ELSEVIER

Biochimica et Biophysica Acta 1236 (1995) 163–169

BBA Biochimica et Biophysica Acta

Proliferation of intracellular membrane structures upon homologous overproduction of cytochrome *P*-450 in *Candida maltosa*

Moriya Ohkuma [a,*], Sun Mee Park [a], Thomas Zimmer [b], Ralph Menzel [b], Frank Vogel [b], Wolf-Hagen Schunck [b], Akinori Ohta [a], Masamichi Takagi [a]

[a] *Department of Agricultural Chemistry, The University of Tokyo, Bunkyo-ku, Tokyo 113, Japan*
[b] *Max Delbrück Center for Molecular Medicine, Robert-Rössle-Str. 10, 13122 Berlin-Buch, Germany*

Received 29 August 1994; revised 24 January 1995; accepted 30 January 1995

Abstract

In an alkane-assimilating yeast, *Candida maltosa*, a cultivation on alkane causes both induction of endoplasmic reticulum (ER)-resident membrane proteins, such as cytochrome *P*-450, and proliferation of ER. In this study, individual genes for alkane-inducible forms of cytochrome *P*-450 (*P*-450alk) were homologously overexpressed in *C. maltosa* using a galactose-inducible expression system developed in this yeast. Immunoelectron microscopy revealed that, upon the overexpression, a dramatic proliferation of ER occurred, in which overproduced *P*-450alk protein accumulated. The proliferated membranes were mainly tubular forms and stacks of paired membranes were also observed after prolonged expression. The tubular forms were morphologically very similar to the proliferated ER in alkane-induced *C. maltosa* cells. The observed proliferation of ER membranes by homologous overproduction of *P*-450alk, here depicted, will provide a unique opportunity for investigating the mechanisms by which cells regulate ER biogenesis, in comparison with the intrinsic form of ER proliferation.

Keywords: Cytochrome *P*-450; Membrane proliferation; Endoplasmic reticulum; Immunocytochemistry; (*C. maltosa*)

1. Introduction

In a yeast *Saccharomyces cerevisiae*, proliferation of the endoplasmic reticulum (ER) is induced in response to elevated expression of certain ER-resident membrane proteins, such as HMG-CoA reductase [1], cytochrome *P*-450 [2,3], and cytochrome b_5 [4]. This artificial induction of ER proliferation [5] provides a suitable model to study so far largely unknown mechanisms coordinating the synthesis and assembly of protein and lipid components of membranes in ER biogenesis. A system would be of particular interest that allows a direct comparison between physiologically induced forms of ER and those elicited by artificial overproduction of individual membrane proteins.

In an alkane-assimilating yeast *Candida maltosa*, ER proliferation is induced by a carbon-source transition from carbohydrates such as glucose to long-chain alkanes. It results in the formation of largely extended membrane tubules surrounding the peroxisomes or being in close vicinity to plasma membranes [6,7]. With respect to the subcellular organization of alkane metabolism, ER is the site where the first step of the assimilation pathway is catalyzed by an alkane monooxygenase system [7], whereas the subsequent oxidation steps proceed in peroxisomes [6]. This suggests a direct physiological importance of ER proliferation and of their association with peroxisomes during growth on alkanes.

An ER-resident alkane monooxygenase system consists of an alkane-inducible cytochrome *P*-450 (*P*-450alk) component and of NADPH-cytochrome *P*-450 reductase [8,9]. Both components are integral membrane proteins and together represent up to 10% of the total microsomal protein of alkane-grown cells [10]. Interestingly, stable formation of native *P*-450alk protein during induction by alkanes was found to be repressed by cerulenin, a potent inhibitor of fatty acid de novo synthesis [11]. This result may

Abbreviations: ER, endoplasmic reticulum; *P*-450, cytochrome *P*-450; *P*-450alk, alkane-inducible *P*-450; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; PBS, phosphate-buffered saline.

* Corresponding author. Fax: +81 3 38129246.

SSDI 0005-2736(95)00040-2

indicate that *C. maltosa* cells actively increase the capacity of their ER membrane system to accommodate the alkane-induced membrane proteins.

