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Overexpression of the ER-membrane protein P-450 CYP52A3 mimics *sec* mutant characteristics in *Saccharomyces cerevisiae*

Brigitte Wiedmann [a,*], Pamela Silver [b,1], Wolf-Hagen Schunck [c] and Martin Wiedmann [a]

[a] *Cellular Biochemistry and Biophysics Program, Memorial Sloan-Kettering Cancer Center, 1275 York Ave., New York, NY 10021 (USA),* [b] *Princeton University, Department of Molecular Biology, Princeton, NJ 08544 (USA) and* [c] *Max-Delbrück-Center for Molecular Medicine, Robert-Rössle-Straße 10, 1115 Berlin (Germany)*

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High expression of microsomal cytochrome P-450 CYP52A3 from *Candida maltosa* induces the formation of membrane stacks in *Saccharomyces cerevisiae*. Membrane proliferation is accompanied by coinduction of the ER proteins KAR2p and SEC61p and accumulation of precursor forms of proteins that have to translocate across the ER membrane (KAR2p, α factor). Cytosolic proteins (SSA1p and 2p) and mitochondrial proteins (CYT c_1p and $F_1\beta$p) are not affected. N-terminal truncated P-450 proteins remain in the cytoplasm and fail to induce membrane proliferation, KAR2p/SEC61p expression, and precursor accumulation. Membrane and precursor protein accumulation are typical features of *sec* mutants. We assume that the high amounts of P-450p block one or more factor(s) of the transport machinery and thereby cause the observed phenomena.

Introduction

Cytochrome P-450 CYP52A3 [1] (referred to as P-450p) is the major alkane-inducible P-450 protein of the yeast *Candida maltosa*. It is an integral membrane protein of the endoplasmic reticulum [2] and catalyzes, in a complex with NADPH–P-450 reductase, the first and rate-limiting step of alkane metabolism [3,4]. The *C. maltosa* P-450 protein expressed in the distantly related *S. cerevisiae* is able to catalyze the same reaction in conjunction with the host's reductase [5]. P-450p is not degraded rapidly in *S. cerevisiae*, as might be expected for a foreign protein without any function during growth on galactose. Instead, P-450p accumulates in the ER membrane and causes its proliferation [5]. Cells expressing P-450p are viable and grow as fast as cells without the plasmid at 28°C (data not shown).

Membrane proliferation is known to occur in prokaryotes [6,7], yeast cells [5,8,9], and higher eukaryotes [10–15] as a response to increased levels of ER- and/or secreted proteins. An over 100-fold induction of P-450 CYP52A3 in *C. maltosa* causes the appearance of extended membrane tubules [9]. In *S. cerevisiae* overexpression of this protein leads to a more pronounced proliferation of membranes of a distinctive structure [5].

In higher eukaryotes, two morphological and functional distinctive ER-forms exist: rough and smooth ER [16]. Secretion and membrane biogenesis in yeast appears to be analogous to that in higher eukaryotes [17]. However, the two ER-membrane types have not been identified in yeast to date. We report here our efforts to determine whether the proliferated membrane structures in *S. cerevisiae* represent rough or smooth ER. To test this, we investigated the localization of typical marker proteins. SEC61p, an integral ERmembrane protein served as marker of rough ER. KAR2p is a luminal ER protein. Both proteins are necessary for the translocation of proteins into the endoplasmic reticulum [18–23]. In mammalian cells the non-mitochondrial P-450 proteins reside in the smooth ER. Assuming a similar distribution in yeast, the ER-membrane protein P-450 CYP52A3 may represent a marker to localize smooth ER.

A second aim of this paper was to analyze the signal that leads to membrane proliferation. The signal may be overproduction per se of the inducing protein, or it might require incorporation of the protein into the

* Corresponding author. Fax: +1 (212) 7173604.
[1] Present address: Division of Molecular Biology, Dana Farber Cancer Institute, Mayer 849, 44 Binney Street, Boston, MA 02115, USA.

membrane. In order to test this, we constructed N-terminal truncated proteins and investigated their location. We analyzed whether cytoplasmic P-450-constructs were able to induce membrane proliferation.

Materials and Methods

Cells and plasmids. Cytochrome P-450 cDNA was subcloned from the p12T plasmid (pUC119 containing at its *Hin*dII site the P-450 CYP52A3 cDNA [24]) into the yeast expression plasmid YEP 51 [5] (provided by J.R. Broach, Princeton University, NJ, USA [25]). This plasmid contains the *GAL10* promotor which is repressed by glucose and induced by galactose. Raffinose may serve as an inert carbon source. N-terminal deletion plasmids (see Fig. 1) were constructed by standard DNA techniques [26] using naturally occurring restriction sites. Yeast transformation was performed with lithium acetate [27]. Data in this paper were obtained with the *Saccharomyces cerevisiae* strain GRF18 (*α*, *his3-11, his3-15, leu2-3, leu2-112, can*^{R}), kindly provided by D. Sanglard, ETH, Zürich, Switzerland [28]. We observed the same phenomena also in other *S. cerevisiae* strains.

Cell fractionation. Cells were precultured for 36 h at 28°C in selective medium lacking leucine with 2% raffinose or glucose to select for plasmid-bearing cells. The cells usually reached a density of $5 \cdot 10^7$ to 10^8 cells/ml. These cells were diluted 1:100 into YP medium (2% peptone, 1% yeast extract) plus 2% raffinose and allowed to grow to late log-phase (about 10^8 cells/ml). The cells were then diluted 1:3 with YP and galactose or glucose was added (each at 2% final concentration) to induce or repress the expression of the plasmid. We chose complete medium even though the cells might lose their plasmid under these nonselective growth conditions. The cells grew much faster and we wanted to omit any condition which could induce stress. The observation that most of the cells expressed P-450p (as judged by immunofluorescence) seemed to justify the chosen culture conditions. Additionally, we did not find differences when we grew the cells the whole time on selective medium. The culture time is indicated in each figure. The cells were harvested and aliquots were processed for immunofluorescence.

