A model system for studying membrane biogenesis

Overexpression of cytochrome \( b_5 \) in yeast results in marked proliferation of the intracellular membrane*

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SUMMARY

Cytochrome \( b_5 \) is an amphipathic microsomal protein that is anchored to the endoplasmic reticulum by a single hydrophobic transmembrane \( \alpha \)-helix located near the carboxyl terminus of the protein. In yeast, cytochrome \( b_5 \) provides electrons for fatty acid desaturation and ergosterol biosynthesis. High level expression of cytochrome \( b_5 \) in \textit{Saccharomyces cerevisiae} was achieved using the yeast metallothionein promoter and a synthetic cytochrome \( b_5 \) gene.

In order to accommodate the markedly increased amount of the membrane-bound cytochrome \( b_5 \), the yeast cell proliferated its nuclear membrane. As many as 20 pairs of stacked membranes could be observed to partially encircle the nucleus. This morphological arrangement of membrane around the nucleus is known as a karmella. In an effort to understand which part of the cytochrome \( b_5 \) molecule, i.e. the membrane anchor or the soluble heme domain, which is competent in electron transfer, provided the signal for the de novo membrane biosynthesis, a series of studies, including site-directed mutagenesis, was undertaken. The results of these experiments demonstrated that the inactive heme-deficient apo form of the membrane-bound protein stimulates membrane proliferation to the same extent as the holo wild-type protein, whereas cytosolic forms of cytochrome \( b_5 \) did not induce membrane synthesis. These data demonstrate that membrane proliferation is a consequence of the cell’s ability to monitor the level of membrane proteins and to compensate for alterations in these levels rather than the result of the ability of the extra cytochrome \( b_5 \) to catalyze synthesis of extra lipid that had to be accommodated in new membrane.

Site-directed mutagenesis studies of the membrane binding domain of cytochrome \( b_5 \) provided additional clues about the nature of the signal for membrane proliferation. Replacement of the membrane anchor by a non-physiological nonsense sequence of 22 leucines gave rise to a mutant protein that triggered membrane biosynthesis. The conclusion from these experiments is clear; the signal for membrane proliferation does not reside in some specific amino acid sequence but instead in the hydrophobic properties of the proliferant. Interestingly, these membranes are somewhat diminished in quantity and have a slightly altered morphology compared to those induced by the wild-type protein. It was also observed that disruption of the putative \( \alpha \)-helix of the membrane anchor by an Ala116Pro mutation, which gives rise to two sequential prolines at positions 115 and 116 results in a protein with diminished capacity to induce membrane formation. This finding suggests that the secondary structure of the membrane binding domain may be an important component of the signal for membrane biosynthesis. In summary, cytochrome \( b_5 \), with its single-helical transmembrane anchor is an excellent model system in which to investigate the molecular mechanism whereby the cell regulates membrane biogenesis.

Key words: cytochrome \( b_5 \), membrane formation, endoplasmic reticulum, membrane proliferation

INTRODUCTION

Cytochrome \( b_5 \) is a microsomal amphipathic protein consisting of two domains: one is the water soluble heme-binding domain while the second is the carboxy-terminal hydrophobic membrane-anchoring domain (Fig. 1). In mammals the membrane-bound protein is an essential component of the NADH-dependent \( \Delta 9 \) stearoyl CoA desaturase system (Oshino, 1982; Strittmatter et al., 1974). It also par-
participates in the Δ6 desaturation of linoleic and oleic acid (Lee et al., 1977), cholesterol biosynthesis (Grinstead and Gaylor, 1982) and fatty acid chain elongation (Nagi et al., 1983). Cytochrome b5 has similar properties in yeast where it is also involved in fatty acid desaturation (Osumi et al., 1979; Tamura et al., 1976) and biosynthesis of ergosterol, the yeast equivalent of cholesterol (Aoyama et al., 1981). In mammalian microsomes the protein may also provide the second electron to cytochrome P-450 for its NADPH-dependent mixed function oxidations (Aoyama et al., 1990; Canova-Davis and Waskell, 1984; Hildebrandt and Estabrook, 1971; Vatsis et al., 1982). A soluble form of cytochrome b5 is found in red blood cells, where it maintains hemoglobin in a reduced state (Hegesh et al., 1986).

In order to be able to study the molecular basis of the requirement for cytochrome b5 for the oxidation of some substrates by cytochrome P-450 (Canova-Davis and Waskell, 1984), an expression system for the amphipathic form of cytochrome b5 was required. Since Saccharomyces cerevisiae is a well-characterized eukaryote with an endoplasmic reticulum (ER), it was chosen as the expression host (Vergères and Waskell, 1992).