In *C. maltosa*, multiple forms of *P*-450alk have been identified and characterized by means of cloning and sequencing of the respective genes [12–15]. First evidence demonstrating that a *P*-450alk protein itself can provide a sufficient signal to induce ER proliferation was obtained by means of heterologous overproduction of individual *P*-450alk forms in *S. cerevisiae* [2]. Taking an advantage of the recently developed host-vector systems for *C. maltosa* [16–20], it will now be possible to test the ability of *P*-450alk proteins and of other ER-resident membrane proteins to induce ER proliferation in the homologous system and to compare the artificially induced forms of ER with the physiologically proliferated forms of ER present in alkane-grown *C. maltosa* cells. As a first step, the present paper describes the effect of homologous overproduction of two *P*-450alk proteins on the ultrastructure of ER in *C. maltosa* cells.

2. Materials and methods

2.1. Strains and culture conditions

The yeast strain *C. maltosa* CHA1 (*his5*, *ade1*) [19] was used in this study. The yeast minimal medium contained 0.67% yeast nitrogen base without amino acids (Difco) and 1% glucose or 2% galactose. It was supplemented with 24 mg/l adenine. Cultivation was carried out using a Bioreacter (Biostat MD, B. Braun Biotech International), maintaining pH of the medium at 4.7. *Escherichia coli* MV1190 (*Δ(srl-recA)*306::Tn10(*tet*^{r}) *Δ(lac-pro) thi*$^-$, *supE* (*F'*, *proAB*, *lacI*^{q}, *lacZΔM15*, *traD36*)) was used as a recipient for plasmid construction and was grown on LB-medium.

2.2. Construction of plasmids for overexpression and transformation of C. maltosa

An expression vector pNGH2 having the galactose-inducible *GAL1* promoter from *C. maltosa* constructed by using a multi-copy vector (more than 20 copies per cell) was used for construction of overexpression plasmids. Two of the *P*-450alk genes, *ALK1-A* [13] and *ALK3-A* [15], were subcloned into pUC119; a 2.8-kb *Eco*T22I fragment of *ALK1-A* was inserted into the *Pst*I site of pUC119, and a 2.2-kb *Bgl*II-*Cla*I fragment of *ALK3-A* was blunt-end ligated into the *Sma*I site of pUC119. The *Sal*I sites were introduced into just upstream of the translational initiation codons of *ALK1-A* and *ALK3-A* by site directed mutagenesis using primers, 5'-CTATAGCCATGTCGACCA-GATG-3' and 5'-CGGCATATTTTGTCGACGTTGCACT-3', respectively. Then, each *Sal*I fragment carrying the entire coding region and the 3'-noncoding region (each of the 3'-*Sal*I site was derived from the multiple cloning site of pUC119) was inserted into the *Sal*I site of pNGH2. The resulting plasmids carrying *ALK1-A* and *ALK3-A* in the proper orientation relative to the *GAL1* promoter were designated pNGH2-ALK1A and pNGH2-ALK3A, respectively. These plasmids were transformed into the *C. maltosa* strain CHA1 by a modified lithium acetate method [16], in which the cells were suspended in 0.05 M lithium acetate and shaken at 30° C for 90 min instead of 0.1 M lithium acetate and 60 min of the original method for *S. cerevisiae*.

2.3. Analytical methods

P-450 content was estimated in intact yeast cells by means of CO-difference spectra using an extinction coefficient of 91 mM^{-1} cm^{-1} [21]. Western blot analysis was done as described previously [22].

2.4. Immunocytochemistry

Fixation and processing for electron microscopy of *C. maltosa* cells were done by a modified cryosectioning method [7]. In particular, yeast cells were harvested and fixed in 0.1 M sodium citrate buffer, pH 4.7, using a mixture of 0.5% glutaraldehyde and freshly prepared 4% paraformaldehyde at 30° C for 1 h. After washing the cells with phosphate buffered saline (PBS), 1% sodium metaperiodate (Sigma-Aldrich, Deisenhofen/Germany) was added to promote the cryoprotectant sucrose to penetrate the cell wall. After incubation for 1 h at 4° C, cells were washed with PBS and the hydrated specimens were immersed with a mixture of 25% polyvinylpyrrolidone K15 (M_r 10000, Fluka, Buchs/Switzerland) and 1.6 M sucrose according to Tokuyasu [23] for 2 h. Samples were mounted on specimen holders, frozen in liquid nitrogen, and sectioned at −115° C with an ultracryotome (Leica, Vienna/Austria). Ultrathin thawed-cryosections were prepared with glass knifes and placed on formvar/carbon-coated copper grids (200 mesh, hexagonal).