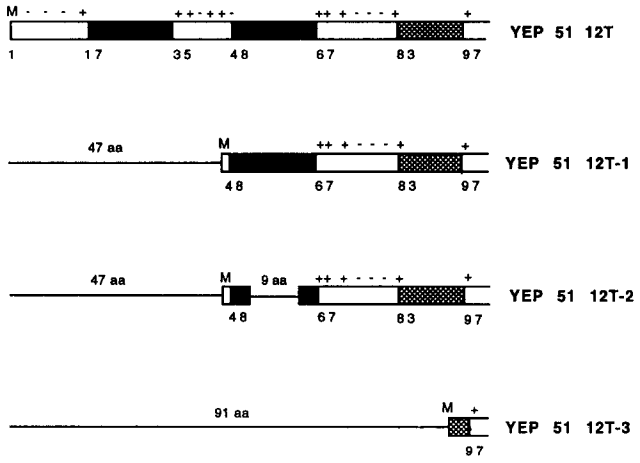


Fig. 1. Structures of plasmids used in this study. YEP 51 12T expresses the full length P-450p under control of the *Gal10* promotor. Using standard DNA-techniques three truncated versions were constructed (YEP 51 12T-1, 2, and 3). Open bars represent amino acid sequences containing the above with − and + indicated charged amino acids. Black bars are hydrophobic sequences. The hatched bars indicate a third hydrophobic region, which is known to be in the cytoplasm. M is the first methionine. The solid lines indicate deleted amino acids in comparison with full length P-450p. Numbers under the bars indicate the numbers of amino acids.

The cells were washed with 1/3 of the original volume YP medium and resuspended at 1 g cells/3 ml in buffer A (50 mM Tris-HCl pH 7.4, 0.5 mM DTT, 1 mM EDTA, 1.2 M sorbitol). 1 mg Zymolyase 100T/g cells was added and the suspension stirred at room temperature for 30 min. The spheroplasts were harvested and washed with buffer A. All following steps were done at 4°C. The spheroplasts were resuspended in 50 mM Tris-HCl (pH 7.4), 0.5 mM DTT, 1 mM EDTA, 0.4 M sorbitol (1 g/2–3 ml), homogenized in a Dounce homogenizer, pestle A, 40 strokes (designated 'cell lysate'), and centrifuged at $3000 \times g$ for 10 min. The supernatant was centrifuged for 20 min at 10000 $\times g$. The pellet ('mitochondria') was resuspended in buffer B (50 mM Tris-HCl (pH 7.4), 0.5 mM DTT, 1 mM EDTA, 150 mM KOAc). The supernatant was centrifuged at $204000 \times g_{av}$ for 15 min, the supernatant ('cytosol') was removed and the pellet resuspended in buffer B ('microsomes'). We used antibodies against the following marker enzymes to test the purity of the fractions by Western blotting: CYT c_1 and $F_1\beta$ proteins for mitochondria; the HSP70 proteins SSA1 and SSA2 for the cytosol; KAR2p, SEC61p, and P-450p for the ER. The ER fraction and the cytosol used in this report were free of any contaminants. It is almost impossible to isolate nuclei or mitochondria free of ER contamination from yeast. The amount of this contamination in our fractionation procedure did not change due to the expression of the foreign protein.

Indirect immunofluorescence. We followed a procedure reviewed in [29]. Exponentially growing cells (5 ml, $OD_{600} \approx 1$) were harvested, washed, and resuspended in 1 ml of 0.1 M potassium phosphate buffer (pH 6.5) with 1.2 M sorbitol. After 90 min fixation in formaldehyde (4% final concentration), cells were washed three times in the same buffer. The cell wall was partially removed with Zymolyase 100T (15 min, 50 μg/ml). The cells were extensively washed and allowed to attach to polylysine coated slides. The slides were incubated at −20°C for 6 min in methanol followed by treatment with acetone (30 s). Dried slides

were incubated overnight with primary antibodies and with the secondary FITC-labeled antibody for at least 2 h. Nucleic acids were stained with 4',6-diamidinophenylindole-HCl (DAPI, 0.1 mg/ml).

Wash of microsomes. Equal amounts of microsomal proteins were diluted with the same volume of either 2-fold low salt buffer (100 mM Hepes/KOH (pH 7.5), 2 mM DTT, 5 mM $Mg(OAc)_2$, 200 mM KOAc), high salt buffer (100 mM Hepes/KOH (pH 7.5), 2 mM DTT, 5 mM $Mg(OAc)_2$, 1 M KOAc), or 0.2 N Na_2CO_3 (pH 11.5). The cells expressing the truncated proteins contained only very small amounts of P-450 protein (see Fig. 5, part A). Therefore, we applied 10 μg microsomal protein from cells carrying plasmid 51 12T, and 70 μg of cells with the plasmids 51 12T-1 and 51 12T-2 in the described washing procedures.

The microsomal pellet was separated from the cytosolic supernatant by a $190\,000 \times g_{av}$ centrifugation step for 10 min. Proteins in the pellet fraction were dissolved immediately in Laemmli sample buffer [30]. Supernatant proteins were collected by TCA precipitation, rinsed with acetone, and then dissolved in Laemmli sample buffer.