The first experimental evidence demonstrating that the cell actively regulates membrane formation was provided by Jones and coworkers (Jones and Fawcett, 1966) when they established that the hepatic endoplasmic reticulum proliferated in response to an increase in the microsomal protein, cytochrome P-450, following administration of phenobarbital. Very recent studies have shown that yeast also proliferate their endoplasmic reticulum in response to expression of heterologous cytochrome P-450 (Schunck et al., 1991). A second more striking example results from a >500-fold increase in the expression of 3-hydroxyl-3-methylglutaryl-coenzyme A reductase (HMG-CoA reductase), the enzyme that catalyzes the rate-limiting step of cholesterol biosynthesis (Chin et al., 1982), in a Chinese hamster ovary cell line. This high-level expression, induced by the HMG-CoA reductase inhibitor compactin, promotes the assembly of endoplasmic reticulum-like tubules organized in a characteristic hexagonal array known as the crystallloid endoplasmic reticulum (Orci et al., 1984; Pathak et al., 1986). Overproduction of the water-soluble catalytic domain of HMG-CoA reductase elevates cellular levels of enzyme activity without crystallloid endoplasmic reticulum formation (Jingami et al., 1987). Treatment of live rats with mevinolin, a potent HMG-CoA reductase inhibitor, also results in crystallloid endoplasmic reticulum formation in the liver, demonstrating the generality of the phenomenon both in vivo and in vitro (Singer et al., 1984). Recently, Wright and coworkers (1988) demonstrated that moderate levels of expression of HMG-CoA reductase isozyme 1 in
S. cerevisiae induced striking perinuclear proliferation of membranes, which they called karmellae.

There are two examples of prokaryotes being able to respond to elevated levels of membrane proteins. In Escherichia coli overproduction of ATP synthase and fumarate reductase leads to the formation of intracellular membrane tubules and cisternae to accommodate these two different membrane proteins (von Meyenburg et al., 1984; Weiner et al., 1984). In cells overproducing fumarate reductase the lipid/protein ratio remained constant; however, alterations in the fatty acid composition, as well as a large increase in cardiolipin content, were detected. Further characterization of such alterations in membrane composition and detailed analysis of the kinetics of these changes promise to provide important structural insights into the assembly of the most complex of the cellular supramolecular structures.

These reports indicate that the cell possesses mechanisms that monitor the level of membrane proteins and are competent to respond to changes in membrane composition by inducing synthesis of the appropriate membrane. However, the nature of the signal for membrane proliferation and the identity of its sensor are not known. In this report we describe an inducible system of membrane biogenesis that, in conjunction with the recent advances in the knowledge of lipid biosynthetic enzymes and of cellular biology, can be used as a probe to investigate membrane synthesis and structure. We show that the primary trigger for membrane proliferation is not a specific amino acid sequence but appears to be a hydrophobic helix.

MATERIALS AND METHODS

Construction and expression of plasmids containing wild-type and mutant cytochrome bs genes

The rat cytochrome bs mutants discussed in this paper were designed, constructed and expressed as previously described (Vergères and Waskell, 1992). The proteinase-deficient yeast strain 20B-12 (α, pep4-3, trpl) was transformed with the vector pCGY1444 containing the gene coding for cytochrome bs downstream of the metallothionein promoter. Yeast were grown in liquid culture in minimal medium (6.7 g/l yeast nitrogen base without amino acids, 20 g/l dextrose, 0.5% Casamino acids, 50 mg/l ampicillin) until early stationary phase. Cytochrome bs expression was induced in two ways. The first procedure involved adding a 300 mM stock solution of CuSO4 in water to a final concentration of 0.3 mM directly to the stationary phase culture and incubating for an additional 24 hours while shaking at 350 rpm at 30°C. In the second procedure the yeast cells were first pelleted and then resuspended in the same volume of fresh medium, at which time CuSO4 was added to a 0.3 mM final concentration and incubation was continued for 7.5 to 24 additional hours as described above. In the experiments where heme synthesis was inhibited the yeast cells were grown as just described. The cells were pelleted and resuspended in an equal volume of fresh medium at which time succinyl acetone (Sigma) was added to a final concentration of 250 µM. CuSO4 (final concentration 0.3 mM) was added simultaneously and the incubation was continued for 24 hours at 30°C.

Quantification of cytochrome bs as a percentage of total yeast cellular protein

Yeast lysates were prepared as described previously (Vergères and Waskell, 1992). A volume equivalent to the lysate of 2×10^7 cells was loaded onto a 13.5% SDS-polyacrylamide gel and stained with Coomassie blue (Laemmli, 1970). Scanning densitometry was used to estimate the amount of cytochrome bs as a percentage of the total cellular protein. This was possible because only an insignificant amount of endogenous yeast protein co-migrates with rat cytochrome bs (Vergères and Waskell, 1992). The precision of the scanning densitometry was evaluated by determining the amount of protein in a control band as a percentage of the total amount of protein in the gel. A band with a RF value of 0.32 (see Fig. 5B) was used for this purpose. Repeated scanning estimated this value to be 10.8 ± 0.9%.