Labeling with antibodies and protein A-gold (10 nm) complexes was performed according to Griffiths et al. [24]. Finally, the frozen-thawed sections were stained and stabilized using a mixture of 0.3% uranyl acetate and 2% methyl cellulose (25 cps, Sigma-Aldrich, Deisenhofen/Germany). As controls, the sections were incubated directly with protein A-gold omitting reaction with primary antibodies, or cells not expressing the antigen were used to check the specificity of primary antibody reaction. Antibodies against *ALK1-A* and *ALK3-A* products were raised in rabbits with SDS-denatured *P*-450 Cm1 and Cm2, respectively. *P*-450Cm1 and *P*-450Cm2 were derived from *C. maltosa* strain EH15 and nucleotide sequence analysis revealed that *ALK1-A* and Cm1, and *ALK3-A* and Cm2 were orthologous to each other [2,15]. The IgG fractions were prepared as described previously [22] and pread-

sorbed to washed *C. maltosa* cells to remove antibodies binding to cell wall carbohydrates. Affinity purification of antibodies was performed according to Pringle et al. [25] using purified *P*-450 protein bound to nitrocellulose.

3. Results

In order to study the effect of the overproduction of individual *P*-450alk forms on the ultrastructure of ER in *C. maltosa*, a galactose-inducible expression system was developed. The plasmid pNGH2-ALK1A (Fig. 1) carried one of *P*-450alk genes of *C. maltosa*, *ALK1-A*, which encodes a major *P*-450alk protein found in alkane-grown cells [12,13]. It was introduced into *C. maltosa* strain CHA1. Transformants were cultivated on 1% glucose in a bioreactor until glucose in the medium was completely consumed, and then galactose was added to a final concentration of 2%. Expression of *P*-450 during growth on galactose was judged by CO-difference spectra (Fig. 2) and Western blotting (Fig. 3). As shown in Fig. 2A, *P*-450 heme-protein was detected at a high amount (27 pmol/10^8 cell) for 6 h cultivation after galactose-induction. The *ALK1-A* product was not detectable in glucose-grown cells (Fig. 3, lane 3) and its formation was specifically induced by galactose (Fig. 3, lanes 5–7; 2, 6, and 10 h galactose-induction). Control cells carrying only the vector pNGH2 cultivated under the same galactose condition showed neither immunoreactive proteins (Fig. 3, lane 4) nor *P*-450 heme-proteins as judged by CO-difference spectra (Fig. 2C), confirming that the genomic genes encoding *P*-450s were not induced by galactose. Moreover, Western blot analysis demonstrated that the amount of immunodetectable *ALK1-A* product was in good agreement with that determined by CO-difference spectra (Fig. 3, lane 1; 0.5 pmol purified *P*-450Cm1, lane 2; cell extract after 6 h galactose-induction, which contained 0.5 pmol *P*-450 calculated from CO-difference spectra). This result indicates that most if not all of the *P*-450 produced was present in its native heme-containing state. Another *P*-450alk gene, *ALK3-A* [9], was also overexpressed in *C. maltosa* carrying

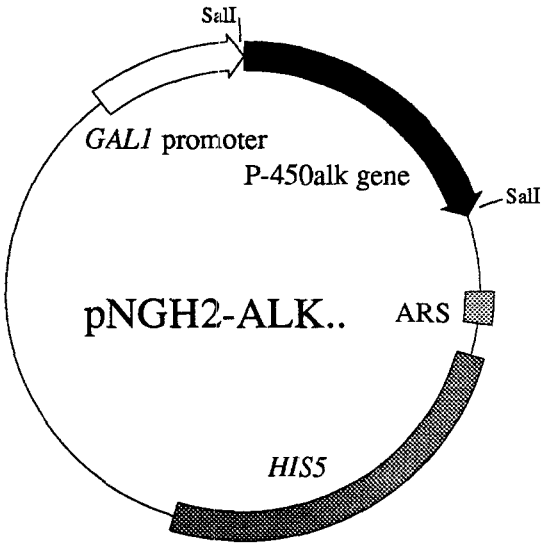


Fig. 1. Structure of the expression vector for overproduction of individual *P*-450 forms in *C. maltosa*. Two *P*-450alk genes, *ALK1-A* and *ALK3-A*, were cloned into the *Sal*I site of the vector pNGH2 and thus placed under the control of the galactose-inducible *GAL1* promoter to yield the plasmids pNGH2-ALK1A and pNGH2-ALK3A, respectively. Thin line indicates the pBR322 sequence; ARS, an autonomously replicating sequence of *C. maltosa*; *HIS5*, a selectable marker gene for *C. maltosa* transformation.