Electrophoresis, protein estimation, cell labeling, and Western blotting. SDS-PAGE was performed according to Laemmli, usually on 7.5% gels [30]. The protein content of the samples was determined with the Bio-Rad protein assay kit (Bio-Rad Laboratories) according to the manufacturer's instructions. In vivo labeling of cells with [^{35}S]methionine was essentially as described by Hann and Walter [31]. We used equal amounts of radioactivity for the immunoprecipitations since the cells were not labeled equally. Western blots were developed with the ECL system according to the manufacturer's instructions (Amersham).

Results

Sub-cellular localization and relative quantitation of CYP52A3p, KAR2p, and SEC61p in cells that overproduce CYP52A3p

We have chosen indirect immunofluorescence (Figs. 2 and 3) and cell fractionation followed by Western blotting (Figs. 4 and 5) to investigate the localization of certain proteins. The alkane inducible P-450p of the yeast *C. maltosa* expressed in *S. cerevisiae* is an integral ER-membrane protein: P-450p is maintained in the microsomal pellet even at high pH conditions (Fig. 4). P-450p appears by immunofluorescence to be localized in a typical ER-protein pattern which is a perinuclear ring with occasional thin filaments extending into the cytoplasm (compare KAR2p, SEC61p, and P-450p in Fig. 3). This localization was shown previously by immunoelectron microscopy [5]. Extending the expression time over more than 3 h results in an accumulation of the protein (Fig. 5, Western blots and Coomassie-stained gel). This is accompanied by a proliferation of membranes. We were able to show the formation of membrane stacks by electronmicroscopy recently [5]. These membrane stacks appear in immunofluorescence studies as clumps, first associated with the nucleus and later dispersed over the whole cell (Fig. 3).

Induction of membrane proliferation did not drastically change the protein pattern of the cell or microsomes (Fig. 5, part D). By Western blotting we observed a 2–5-fold increase of KAR2p and SEC61p (Fig. 5, part A corresponds to the same samples loaded in part D) in association with the P-450p induction. The cytosolic HSP70 proteins SSA1p and SSA2p and the mitochondrial proteins CYT c_1 (Fig. 5) and $F_1\beta$ (data not shown) remained unaffected.

We detected by immunfluorescence the same unusual membrane structures when we applied KAR2p or SEC61p antibodies instead of P-450p antibodies to cells grown on galactose. Under repressive growth conditions, e.g., on glucose, no membrane clumps were detectable with any of these antibodies.

Cells that overproduced P-450p accumulated nontranslocated preKAR2p and prepro-α factor

P-450p expression caused an accumulation of KAR2 (Fig. 5, in cell lysates) and α-factor precursor proteins (cell labeling, Fig. 6) in the cytosol. Both proteins are known to be synthesized as larger precursor molecules. KAR2p contains a 3 kDa ER-signal sequence which is cleaved off when the protein enters the lumen of the ER [19,31]. Prepro-α factor is processed to pro-α factor inside the ER and then becomes glycosylated at three sites on the protein. After transport to the Golgi compartment, the glycosylated pro-α factor is cleaved to mature size and secreted [32,33].

We performed control experiments which rule out that the induction of KAR2p/SEC61p and accumulation of precursor proteins is caused by the carbon source: cells containing only the YEP 51 vector do not show these phenomena when cultured on galactose (Fig. 5, part C), neither do cells with the plasmid 51 12T on glucose or raffinose (Fig. 5, parts A, B, and C).

The N-terminal hydrophobic sequences of P-450p are important for the location of P-450p and the coinduction of KAR2p and SEC61p

N-terminal deletion mutants of P-450p were used to test whether membrane incorporation is necessary for induction of KAR2p/SEC61p and membrane proliferation. Plasmids were constructed which encoded variants of P-450p in which either the first hydrophobic sequence was deleted (5112T-1), the first sequence plus 9 amino acids of the second were deleted (5112T-2), or the start AUG codon was removed, so that translation started at a second AUG codon occurring

later in the P-450 DNA sequence (5112T-3). There exist data in the literature where mammalian N-terminal truncated P-450 proteins nevertheless become integrated into the *E. coli* inner cell membrane [34–36]. The truncated P-450 proteins studied in this report were not incorporated into the membrane in *S. cerevisiae*. The proteins encoded by the plasmids 51 12T-1 and 51 12-2 were found in the 190000 × *g* supernatant. A very small amount of these proteins is detectable in the microsomal fraction. It becomes soluble in high salt buffer and is found exclusively in the supernatant at high pH (Fig. 4). These observations were reinforced by immunofluorescence data. We observed these proteins in the cytoplasm (Fig. 2, the left of the two samples in each panel) as shown by the overall staining of the cells independent of the focal plane. Additional membrane staining occurred in some cells which might account for the small amount of membrane associated

