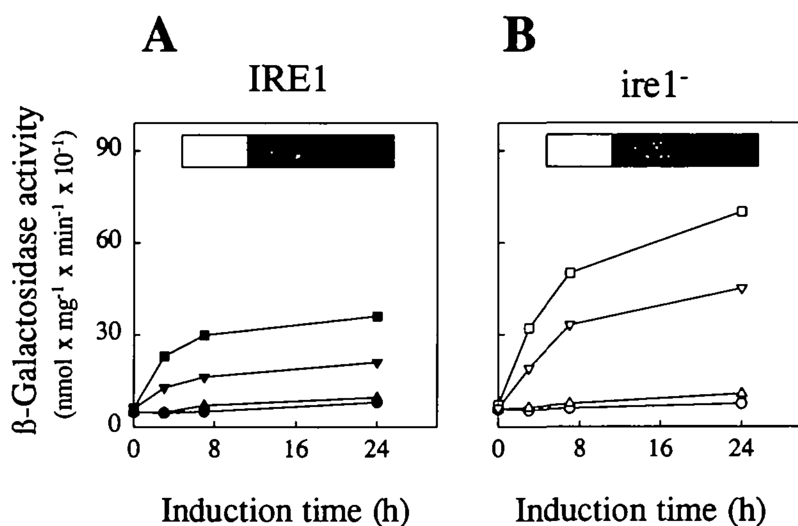

Fig. 5. β-Galactosidase activities using a *KAR2* promoter element lacking the UPRE in wild-type (A) and *ire1* disruptant strain (B). Cultivation of the *S. cerevisiae* strains as well as sampling for the determination of β-galactosidase activities were done as described in the legend to Fig. 4. ●, ○, control plasmid; ▲, △, P450Cm2; ▽, ▼, P450M2; ■, □, P450M4.

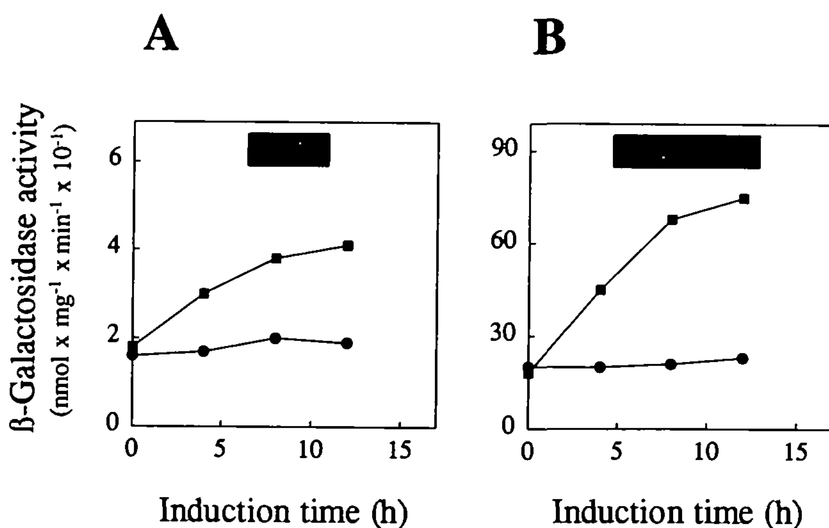

Fig. 6. HSE-dependent β-galactosidase activities (A) and the effect of the GC-rich region on *lacZ* expression (B). For cultivation and sampling to determine the β-galactosidase activities, see the legend to Fig. 4. ●, control plasmid; ■, P450M4.

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strains (compare lanes 4 in Fig. 3, A and B), indicating that the induction of *KAR2* transcription occurs *via* a mechanism that functions independently of the UPR pathway, and that can completely substitute for the lack of a functional UPR pathway.

Transcription of KAR2 Is Induced in Response to the Accumulation of Mutant P450 via an HSE-dependent Mechanism—To confirm these results, a *KAR2* promoter-*lacZ* fusion was constructed that lacked parts of the 3′-site and the UPRE. This construct (see pJS-5HG in Fig. 1B), which still contained the GC-rich region as well as the HSE, was co-transformed with the P450-containing plasmids, and β-galactosidase activities were measured (according to the strategy given in Fig. 1C) for both the wild-type *S. cerevisiae* strain (Fig. 5A) and the *ire1* disruptant (Fig. 5B). As shown in Fig. 5A, a strong induction was found. In contrast to the UPRE-dependent pathway, the induction was already very strong after a short induction time, suggesting that this pathway is activated at an early step. Interestingly, the β-galactosidase activities were significantly lower in the strain expressing mutant P450M2, compared with the data obtained for P450M4, demonstrating that this *KAR2* induction response is correlated with the P450 folding state, in contrast to the UPRE-dependent mechanism.