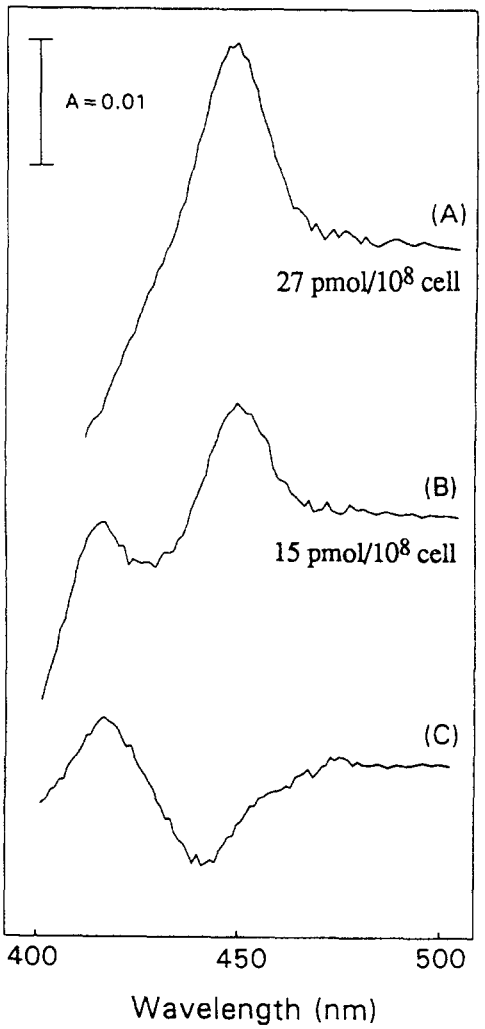


Fig. 2. Reduced CO-difference spectra of whole yeast cells demonstrating the overproduction of *P*-450alk in *C. maltosa*. (A) The *C. maltosa* CHA1 cells transformed with pNGH2-ALK1A, (B) with pNGH2-ALK3A, and (C) with the parental plasmid pNGH2. The estimated amount of *P*-450 protein based on the spectra is written in (A) and (B). The cells were cultivated for 6 h after galactose-induction, harvested and resuspended in 0.1 M potassium phosphate buffer, pH 7.25 containing 20% glycerol at the cell concentration of $6.6 \cdot 10^8$ cells/ml (A), $6.2 \cdot 10^8$ cells/ml (B), and $6.7 \cdot 10^8$ cells/ml (C) for measurement.

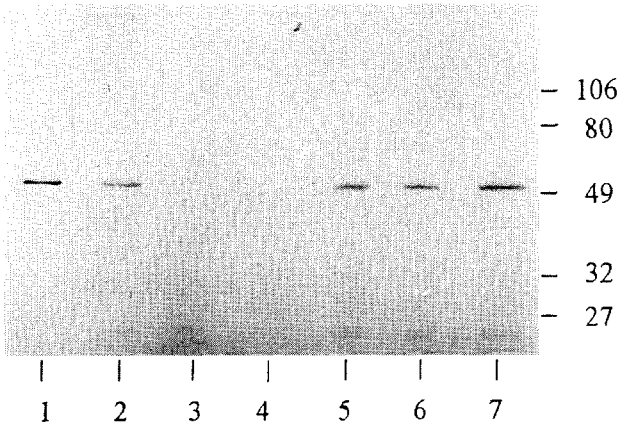


Fig. 3. Western blot analysis of *P*-450alk (*ALK1-A* product)-overproduction in *C. maltosa*. Crude extracts were separated by 10% gel, transferred to nitrocellulose membrane and probed with anti-*P*-450Cm1 IgG as described previously [22]. Lane 1, purified *P*-450Cm1 (close relatives of *ALK1-A* product, 0.5 pmol); lane 2, extract of *C. maltosa* strain CHA1 cells carrying pNGH2-ALK1A and harvested 6 h after galactose-induction, which contained 0.5 pmol *P*-450 calculated from CO-difference spectra; lane 3, control strain CHA1/pNGH2, 6 h after addition of galactose; lanes 4–7, strain CHA1/pNGH2-ALK1A harvested during growth on glucose (lane 4) and 2 h (lane 5), 6 h (lane 6), and 10 h (lane 7) after galactose-induction. On lanes 3–7, 20 μg protein of total cellular extracts were loaded. On the right side, the molecular masses (in kDa) of standard proteins are shown.