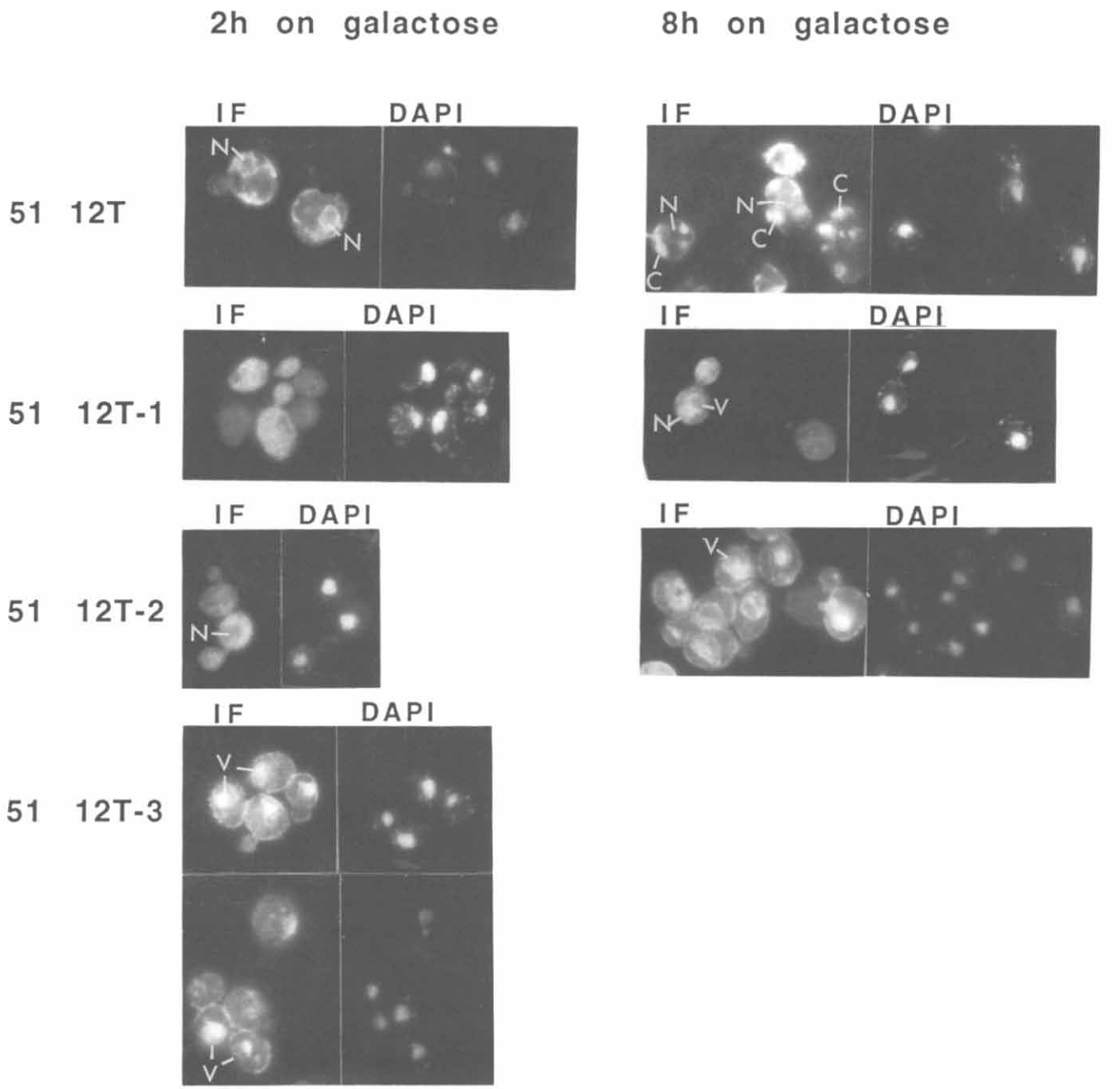


Fig. 2. Full length and N-terminal truncated P-450 proteins are localized in different compartments of the cell. Indirect immunofluorescence was performed with P-450 antibody as described in Materials and Methods. On the right site of each immunofluorescence picture (IF) are the corresponding nucleic acid stains of the same cells by DAPI. The induction times are indicated at the top. There was no immunofluorescence signal detectable after 8 h growth on galactose of cells expressing the plasmid 51 12T-3. Nuclei are marked by N; vacuoles by V; and membrane clumps by C.