The amount of heme-containing cytochrome bs in intact cells was determined spectroscopically as follows: 1 ml of cell culture was added to an Eppendorf tube. The cells were washed twice in ice-cold buffer (10 mM potassium phosphate, pH 7.4, 20% glycerol) by centrifugation and resuspended in the same buffer at a density 1×10^9 cells/ml. Cytochrome bs was reduced in the sample cuvette by the addition of a few grains of dithionite and 5 µl of a 30% H2O2 solution was added to the reference cuvette to completely oxidize cytochrome bs. The holo cytochrome bs content was calculated using a difference spectrum (reduced minus oxidized) with a molar absorption coefficient (ε424-410) of 190 mM⁻1 cm⁻¹ (Beck von Bodman et al., 1986). The contribution of endogenous proteins to the heme spectrum was estimated in the control strain, which contained the plasmid with the cytochrome bs gene in the inverted orientation relative to the chelatin promoter and this value was subtracted from the value of the cytochrome bs-producing strains.

Electron microscopy

The processing of yeast cells for electron microscopy was performed in two different ways.

Method A

A 100 µl sample of a 10x fixative (10% glutaraldehyde, 2% paraformaldehyde in 0.4 M potassium permanganate, pH 7) was added to 900 µl of a yeast cell culture in stationary phase and incubated at room temperature for 5 min. The cells were then pelleted and resuspended in 1 ml of a 1x fixative and incubated for 30 min on ice. Subsequently the cells were treated with 2% potassium permanganate and embedded in Spur resin (Wright et al., 1988).

Method B

The pellet of 1 ml of yeast cell culture in stationary phase was resuspended in 1 ml of 1x fixative (2.5% glutaraldehyde in phosphate buffered saline). After a 5 min incubation at room temperature, the cells were centrifuged and the supernatant discarded. The pellet was resuspended in 1 ml of a 1x fixative and incubated at 4°C overnight. The cells were post-fixed in osmium tetroxide and embedded in Epon. Thin sections were stained with uranyl acetate and lead citrate and examined under a Philips 201 electron microscope.

Cellular morphology and intracellular organelles were optimally examined following fixation using Method A, while Method B was best for revealing the structure of karmellae at higher magnification.

Quantification of the karmellar membrane by electron microscopy

The overall strategy for quantifying the karmellar membrane was...
to determine the proportion of cells in thin sections of a yeast cell pellet with karmellae, then to count the average number of karmellae in cells whose nuclei were surrounded by the extra membrane. These two values were multiplied to provide an approximation of the relative amount of membrane formed.

Two separate procedures were used in these calculations. In the first procedure (used for points 3-5 in Fig. 6) thin sections of yeast cells fixed by Method A were photographed to give a final magnification of ×10,000. The micrographs were then examined to determine if karmellae were present. A total of 110 to 160 cells were examined; cells with a diameter <1 µm or unusual cell wall thickness were not counted. Dividing cells were considered as two if they were separated by a plasma membrane and as one if they had a common cytoplasm. The number of karmellae per cell (one karmella is defined as a stacked pair of membranes with a central thick line and two lateral thin lines; see Fig. 4), was determined by measuring the total thickness of all karmellar layers. The apparent width of the nuclear envelope, 25.5 ± 7 nm, was subtracted from the total thickness. This value was divided by the average thickness of a single karmellar layer, which was taken as the distance between the thick lines (15.4 ± 2.5 nm for fixation by Method A) to approximate the average number of karmellae per cell. If this calculation indicated that the cell had zero or one karmella, the cell was not counted, since this method cannot discriminate accurately between a cell with a normal nuclear envelope and one with a single extra pair of membranes.

In the second method (used for points 1 and 2 in Fig. 6 and to generate all the data in Table 1), thin sections of yeast cells processed by Method B were examined directly under the electron microscope at ×30,000 magnification. The fraction of cells with a diameter >1 µm containing karmellae was determined by direct examination of at least 100 consecutive cells in the grid on a minimum of two separate occasions (four for the polyleucine and AAl16Pro mutants). The number of karmellae was determined at ×45,000 magnification by counting the individual layers of karmellae in 16 consecutive cells whose nuclei were surrounded by karmellae. The microscopist examined all samples without knowledge of their identity.