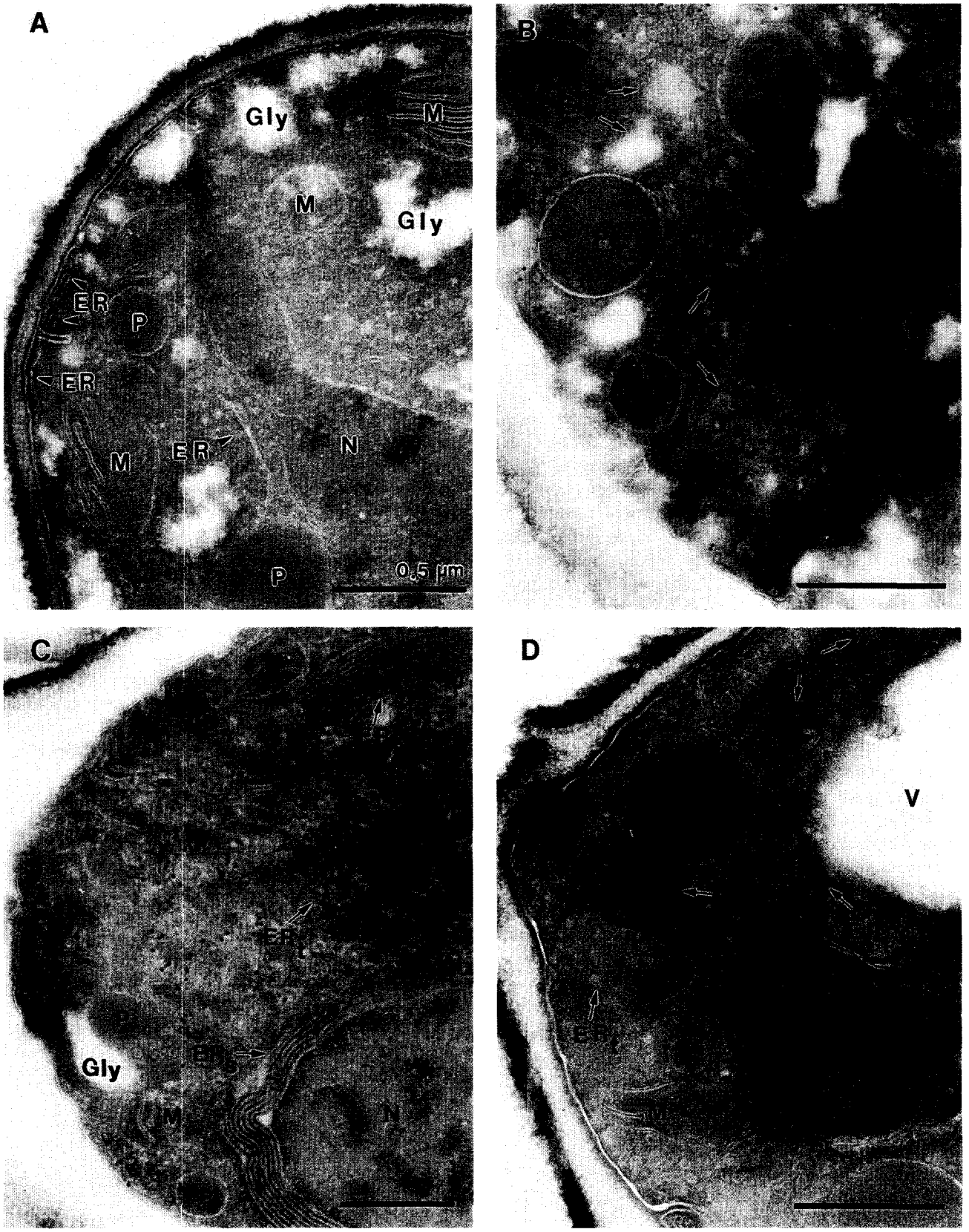
the plasmid pNGH2-ALK3A. In this case, the amount of overexpressed *P*-450 heme-protein (15 pmol/10^8 cell) was slightly smaller than that of pNGH2-ALK1A carrying cells (Fig. 2B).