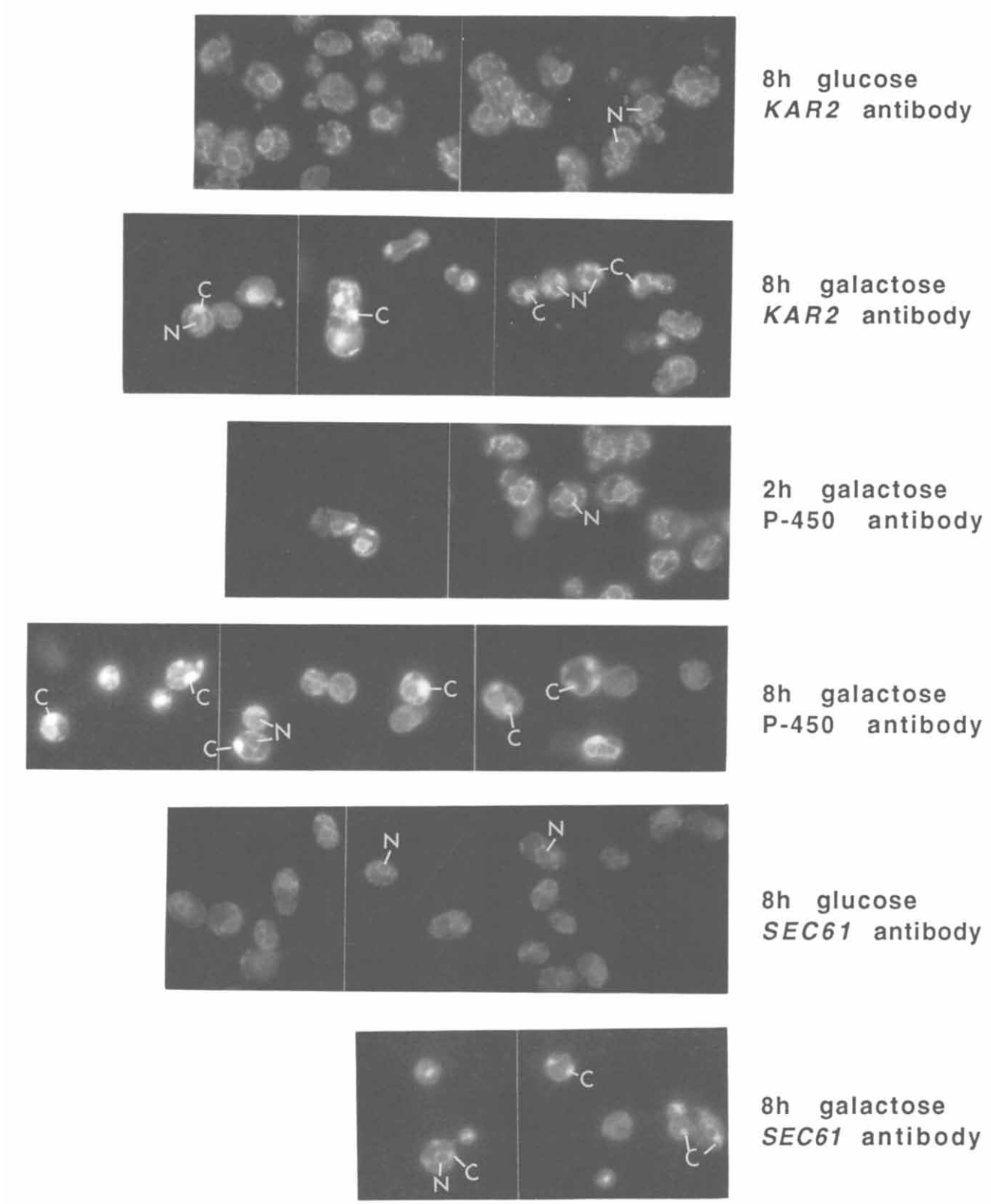


Fig. 3. P-450p, KAR2p, and SEC61p are localized in proliferated membranes of the endoplasmic reticulum. Indirect immunofluorescence was done with cells containing the plasmid 51 12T after growth on the indicated media. The length of induction (h on galactose) or repression (h on glucose), and the first antibody used in the procedure are shown at the right site of the figure. Using the antibody directed against P-450p after growth on glucose gave no immunofluorescence signal. Therefore we represent here induced cells after 2 h growth on galactose where the membranes started to proliferate (see the strong staining of the perinuclear ring). Nuclei are marked by N; vacuoles by V; and membrane clumps by C.

