

The Role of the 3-Hydroxy 3-Methylglutaryl Coenzyme A Reductase Cytosolic Domain in Karmellae Biogenesis

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In all cells examined, specific endoplasmic reticulum (ER) membrane arrays are induced in response to increased levels of the ER membrane protein 3-hydroxy 3-methylglutaryl coenzyme A (HMG-CoA) reductase. In yeast, expression of Hmg1p, one of two yeast HMG-CoA reductase isozymes, induces assembly of nuclear-associated ER stacks called karmellae. Understanding the features of HMG-CoA reductase that signal karmellae biogenesis would provide useful insights into the regulation of membrane biogenesis. The HMG-CoA reductase protein consists of two domains, a multitopic membrane domain and a cytosolic catalytic domain. Previous studies had indicated that the HMG-CoA reductase membrane domain was exclusively responsible for generation of ER membrane proliferations. Surprisingly, we discovered that this conclusion was incorrect: sequences at the carboxyl terminus of HMG-CoA reductase can profoundly affect karmellae biogenesis. Specifically, truncations of Hmg1p that removed or shortened the carboxyl terminus were unable to induce karmellae assembly. This result indicated that the membrane domain of Hmg1p was not sufficient to signal for karmellae assembly. Using β -galactosidase fusions, we demonstrated that the carboxyl terminus was unlikely to simply serve as an oligomerization domain. Our working hypothesis is that a truncated or misfolded cytosolic domain prevents proper signaling for karmellae by interfering with the required tertiary structure of the membrane domain.

INTRODUCTION

Assembly of specific membranes is an essential process throughout cell growth and development, but the molecular mechanisms responsible for specific membrane biogenesis are not understood in even a single case. Specific endoplasmic reticulum (ER) membrane arrays are induced in yeast by increasing the levels of certain ER membrane proteins, such as 3-hydroxy 3-methylglutaryl coenzyme A (HMG-CoA) reductase (Wright *et al.*, 1988), cytochrome P450 (Schunck *et al.*, 1991), cytochrome *b*₅ (Vergeres *et al.*, 1993), Sec12p (Nishikawa *et al.*, 1994), the canine ribosome receptor (Wanker *et al.*, 1995), and Pbn1p (protease B negative 1 protein) (Naik and Jones, 1998). Analysis of these inducible membranes

provides an opportunity to discover the molecular mechanisms cells use to regulate the synthesis and organization of new membrane arrays.

HMG-CoA reductase is an integral ER membrane protein that catalyzes the production of mevalonate, a key intermediate in the synthesis of sterols and nonsterol isoprenoid compounds. The structure of HMG-CoA reductase can be divided into two domains: a complex membrane-spanning domain at the amino terminus followed by a cytosolic catalytic domain (Liscum *et al.*, 1985; Basson *et al.*, 1986; Roitelman *et al.*, 1991). The membrane domain of mammalian and yeast HMG-CoA reductases spans the membrane seven or eight times and is connected to the catalytic domain through a flexible linker sequence (Liscum *et al.*, 1985; Lum *et al.*, 1996; Roitelman *et al.*, 1992). The membrane domain is essential for both the proliferation of ER membranes (Jingami *et al.*, 1987; Parrish *et al.*, 1995) and the regulated degradation of both the mammalian and yeast HMG-CoA reductases (Gil *et al.*, 1985; Skalnik *et al.*, 1988; Hampton *et al.*, 1996). The cytosolic domain is responsible for catalysis and is thought to

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Abbreviations: β -gal, β -galactosidase; CHX, cycloheximide; CoA, coenzyme A; DiOC6, 3,3'-dihexyloxycarbocyanine iodide; ER, endoplasmic reticulum; GFP, green fluorescent protein; HA, hemagglutinin; HMG, 3-hydroxy 3-methylglutaryl.

mediate protein dimerization (Edwards *et al.*, 1985; Basson *et al.*, 1987; Frimpong and Rodwell, 1994).

Unlike mammalian cells, which have one HMG-CoA reductase, yeast express two functional HMG-CoA reductase isozymes, Hmg1p and Hmg2p (Basson *et al.*, 1986). Each isozyme triggers the proliferation of distinct sets of membrane arrays that reflect the localization of the particular isozyme (Koning *et al.*, 1996). Specifically, Hmg1p triggers the formation of karmellae, which are stacked pairs of membranes associated with the nucleus (Wright *et al.*, 1988). Hmg2p induces peripheral ER membrane stacks and short karmellae (Koning *et al.*, 1996). The ability to respond to HMG-CoA reductase elevations by generating ER membrane arrays is not unique to yeast but occurs in all cell types that have been examined. For example, in mammalian cells, HMG-CoA reductase induces the formation of hexagonal arrays of smooth ER tubules called crystalloid ER (Chin *et al.*, 1982; Anderson *et al.*, 1983; Orci *et al.*, 1984; Kochevar and Anderson, 1987). In addition, many tissues that produce sterol derivatives, such as steroid hormones, contain characteristic ER membrane arrays (Fawcett, 1981).

Results of our laboratory and others using chimeric fusion proteins indicated that the catalytic domain of HMG-CoA reductase played no role in either induction of ER membranes or controlling their morphology. For example, proteins containing the HMG-CoA reductase membrane domain fused to unrelated carboxyl-terminal sequences such as β -galactosidase (β -gal) (Skalnik *et al.*, 1988) or Suc2His4Cp (containing a portion of the invertase protein and histidinol dehydrogenase) induced the proliferation of ER membrane arrays that were indistinguishable from the membranes generated by the wild-type HMG-CoA reductase (Parrish *et al.*, 1995). To determine whether the membrane domain of Hmg1p was sufficient to induce karmellae, we generated truncations of Hmg1p that shortened or completely removed the carboxyl terminus. Surprisingly, although the truncated proteins were expressed at high levels, yeast did not generate karmellae in response to them. Therefore, sequences beyond the membrane domain must be playing some role in the process of signaling for karmellae. One postulated role for the carboxyl-terminal sequences is to provide an oligomerization domain that would allow HMG-CoA reductase monomers to associate with each other. This hypothesis was tested by fusing the Hmg1p membrane domain to an oligomerization-competent β -gal and to an oligomerization-incompetent truncated β -gal (Tsuneoka and Mekada, 1992). Both β -gal chimeras induced karmellae, indicating that oligomerization of the cytosolic domain was not a requirement. Our experiments suggested that the carboxyl terminus of HMG-CoA reductase was not neutral for karmellae assembly and that the role of the cytosolic domain in karmellae assembly was unlikely to be oligomerization.

MATERIALS AND METHODS

Strains and Media

The yeast strains used in this study are listed in Table 1. Strains were grown at 30°C on rich minimal medium (0.67% yeast nitrogen base without amino acids, 2% casamino acids, and 2% glucose or 2% galactose plus 3% sucrose) supplemented with the appropriate acids or nucleotide bases (Sherman *et al.*, 1986) or complete synthetic medium lacking histidine and uracil from Bio 101 (La Jolla, CA). Solid medium contained 2% agar (Sherman *et al.*, 1986).

Table 1. Yeast strains

Strain	Genotype
JRY527 ^a	<i>MATa ade2-101 his3Δ200 lys2-801 met ura3-52</i>
RWY410 ^b	JRY527 + pAK266
RWY406	JRY527 + pAK260
RWY614	JRY527 + pDP428
RWY572	JRY527 + pCS40
RWY621 ^b	JRY527 + pCR425
RWY626	JRY527 + pCR427
RWY900	JRY527 + pRH127-3
RWY1494	JRY527 + pDP586
RWY1497	JRY527 + pDP586 + pRH127-3
RWY943	JRY527 + pDP601
RWY944	JRY527 + pDP602

Plasmid	Description
pAK266 ^b	<i>pGAL:Hmg1p</i> in pRS316; <i>CEN6 URA3</i>
pAK260	<i>pGAL:Hmg1Δ29p</i> in pRS316; <i>CEN6 URA3</i>
pDP428	<i>pHMG1:HMG1:HA</i> in YEp352; 2 micron origin <i>URA3</i>
pCS40	<i>pHMG1:Hmg1^{mem}:Hmg2^{cat}</i> in YEp352; 2 micron origin <i>URA3</i>
pCR425 ^b	<i>pGAL:Hmg1^{mem}:HMG1⁵²⁵⁻⁹⁸⁷:GFP</i> in pRS316; <i>CEN6 URA3</i>
pCR427	<i>pGAL:Hmg1^{mem}:GFP</i> in pRS316; <i>CEN6 URA3</i>
pRH127-3 ^c	<i>pGAPDH:Hmg1 catalytic domain 2</i> in Yplac195; micron origin <i>URA3</i>
pDP586	<i>pGAL:Hmg1p</i> in pRS313; <i>CEN6 HIS3</i>
pDP601	<i>pHMG1:Hmg1:β-galactosidase</i> in YEp352; 2 micron origin <i>URA3</i>
pDP602	<i>pHMG1:Hmg1:β-galΔ20</i> in YEp352; 2 micron origin <i>URA3</i>

^a Basson *et al.* (1986).
^b Koning *et al.* (1996).
^c Donald *et al.* (1997).

Plasmids

To truncate Hmg1p after the membrane domain, pCS4-14, a derivative of pCS4 with an *XhoI* site immediately after the membrane domain coding region (Sengstag *et al.*, 1990), was digested with *XhoI* and *Clal* to add a synthetic double-stranded oligonucleotide with the hemagglutinin (HA) epitope and a stop codon, resulting in plasmid pDP428. The HA epitope is a nonapeptide sequence derived from the influenza HA protein (Wilson *et al.*, 1984). This plasmid encodes the N-terminal 523 amino acids of Hmg1p (the entire membrane domain), followed by 14 amino acids that included the HA epitope and a stop codon. The following two synthetic oligonucleotides were annealed to provide the 14 amino acids: 5'-TCGAGCATACCAGTTACCCATACGATGTTCAGATTACGCTTAACTAGTTGA-3' and 5'-CGTCAACTAGTTAAGCGTAATCTGGAACATCGTATGGGT AACTGGTATGC-3'.

To create Hmg1: β -gal fusion proteins, pDP428 was digested with *XhoI* and *SpeI* to insert an *XhoI*-*SpeI* fragment containing wild-type *lacZ* (pDP598) or to insert an *XhoI*-*SpeI* fragment containing a truncated *lacZ* (pDP600). The full-length β -gal was obtained from the plasmid pMKITNeo-*XhoI*-HMGal δ 5', and the truncated β -gal was obtained from the plasmid pMKITNeo-*XhoI*-HMGal δ 20 (both provided by Helen Cheng and Robert Simoni, Stanford University, Palo Alto, CA). These plasmids contain Syrian hamster HMG-CoA reductase fused to β -gal or a truncated β -gal missing the last 20 amino acids needed for tetramerization (Cheng *et al.*, 1999). Both

intermediate plasmids, pDP598 and pDP600, needed a correction in reading frame to encode the N-terminal 523 amino acids of Hmg1p, followed by 76 amino acids of Syrian hamster HMG-CoA reductase linker region, and then ending with the entire β -gal or β -gal missing the last 20 amino acids (β -gal Δ 20). The reading frame between the Hmg1p membrane domain and the hamster HMG-CoA reductase linker region was corrected by inserting a double-stranded oligonucleotide. The double-stranded oligonucleotide was prepared by annealing two oligonucleotides: 5'-TCGAACATACCAGTACG-3' and 5'-TCGACGTACTGGTATGT-3'. The final plasmids are pDP601 with the full-length β -gal fusion and pDP602 with the truncated β -gal fusion Δ 20.