Immunoelectron microscopy was applied in order to examine the intracellular localization of the overproduced *P*-450alk and the effect on intracellular membrane structures. Ultrathin frozen-thawed sections of galactose-grown cells were incubated with antibodies against *ALK1-A* or *ALK3-A* protein followed by gold-conjugated protein A. As shown in Fig. 4, the micrographs demonstrated clearly distinguishable membranes of ER, the nuclear envelope and those of mitochondria, peroxisomes and vacuoles. The immunolabel was strictly localized over ER membranes when *P*-450alk was expressed. Control cells that carried only the vector pNGH2 did not display any specific labeling of the ER membranes (Fig. 4A). In these *P*-450 non-producing cells, ER membranes appeared at low frequency and mostly as single tubules scattered over various parts of the cytoplasm (Fig. 4A).

In comparison, *P*-450alk overproducing cells were characterized by an ultrastructural abnormality consisting of a marked proliferation of intracellular membranes (a dramatic alteration in the amount and organization of membranes) morphologically belonging to ER. The proliferation of ER membranes was a time dependent event. When cells carrying the plasmid pNGH2-ALK1A were cultivated on galactose-containing medium for 6 h after induction, a proliferation of ER-like tubular membranes was observed. The small ring-shaped membrane structures represented cross-sections of membrane tubules. These tubular membranes were distributed over the cytoplasm and exhibited the immunogold labels for *P*-450alk protein when the sections were incubated with antibodies against *ALK1-A* protein (Fig. 4B). After prolonged growth on galactose-containing medium, the morphological appearance of the newly-built ER membranes was changed; besides the increased number of tubular structures, a stacking of membranes consisting of up to 15 single membranes became visible. Deduced from clear appearance of membrane layers, we suppose that these membrane stacks consist of flattened cisternae. This kind of membrane stacks were usually localized in tight vicinity to the nucleus but were also observed in various parts of the cytoplasm (Fig. 4C). The membrane stacks were indicated by a highly concentrated immunolabel when incubated with antibodies against *ALK1-A* protein.

In the case of the overexpression of *ALK3-A* in *C. maltosa* using the same strategy as described for *ALK1-A*, we observed an accumulation of ER structures after induction by galactose. In contrast to *ALK1-A* protein producing cells, no clear distinction between tubular and stacked membranes can be made. There seemed to be a tendency to form both a tubular network and small irregularly shaped flattened cisternae as indicated in Fig. 4D. A favored localization in vicinity to the nucleus could not be detected. Although the antibodies exhibited a weak cross-reactivity for *ALK3-A* protein by Western-blot experiments, a faint but specific immunolabeling of the proliferated ER structures could be observed, indicating that the overexpressed *ALK3-A* protein was present in the newly formed ER membranes consistent with the results using the *ALK1-A* gene.

Fig. 4. Immunolocalization of cytochrome *P*-450alk overproduced in the yeast strain *C. maltosa* CHA1. Frozen-thawed sections were immunolabeled with anti-*P*-450 antibodies followed by 10 nm colloidal gold-conjugated protein A. (A) Control cell carrying only the vector pNGH2. (B,C) *P*-450alk (the *ALK1-A* gene product) overproducing cells, 6 h (B) and 10 h (C) after galactose-induction, immunolabeled with antibodies against *ALK1-A* protein. (D) *P*-450alk (the *ALK3-A* gene product) overproducing cell 6 h after galactose-induction, immunolabeled with antibodies raised against the closely related *P*-450 form, *P*-450Cm2. These antibodies showed only a weak cross-reactivity with the *ALK3-A* product in Western blotting, resulting in the low but specific labeling visible. Compare the different organization of the proliferated ER, when *ALK1-A* and *ALK3-A* is overexpressed, respectively. N, nucleus; M, mitochondrion; V, vacuole; Gly, glycogen; P, peroxisome; ER_t, tubular endoplasmic reticulum; ER_s, stacked endoplasmic reticulum. Bars represent 0.5 μm.