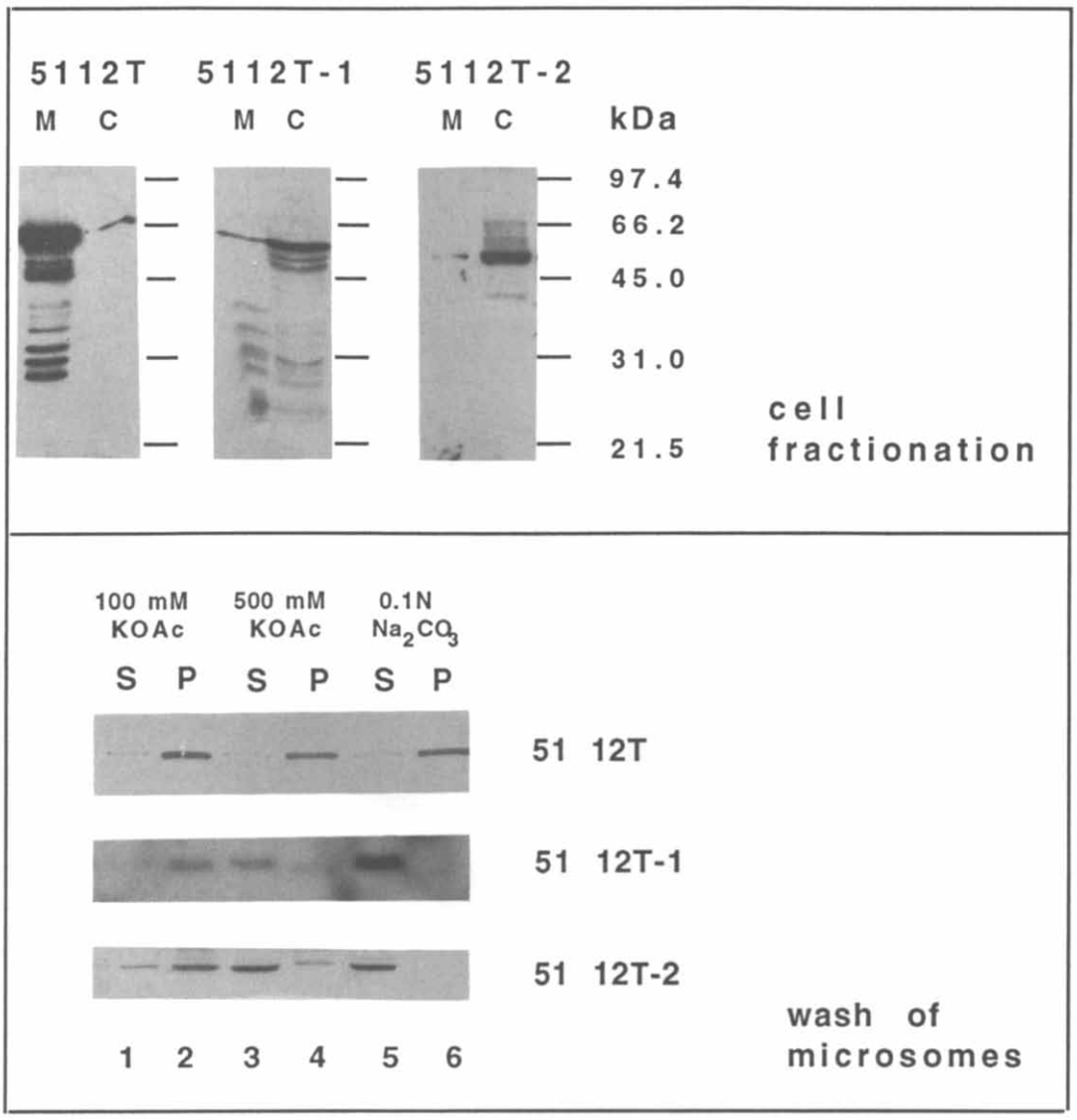
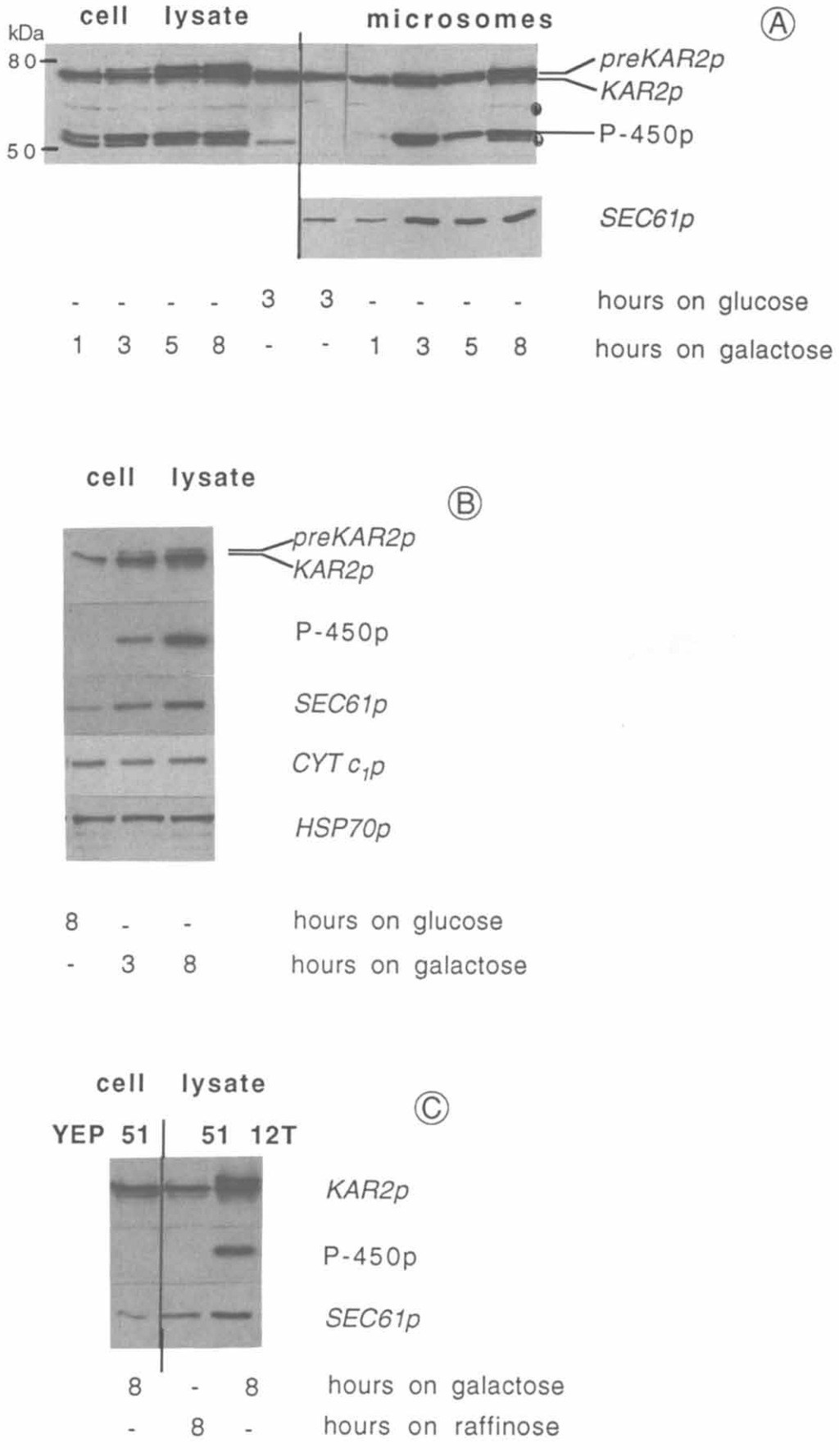


Fig. 4. The first N-terminal hydrophobic amino acid sequence of P-450p is necessary to anchor the protein into the ER membrane. Western blots of microsomes (M) and cytoplasm (C) of cells expressing full length P-450p (51 12T) and the truncated proteins (51 12T-1 and 2). Microsomes of different constructs were diluted into low and high salt buffer or 0.1 N Na_2CO_3, respectively. After separation at 190000 × g the supernatants (S) and pellets (P) were analyzed by Western blotting with P-450 antibodies as described under Materials and Methods.

protein detected by Western blotting. After 8 h both truncated proteins were detectable in vacuoles under our growth conditions (Fig. 2, the samples on the right side). This is most likely their site of degradation, because at that time the amount of truncated proteins starts to decrease (detected by Western blots, data not shown). The shortest protein (encoded by 5112T-3) was not detectable by Western blots. We found this protein by immunofluorescence only in association with the vacuole (Fig. 2).