The galactose-inducible HMG-CoA reductase plasmid pGAL-HMG1 (pAK266) has been described previously (Koning *et al.*, 1996). To create a catalytically inactive HMG-CoA reductase mutant, pAK266 was digested with *KpnI* and then religated. This plasmid (pAK260) encodes the *GAL1/10* promoter, N-terminal 957 amino acids of Hmg1p (which includes the entire membrane domain and most of the catalytic domain), followed by a 29-amino-acid gap, and then the remaining 68 amino acids of Hmg1p. The green fluorescent protein (GFP) (Chalfie *et al.*, 1994; Chalfie, 1995; Prasher *et al.*, 1992; Prasher, 1995) expression constructs used in these studies were derived from the plasmid pJC81 (provided by Jeff Cox and Peter Walter, University of California San Francisco, San Francisco, CA). This plasmid contains a mutant version of *GFP10* in which the first two codons have been changed to encode a *Bam*HI site. To create a membrane domain Hmg1:GFP fusion, a 1.5-kb *Bam*HI-*Eag*I fragment of pJC81 (containing the 710 bp *GFP10* ORF, 400 bp of the *ACT1* gene containing the transcriptional terminator, and 390 bp of the *tet* gene) was subcloned into the *Bam*HI-*Eag*I sites of pRS316 (Sikorski and Hieter, 1989) creating plasmid pCR415. This plasmid, pCR415, was then digested with *Bam*HI and *Sma*I, treated with Klenow to fill in the ends, and religated, creating pCR426. A 2.2-kb *Eco*RI fragment containing pGAL-HMG1 from pJR435 (Basson *et al.*, 1988) was ligated into the *Eco*RI site of pCR426, creating plasmid pCR427. This plasmid encodes an Hmg1^{mem}:GFP fusion, consisting of the *GAL1/10* promoter, N-terminal 525 amino acids of Hmg1p, followed by three linker residues and all of GFP except the initiating methionine. The cytosolic Hmg1:GFP fusion encoded by pCR425 has been described previously (Koning *et al.*, 1996). This plasmid encodes the *GAL1/10* promoter, N-terminal 987 amino acids of Hmg1p, followed by two linker residues and all of GFP except the initiating methionine.

To exchange the Hmg1p catalytic domain with the catalytic domain of Hmg2p, pA7, which contains an introduced *Xho*I site after the sequences encoding the Hmg1p last transmembrane domain (Sengstag *et al.*, 1990), was digested with *Hind*III, treated with Klenow, and then cut with *Xho*I. The resulting 9-kb fragment contained the *HMG1* promoter and all the membrane domain coding sequences as well as vector sequences. A 2-kb fragment that contains the Hmg2p linker region and catalytic domain coding sequences was isolated from pB7, which contains an introduced *Sall* site after the last transmembrane domain of Hmg2p (Sengstag *et al.*, 1990). This fragment was isolated after digestion with *Eco*RI and treatment with Klenow and then digested with *Sall*I. Ligation of the fragments produced pCS40, which encodes the *HMG1* promoter, N-terminal 525 amino acids of Hmg1p, fused to the Hmg2p linker and catalytic domain.

The multicopy (2 μ) plasmid pRH127-3, which expressed the Hmg1p catalytic domain under control of the constitutive glyceraldehyde-3-phosphate dehydrogenase promoter, has been previously described (Donald *et al.*, 1997). To coexpress full-length Hmg1p with the soluble Hmg1p catalytic domain, a galactose-inducible *HMG1* plasmid, pAK266, was digested with *Xho*I and *Spe*I, yielding a 4.3-kb fragment with the *GAL1/10* promoter and *HMG1*. This fragment was ligated into the *Xho*I and *Spe*I sites of pRS313 (Sikorski and Hieter, 1989) to produce a *CEN-GAL1/10-HMG1* plasmid in a vector that contains the *HIS3* selectable marker (pDP586).

3,3'-Dihexyloxycarbocyanineiodide (DiOC₆) Staining

DiOC₆ staining was performed as described previously (Koning *et al.*, 1993). Cells in log phase growth were stained with 10 μ g/ml DiOC₆ (Kodak, Rochester, NY) per 10⁷ cells using a 1 mg/ml ethanolic stock. Stained cells were observed with conventional fluorescence optics, using a Nikon (Melville, NY) Microphot-FXA epifluorescence microscope with excitation (480 \pm 20 nm) and barrier (535 \pm 40 nm) filters appropriate for fluorescein.

Electron Microscopy

Preparation of cells for electron microscopy was a variation on methods described previously (Wright and Rine, 1989). Specifically, cells were grown to 1 OD₆₀₀/ml, and the culture was fixed in 2% glutaraldehyde in buffer [0.1 M piperazine-*N,N'*-bis(2-ethanesulfonic acid), pH 6.8, 1 mM CaCl₂, 1 mM MgCl₂], postfixed in 2% KMnO₄, and stained en bloc with 1% uranyl acetate. The cells were dehydrated through a graded ethanol series and embedded in Spurr's resin (Spurr, 1969). Sections were stained with Reynolds' lead citrate (Reynolds, 1963). Observations were made on a Philips (Eindhoven, The Netherlands) CM100 transmission electron microscope at 60–80 kV.

Immunofluorescence

Immunofluorescence was performed using a procedure similar to that described by Pringle *et al.* (1989). Log phase cells were fixed with 3.7% formaldehyde, treated with Zymolyase (United States Biological, Swampscott, MA) to partially remove their cell walls, and applied to multiwell slides (Koning *et al.*, 1996). Antisera generated against the carboxyl terminal 15 amino acids of Hmg1p (LDII) was used at a 1:100 dilution. Kar2p antibodies were a gift of Mark Rose (Princeton University, Princeton, NJ) or Jeff Brodsky (University of Pittsburgh, Pittsburgh, PA) and were used at a 1:2000 dilution. GFP antibody was purchased from Clontech (Palo Alto, CA) and used at a 1:100 dilution. HA antisera (12CA5) was purchased from Boehringer Mannheim (Indianapolis, IN) and used at a 1:100 dilution. A 1:800 dilution of rabbit anti- β -gal antibody (Cappel Organon Technika, Durham, NC) was used after pretreatment with a yeast cell lysate to decrease nonspecific binding. In all cases, the incubation in primary antisera was for 1 h at room temperature. The antibody solution was gently aspirated away, and the cells were washed five times with TBST (25 mM Trizma base, 3 mM KCl, 140 mM NaCl, 0.05% Tween 20). Then 10 μ l of blocking solution (TBST and 1% ovalbumin) was applied to each well. In all cases, 10 μ l of secondary antibody was diluted in blocking solution, centrifuged for 10 min at 12,000 rpm in a microcentrifuge, and applied to the appropriate wells. The secondary antibodies used were 1:1000 goat anti-rabbit fluorescein conjugated, 1:200 goat anti-mouse fluorescein conjugated, and 1:500 goat anti-rabbit Texas Red conjugated (Cappel Organon Technika). After 45 min, the secondary antibody was washed five times with TBST. Ten microliters of a 1:1000 dilution of 1 mg/ml DAPI (Sigma, St. Louis, MO) in Tris-buffered saline was added to each well for 1 min. After one rinse with Tris-buffered saline, a drop of Citifluor (Ted Pella, Redding, CA) was applied to each well, and the slide was sealed with a coverslip and nail polish. Each experiment included a positive control using an antibody to detect tubulin (Yol1/34; Accurate Chemical, Westbury, NY) used at 1:10 dilution and a negative control lacking primary antiserum.

Protein Preparation and Immunoblotting

After growth in rich minimal medium containing 2% galactose and 3% sucrose for 12 h, the cultures were diluted into fresh medium and grown for 4–6 h until they reached 1 OD₆₀₀/ml. A 16- to 18-h galactose induction was used because a high level of karmellae is observed in cells after 12 h in galactose. The cells were then pelleted for 5 min at 834 \times g in a clinical centrifuge, and the cell pellets were frozen at -80°C until lysed. Strains that did not contain galactose-

inducible plasmids were grown overnight in rich minimal medium containing 2% glucose medium. The next morning, the cells were diluted into fresh glucose medium and grown to 1 OD₆₀₀/ml before harvesting a cell pellet. A total membrane fraction was prepared from the cells using modifications of previously described methods (Deschenes and Broach, 1987; Koning *et al.*, 1996). The pelleted membranes were resuspended in 100 μ l of lysis buffer (0.3 M sorbitol, 0.1 M NaCl, 5 mM MgCl₂, 20 mM MOPS, pH 7.4) containing protease inhibitors (2 μ g/ml each *N*-tosyl-L-phenylalanine-chloromethyl ketone, leupeptin, pepstatin A, aprotinin) found in Complete Protease Inhibitor tablets (Boehringer Mannheim). The absorbance at 280 nm was measured for each sample in 1% SDS, and 100 A₂₈₀ units were loaded on duplicate gels after heating at 65°C in 1× Laemmli sample buffer (0.03 M Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% β -mercaptoethanol, 0.005% bromophenol blue) for 10 min, followed by a 5 min centrifugation at top speed in a microcentrifuge (Laemmli, 1970). One gel was blotted to nitrocellulose and processed for immunodetection as described (Lum and Wright, 1995), and the duplicate gel was stained with colloidal blue from Novex (San Diego, CA). The duplicate gel and Western blot were digitized by scanning and analyzed using NIH Image 1.60 software as described (Lum and Wright, 1995). The same membrane preparation procedure was used to determine the stability of the Hmg1p or Hmg1 fusion protein pool by measuring the relative amount of Hmg1p immunoreactivity remaining at various time points after blocking protein synthesis with 50 μ g/ml cycloheximide (CHX) (Hampton and Rine, 1994).

β -Gal Activity Assays

The strains RWY 943 and RWY 944 were assayed for β -gal activity according to the protocol of Guarente and Ptashne (1981). Briefly, log phase cells were resuspended in buffer, SDS, and chloroform. Then *o*-nitrophenyl- β -D-galactoside was added as the substrate for β -gal. The reaction was stopped by adding Na₂CO₃, and the cell debris was removed by centrifugation. The OD₄₂₀ was measured, and the results were normalized to the OD₆₀₀ of the culture and to the assay time. Duplicate assays were run in each case, and cell dilutions were determined to be in the linear range of the assay.

RESULTS

Truncating the Hmg1p Carboxyl Terminus Reduced or Eliminated the Protein's Ability to Induce Karmellae

Yeast cells respond to increased levels of Hmg1p by proliferating stacked pairs of nuclear-associated membranes called karmellae (Wright *et al.*, 1988). To determine whether the cytosolic domain of Hmg1p was required for karmellae biogenesis, we examined the organization of the ER in cells expressing wild-type or truncated Hmg1p proteins, using fluorescence microscopy (summarized in Figure 1) and electron microscopy (Figure 2). In this study Hmg1p levels were elevated by use of either a galactose-inducible promoter or a multicopy plasmid, resulting in an ~10-fold increase in protein in both cases. As expected, karmellae were observed in 35% of the cells expressing wild-type Hmg1p (Figure 2A). Note that karmellae are never observed in 100% of the cell population in part because karmellae remain in the mother cell at mitosis (Wright *et al.*, 1988). Surprisingly, the truncated Hmg1p, which lacked a catalytic domain (Hmg1^{mem}:HA), failed to induce karmellae or any other type of ER membrane proliferation (Figure 2B).