When testing the same *lacZ* construct in the *ire1* disruptant (see Fig. 5B), up to 1.5 to 2-fold higher β-galactosidase activities were measured when both mutant enzymes were expressed (compare Fig. 5, A and B). The significantly enhanced β-galactosidase activities in the *ire1* disruptant strain strongly suggest that the UPRE-independent *KAR2* induction pathway can compensate for the lack of the UPR pathway, finally leading to equal amounts of Kar2p after the expression of mutant P450 variants (compare Fig. 3, A and B). When expressing wild-type P450Cm2, the β-galactosidase activities were only slightly increased using the same *KAR2* promoter construct.

To further specify the sequence in the *KAR2* promoter that is activated in response to mutant P450, we inserted the *KAR2* HSE into the same *ΔICL1-lacZ*-containing plasmid and tested the ability of the HSE region to induce *KAR2* when the P450 variants were expressed. The short sequence of the *KAR2* HSE mediated *lacZ* transcription when mutant P450M4 was expressed (Fig. 6A). The GC-rich region alone did not induce this response (data not shown), but contributed to the HSE-dependent induction as a strong enhancer (Fig. 6B). Furthermore, replacement of both highly conserved nucleotide segments (GAANNTTC) in the HSE region by random sequences completely repressed *lacZ* transcription even in the presence of the GC-rich region (Fig. 7; for constructs see Fig. 1B). These results demonstrate that the activation of the UPRE-independent pathway in response to the presence of mutant P450s occurs *via* an HSE-dependent pathway.

Lack of the P450 Membrane Anchor Abolishes KAR2 Induction via the UPR Pathway, but Not via the HSE-Dependent Mechanism—To gain more insight into the nature of the two different *KAR2* induction pathways activated upon the expression of the mutated P450 forms, and to discover the role of the membrane spanning region and the misfolded cytosolic P450 domain in this process, a P450

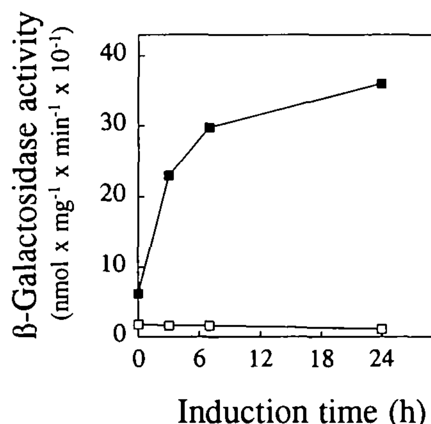

Fig. 7. **Effect of replacement of both conserved nucleotide segments (GAANNTTC) in *KAR2* HSE on β-galactosidase activities after the expression of P450M4.** Plasmid pJS-5HG (■, intact HSE) and plasmid pJS-5ΔHG (□, modified HSE) were cotransformed with YEp51M4 into *S. cerevisiae* YS18 according to Fig. 1C, and the β-galactosidase activities were measured after the addition of galactose to the cultures. Replacement of the conserved HSE nucleotides completely abolished *lacZ* expression (□).