Fig. 5. Expression of P-450p is accompanied by coinduction of some ER proteins. Equal amounts of cell lysates or microsomes were analyzed in Western blots with the indicated antibodies. The time of induction/repression and the carbon source are given at the bottom in each part of the figure. Parts A, B, and C represent three independent experiments. Part A shows the analysis of cell lysates and microsomes of cells with the plasmid 51 12T in dependence of the induction time. Part D shows the corresponding Coomassie-stained gel: the same samples as applied in part A were separated on a 7.5–15% SDS-polyacrylamide gradient gel. KAR2p and P-450p antibodies were applied together in the part A samples so that the relation between the protein levels of P-450p and KAR2p is not desturbed by an accidentially different amount of loaded protein. The bands marked with • are degradation products of KAR2p. We could not apply several antibodies together on one Western blot because of the similarity in size of some proteins and because of the different sensitivity of the antibodies. Analysis of the amount of HSP70p and CYT C_1p in correlation to P-450p, KAR2p, and SEC61p is shown in part B. Part C represents a control, in cells containing the vector only (YEP 51) no changes were observed during growth on galactose.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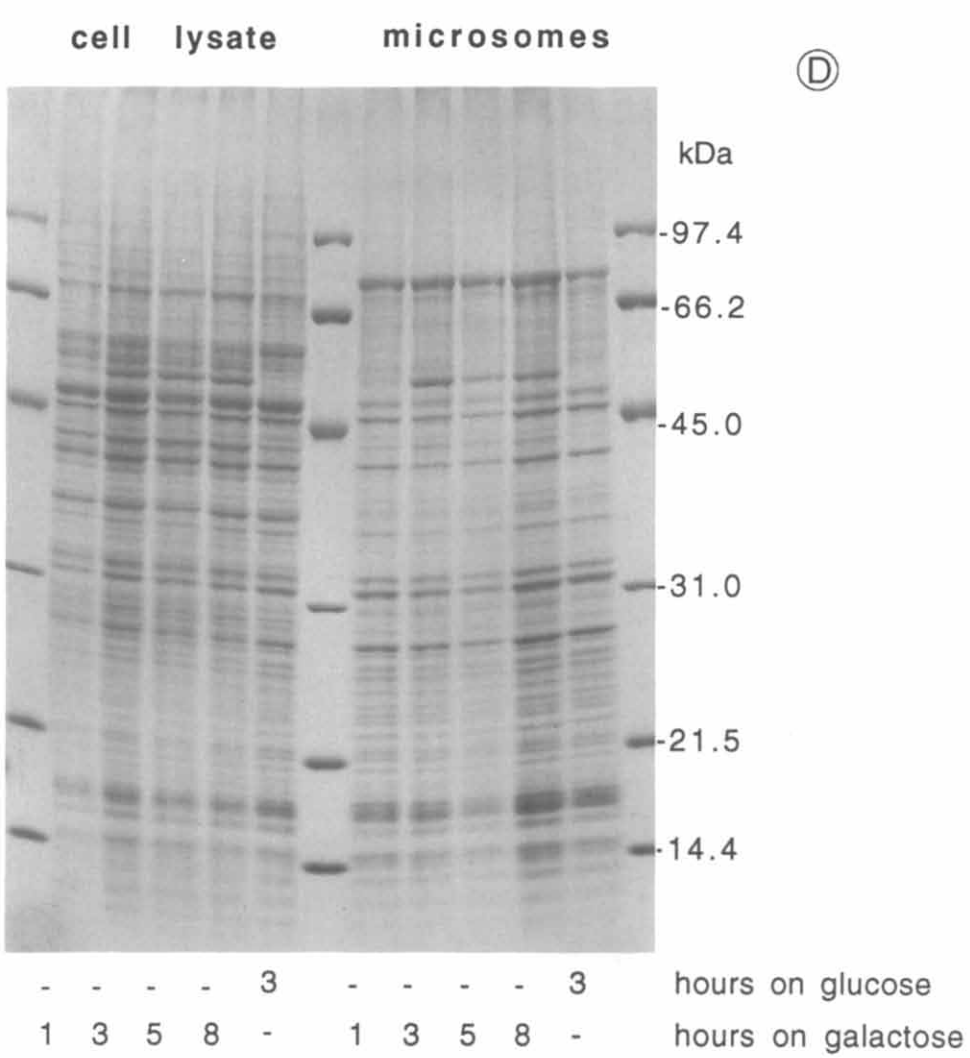

Fig. 5 (continued).

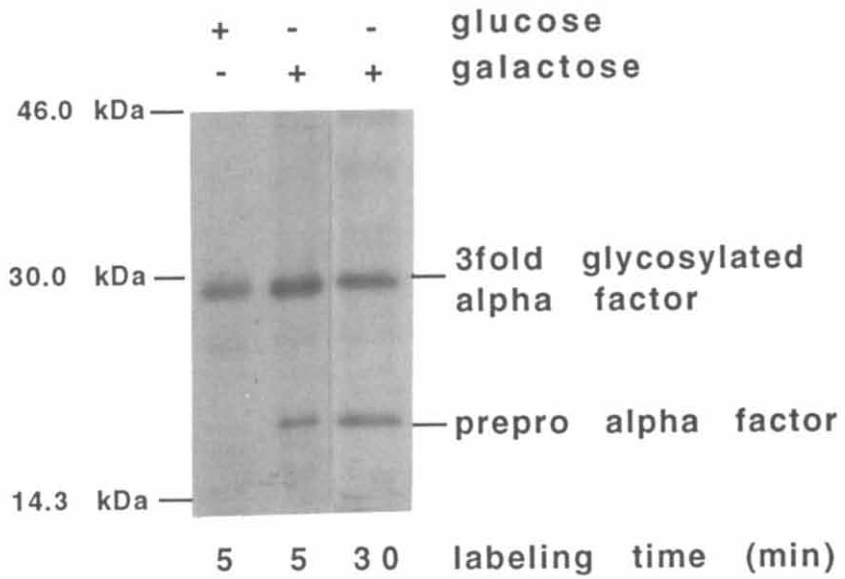

Fig. 6. Expression of P-450p causes accumulation of prepro alpha factor. Cells with the plasmid 51 12T were grown for 8 h on glucose or galactose and labeled with [^{35}S]methionine for 5 or 30 min. After mechanical disruption of the cells immunoprecipitation with antibodies against alpha factor was performed as described in Materials and Methods.