We considered the possibility that removal of the entire catalytic domain might have profound effects on the folding of the Hmg1p membrane domain. If so, less dramatic

changes to the catalytic domain should allow the protein to remain karmellae competent. To test this idea, we examined the ultrastructure of cells expressing a catalytically inactive Hmg1p missing only 29 amino acids within the catalytic domain (Hmg1 Δ 29p) and determined that only 1% of the cell population contained karmellae (Figure 2C). In an additional 1% of the cell population, the Hmg1 Δ 29p induced tubular ER stacks, which resembled the mammalian crystalloid ER (Figure 2D) (Anderson *et al.*, 1983). This dramatic reduction in karmellae was unexpected, because the catalytic domain can be completely replaced with unrelated carboxyl-terminal sequences without adversely affecting the ability of the protein to induce karmellae (Skalnik *et al.*, 1988; Parrish *et al.*, 1995). In addition, we had previously confirmed that the catalytic activity of Hmg1p was not required for karmellae biogenesis by examining the ultrastructure of cells expressing a catalytically inactive allele of *HMG1* in which a single amino acid had been changed; this mutant Hmg1p induced karmellae at the wild-type level (Profant, unpublished observations). Thus, neither a specific protein sequence nor enzymatic activity appeared to be necessary for the protein's ability to induce karmellae, yet even rather minor changes to the Hmg1p carboxyl terminus could interfere with karmellae assembly.

We also examined cells expressing an Hmg1 membrane: Hmg2 catalytic domain fusion, in which the Hmg2p catalytic domain replaced the Hmg1p catalytic domain (Hmg1^{mem}:Hmg2^{cat}). If the information in the Hmg1p membrane domain dictates the cell's response as predicted, the Hmg1^{mem}:Hmg2^{cat} should generate karmellae. In addition, the catalytic domain is highly conserved between Hmg1p and Hmg2p (Basson *et al.*, 1988), making it an unlikely source of the membrane morphology differences induced by Hmg1p versus Hmg2p. As expected, the Hmg1^{mem}:Hmg2^{cat} induced predominantly karmellae (Figure 2H). However, in 4% of the cell population, the Hmg1^{mem}:Hmg2^{cat} induced Hmg2-type membranes. Therefore, information present in the Hmg2 catalytic domain continued to exert some influence on the membranes generated even in the context of the Hmg1 membrane domain.

To investigate the role of carboxyl-terminal sequences in karmellae biogenesis further, we characterized the membrane proliferations induced by two different Hmg1:GFP fusion proteins. The first fusion included the linker sequence and a portion of the Hmg1 catalytic domain (Hmg1^{mem}:Hmg1⁵²⁵⁻⁹⁸⁷:GFP). In the second fusion, the cytosolic domain of Hmg1p was completely replaced by GFP (Hmg1^{mem}:GFP). Karmellae were induced by Hmg1^{mem}:Hmg1⁵²⁵⁻⁹⁸⁷:GFP (Figure 2G). In contrast, as for the Hmg1:HA protein, karmellae were never observed in cells expressing the Hmg1^{mem}:GFP; instead 10% of the cells assembled disorganized ER membrane stacks (Figure 2, E and F). The disorganized ER consisted of either portions of the nuclear envelope folding away from the nucleus or as peripheral membrane stacks. These observations are consistent with an essential, but unexpected, role for the carboxyl terminus in karmellae biogenesis.

Differences in Protein Amounts Did Not Account for the Inability to Induce Karmellae

Elevated levels of Hmg1p are required to induce karmellae (Wright *et al.*, 1988). Thus, mutations that decrease the

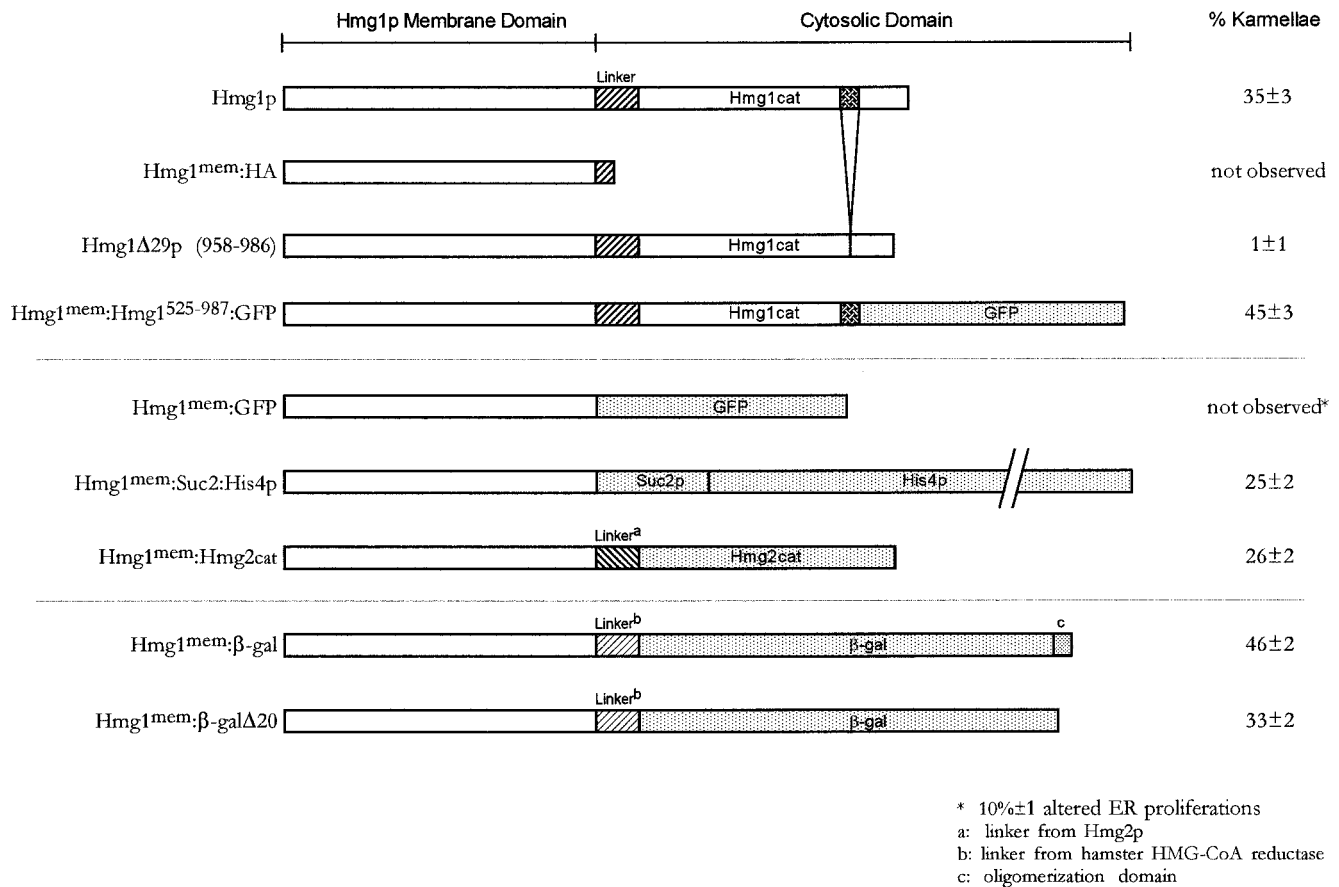


Figure 1. Summary of karmellae levels in cells expressing intact Hmg1p or the different Hmg1 fusion proteins. The fusion proteins used in this study are diagrammed together with the percentage of karmellae observed for the strains expressing each protein. These karmellae percentages were determined by scoring cells from three independent experiments. At least 400 cells were scored per experiment.

amount of Hmg1p would result in proteins that are unable to induce karmellae assembly. We determined that the steady-state levels of all of the fusion proteins were 70–94% of wild type, verifying that adequate protein was expressed to induce karmellae assembly in each case (Figure 3 and Table 2).

An alternate explanation for the inability of certain *HMG1* constructs to generate karmellae is that Hmg1 fusion proteins that cannot induce karmellae may have decreased stability. To investigate this possibility, we compared levels of Hmg1p that remain after addition of CHX. Because CHX inhibits new protein synthesis, the amount of protein remaining at various times after treatment can be used to assess the degradation rate of the protein in question (Hampton and Rine, 1994). As expected (Hampton and Rine, 1994), wild-type Hmg1p was stable, with little degradation observed after 4 h of CHX treatment (Figure 4A). The truncated Hmg1p that lacked a cytosolic domain (Hmg1:HA) (Figure 4B) and the Hmg1Δ29p (Figure 4D) were also stable after 4 h of CHX treatment. In addition, both the Hmg1^{mem}:GFP protein (Figure 4C) and the Hmg1^{mem}:Hmg1⁵²⁵⁻⁹⁸⁷:GFP protein (Figure 4E) were stable after 4 h in CHX. In contrast to Hmg1p, intact Hmg2p is quite unstable, with a

half-life of 60 min (Hampton and Rine, 1994). Unlike wild-type Hmg2p, the Hmg1^{mem}:Hmg2^{cat} protein was stable after 4 h of CHX treatment (Figure 4E), confirming that the membrane domain of the protein determines its half-life (Gil *et al.*, 1985; Skalnik *et al.*, 1988; Hampton and Rine, 1994). In Figure 4E, the percent decrease in protein after 4 h was plotted for the different Hmg1 fusions, showing that the fusion proteins had similar stabilities. These results eliminated the possibility that the fusions were unable to signal for karmellae because of inadequate steady-state protein levels or instability of the Hmg1p variants.

Alteration of the Cytosolic Domain of Hmg1p Did Not Cause Mislocalization of the Protein to the Peripheral ER

The ability of a protein to induce karmellae might depend on its specific localization within the cell (Koning *et al.*, 1996). Because karmellae arise from the nuclear envelope, a protein capable of signaling for karmellae assembly would be expected to be localized in the nuclear envelope. Consistent with this hypothesis, increased levels of wild-type Hmg1p are present predominantly in the nuclear envelope; in con-