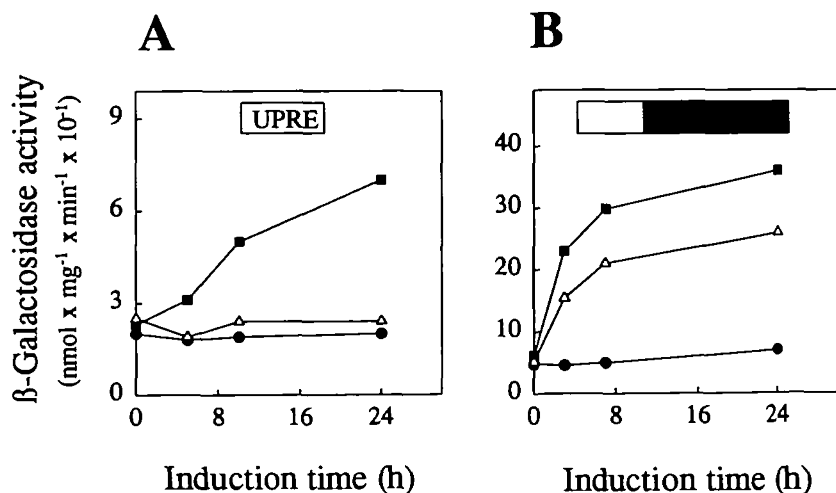

Fig. 8. **Effect of the P450 membrane spanning segment on UPRE-dependent (A) and HSE-dependent (B) *KAR2* induction.** Cultivation of co-transformed yeast strains (see Fig. 1C), P450 induction, and the determination of β-galactosidase activities were done as described in "EXPERIMENTAL PROCEDURES" and in the legend to Fig. 4. ●, control plasmid; ■, P450M4; △, P450V1.

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form lacking the hydrophobic N-terminal membrane anchor was constructed and tested for its ability to induce *KAR2*. Previously, it was clearly shown that deletion of the N-terminal sequence of another member of the same P450 family, P450Cm1, which is highly homologous to P450Cm2 (56% amino acid sequence identity), results in a soluble P450 protein (*5, 6*).

When this deletion variant was overexpressed in *S. cerevisiae* we detected large amounts of the shortened P450 protein by means of Western blotting. At the same time, we could not detect a signal at 450 nm in the CO difference spectra, and cell extracts did not exhibit P450-specific hydroxylation activities. Instead, a large peak at 420 nm was observed in the CO difference spectra, similar to that obtained for mutant P450M4. Thus, this P450 variant accumulates in the cells as a spectrally and enzymatically inactive form and fulfills the requirements of a misfolded cytosolic P450 domain which should induce *KAR2* transcription.

As shown in Fig. 8A, the UPR pathway is not activated in response to the expression of the shortened P450 form. When testing the *KAR2* promoter construct containing the HSE, however, significant β-galactosidase activities were found even after a short time of P450 induction (Fig. 8B). These results show that (i) the membrane spanning domain is required to induce *KAR2* transcription *via* the UPR pathway, a process mediated by Ire1p, and (ii) that a misfolded cytosolic P450 protein domain is already sufficient to induce *KAR2* by a pathway independent of UPRE but mediated by HSE.

DISCUSSION

The present study demonstrates a significant induction of *KAR2* in response to a misfolded ER membrane protein, cytochrome P450. It further reveals that both the UPR pathway as well as an HSE-dependent pathway are activated. The latter pathway was obviously sufficient to maintain the high Kar2p level even in the absence of the UPR pathway, and was found to sense P450 quality, as determined by the CO difference spectra. Activation of the UPR pathway requires membrane integration and functions independently of the folding state of the mutated P450 forms expressed. To our knowledge, this is the first report demonstrating such a selective induction of the major ER chaperone in response to a misfolded ER membrane protein.

By overexpressing wild-type P450 forms (P450Cm1, P450Alk1), previous studies demonstrated elevated levels of Kar2p, which has been discussed as a response to massive ER proliferation caused by an alteration in the protein/lipid ratio (*10, 11*). In these cases, *KAR2* induction was due to activation of the UPRE. Similarly, the overexpression of a wild-type form of HMG-CoA reductase, and thus karmellae formation, also leads to activation of UPRE-dependent *KAR2* induction (*45*). In our study, we also found a slight increase in Kar2p when overexpressing wild-type P450Cm2 (see Figs. 3A and 4A). We propose that the proliferated tubular ER network, which is a characteristic of such yeast cells (*12*), may require not only a higher ER protein and lipid content, but also an elevated level of Kar2p. Compared to data previously obtained for P450Cm1 (*8*), the tubular ER network containing P450-Cm2 is less densely packed in the cytosol (*8, 12*) which is likely due to the significantly lower P450Cm2 expression level (*8, 35*). This may explain the difference in the Kar2p levels between *S. cerevisiae* cultures overexpressing P450-Cm1 or P450Cm2. Mutations in the cytosolic domain of P450Cm2, however, led to a dramatic increase in the intracellular amount of Kar2p, without an increase in the expression level of the mutant P450 forms (Fig. 3). This indicates that *KAR2* induction is, directly or indirectly, an unfolded protein response, due to the activation of two independent pathways. The level of Kar2p found after the expression of mutant P450M4 was clearly higher than that obtained upon overexpression of wild-type P450Cm1. Moreover, we also found significantly higher HSE- and UPRE-dependent β-galactosidase activities when expressing a mutant P450 form, compared to data obtained for P450Cm1 (unpublished results).