A
Gly
M
M
Gly
ER
P
ER
M
ER
N
P
0.5 μm
B
C
ER
Gly
ER
M
N
D
V
ER

4. Discussion

Our results with the homologous expression system in *C. maltosa* showed that overproduction of an ER-resident membrane protein, *P*-450alk, can induce a marked proliferation of ER membranes in which the overproduced proteins accumulated. This observation and those reported in *S. cerevisiae* [1–4] disclose the existence of cellular mechanisms that monitor the levels of membrane proteins and regulate membrane biogenesis and morphology. The artificial increase in levels of membrane proteins can provide a convenient system for investigating the mechanisms by which cells regulate membrane biogenesis and morphology as discussed by R. Wright [5]. Our results show that *C. maltosa* as well as *S. cerevisiae* is a proper system. Moreover, using *C. maltosa* as a host, it is now possible to compare physiologically proliferated forms of ER with those induced artificially.

A remarkable difference of proliferated ER-membranes between *P*-450alk-overproducing cells and other ER-resident membrane proteins-overproducing cells was their morphology. In the cases of overproducing HMG-CoA reductase and cytochrome b_5 in *S. cerevisiae*, the elevated level of each ER-resident membrane protein was found to result in the formation of stacks of paired membranes named karmellae, which were usually found in the close vicinity of nuclear envelope [1,4]. In contrast, the morphology of the proliferated ER membranes with the overproduction of *P*-450alk for 6 h in *C. maltosa* was mainly tubular forms, which were distributed in the cytoplasm. Although some karmellae-like structures of membrane stacks were actually found with *P*-450alk in *C. maltosa*, they appeared only in the late phase of induction and their distribution inside the cells (not restricted in perinuclear region but also present in various parts of the cytoplasm) was also different from that of the karmellae. In *S. cerevisiae*, overproduction of *P*-450alk also resulted in the formation of tubular forms of ER membranes [2]. Thus, the tubular forms of ER membranes with overproduction of *P*-450alk were actually unique organization when compared with the karmellae structures found with HMG-CoA reductase and cytochrome b_5. However, the stacks of paired membranes like karmellae were frequently observed in *P*-450alk-overproducing *S. cerevisiae*. As in the case in *C. maltosa*, they were not restricted in the perinuclear region but were also present in various part of the cytoplasm. Interestingly, the morphology of the proliferated ER membranes in *ALK3-A*-overexpressed cells seemed to be different that observed in *ALK1-A*-overexpressed cells. Especially, the flattened cisternae observed in *ALK3-A*-overexpressed cells had irregular shapes. In *S. cerevisiae*, distinctly organized and distributed karmellae-like stacks also appeared for overproduction of different kinds of *P*-450alk proteins [2]. Thus, the organization and distribution of the proliferated karmellae-like membranes were dependent on *P*-450alk forms overproduced in both yeasts. Whether these morphological differences in ER-proliferation in relation to the overproduction of different ER-membrane proteins are related to different physiological functions of the overproduced proteins remains to be clarified.

Since ER proliferation is a intrinsic nature of *C. maltosa* in response to alkane-induction, the comparative analysis of membrane proliferation between alkane-induced and *P*-450alk-overproduced cells is thought to be informative. In alkane-growing cells of *C. maltosa*, the formation of largely extended membrane tubules was observed [6,7]. The karmellae-like membrane stacks hardly appeared. The formation of the tubular ER structures was the common feature in alkane-growing cells and in artificially *P*-450alk-overproducing cells, indicating that the regulatory mechanisms underlying in membrane biogenesis and morphology determination could be common in both cases.

Our results give us an important information in considering the mechanisms of the alkane-induced membrane proliferation. In alkane-grown *C. maltosa* cells, ER proliferation is accompanied by the induction of a number of membrane proteins. One possible mechanism for alkane-induced membrane proliferation is that the activity of membrane biogenesis including the expression of various membrane proteins and the synthesis of membrane lipids is directly stimulated by alkane. Another possibility is a so-called successive induction mechanism in which alkane induces the expression of a limited number of membrane proteins and then these induced membrane proteins stimulate membrane biogenesis. Considering that the same type of membranes was induced both in alkane-grown cells and in *P*-450alk-overproducing cells, it is indicated that, at least in part, such a successive induction mechanism is really working. Further experiments are necessary to clarify what kind of a signal is produced, how this signal is transduced, and which genes and/or enzymes are switched on upon overproducing a *P*-450alk.