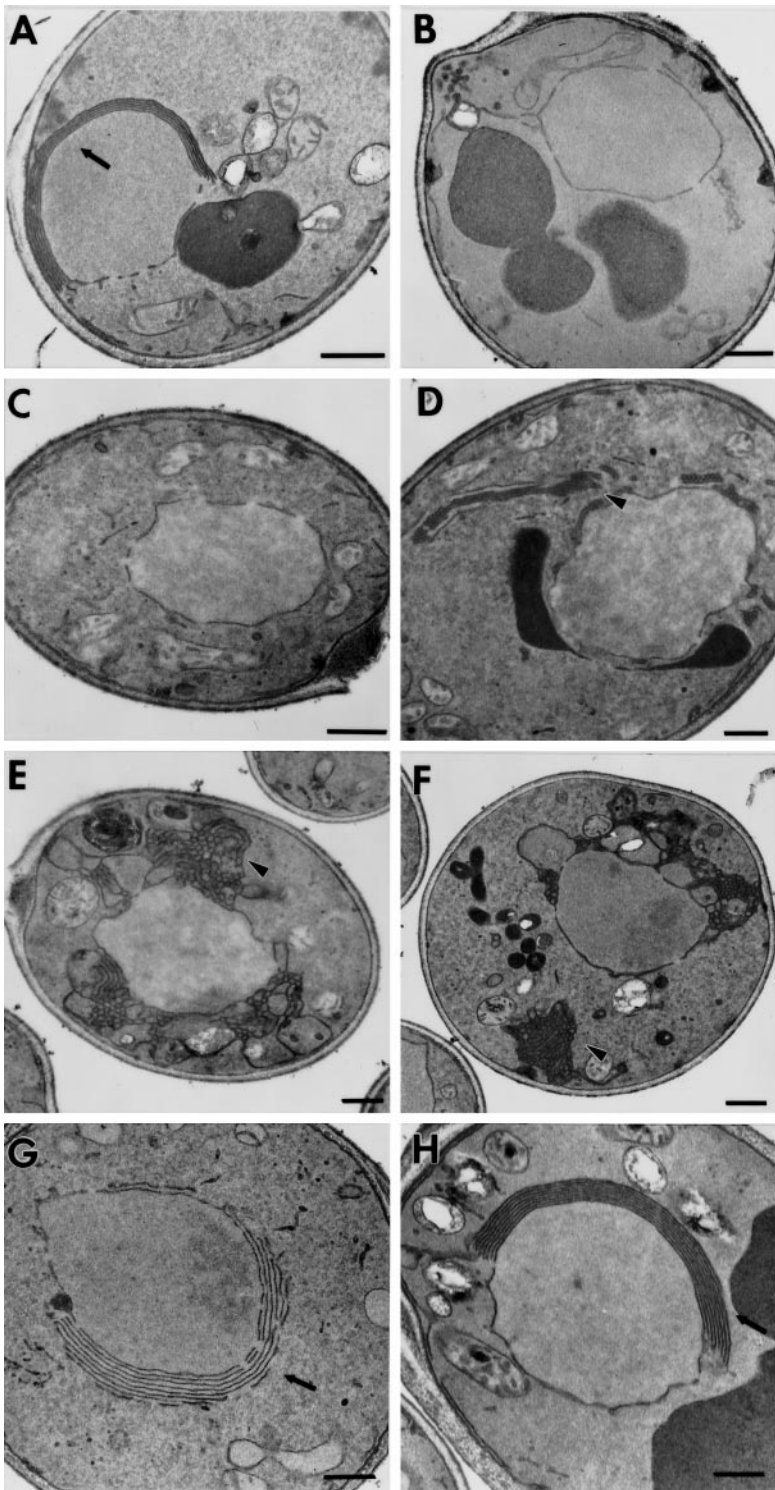


Figure 2. The ultrastructure of the nuclear envelope in a wild-type strain and in strains expressing the Hmg1 fusion proteins was compared. (A) A wild-type strain expressing intact Hmg1p (RWY 410) contains karmellae membranes in an ordered array on the nucleus. (B) Cells expressing the truncated Hmg1:HA protein (RWY 614) lack any ER membrane proliferations. (C and D) Cells expressing Hmg1 Δ 29p (RWY 406) usually lack karmellae and sometimes produce extended ER networks. (E and F) Cells expressing the Hmg1^{mem}:GFP (RWY 626) do not generate karmellae but have altered ER arrays. (G) Cells expressing the Hmg1^{mem}:Hmg1⁵²⁵⁻⁹⁸⁷:GFP (RWY 621) generate karmellae. (H) Cells expressing the Hmg1^{mem}:Hmg2^{cat} (RWY 572) generate karmellae predominantly. Occasionally, Hmg2-type membranes are found in the population of cells expressing the Hmg1^{mem}:Hmg2^{cat}. Arrows point to karmellae; arrowheads point to proliferated ER. Bars, 0.5 μ m.

trast, increased levels of Hmg2p localize predominantly to the peripheral ER (Koning *et al.*, 1996).

The subcellular localization of the fusion proteins was examined by immunofluorescence to assess whether alter-

ations in the cytosolic domain of Hmg1p influenced the distribution of the protein within the ER. As expected, intact Hmg1p was present predominantly in the nuclear envelope, with increased fluorescence found in cells generating kar-

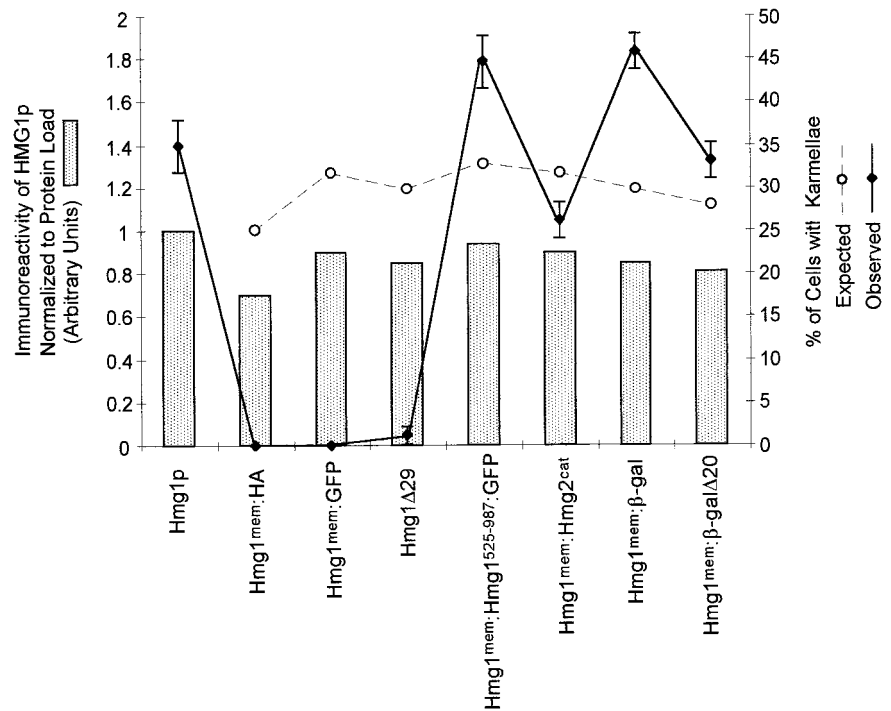


Figure 3. Comparison of relative protein levels and ability to induce karmellae for the Hmg1 fusion proteins. The graph compares the relative protein levels of the Hmg1 fusion proteins with the wild-type Hmg1p level. The wild-type Hmg1p steady-state level was set to 1.0. For each strain, the percentage of cells in the population with karmellae was observed using DiOC₆, which stains the ER. The expected percentage of cells with karmellae was determined by adjusting the observed percentage of karmellae in the wild-type Hmg1p strain for the relative HMG-CoA reductase protein level in each subsequent strain.

mellae (Figure 5, A and B). The localization of the luminal ER protein Kar2p demonstrates the pattern for proteins found throughout the ER (Rose *et al.*, 1989; Preuss *et al.*, 1991). In Figure 5, C and D, Kar2p staining appeared in the nuclear envelope and in a network stretching to the cell periphery. The Hmg1Δ29p had a localization pattern indistinguishable from wild-type Hmg1p (Figure 5, E and F), indicating that the inability of this protein to generate karmellae was not due to an altered distribution within the ER. The localization pattern for Kar2p in the strain expressing Hmg1Δ29p was unaltered from wild-type Kar2p localization (Figure 5, G and H).

In Figure 6, the truncated Hmg1p lacking a catalytic domain (Hmg1:HA) was localized using a monoclonal antibody (12CA5) to the HA epitope. The protein was detected in the nuclear envelope and in the ER network extending throughout

the cytosol (Figure 6, A and B). Thus, the truncated Hmg1:HA protein appeared to be localized throughout the ER rather than displaying a predominantly nuclear envelope pattern. The Kar2p localization pattern is shown for comparison in Figure 6, C and D. To confirm the specificity of the HA antibody, immunofluorescence was performed on a yeast strain that was not expressing an HA-tagged protein. No immunofluorescence pattern was observed in these cells, but the entire cell was faintly visible (our unpublished data).

Next, we investigated the localization patterns of the two Hmg1:GFP fusions. The Hmg1^{mem}:Hmg1⁵²⁵⁻⁹⁸⁷:GFP protein, which generates karmellae, was localized in the nuclear envelope (Figure 7, E and F; Koning *et al.*, 1996) similar to wild-type Hmg1p. The Hmg1^{mem}:GFP fusion, which failed to generate karmellae, had a nuclear envelope localization pattern with some peripheral ER staining (Figure 7, A and B), again appearing similar to that of wild-type Hmg1p.

Taken together, the localization patterns demonstrated that the failure to assemble karmellae in response to the Hmg1 fusions was not due to a large proportion of the protein being mislocalized out of the ER or mislocalized exclusively to the peripheral ER. However, from our data we cannot rule out the possibility that subtle alterations in the localization of the protein, such as that observed for Hmg1p:HA, might influence the ability of the protein to induce karmellae.

Competition with the Soluble Catalytic Domain Reduced the Overall Level of Karmellae Generated by the Wild-Type Hmg1p

Our results suggested that the membrane domain and the cytosolic domain of Hmg1p cooperate in some way to generate karmellae. To test this idea, we examined karmellae

Table 2. The inability to generate karmellae did not correlate with relative amount of HMG-CoA reductase protein expressed

Protein expressed	Relative protein amount	Karmellae observed (%)	Karmellae expected (%)
wild-type Hmg1p	1.00	35	35
Hmg1 ^{mem} :HA	0.70	0	25
Hmg1 ^{mem} :GFP	0.90	0	32
Hmg1Δ29p	0.85	1	30
Hmg1 ^{mem} :Hmg1 ⁵²⁵⁻⁹⁸⁷ :GFP	0.94	45	33
Hmg1 ^{mem} :Hmg2 ^{cat}	0.90	26	32
Hmg1 ^{mem} :β-galactosidase	0.85	46	30
Hmg1 ^{mem} :β-galΔ20	0.81	33	28

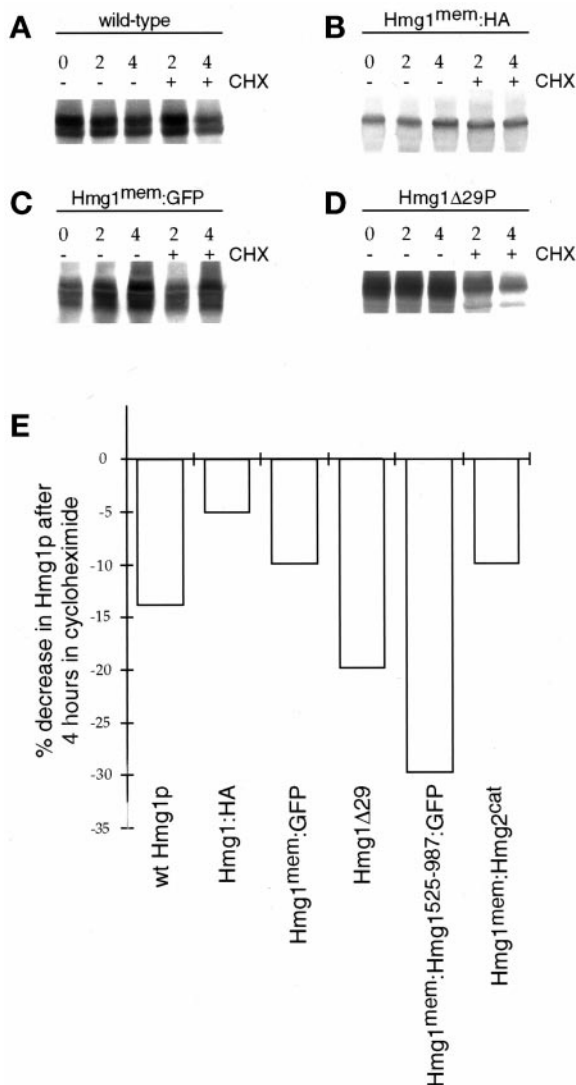


Figure 4. Comparison of Hmg1p levels in wild-type cells and cells expressing the Hmg1 fusion proteins. Total cellular membranes were isolated and prepared for immunoblotting. At time zero, the culture was split into two flasks with one flask receiving CHX. At subsequent times (2 and 4 h), aliquots were removed for membrane isolation. (A) Intact Hmg1p (RWY 410) was detected using an antisera against the Hmg1p catalytic domain. (B) The truncated Hmg1^{mem}:HA protein (RWY 614) was detected using the 12CA5 anti-HA antibody. (C) The Hmg1^{mem}:GFP fusion (RWY 626) was detected using anti-GFP antisera. (D) Hmg1Δ29p (RWY 406) was detected using an antisera against the Hmg1p catalytic domain. (E) The graph compared the fusion protein levels, normalized to total protein and to designation of the zero time point level for each protein set to 1.0. The relative levels of protein were determined for all time points, and the percent decrease was calculated by comparing the 0-h protein level with the 4-h with-CHX protein level.