RESULTS

High-level expression of the membrane-bound form of cytochrome b5 induces karmellae

To assess the effect of overexpressing cytochrome b5 on the ultrastructure of yeast cells, strains expressing the membrane-bound form of cytochrome b5 were examined by electron microscopy (Fig. 2). Panel a illustrates the normal morphology of uninduced yeast cells which harbor the plasmid for the amphipathic form of cytochrome b5. Note the single membrane surrounding the nucleus. Panels b, c and d illustrate the marked morphological changes that occur in yeast upon induction of rat cytochrome b5 using the copper-inducible yeast metallothionein promoter. The marked proliferation of the membrane around the nucleus is immediately obvious. The structure of these membranes is identical to that of the karmellae resulting from overproduction of HMG-CoA reductase isozyme 1 in yeast (Wright et al., 1988). In some cells the karmellae completely surround the nucleus (panels b and c) whereas in other cells the nucleus is only partially surrounded by the karmellae (panel d). Wright and coworkers (1988) made similar observations in yeast overexpressing HMG-CoA reductase. When they analyzed serial sections, most, if not all, karmella-containing nuclei also had a region where only the single nuclear envelope could be observed. We observed that under conditions that maximize the amount of karmellae (see Fig. 6, point 5), 70-80% of the karmellae are in contact with the plasma membrane (panels c and d). A statistical analysis of the distance between the nuclear and plasma membranes did not reveal any significant differences between cells with and without karmellae. Nonetheless, such contact sites might function in membrane assembly, since lipid transport can be mediated by direct contact between donor and acceptor membranes (Voelker, 1985). Circular cross-sections of membrane similar to those of the crystallloid endoplasmic reticulum induced by overexpression of HMG-CoA reductase in a mutant CHO cell line were never observed (Chin et al., 1982; Orci et al., 1984; Pathak et al., 1986).

Fig. 3 is an illustration of a tangential section through 20 concentric layers of karmellae. The nucleus is not visible. The outermost membrane is detached from the rest of the karmellae at different sites (arrowheads), which might be an artifact due to the processing of the cells. However, since vesicle-mediated transport of lipids from a donor to an acceptor membrane is a major pathway in membrane assembly (Voelker, 1985), it is also possible that these sites are involved in lipid transport.

The high-resolution structure of the karmellae is best examined when cells have been post-fixed with osmium tetroxide (Method B) versus processing using potassium permanganate (Method A, Materials and Methods). Each karmella is a 100-120 Å thick membranous structure composed of an electron-dense central line (Fig. 4, arrow) bordered by two lines with a lower electron density (arrowheads). The karmellae represent two apposed bilayers, devoid of ribosomes, morphologically similar to the normal double membrane of the nuclear envelope, in which the luminal space has been eliminated, presumably since these membrane are not actively secreting large amounts of protein. Each stacked pair of membranes is separated by a cytosolic space with an average distance of 100 Å, which is sufficient to accommodate the heme-containing domain of cytochrome b5 ≅25 Å (Mathews and Czerwinski, 1976). Microscopically, the karmellae bear a striking similarity to the myelin sheath of peripheral nerves (Fawcett, 1981), the difference being that the plasma membrane of the Schwann cell is wrapped in a spiral around the nerve axon, whereas the karmellae result from the contact of the two bilayers of the redundant nuclear membrane. These observations and the absence of tubular cross-sections indicate that the karmellae surround the nucleus in an ‘onion-like’ structure.

The cytosolic form of cytochrome b5 does not induce karmellae

Karmellae are not observed in yeast expressing the cytosolic hydrophilic heme-containing domain of cytochrome b5 (Table 1), nor are they found in yeast harboring the plasmid with the amphipathic cytochrome b5 gene in the inverted orientation relative to the chelatin promoter. It could also be demonstrated that yeast expressing a cytochrome b5 molecule that had been truncated by 19 amino acids at its carboxyl terminus (Pro115Stop) and hence missing approximately one-half of its putative mem-
brane binding domain (see Fig. 1) did not stimulate karra formation (Table 1). Previous studies have shown that this Pro115Stop mutant protein does not integrate into the endoplasmic reticulum and can be isolated from the cytosol (Vergères and Waskell, 1992).

The apo form of membrane-bound cytochrome \(b_5\) induces the formation of karra

In order to determine whether the formation of karra is related to the lipid biosynthetic activity of the overexpressed cytochrome \(b_5\), we expressed the apo form of the protein by inducing cytochrome \(b_5\) synthesis in the presence of succinyl acetone. Succinyl acetone is a specific inhibitor of aminolevulinate dehydratase and hence of heme synthesis (Sassa and Kappas, 1983). It has also been used to inhibit heme biosynthesis in hepatocytes (Sinclair et al., 1990). Fig. 5 shows a difference spectrum (reduced minus oxidized) of a cell suspension in which cytochrome \(b_5\) expression has been induced with copper sulfate in both the absence and presence of succinyl acetone. The synthesis of heme is completely inhibited by succinyl acetone as indicated by the flat spectrum in the vicinity of the Soret band of heme proteins. Previous experiments (Vergères and Waskell, 1992) have demonstrated that 80% of the heme protein in the top spectrum of Fig. 5 is due to rat cytochrome \(b_5\). The absence of detectable heme protein in the yeast treated with succinyl acetone indicates that the yeast heme proteins that were present before succinyl acetone was added were either degraded or lost their heme and were unable to replenish it. Yeast grown in the absence or