Acknowledgements

This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan, and by a grant from Deutsche Forschung-Gemeinschaft to W.H.S. We thank Dr. E. Kärgel for providing the antibodies and advice, Mrs. M. Vogel and Mrs. E. Honeck for excellent technical assistance. This work was partly performed using the facilities of the Biotechnology Research Center, The University of Tokyo.

References

[1] Wright, R., Basson, M., D'Ari, L. and Rine, J. (1988) J. Cell Biol. 107, 101–114.

[2] Schunck, W.-H., Vogel, F., Gross, B., Kärgel, E., Mauersberger, S., Köpke, K., Gengnagel, C. and Müller, H.-G. (1991) Eur. J. Cell Biol. 55, 336–345.
[3] Wiedmann, B., Silver, P., Schunck, W.-H. and Wiedmann, M. (1993) Biochem. Biophys. Acta 1153, 267–276.
[4] Vergeres, G., Yen, T.S.B., Aggeler, J., Lausier, J. and Waskell, L. (1993) J. Cell Sci. 106, 249–259.
[5] Wright, R. (1993) Curr. Biol. 3, 870–873.
[6] Mauersberger, S., Kärgel, E., Matyashova, R.N. and Müller, H.-G. (1987) J. Basic Microbiol. 27, 565–582.
[7] Vogel, F., Gengnagel, C., Kärgel, E., Müller, H.-G. and Schunck, W.-H. (1992) Eur. J. Cell Biol. 57, 285–291.
[8] Honeck, H., Schunck, W.-H., Riege, P. and Müller, H.-G. (1982) Biochem. Biophys. Res. Commun. 106, 1318–1324.
[9] Schunck, W.-H., Mauersberger, S., Huth, J., Riege, P. and Müller, H.-G. (1987) Arch. Microbiol. 147, 240–244.
[10] Mauersberger, Schunck, W.-H. and Müller, H.-G. (1984) Appl. Microbiol. Biotechnol. 19, 29–35.
[11] Takagi, M., Moriya, K. and Yano, K. (1980) Cell. Mol. Biol. 25, 371–375.
[12] Schunck, W.-G., Kärgel, E., Gross, B., Wiedmann, B., Mauersberger, S., Köpke, K., Kießling, K., Strauss, M., Gaestel, M. and Müller, H.-G. (1989) Biochem. Biophys. Res. Commun. 161, 843–850.
[13] Takagi, M., Ohkuma, M., Kobayashi, N., Watanabe, M. and Yano, K. (1989) Agric. Biol. Chem. 53, 2217–2226.
[14] Ohkuma, M., Hikiji, T., Tanimoto, T., Schunck, W.-H., Müller, H.-G., Yano, K. and Takagi, M. (1991) Agric. Biol. Chem. 55, 1757–1764.
[15] Ohkuma, M., Tanimoto, T., Yano, K. and Takagi, M. (1991) DNA and Cell Biol. 10, 271–282.
[16] Takagi, M., Kawai, S., Chang, M.C., Shibuya, I. and Yano, K. (1986) J. Bacteriol 167, 551–555.
[17] Kawai, S., Hwang, C.W., Sugimoto, M., Takagi, M. and Yano, K. (1987) Agric. Biol. Chem. 51, 1587–1591.
[18] Hikiji, T., Ohkuma, M., Takagi, M. and Yano, K. (1989) Curr. Genet. 16, 261–266.
[19] Kawai, S., Hikiji, T., Murao, S., Takagi, M. and Yano, K. (1991) Agric. Biol. Chem. 55, 59–65.
[20] Ohkuma, M., Muraoka, S.-I., Hwang, C.W., Ohta, A. and Takagi, M. (1993) Curr. Genet. 23, 205–210.
[21] Omura, T. and Sato, R. (1964) J. Biol. Chem. 239, 2379–2385.
[22] Kärgel, E., Schunck, W.-H., Riege, P., Honeck, E., Claus, R., Kleber, H.P. and Müller, H.-G. (1985) Biochem. Biophys. Res. Commun. 128, 1261–1267.
[23] Tokuyasu, K.T. (1989) Histochem. J. 21, 163–171.
[24] Griffiths, G. and Hoppler, H. (1984) Ultrastruct. Res. 89, 65–78.
[25] Pringle, J.R., Adams, A.E.M., Drubin, D.G. and Haarer, B.K. (1991) Methods Enzymol. 194, 565–602.