assembly in a strain expressing both full-length Hmg1p and a truncated protein containing only the linker and the catalytic domain of Hmg1p. Because HMG-CoA reductase is thought to act as a dimer using contact sites in the catalytic

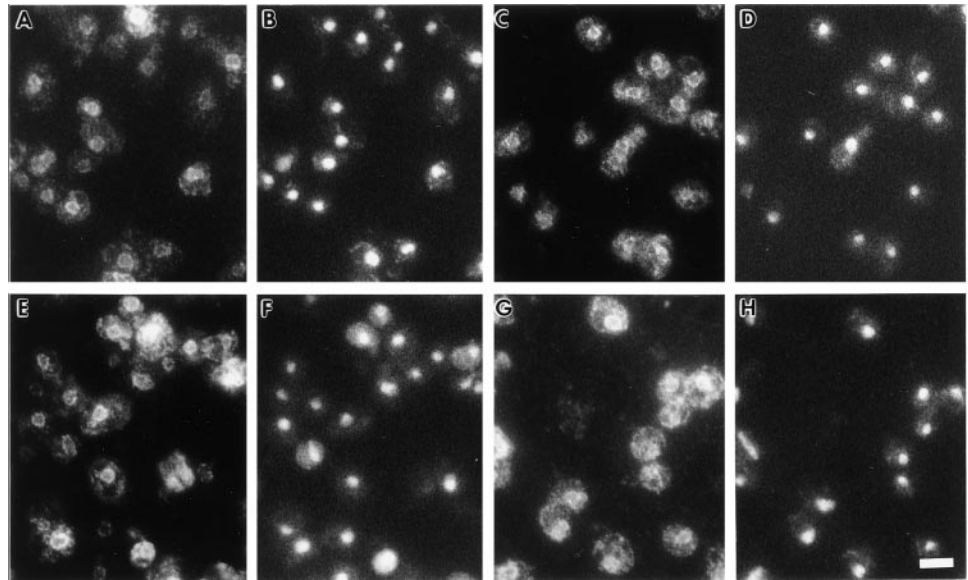
domain (Edwards *et al.*, 1985; Basson *et al.*, 1987; Frimpong and Rodwell, 1994; Lawrence *et al.*, 1995; Rogers *et al.*, 1997; Taberero *et al.*, 1999), we hypothesized that expression of a soluble catalytic domain would interfere with the ability of the holoprotein to dimerize and to induce karmellae. It is important to note that the catalytic domain is capable of proper folding and oligomerization in the absence of attachment to the membrane domain, because it is enzymatically active, and the amount of activity parallels the protein amount (Donald *et al.*, 1997). As expected, elevated expression of the soluble catalytic domain alone was incapable of inducing karmellae (Table 3). In the strain expressing both the holoprotein and the soluble catalytic domain, the soluble Hmg1p catalytic domain was expressed at threefold higher levels than the holoprotein (Table 3). The Hmg1p holoprotein was expressed at levels similar to strains that are not simultaneously expressing the catalytic domain. Consequently, by the law of mass action, and assuming that the proteins dimerized via carboxyl-terminal sequences, we expected that 86% of the holoprotein would be complexed with a soluble catalytic domain and that 14% of the holoprotein would form holoprotein-holoprotein dimers. Thus, if dimerization between holoproteins were important for karmellae assembly, a dramatic drop in the level of karmellae should be seen. Specifically, we would predict that the proportion of cells containing karmellae should decrease by 85% (from 41 to <6%). Unexpectedly, karmellae assembly was decreased by only 33% in the strain expressing both the holoprotein and the soluble catalytic domain (Table 3). This result suggested that carboxyl terminus-mediated dimerization between holoproteins may not be essential for karmellae assembly.

Oligomerization of the Cytosolic Domain of Hmg1p Was Not Required for Karmellae Formation

To further test the hypothesis that oligomerization via the cytosolic domain was not required for karmellae assembly, we fused the Hmg1p membrane domain to β -gal or to a truncated β -gal missing the last 20 amino acids (β -galΔ20). Full-length β -gal forms tetramers, which are the enzymatically active form of the enzyme; dimers and monomers are not catalytically active (Fowler and Zabin, 1983). Truncated β -gal missing the last 10 amino acids is found as a monomer (Fowler and Zabin, 1966, 1968). In addition, β -gal missing the last 16 amino acids lacks enzyme activity and runs on a nondenaturing gel as a monomer (Tsuneoka and Mekada, 1992). Although we did not directly assess the oligomerization state of either β -gal fusion, the lack of β -gal activity (Table 4) exhibited by Hmg1: β -galΔ20 was consistent with this carboxyl terminus being monomeric. Additionally, because Hmg1: β -gal was enzymatically active (Table 4), the carboxyl termini of separate fusion proteins were capable of forming tetramers. If oligomerization of the cytosolic region on Hmg1p is critical for karmellae, then the Hmg1: β -galΔ20 will fail to induce karmellae.

We performed immunoblots to measure expression of the β -gal fusion proteins and to confirm the stability of the proteins. Hmg1: β -gal was present at 85% and Hmg1: β -galΔ20 was present at 81% of the wild-type steady-state Hmg1p level, and both fusions were stable during the 4-h treatment with CHX (Figure 8). Both Hmg1: β -gal and Hmg1: β -gal Δ20 generated karmellae (Figure 9) and were

Figure 5. Localization of elevated levels of wild-type Hmg1p and the Hmg1 fusion proteins with altered cytosolic domains by indirect immunofluorescence. Indirect immunofluorescent localization of Hmg1p or Kar2p is shown in A, C, E, and G. Localization of the nucleus in the same cells is shown by DAPI staining in B, D, F, and H. (A and B) Localization of elevated levels of wild-type Hmg1p was determined in cells of strain RWY 410 using an antibody to the catalytic domain of Hmg1p. (C and D) Localization of Kar2p in strain RWY 410 is shown for comparison. (E and F) Localization of elevated levels of Hmg1 Δ 29p in strain RWY 406 was determined by using an antibody to the catalytic domain of Hmg1p. (G and H) Localization of Kar2p in strain RWY 406 is shown for comparison. Bar, 5 μ m.



localized predominantly to the nuclear envelope of the ER (Figure 10). Because the Hmg1: β -gal Δ 20 induced karmellae, oligomerization of the cytosolic domain did not appear to be required for karmellae signaling. However, cells expressing the Hmg1: β -gal generated a higher level of karmellae compared with the cells expressing the Hmg1: β -gal Δ 20 (Table 4).

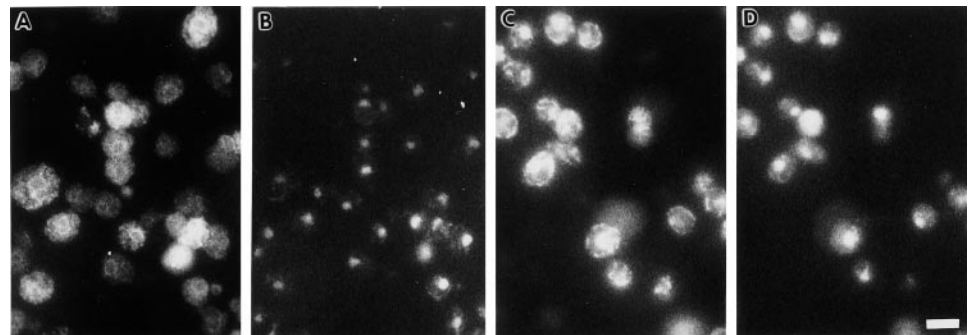
DISCUSSION

To explore how cells regulate the synthesis and organization of new membrane arrays, we sought to define the features of Hmg1p that are necessary for this protein to act as a signal for karmellae biogenesis. In eukaryotes, the carboxyl terminus of HMG-CoA reductase contains the cytosolic catalytic domain, and the amino terminus contains the membrane domain (Liscum *et al.*, 1985). Although details concerning the structure and function of the HMG-CoA reductase catalytic domain are beginning to be solved (Darnay *et al.*, 1992; Lawrence *et al.*, 1995; Rogers *et al.*, 1997; Taberner *et al.*, 1999), the HMG-CoA reductase membrane domain remains much more cryptic. In plants, the membrane domain contains two transmembrane helices (Denbow *et al.*, 1996; Re *et al.*, 1997), whereas in animals and fungi, it contains of a

series of seven or eight transmembrane helices (Liscum *et al.*, 1985; Basson *et al.*, 1988; Sengstag *et al.*, 1990; Olender and Simon, 1992; Roitelman *et al.*, 1992). Although there is little obvious primary sequence conservation, the structural conservation of this complex membrane domain over the 1 billion years of evolution since divergence of fungi and animals (Doolittle *et al.*, 1996; Lum *et al.*, 1996; Feng *et al.*, 1997) suggests that the structure has an important functional role. However, the molecular logic of tethering the catalytic activity to a membrane appears enigmatic, because prokaryotic HMG-CoA reductase proteins are soluble (Beach and Rodwell, 1989; Bischoff and Rodwell, 1996; Baltscheffsky *et al.*, 1997; Bochar *et al.*, 1997; Takahashi *et al.*, 1999), and the catalytic domain of eukaryotic HMG-CoA reductase can support apparently normal growth of cells when it is freed from the membrane domain (Gil *et al.*, 1985; Donald *et al.*, 1997).

Although the molecular details are not yet understood, two functions can be assigned to the HMG-CoA reductase membrane domain. First, it mediates regulation of protein half-life in response to sterol levels (Skalnik *et al.*, 1988; Chun and Simoni, 1992; Hampton and Rine, 1994; Kumagai *et al.*, 1995; Sekler and Simoni, 1995). Second, it is required for

Figure 6. Localization of the truncated Hmg1:HA protein by indirect immunofluorescence. Indirect immunofluorescent localization of the truncated Hmg1:HA protein or Kar2p is shown in A and C. Localization of the nucleus in the same cells is shown by DAPI staining in B and D. (A and B) Localization of elevated levels of the truncated Hmg1:HA protein in strain RWY 614 was detected by using 12CA5 anti-HA antibody. (C and D) Localization of Kar2p in strain RWY 614 is shown for comparison. Bar, 5 μ m.