Compared to the UPR pathway, *KAR2* induction through HSE is very strong and occurs after a short time of P450 expression. Moreover, this response is independent of the P450 membrane anchor sequence, but strongly related to P450 quality, *i.e.* the ability of the P450 form to generate a spectrally and enzymatically active form. Interestingly, this pathway could obviously substitute for the lack of a functional UPR pathway, as suggested from Western blotting data and measurements of β-galactosidase activities using an *ire1* disruptant strain (see Figs. 3 and 5). Therefore, we conclude that the presence of the HSE/GC-rich region in the *KAR2* promoter allows the generation of an ER-independent *KAR2* induction, and that this pathway does not originate in the ER lumen, but in the cytosolic compartment at a posttranslational step, and should occur already before integration of the P450 form into the ER membrane. Supporting this view, Oka *et al.* found that already a genetically induced loss of cytosolic Ssa1p induces *KAR2* gene expression *via* an HSE-mediated pathway (*46*). It is reasonable to assume that misfolded P450 is also recognized and bound by Ssa1p after its translation, which may initially cause a significant depletion of free Ssa1p molecules. This could be the signal for the following activation of an HSE-dependent pathway, which should lead to the enhanced transcription of several chaperone genes. Besides the enhanced induction of *KAR2, LHS1*, and *MDJ1* (see Table I), we also found increased β-galactosidase activities when testing an *ΔICL1-lacZ* fusion construct containing the HSE of *SSA1* (data not shown). The fact that the induction of SSA gene transcription was not found by the differential display approach is likely due to the limited number of primer combinations used in this study (see "EXPERIMENTAL PROCEDURES"). Clearly, the actual function of Kar2p induced by the HSE-dependent pathway in response to a misfolded membrane protein remains to be established. However, the finding that both Ssa1p depletion (*46*) and the accumulation of a misfolded cytosolic P450 domain can induce *KAR2* necessarily raises the question of whether Kar2p chaperone function is indeed strictly limited to the lumen of the ER, but might be extended to the cytoplasmic compartment.

In contrast to this early unfolded protein response mediated by HSE, a significant activation of the UPR pathway occurs only after several hours of P450 expression and at a lower level. It depends on the membrane anchor sequence of the P450 forms and is independent of the

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quality of the P450 protein. Therefore, we suggest that this *KAR2* induction mechanism is a secondary cellular response to unfolded P450 forms. The signal for *KAR2* induction could simply originate by an extension of the ER lumen during ER proliferation followed by the depletion of Kar2p. As shown previously, both mutant P450s induce the same karmellae-like ER stacks when expressed in *S. cerevisiae* (*12*), and despite the obvious differences in their protein conformations, they are both subject to accelerated degradation in host cells. This ER subcompartment bearing the mutant P450 variants might therefore be closely related to the P450 degradation compartment and might be characterized by a concentration of Kar2p that is specific for such nucleus-associated membrane stacks. This level of Kar2p maintained *via* the UPR pathway could be required for efficient P450 degradation, possibly *via* a role for Kar2p in P450 translocation out of the ER membrane. The fact that the *KAR2* HSE response is enhanced in the ire1 disruptant is consistent with such a requirement for UPR signaling for the disposal of malfolded P450. We argue that the mislocalization of the cytosolic P450 domain to the ER lumen, and the resulting direct recognition of the unfolded P450 domain by Kar2p can not be the *KAR2* induction signal, since both mutant P450 variants differ significantly in their structural quality while inducing *KAR2* expression *via* the UPR pathway to the same extent (see Fig. 4A).

In conclusion, HSE and UPRE control the expression of Kar2p upon the accumulation of misfolded ER membrane protein, cytochrome P450. Both induction pathways are characterized by specific kinetics, that depend either on the protein folding state (HSE) or on membrane integration (UPRE). Although they function in parallel, the HSE-mediated pathway can sense alterations in the UPR pathway, suggesting the existence of a still unknown auto-regulatory mechanism. Thus, *KAR2* induction in response to a misfolded membrane protein seems to be a complex process. Further biochemical and immunological approaches should reveal the functional significance of this complexity for a proper cellular stress response.

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