![Fig. 2. Electron micrographs of yeast cells expressing rat cytochrome \(b_5\). Yeast cells containing the plasmid with the gene coding for the membrane-bound form of cytochrome \(b_5\) have been grown and fixed by Method A as described under Materials and Methods. (a) Cell grown in the absence of copper. (b,c,d) Cells grown in the presence of copper, and overexpress the cytochrome \(b_5\). Nu, nucleus; K, karra. Bar, 1 \(\mu\)m.](image-url)
The presence of succinyl acetone express similar amounts of cytochrome $b_5$. The structure and the relative amount of the karmellae is also identical in the presence (Table 1, column E) and absence (Table 1, column E) of succinyl acetone indicating that enzymatically active cytochrome $b_5$ is not necessary for proliferation of the membranes. Thus the synthesis of excess ergosterol or unsaturated lipids is not the signal for membrane biogenesis in this system.

The experimental results with succinyl acetone-treated cells were confirmed by expressing three mutant proteins of cytochrome $b_5$ (Tyr78Lys, Tyr78Glu, Tyr78His) under identical conditions. Tyr78 is located in the center of a hydrophobic patch on the surface of the otherwise hydrophilic catalytic domain of cytochromes $b_5$ (see Fig. 1) (Mathews and Czerwinski, 1976). These mutant proteins vary in their ability to incorporate heme in vivo in *S. cerevisiae*: Tyr78Lys and Tyr78His incorporate wild-type levels of heme whereas the Tyr78Glu mutant results in an apo protein (Vergères et al., 1993). However, these three mutants express close to wild-type levels of karmellae (Table 1, column E) with normal morphology (micrograph not shown).

These results indicate that the formation of karmellae is not dependent on the structure or function of a heme-containing electron transport-active cytochrome $b_5$ but rather is triggered by incorporation of protein into the existing membrane. The signal for membrane biosynthesis must therefore be in the single transmembrane segment of cytochrome $b_5$. Once embedded in the membrane the apo protein directs membrane assembly in a manner indistinguishable from the holoenzyme. These results also demonstrate that apocytochrome $b_5$ undergoes intracellular processing similar to that of the holoenzyme, which has been previously shown to involve synthesis on cytosolic ribosomes and insertion into the endoplasmic reticulum via a signal recognition particle independent process (Anderson et al., 1983; Okada et al., 1982; Rachubinski, 1980).

**The quantity of membrane produced is proportional to the level of expression of wild-type cytochrome $b_5$**

In order to determine whether the cytochrome $b_5$-induced membrane proliferation is proportional to the level of cytochrome $b_5$ expression, the amount of membrane formed by yeast expressing varying amounts of cytochrome $b_5$ was estimated by counting the fraction of cells with karmellae and the average number of karmellae in cells where karmellae could be observed. Multiplication of these values provides an estimate of the relative amount of karmellae synthesized by the cell. The two procedures used to quantitate the karmellar membrane (see Materials and Methods) undoubtedly underestimate the amount of karmellae per cell, since a thin section only samples a 80 nm slice of a yeast cell which is 2.5–10 µm in width and 4.5–21 µm in length (Berry, 1982). For example, micrographs of thin sections of the yeast cells demonstrate that only 48% (±8%)
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of the cells are sectioned through the nucleus. Furthermore, the karmellae never entirely encircle the nucleus (Wright et al., 1988) (see also Fig. 2d). Some of the cells sectioned through the nucleus might therefore not reveal the presence of karmellae. In spite of these limitations, our sampling technique allows us to compare the relative amount of membrane produced by yeast grown under different experimental conditions.

Under conditions of maximal expression (Fig. 6, point 5), 36% of the cells in a thin section had karmellae. This number increased to 74% if only cells with visible nuclei were considered, indicating that under these conditions the vast majority of cells have karmellae. Fig. 6 clearly demonstrates that yeast, grown under slightly different conditions (see Materials and Methods) and expressing ±5-15% of the total cellular proteins as cytochrome \( b_5 \), synthesizes an amount of membrane proportional to the quantity of cytochrome \( b_5 \) present. Although the data illustrated in Fig. 6 suggest a linear relationship between the level of cytochrome \( b_5 \) expression and the amount of membrane synthesized, additional detailed experiments are required to further characterize this relationship. These findings are a clear example of the cells’ ability to regulate membrane assembly in response to an increase in the quantity of membrane protein. The fact that a rat liver endoplasmic reticulum protein induces membrane proliferation in yeast indicates that signals for this basic physiological process are likely to be similar in yeast and in mammals.