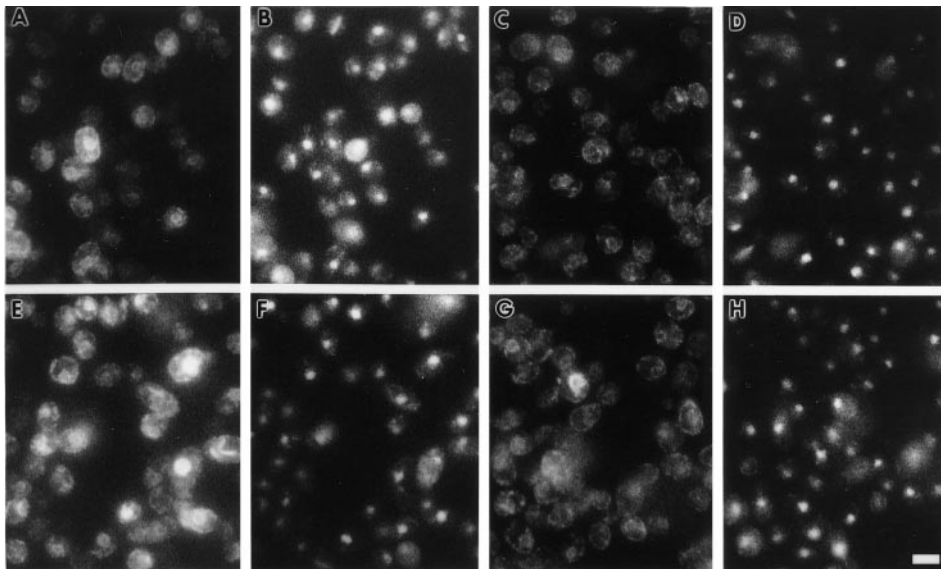


Figure 7. Localization of the Hmg1^{mem}:GFP fusion and the Hmg1^{mem}:Hmg1⁵²⁵⁻⁹⁸⁷:GFP fusion by indirect immunofluorescence. Indirect immunofluorescent localization of the GFP fusion proteins or Kar2p in shown in A, C, E, and G. Localization of the nucleus in the same cells is shown by DAPI staining in B, D, F, and H. (A and B) Localization of elevated levels of the Hmg1^{mem}:GFP protein in strain RWY 626 was determined by using a GFP antibody. (C and D) Localization of Kar2p in strain RWY 626 is shown for comparison. (E and F) Localization of elevated levels of the Hmg1^{mem}:Hmg1⁵²⁵⁻⁹⁸⁷:GFP protein was determined in cells of strain RWY 621 using a GFP antibody. (G and H) Localization of Kar2p in strain RWY 621 is shown for comparison. Bar, 5 μ m.

stimulation of ER expansion in response to increased HMG-CoA reductase levels (Chin *et al.*, 1982; Jingami *et al.*, 1987; Wright *et al.*, 1988; Parrish *et al.*, 1995). For both functions, regions of the membrane domain, including transmembrane segments and the loops that connect them, have been identified as playing particularly critical roles (Gil *et al.*, 1985; Pathak *et al.*, 1986; Jingami *et al.*, 1987; Hampton and Rine, 1994; Kumagai *et al.*, 1995; Parrish *et al.*, 1995; Sekler and Simoni, 1995; Koning *et al.*, 1996). For example, at least some aspects of protein half-life control may function via a sterol-sensing domain located within the region of the membrane domain extending from transmembrane segments 2–6 (Lange and Steck, 1998; Osborne and Rosenfeld, 1998; Cheng *et al.*, 1999). In yeast, the amino-terminal region before transmembrane segment 1 is important for regulated degradation (Gardner *et al.*, 1998), and the final ER-luminal loop between predicted seventh and eighth transmembrane domains (loop G) carries information required for the assembly of karmellae (Parrish *et al.*, 1995). We hypothesize that loop G mediates karmellae assembly via interactions with other proteins, perhaps other HMG-CoA reductase molecules. Consequently, an obvious prediction is that alterations of HMG-CoA reductase that prevent proper folding or tertiary struc-

ture of this ER-luminal loop would interfere with necessary protein-protein interactions, resulting in failure to induce karmellae assembly. This prediction is supported by the identification of point mutations in loop G that can nearly abolish the protein's ability to induce karmellae without affecting the protein's stability or steady-state levels (Profant and Wright, unpublished observations).

Studies of chimeric Hmg-CoA reductase proteins in which the carboxyl terminus was replaced with unrelated sequences suggested that the ability of this protein to induce membrane proliferation resided solely with the HMG-CoA reductase membrane domain (Skalnik *et al.*, 1988; Parrish *et al.*, 1995). Thus, our initial observation that removal of the carboxyl terminus prevented karmellae assembly was surprising and suggested that sequences present at the carboxyl terminus were important for membrane biogenesis. Pursuing this initial observation, we found that the inability of certain Hmg1p carboxyl-terminal mutants to induce karmellae was not due to mislocalization of the protein, an insufficient level of protein, an unstable protein, or the inability of the protein to oligomerize via its carboxyl terminus. How, then, does the carboxyl terminus exercise its effects on the ability of Hmg1p to induce karmellae? Our working hypoth-

Table 3. Expression of the soluble catalytic domain interfered with the production of karmellae without lowering the level of Hmg1p holoprotein

Strain	Protein expressed		Karmellae (%) ^a	Relative holoprotein level ^b	Relative catalytic domain level ^b
	pGAL:Hmg1p	pGAPDH:Hmg1p			
RWY1494	X		41 \pm 1	1.00	
RWY900		X	Never seen		3.60
RWY1497	X	X	27 \pm 1	1.05	3.30

^a The percent karmellae was determined from three independent experiments.

^b After adjusting for total protein loaded, the amount of holoprotein or catalytic domain was normalized to holoprotein in RWY1494.

Table 4. Karmellae biogenesis in response to β -galactosidase fusion proteins

Protein	Karmellae (%) ^a		β -gal activity ^b (U/OD/min)
	4 h	8 h	
Hmg1: β -galactosidase	46 \pm 2	56 \pm 2	236 \pm 12
Hmg1: β -gal Δ 20	33 \pm 2	37 \pm 2	0.7 \pm 0.4

^a The percent karmellae was determined from three independent experiments.

^b β -Galactosidase activity was determined from three independent experiments, running duplicate tubes for each experiment.

esis is that the conformation of the carboxyl terminus of HMG-CoA reductase affects the folding of the membrane domain, particularly loop G, in certain cases rendering it unable to achieve the conformation needed to stimulate karmellae assembly.

This hypothesis reflects the well-established observation that membrane protein function typically involves conformational changes. For example, conformational changes are required for the function of ion channels and pumps, as well as receptors for extracellular matrix proteins, hormones, neurotransmitters, light, and odorant molecules. In many cases the necessary conformational change is at least initiated by subtle alterations in one or more transmembrane-spanning regions of the protein. For example, fluorescence spectroscopy reveals that agonist binding to the β_2 adreno-receptor causes conformational changes in two of the seven transmembrane helices that comprise the membrane domain (Gether *et al.*, 1997). These changes are then believed to alter cytoplasmic regions of the protein, allowing interactions with the associated G-protein α subunit necessary for signal transduction. Another potentially relevant example is the "inside-out" signaling of integrins (Ginsberg *et al.*, 1992; Dedhar, 1999). In the absence of thrombin, $\alpha_{IIb}\beta_3$ integrin of platelets is inactive and has poor affinity for fibrinogen. Upon activation of platelets by exposure to thrombin or ADP, the $\alpha_{IIb}\beta_3$ integrin takes on an altered conformation that greatly increases its affinity for fibrinogen. This inside-out signaling is believed to involve changes in the $\alpha_{IIb}\beta_3$ integrin cytoplasmic domain that are transmitted to the extracellular domain, exposing previously hidden fibrino-

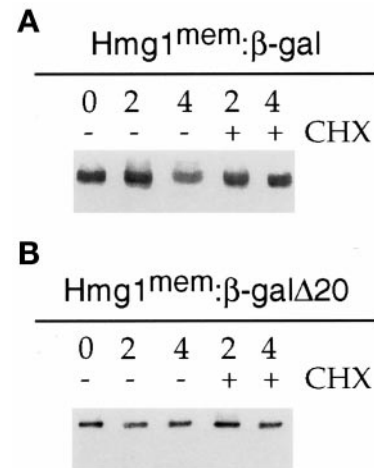


Figure 8. Comparison of the stability of the Hmg1: β -gal fusion proteins by CHX chase. Total cellular membranes were isolated and prepared for immunoblotting. At time zero, the culture was split into two flasks with one flask receiving CHX. At subsequent times (2 and 4 h), aliquots were removed for membrane isolation. (A) The Hmg1: β -gal protein expressed in RWY 943 was detected using an antisera generated against the membrane domain of Hmg1p. (B) The Hmg1: β -gal Δ 20 protein level in RWY 944 was detected using the same antisera against the Hmg1p membrane domain.

gen binding sites (O'Toole *et al.*, 1994; Hughes *et al.*, 1995; Leisner *et al.*, 1999). Deletions or point mutations within the cytoplasmic domain can constitutively activate $\alpha_{IIb}\beta_3$ integrin for fibrinogen binding, indicating that the intracellular and extracellular domains are "conformationally and functionally coupled" (Leisner *et al.*, 1999). Thus, the mechanics of transmembrane signaling illustrate the ability of subtle changes in protein conformation on one side of a membrane to be transmitted across a membrane bilayer, ultimately changing the protein's function or interactions.

We envision a similar effect of different carboxyl terminal sequences on the folding of the Hmg1p membrane domain. One possibility is that carboxyl terminal sequences that are incompatible with karmellae assembly affect the packing of the transmembrane helices so that loop G cannot properly fold. Consistent with this notion, we have been unsuccessful in using the yeast two-hybrid system to identify proteins

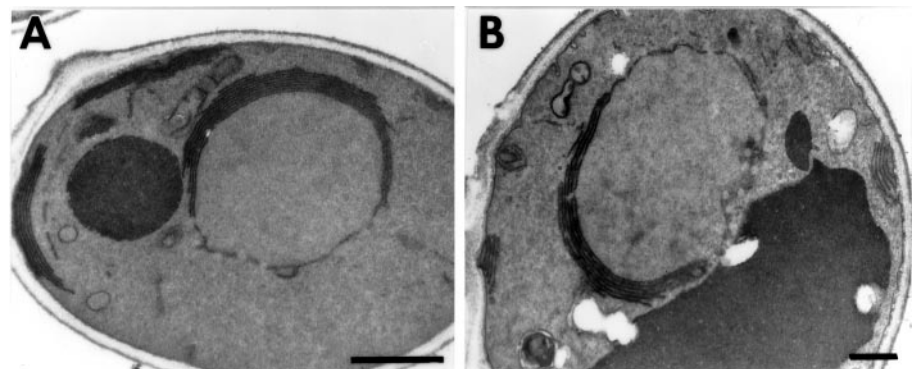


Figure 9. Comparison of the karmellae induced by either Hmg1: β -gal or Hmg1: β -gal Δ 20. Cells from both strains were fixed for electron microscopy. (A) RWY 943 cells expressing Hmg1: β -gal generated karmellae. (B) RWY 944 cells expressing the Hmg1: β -gal Δ 20 also generated karmellae. Bars, 0.5 μ m.