The amount of full length P-450p continues to increase after 8 h induction and we never found an accumulation in vacuoles, even after 17 h repression on glucose medium following an 8 h induction (data not shown).

The truncated P-450 proteins failed to induce KAR2p, SEC61p and precursor accumulation. This was tested by Western blotting and cell labeling. We never found membrane clumps in micrographs, neither with P-450p antibody nor with KAR2p orSEC61p antibody. So, if membranes had proliferated, they had to have been different in their protein content than the membrane stacks induced by full length P-450p. This seemed very unlikely but can be proved by immunoelectron microscopy.

Discussion

Overexpression of the microsomal protein P-450 *CYP52A3* in *S. cerevisiae* causes membrane proliferation. These membrane stacks contain SEC61p and KAR2p which participate in protein translocation. Besides membrane proliferation, an accumulation of precursor proteins was observed. The N-terminal hydrophobic sequences of P-450p appeared to be crucial for the localization of the protein as well as for the induction of membrane proliferation.

The precise mechanism that leads to extended membrane synthesis is unknown. Membrane proliferation occurs in connection with overexpression of certain ER or secreted proteins [5–15]. The membranes differ in their morphology dependent upon the inducing protein and the cell type [14,15]. This lead Wright and coworkers [14] to the suggestion that qualitative rather then quantitative factors are responsible for the stimulation of membrane synthesis. Another possibility is that membranes proliferate in order to increase the membrane volume in relation to the elevated protein quantity. The finding that some *sec* mutants accumulate membranes [37] lead to a third hypothesis: factors of the transport machinery become limited because they are blocked by the high amount of protein which has to be transported. Precursor proteins accumulate in the cytoplasm and induce membrane synthesis to compensate.

Our results support this latter hypothesis. Precursor forms of proteins which have to cross the ER membrane, i.e., KAR2p and α factor, accumulated in cells overexpressing full length P-450p. In this respect cells overexpressing the membrane protein P-450 resemble *sec* mutants where a missing transport factor causes precursor accumulation [18–22,38,40,41] and membrane proliferation [37]. It remains to be tested which factors are blocked. Candidates include membrane proteins like SEC62p [38] and TRAM [39], and cytosolic factors. Young et al. [40] demonstrated that expression of a fusion protein of acid phosphatase and bacterial β-galactosidase in *S. cerevisiae* depletes a cytosolic factor and prevents the effective translocation of other proteins. This factor is not any of the *SSA* gene products. We also observed no change in the SSA1 and SSA2 protein levels under our conditions. Depletion of SSA proteins causes the accumulation of mitochondrial precursors like the precursor form of the β subunit of mitochondrial F_1-ATPase [41]. Neither by cell labeling nor Western blotting were we able to detect pre$F_1\beta$p. Another formal possibility is that SRP is depleted when P-450p is overexpressed. Hann and Walter [31] reported that SRP mutants accumulate precursor forms of secreted, mitochondrial, and ER proteins. We have shown that translocation of P-450p from yeast into dog pancreatic microsomes in vitro is SRP dependent. However, in a homologous yeast system this protein can be efficiently translocated posttranslationally without SRP [33]. Therefore it is unlikely that high levels of P-450p lead to depletion of SRP and thereby cause precursor accumulation.

In eukaryotic cell lines ER proliferation as a response to increased traffic of secretory proteins into the ER has been reported to be accompanied by coinduction of BiP (Grp 78p) [11,12]. BiP, which is a major component of the ER lumen of eukaryotic cells, is believed to play a role in the folding and assembly of proteins that are translocated across the ER membrane. The BiP protein of *S. cerevisiae* is encoded by the essential *KAR2* gene [19]. Mori et al. [42] showed recently that there exist three independent cis-acting elements in the *KAR2* promoter. One of them (called *UPR*) is involved in the induction of *KAR2* mRNA by unfolded proteins. Since the first hydrophobic sequence is able to anchor a fusion protein of the P-450 N-terminus and mature invertase into the membrane (data not shown), we assume that this is the membrane anchor of P-450p. This type I membrane protein conformation [43] causes 17 amino acids to face the ER lumen. It is unlikely that these 17 amino acids are unfolded and therefore stimulate *KAR2* transcription through the proposed transcription factor. This implicates the existence of another induction mechanism for KAR2p, probably indirectly via increased membrane synthesis. Increased levels of SEC61p, which also participates in protein translocation [18–20], underline this assumption. Wiest et al. [11] reported a coordinated expression of most ER proteins during amplification of rough ER in a eukaryotic cell line. Our finding that the cytosolic truncated P-450 proteins did not stimulate KAR2p/SEC61p synthesis or membrane proliferation supports the idea that both processes are coupled.

As judged by immunofluorescence, P-450p and KAR2p colocalize with SEC61p in the proliferated membranes. This suggests that at least parts of the

membrane structures represent rough ER. There might exist different ER fractions in yeast, like in higher eukaryotes but this has to be tested by other methods.

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