Role of the amino acid sequence of the membrane binding segment of cytochrome \( b_5 \) in the formation of karmellae

Amphipathic cytochrome \( b_5 \) is anchored to the endoplasmic reticulum via a single \( \alpha \)-helical hydrophobic peptide at its carboxyl terminus (Ozols, 1989). In order to determine whether a specific amino acid sequence or the topology of the membrane anchor is the signal for synthesis of karmellae, we constructed a series of mutants (see Fig. 1), whose design, expression, partial characterization and intracellular localization have been described in detail elsewhere (Vergères and Waskell, 1992), and will only be briefly described here as they relate to karmella formation.

![Fig. 4](image-url). Electron microscopic analysis of the structure of karmellae. High-power magnification of the karmellae from cells expressing cytochrome \( b_5 \) that have been grown and fixed by Method B as described in Materials and Methods. The arrow points toward the central electron dense line and the arrowheads toward the bordering lines with lower electron density. Nu, nucleus. Note the absence of ER lumen separating the membrane bilayers. Bar, 0.1 \( \mu \)m.

### Table 1. The ability of various constructs of cytochrome \( b_5 \) to induce membrane proliferation

<table>
<thead>
<tr>
<th>Cytochrome ( b_5 )</th>
<th>A: Cytochrome ( b_5^{*} ) expression (%)</th>
<th>B: Fraction of cells with karmellae</th>
<th>C: Average number of layers</th>
<th>D: ( \frac{B \times C^{*}}{} )</th>
<th>E: ( \frac{D}{A} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type (WT)</td>
<td>5.4</td>
<td>0.19</td>
<td>7.9±2.6§</td>
<td>1.5</td>
<td>0.27</td>
</tr>
<tr>
<td>WT + succinyl acetone</td>
<td>3.4</td>
<td>0.16</td>
<td>5.7±1.6</td>
<td>0.9</td>
<td>0.26</td>
</tr>
<tr>
<td>Tyr78His</td>
<td>1.9</td>
<td>0.10</td>
<td>4.7±2.3</td>
<td>0.5</td>
<td>0.26</td>
</tr>
<tr>
<td>Tyr78Lys</td>
<td>2.8</td>
<td>0.09</td>
<td>6.3±2.3</td>
<td>0.6</td>
<td>0.21</td>
</tr>
<tr>
<td>Tyr78Glu</td>
<td>3.0</td>
<td>0.10</td>
<td>6.3±2.3</td>
<td>0.6</td>
<td>0.20</td>
</tr>
<tr>
<td>Pro115Ala</td>
<td>3.0</td>
<td>0.09</td>
<td>6.3±2.7</td>
<td>0.6</td>
<td>0.20</td>
</tr>
<tr>
<td>Ala131Lys, Glu132Lys</td>
<td>3.5</td>
<td>0.10</td>
<td>6.1±2.4</td>
<td>0.6</td>
<td>0.17</td>
</tr>
<tr>
<td>Ala116Pro</td>
<td>3.3</td>
<td>0.05</td>
<td>6.4±2.7</td>
<td>0.3</td>
<td>0.09</td>
</tr>
<tr>
<td>Polyleucine mutant</td>
<td>&gt;1.8§</td>
<td>0.05</td>
<td>3.7±1.3</td>
<td>0.2</td>
<td>&lt;0.11</td>
</tr>
<tr>
<td>Pro115Stop</td>
<td>4.6</td>
<td>0.00</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cytosolic ( b_5 )</td>
<td>5.3</td>
<td>0.00</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

The cells in these experiments were grown to stationary phase and induced with CuSO\(_4\) (see Materials and Methods). The cell samples were divided and protein and membrane expression were evaluated separately. The results are the average of two different preparations.

*The level of cytochrome \( b_5^{*} \) expression is given as a percentage of the total cellular protein as measured by scanning densitometry of Coomassie blue-stained SDS-gels of the total cellular protein.

†D expresses the relative amount of membrane formed (see Materials and Methods).

‡The value in column E is obtained by dividing the value for the relative amount of membrane (D) by the percentage of cytochrome \( b_5 \) of the total cellular protein (A). It reflects the amount of membrane produced per amount of cytochrome \( b_5 \) expressed.

§The standard deviation reflects the distribution of the number of layers.

¶The overexpression of the polyleucine mutant cannot be precisely quantified because variable amounts of protein enter the gel.
Pro115Ala and Ala116Pro mutants

Pro115 is presumably located in the middle of the hydrophobic membrane anchor of cytochrome bs and could be critical in determining whether the α-helical transmembrane domain exists in a hairpin loop or spans the membrane. The sequence of the membrane anchor and the two models of its topology in the bilayer are illustrated in Fig. 1 (Ozols, 1989). If Pro115 is in the uncommon cis conformation (10% of the prolines in proteins form cis peptide bonds) a hairpin loop will result (Richardson and Richardson, 1989; Williams and Deber, 1991). Conversely, if Pro115 is in the trans conformation, the α helix should span the bilayer and the carboxyl terminus would be in the lumen of the endoplasmic reticulum. Pro115 was mutated by site-directed mutagenesis (Vergères and Waskell, 1992) to an alanine, which is expected to result in a α-helical domain that spans both leaflets of the membrane. If a hairpin loop exists in the wild-type protein and is important for karmella formation, the substitution of alanine for proline at residue 115 should affect the karmellae in some manner. The electron micrographs of karmella from yeast expressing the Pro115Ala mutant cytochrome b5 are identical in structure (micrographs not shown) and quantity, when adjusted for the amount of cytochrome b5 expressed (column E in Table 1), to the karmellae from the yeast expressing the wild-type protein. It is therefore concluded that Pro115 and the topology it generates are not critical for karmella formation.