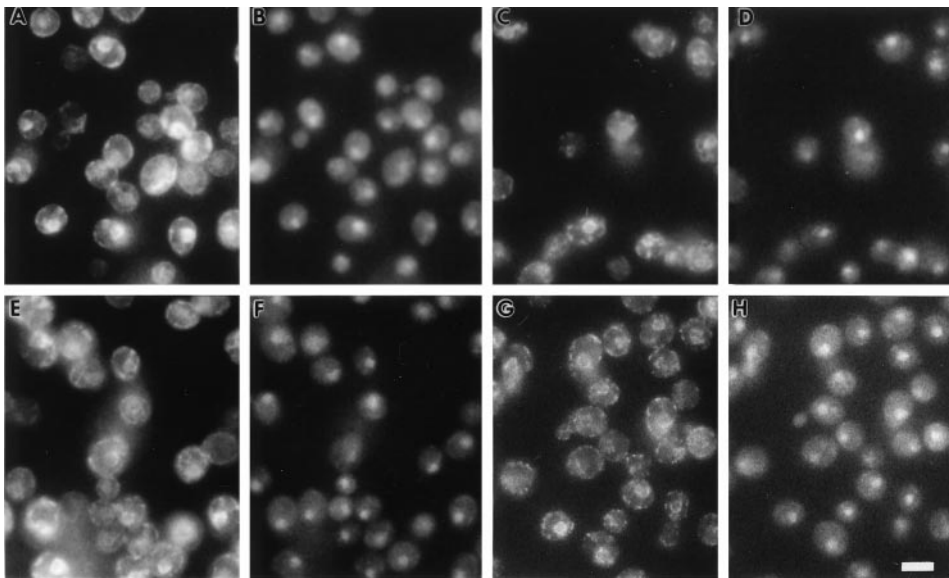


Figure 10. Indirect immunofluorescent localization of the Hmg1:β-gal fusions or Kar2p is shown in A, C, E, and G. Localization of the nucleus in the same cells is shown by DAPI staining in B, D, F, and H. (A and B) Localization of elevated levels of Hmg1:β-gal was determined in strain RWY 943 by using anti-β-gal antibody. (C and D) Localization of Kar2p in strain RWY 943 is shown for comparison. (E and F) Localization of elevated levels of Hmg1:β-gal 20 was determined in strain RWY 944 by using the same anti-β-gal antibody. (G and H) Localization of Kar2p in strain RWY 944 is shown for comparison. Bar, 5 μm.

that interact with loop G. For this analysis, it was necessary to eliminate the transmembrane sequences from the “bait” construct, so that the protein would be soluble and able to enter the nucleus to activate reporter genes. Consequently, the bait consisted solely of loop G sequences, flanked by several hydrophobic residues. Extensive searches using this construct did not reveal interacting proteins. Although the failure of the two-hybrid approach may indicate the absence of proteins that interact with loop G, it is also consistent with the idea that proper folding of loop G may be very sensitive to the status of adjacent transmembrane domains.

The ability of changes in one protein domain to affect transmembrane helix packing is well illustrated by structural studies of receptor tyrosine kinases such as the erythropoietin receptor. Upon ligand binding, the extracellular domains of the receptor homodimer undergo a dramatic shift in conformation that brings transmembrane helices more closely together, allowing activation of marker enzymes that replace the cytoplasmic kinase domains (Remy *et al.*, 1999). The packing of or interactions between the Hmg1p transmembrane helices may have similar sensitivity to the folding status of its carboxyl terminus.

Dimerization of the catalytic domains is critical for HMG-CoA reductase activity, because the enzyme active site is formed by interactions between two monomers (Lawrence *et al.*, 1995; Rogers *et al.*, 1997; Taberner *et al.*, 1999). Recent results also support the importance of oligomerization for regulated degradation of HMG-CoA reductase, such that monomeric fusion proteins are degraded rapidly relative to fusion proteins capable of oligomerizing via their carboxyl termini (Cheng *et al.*, 1999). Cheng and coworkers (1999) did not analyze the ability of their fusion proteins to induce crystalloid ER. However, studies of microsomal aldehyde dehydrogenase, which induces crystalloid ER similar to that induced by HMG-CoA reductase, also indicate the importance of interactions via the carboxyl termini for induction of membrane assembly (Masaki *et al.*, 1994, 1996; Gong *et al.*, 1996; Yamamoto *et al.*, 1996). Cheng *et al.* (1999) propose that dimerization via the HMG-CoA reductase catalytic domains

serves to promote or to stabilize interactions between the attached membrane domains. However, to account for the unequivocal importance of the membrane domain in regulated degradation, they propose that the key focus for regulated degradation is the dimerization status of the protein’s membrane domain. Thus, they propose that carboxyl-terminal fusions capable of oligomerization stabilize the protein by bringing the membrane domains close to one another, facilitating interactions between membrane domains that are critical for regulated degradation.

Although our results do not support an absolute requirement for dimerization via catalytic domains for karmellae assembly, they are consistent with the model proposed by Cheng *et al.* (1999). Thus, interactions via the carboxyl termini may increase the efficiency of membrane domain interactions, thereby increasing the efficiency of karmellae signaling. However, unlike the mammalian protein, the Hmg1p membrane domain may have sufficient affinity for one another to allow efficient interactions in the absence of cytosolic domain oligomerization, provided that the membrane domains can properly fold. If so, only the carboxyl terminus fusions that resulted in altered membrane domain folding would prevent karmellae assembly by preventing the necessary interactions between membrane domains. Our data predict that these proteins include the construct lacking a carboxyl-terminal domain (Hmg1^{mem}:HA), as well as the protein with a truncated catalytic domain (Hmg1^{mem}Δ29p), or in which the carboxyl terminus is replaced by GFP (Hmg1^{mem}:GFP).

It is interesting to note that the Hmg1^{mem}:GFP fusion could not induce karmellae, although the Hmg1^{mem}:β-galΔ20 was able to do so. Both of these fusion proteins are predicted to be unable to dimerize via their carboxyl termini, yet one induced karmellae and the other did not. Although this result supports the conclusion that oligomerization via the carboxyl terminus is not essential for karmellae assembly, it raises questions about how the proteins differ. A possible explanation for the different abilities of Hmg1^{mem}:GFP and Hmg1^{mem}:β-galΔ20 to induce karmellae is the ab-

sence of a linker sequence in Hmg1^{mem}:GFP. In this protein, the cytosolic domain (GFP) immediately follows the membrane domain, whereas the cytosolic domain of Hmg1^{mem}: β -gal Δ 20 is separated from the membrane by a 143-amino-acid linker sequence. Thus, sufficient separation of the membrane and catalytic domains may be important for allowing each domain to achieve its proper conformation. Consistent with this idea, a fusion protein in which the GFP domain was widely separated from the catalytic domain (Hmg1^{mem}:Hmg1⁵⁹⁵⁻⁹⁸⁷:GFP) was capable of generating karmellae. Separation of the membrane and catalytic domain is not sufficient, however, because Hmg1^{mem} Δ 29p has a linker sequence but is unable to induce karmellae assembly.

Recently, another view that emphasizes protein quality has emerged to explain different types of ER proliferations that occur in response to mutant and wild-type cytochrome p450. When expressed in *Saccharomyces cerevisiae*, a wild-type cytochrome P450 of *Candida maltosa* generates tubular stacks of ER throughout the cytoplasm that appear distinct from karmellae (Zimmer *et al.*, 1995). In contrast, mutant forms of *Candida maltosa* cytochrome P450 expressed in *S. cerevisiae* lead to the proliferation of karmellae-like structures (Zimmer *et al.*, 1995). Because the mutant cytochrome P450 forms have lower protein stability than the wild-type P450 enzyme, these authors favor a model in which a quality control system sorts the mutant P450 forms into a stacked ER subcompartment for degradation rather than the subcompartment responsible for tubular expansion (Zimmer *et al.*, 1995). In our study, the Hmg1p variants that failed to induce karmellae did not have lower protein stability than the wild-type Hmg1p, making it unlikely that karmellae are assembled as degradative compartments to remove an unstable protein.

Our ultimate goal is to understand the molecular events that underlie karmellae assembly as a model for how cells modulate membrane biogenesis in response to changing physiological demands. Characterizing the features of Hmg1p that are critical for induction of karmellae is an important step in achieving that goal. Although the molecular details remain to be discovered, our results suggest that proper folding of the Hmg1p membrane domain is a critical first step in the karmellae signaling process and indicate that this folding may be influenced by sequences at the carboxyl terminus. Our results also rule out models that suggest that oligomerization of a membrane protein is sufficient to induce alterations in membrane assembly (Gong *et al.*, 1996). Consequently, identifying *trans*-acting factors that respond to the Hmg1p membrane domain will be essential for learning how this protein stimulates the dramatic changes in membrane biogenesis and organization that accompany karmellae assembly.

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REFERENCES

- Anderson, R.G., Orci, L., Brown, M.S., Garcia-Segura, L.M., and Goldstein, J.L. (1983). Ultrastructural analysis of crystalloid endoplasmic reticulum in UT-1 cells and its disappearance in response to cholesterol. *J. Cell Sci.* 63, 1–20.
- Baltscheffsky, M., Brosche, M., Hultman, T., Lundvik, L., Nyren, P., Sakai-Nore, Y., Severin, A., and Strid, A. (1997). A 3-hydroxy-3-methylglutaryl-CoA lyase gene in the photosynthetic bacterium *Rhodospirillum rubrum*. *Biochim. Biophys. Acta.* 1337, 113–122.
- Basson, M.E., Moore, R.L., O'Rear, J., and Rine, J. (1987). Identifying mutations in duplicated functions in *Saccharomyces cerevisiae*: recessive mutations in HMG-CoA reductase genes. *Genetics* 117, 645–655.
- Basson, M.E., Thorsness, M., Finer-Moore, J., Stroud, R.M., and Rine, J. (1988). Structural and functional comparison between yeast and human 3-hydroxy-3-methylglutaryl CoA reductases, the rate-limiting enzyme of sterol biosynthesis. *Mol. Cell. Biol.* 8, 3797–3808.
- Basson, M.E., Thorsness, M., and Rine, J. (1986). *Saccharomyces cerevisiae* contains two functional genes encoding 3-hydroxy-3-methylglutaryl-CoA reductase. *Proc. Natl. Acad. Sci. USA* 83, 5563–5567.
- Beach, M.J., and Rodwell, V.W. (1989). Cloning, sequencing, and overexpression of *mvaA*, which encodes *Pseudomonas mevalonii* 3-hydroxy-3-methylglutaryl CoA reductase. *J. Bacteriol.* 171, 2994–3001.
- Bischoff, K.M., and Rodwell, V.W. (1996). 3-Hydroxy-3-methylglutaryl-CoA reductase from *Haloferax volcanii*: purification, characterization, and expression in *Escherichia coli*. *J. Bacteriol.* 178, 19–23.
- Bochar, D.A., Brown, J.R., Doolittle, W.F., Klenk, H.P., Lam, W., Schenk, M.E., Stauffacher, C.V., and Rodwell, V.W. (1997). 3-Hydroxy-3-methylglutaryl CoA reductase of *Sulfolobus solfataricus*: DNA sequence, phylogeny, expression in *Escherichia coli* of the *hmgA* gene, and purification and kinetic characterization of the gene product. *J. Bacteriol.* 179, 3632–3638.
- Chalfie, M. (1995). Green fluorescent protein. *Photochem. Photobiol.* 62, 651–656.
- Chalfie, M., Tu, Y., Euskirchen, G., Ward, W.W., and Prasher, D.C. (1994). Green fluorescent protein as a marker for gene expression. *Science* 263, 802–805.
- Cheng, H.H., Xu, L., Kumagai, H., and Simoni, R.D. (1999). Oligomerization state influences the degradation rate of 3-hydroxy-3-methylglutaryl-CoA reductase. *J. Biol. Chem.* 274, 17171–17178.
- Chin, D.J., Luskey, K.L., Anderson, R.G.W., Faust, J.R., Goldstein, J.L., and Brown, M.S. (1982). Appearance of crystalloid endoplasmic reticulum in compactin-resistant Chinese hamster cells with a 500-fold elevation in 3-hydroxy-3-methylglutaryl CoA reductase. *Proc. Natl. Acad. Sci. USA* 79, 1185–1189.
- Chun, K.T., and Simoni, R.D. (1992). The role of the membrane domain in the regulated degradation of 3-hydroxy-3-methylglutaryl CoA reductase. *J. Biol. Chem.* 267, 4236–4246.
- Darnay, B.G., Y. Wang, and V.W. Rodwell. (1992). Identification of the catalytically important histidine of 3-hydroxy-3-methylglutaryl-CoA reductase. *J. Biol. Chem.* 267, 15064–15070.
- Dedhar, S. (1999). Integrins and signal transduction. *Curr. Opin. Hematol.* 6, 37–43.
- Denbow, C.J., Lang, S., and Cramer, C.L. (1996). The N-terminal domain of tomato 3-hydroxy-3-methylglutaryl-CoA reductases. Sequence, microsomal targeting, and glycosylation. *J. Biol. Chem.* 271, 9710–9715.