In contrast to the results with the Pro115Ala mutant, expression of the Ala116Pro mutant protein induced considerably less membrane than expected relative to the amount of the protein expressed (column E in Table 1). The Ala116Pro mutant protein is thought to have an α-helical domain interrupted by an extended polypeptide chain conformation at Pro115 and -116 (Bennett and Steitz, 1980). This result suggests that the secondary structure of the hydrophobic membrane anchor may be an important component of the signal that regulates biogenesis of the endoplasmic reticulum. In spite of the decreased ability of the Ala116Pro mutant protein to induce karmella formation, their morphology was normal (micrographs not shown).

Ala131Lys,Glu132Lys mutant

Dailey and Strittmatter (1981) demonstrated that anionic charges, including Glu132 at the carboxyl terminus of cytochrome b5 played a crucial role in establishing 'tight' binding of cytochrome b5 to phospholipid vesicles and isolated microsomes. In addition, it has been demonstrated (Beltzer et al., 1991; von Heijne, 1991) that the charge of amino acids flanking membrane binding segments of proteins is of importance in regulating the incorporation and transmembrane orientation of proteins. The net negative charge of −3 at the carboxyl terminus of rat cytochrome b5 was altered to a net charge of 0 by constructing the Ala131Lys,Glu132Lys double mutant (see Fig. 1) to determine whether it would be capable of inducing karmellae. This mutant protein gives rise to karmellae of normal morphology (micrographs not shown) and abundance (column E in Table 1), indicating that the negative charges at the carboxyl terminus of the wild-type protein do not appear to be critical in vivo for incorporation of the protein into the endoplasmic reticulum or induction of membrane biosynthesis.

Polyleucine mutant

The putative membrane anchor of cytochrome b5 was replaced by a homopolymer of 22 leucines in order to determine whether a completely nonphysiological α-helical hydrophobic membrane anchor was sufficient to induce karmella formation, or whether a specific amino acid
sequence might be necessary (Chou and Kendall, 1990). This very hydrophobic mutant of the membrane binding domain was indeed able to induce karmellae. However, the abundance (column E in Table 1) and morphology (Fig. 7) of the karmellae were strikingly different from those observed with the wild-type protein. The amount of karmellae formed appears to be decreased to a greater extent than the expression of cytochrome \( b_5 \) (column E in Table 1), indicating that the cytochrome \( b_5 \) mutant has a decreased ability to signal the formation of karmellae. Confirmation of this hypothesis must await the development of a sensitive and accurate assay for the quantitation of the apo and holoheme of the enzyme in the endoplasmic reticulum. The structure of the karmellae in yeast harboring the polyleucine cytochrome \( b_5 \) mutant differs from that of the karmellae induced by the wild type and all of the other cytochrome \( b_5 \) mutants by having the individual membrane bilayers of the endoplasmic reticulum separated by a 60-70Å luminal space (Fig. 7). Note the absence of the thick line representing the apposed endoplasmic reticulum membrane. This increase in the luminal space might be the result of a change in the protein composition of the karmellae or of a different topology of the cytochrome \( b_5 \) mutant. In all other mutants the two membrane bilayers are adherent and there is no space between them.

DISCUSSION

High-level expression of the membrane-bound form of cytochrome \( b_5 \) in \textit{S. cerevisiae} results in the proliferation of membranes surrounding the nucleus identical to the karmellae induced by the expression of HMG-CoA reductase in yeast (Wright et al., 1988). Interestingly, both HMG-CoA reductase and cytochrome \( b_5 \) are required for ergosterol biosynthesis in yeast (Aoyama et al., 1981). Therefore, one of our first goals was to establish whether the lipid biosynthetic activity of cytochrome \( b_5 \) was responsible for karmella formation. The ability of heme-deficient completely inactive cytochrome \( b_5 \) to induce normal levels of karmellae established unambiguously that incorporation of the protein into the membrane was the trigger for membrane proliferation rather than its lipid biosynthetic activity. This conclusion was supported by the finding that the cytosolic form of cytochrome \( b_5 \) and a mutant protein lacking 50% of the membrane anchor, both of which localized to the cytosol, did not induce karmellae. Furthermore, two proteins mutated in the membrane anchor of cytochrome \( b_5 \) (Ala116Pro with its disrupted \( \alpha \)-helix and the polyleucine mutant proteins) were less effective than the wild-type protein in stimulating membrane formation. These results suggest that the sensor that mediates membrane proliferation responds to a hydrophobic sequence of amino acids and therefore may be analogous to the protein translocation machinery in the endoplasmic reticulum, which recognizes hydrophobicity rather than a specific amino acid sequence. The mutant in which the membrane anchor was replaced by a homopolymer of 22 leucines induced a smaller amount of karmellae with a different morphology, suggesting that further studies with this mutant might also provide insight into the determinants of membrane structure in addition to the signal for membrane biogenesis.

A computer search for sequence homologies between the membrane binding domains of the three proteins (HMG-CoA reductase, cytochrome \( b_5 \) and cytochrome P-450 isozymes 1 and 2 from \textit{Candida maltosa}) that lead to marked endoplasmic reticulum proliferation in yeast did not reveal any common sequences. We therefore propose that membranes possess ‘sensors’ that monitor biophysical characteristics such as membrane fluidity, membrane surface charge density or protein density either within or on the membrane surface. Since the principal organelle of lipid biosynthesis is the endoplasmic reticulum, logical candidates for such sensors are the lipid biosynthetic enzymes, for they could immediately and directly detect and correct any imbalance in the properties of the membrane. In spite of an apparent lack of specificity in the amino acid sequences inducing karmellae formation, yeast does not respond uniformly to the expression of cytochrome P-450, HMG-CoA reductase and cytochrome \( b_5 \). The extra membranes can be associated with the nuclear envelope (HMG-CoA reductase isozyme 1, P450\(_{cm2}\) cytochrome \( b_5 \)) or with the plasma membrane (HMG-CoA reductase isozyme 2). They can surround the nucleus (HMG-CoA reductase isozyme 2, cytochrome \( b_5 \)) or can simply be found in the cytoplasm (P450\(_{cm1}\)). The spacing between each pair of membranes also varies (P450\(_{cm2}\) vs P450\(_{cm1}\); wild-type cytochrome \( b_5 \) vs polyleucine mutant). Finally the amount of karmellae produced by different construct (wild type cytochrome \( b_5 \) vs polyleucine and Ala116Pro mutants) is different. Variations in the structure of these membranes appear to reflect different properties of the expressed proteins.

Since it has been demonstrated that the amount of mem-

Fig. 7. Electron micrographs of a yeast cell expressing the polyleucine mutant cytochrome \( b_5 \). Cells expressing the polyleucine mutant were grown and fixed by Method B as described in Materials and Methods. Note that the membrane bilayers are separated by a luminal space. Nu, nucleus. Bar, 0.1 µm.
brane formed was proportional to the amount of cytochrome \( b_5 \) expressed (see Fig. 6), we calculated how much newly formed membrane would be needed to accommodate the expressed cytochrome \( b_5 \) if the molecules were packed next to one another. X-ray crystallography of the hydrophilic head group of cytochrome \( b_5 \) reveals it has dimensions of \( 25 \, \text{Å} \times 25 \, \text{Å} \times 32 \, \text{Å} \) (Mathews et al., 1972). The area occupied by a single molecule of radius 16 Å would be \( \approx 800 \, \text{Å}^2 \). The average number of cytochrome \( b_5 \) molecules per cell is \( 1.8 \times 10^6 \) (Vergères and Waskell, 1992) corresponding to 5.4% of the total cellular protein (average of points 1 and 2 in Fig. 6). Cytochrome \( b_5 \) would therefore cover \( 1.4 \times 10^9 \, \text{Å}^2 \) of membrane. Assuming an average nuclear diameter of \( \approx 2 \, \mu \text{m} \), a spherical shape for the nucleus (area of a sphere = \( \pi d^2 = 1.25 \times 10^9 \, \text{Å}^2 \), where \( d \) is diameter) and a complete packing of cytochrome \( b_5 \) on both sides of the karmellae (see Fig. 4B), the protein would occupy 0.6 layer of karmellae. Although the exact density of protein in the endoplasmic reticulum is not known, this estimate suggests that cytochrome \( b_5 \) does not completely occupy the available membrane and piques one’s curiosity about the remaining components. Is there a preponderance of lipid in the karmellae such as there is in myelin (3-4 lipid/protein ratio; w/w), or is the lipid-to-protein ratio more typical of that in microsomes (0.4 lipid/protein; w/w) (Gennis, 1989)? If the lipid-to-protein ratio proves to be characteristic of that in microsomes, one would wish to know the identity of the other membrane proteins. We speculate that these karmellae may concentrate the microsomal lipid biosynthetic enzymes and the protein machinery required for cytochrome \( b_5 \) membrane insertion.

All of these very interesting questions must await further characterization of the karmellae but they promise to provide a unique opportunity to study critical biological phenomena.

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