- Deschenes, R.J., and Broach, J.R. (1987). Fatty acylation is important but not essential for *Saccharomyces cerevisiae* RAS function. *Mol. Cell Biol.* 7, 2344–2351.
- Donald, K.A., Hampton, R.Y., and Fritz, I.B. (1997). Effects of overproduction of the catalytic domain of 3-hydroxy-3-methylglutaryl CoA reductase on squalene synthesis in *Saccharomyces cerevisiae*. *Appl. Environ. Microbiol.* 63, 3341–3344.
- Doolittle, R.F., Feng, D.F., Tsang, S., Cho, G., and Little, E. (1996). Determining divergence times of the major kingdoms of living organisms with a protein clock. *Science* 271, 470–477.
- Edwards, P.A., Kempner, E.S., Lan, S.-F., and Erickson, S.K. (1985). Functional size of rat hepatic 3-hydroxy-3-methylglutaryl CoA reductase as determined by radiation inactivation. *J. Biol. Chem.* 260, 10278–10282.
- Fawcett, D.W. (1981). *The Cell*, Philadelphia: W.B. Saunders, 301–368.
- Feng, D.F., Cho, G., and Doolittle, R.F. (1997). Determining divergence times with a protein clock: update and reevaluation [see comments]. *Proc. Natl. Acad. Sci. USA* 94, 13028–13033.
- Fowler, A.V., and Zabin, I. (1966). Colinearity of beta-galactosidase with its gene by immunological detection of incomplete polypeptide chains. *Science* 154, 1027–1029.
- Fowler, A.V., and Zabin, I. (1968). Beta-galactosidase: immunological studies of nonsense, missense and deletion mutants. *J. Mol. Biol.* 33, 35–47.
- Fowler, A.V., and Zabin, I. (1983). Purification, structure, and properties of hybrid beta-galactosidase proteins. *J. Biol. Chem.* 258, 14354–14358.
- Frimpong, K., and Rodwell, V.W. (1994). The active site of hamster 3-hydroxy-3-methylglutaryl-CoA reductase resides at the subunit interface and incorporates catalytically essential acidic residues from separate polypeptides. *J. Biol. Chem.* 269, 1217–1221.
- Gardner, R., Cronin, S., Leader, B., Rine, J., Hampton, R., and Leder, B. (1998). Sequence determinants for regulated degradation of yeast 3-hydroxy-3-methylglutaryl-CoA reductase, an integral endoplasmic reticulum membrane protein. *Mol Biol Cell* 9, 2611–2626 (erratum [1999]. 10[3]).
- Gether, U., Lin, S., Ghanouni, P., Ballesteros, J.A., Weinstein, H., and Kobilka, B.K. (1997). Agonists induce conformational changes in transmembrane domains III and VI of the beta2 adrenoceptor. *EMBO J.* 16, 6737–6747.
- Gil, G., Faust, J.R., Chin, D.J., Goldstein, J.L., and Brown, M.S. (1985). Membrane-bound domain of HMG CoA reductase is required for sterol-enhanced degradation of the enzyme. *Cell* 41, 249–258.
- Ginsberg, M.H., Du, X., and Plow, E.F. (1992). Inside-out integrin signaling. *Curr. Opin. Cell Biol.* 4, 766–771.
- Gong, F.C., Giddings, T.H., Meehl, J.B., Staehelin, L.A., and Galbraith, D.W. (1996). Z-membranes: artificial organelles for overexpressing recombinant integral membrane proteins. *Proc. Natl. Acad. Sci. USA* 93, 2219–2223.
- Guarente, L., and Ptashne, M. (1981). Fusion of *Escherichia coli* lacZ to the cytochrome *c* gene of *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. USA* 78, 2199–2203.
- Hampton, R.Y., Koning, A., Wright, R., and Rine, J. (1996). In vivo examination of membrane protein localization and degradation with GFP. *Proc. Natl. Acad. Sci. USA* 93, 828–833.
- Hampton, R.Y., and Rine, J. (1994). Regulated degradation of HMG-CoA reductase, an integral membrane protein of the endoplasmic reticulum, in yeast. *J. Cell Biol.* 125, 299–312.
- Hughes, P.E., O'Toole, T.E., Ylanne, J., Shattil, S.J., and Ginsberg, M.H. (1995). The conserved membrane-proximal region of an integrin cytoplasmic domain specifies ligand binding affinity. *J. Biol. Chem.* 270, 12411–12417.
- Jingami, H., Brown, M.S., Goldstein, J.L., Anderson, R.G., and Luskey, K.L. (1987). Partial deletion of membrane-bound domain of 3-hydroxy-3-methylglutaryl CoA reductase eliminates sterol-enhanced degradation and prevents formation of crystalloid endoplasmic reticulum. *J. Cell Biol.* 104, 1693–1704.
- Kochevar, D., and Anderson, R.G.W. (1987). Purified crystalloid endoplasmic reticulum from UT-1 cells contains multiple proteins in addition to 3-hydroxy-3-methylglutaryl CoA reductase. *J. Biol. Chem.* 262, 10321–10326.
- Koning, A.J., Lum, P.Y., Williams, J.M., and Wright, R. (1993). DiOC₆ staining reveals organelle structure and dynamics in living yeast cells. *Cell Motil. Cytoskeleton* 25, 111–128.
- Koning, A.J., Roberts, C.J., and Wright, R.L. (1996). Different subcellular localization of *Saccharomyces cerevisiae* HMG-CoA reductase isozymes at elevated levels corresponds to distinct endoplasmic reticulum membrane proliferations. *Mol. Biol. Cell* 7, 769–789.
- Kumagai, H., Chun, K.T., and Simoni, R.D. (1995). Molecular dissection of the role of the membrane domain in the regulated degradation of 3-hydroxy-3-methylglutaryl CoA reductase. *J. Biol. Chem.* 270, 19107–19113.
- Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680–685.
- Lange, Y., and Steck, T.L. (1998). Four cholesterol-sensing proteins. *Curr. Opin. Struct. Biol.* 8, 435–439.
- Lawrence, C.M., Rodwell, V.W., and Stauffacher, C.V. (1995). Crystal structure of *Pseudomonas mevalonii* HMG-CoA reductase at 3.0 angstrom resolution. *Science* 268, 1758–1762.
- Leisner, T.M., Wencel-Drake, J.D., Wang, W., and Lam, S.C. (1999). Bidirectional transmembrane modulation of integrin alphaIIb beta3 conformations. *J. Biol. Chem.* 274, 12945–12949.
- Liscum, L., Finer-Moore, J., Stroud, R.M., Luskey, K.L., Brown, M.S., and Goldstein, J.L. (1985). Domain structure of 3-hydroxy-3-methylglutaryl CoA reductase, a glycoprotein of the endoplasmic reticulum. *J. Biol. Chem.* 260, 522–530.
- Lum, P.Y., Edwards, S., and Wright, R. (1996). Molecular, functional, and evolutionary characterization of the gene encoding HMG-CoA reductase in the fission yeast, *Schizosaccharomyces pombe*. *Yeast* 12, 1107–1124.
- Lum, P.Y., and Wright, R. (1995). Degradation of HMG CoA reductase-induced membranes in the fission yeast. *Schizosaccharomyces pombe*. *J. Cell Biol.* 131, 81–94.
- Masaki, R., Yamamoto, A., and Tashiro, Y. (1994). Microsomal aldehyde dehydrogenase is localized to the endoplasmic reticulum via its carboxyl-terminal 35 amino acids. *J. Cell Biol.* 126, 1407–1420.
- Masaki, R., Yamamoto, A., and Tashiro, Y. (1996). Membrane topology and retention of microsomal aldehyde dehydrogenase in the endoplasmic reticulum. *J. Biol. Chem.* 271, 16939–16944.
- Naik, R.R., and Jones, E.W. (1998). The PBN1 gene of *Saccharomyces cerevisiae*: an essential gene that is required for the posttranslational processing of the protease B precursor. *Genetics* 149, 1277–1292.
- Nishikawa, S., Hirata, A., and Nakano, A. (1994). Inhibition of endoplasmic reticulum (ER)-to-Golgi transport induces relocalization of binding protein (BiP) within the ER to form the BiP bodies. *Mol. Biol. Cell* 5, 1129–1143.
- Olender, E.H., and Simon, R.D. (1992). The intracellular targeting and membrane topology of 3-hydroxy-3-methylglutaryl-CoA reductase. *J. Biol. Chem.* 267, 4223–4235.

- Orci, L., Brown, M.S., Goldstein, J.L., Garcia-Segura, L.M., and Anderson, R.G. (1984). Increase in membrane cholesterol: a possible trigger for degradation of HMG CoA reductase and crystalloid endoplasmic reticulum in UT-1 cells. *Cell* 36, 835–845.
- Osborne, T.F., and Rosenfeld, J.M. (1998). Related membrane domains in proteins of sterol sensing and cell signaling provide a glimpse of treasures still buried within the dynamic realm of intracellular metabolic regulation. *Curr. Opin. Lipidol.* 9, 137–140.
- O'Toole, T.E., Katagiri, Y., Faull, R.J., Peter, K., Tamura, R., Quaranta, V., Loftus, J.C., Shattil, S.J., and Ginsberg, M.H. (1994). Integrin cytoplasmic domains mediate inside-out signal transduction. *J. Cell Biol.* 124, 1047–1059.
- Parrish, M.L., Sengstag, C., Rine, J.D., and Wright, R.L. (1995). Identification of the sequences in HMG-CoA reductase required for karmellae assembly. *Mol. Biol. Cell* 6, 1535–1547.
- Pathak, R.K., Luskey, K.L., and Anderson, R.G. (1986). Biogenesis of the crystalloid endoplasmic reticulum in UT-1 cells: evidence that newly formed endoplasmic reticulum emerges from the nuclear envelope. *J. Cell Biol.* 102, 2158–2168.
- Prasher, D.C. (1995). Using GFP to see the light. *Trends Genet.* 11, 320–323.
- Prasher, D.C., Eckenrode, V.K., Ward, W.W., Prendergast, F.G., and Cormier, M.J. (1992). Primary structure of the *Aequorea victoria* green-fluorescent protein. *Gene* 111, 229–233.
- Preuss, D., Mulholland, J., Kaiser, C.A., Orlean, P., Albright, C., Rose, M.D., Robbins, P.W., and Botstein, D. (1991). Structure of the yeast endoplasmic reticulum: localization of ER proteins using immunofluorescence and immunoelectron microscopy. *Yeast* 7, 891–911.
- Pringle, J.R., Preston, R.A., Adams, A.E., Stearns, T., Drubin, D.G., Haarer, B.K., and Jones, E.W. (1989). Fluorescence microscopy methods for yeast. *Methods Cell Biol.* 31, 357–435.
- Re, E.B., Brugger, S., and Learned, M. (1997). Genetic and biochemical analysis of the transmembrane domain of *Arabidopsis* 3-hydroxy-3-methylglutaryl CoA reductase. *J. Cell Biochem.* 65, 443–459.
- Remy, I., Wilson, I.A., and Michnick, S.W. (1999). Erythropoietin receptor activation by a ligand-induced conformation change. *Science* 283, 990–993.
- Reynolds, E.S. (1963). The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *J. Cell Biol.* 17, 208–212.
- Rogers, K.S., Rodwell, V.W., and Geiger, P. (1997). Active form of *Pseudomonas mevalonii* 3-hydroxy-3-methylglutaryl CoA reductase. *Biochem. Mol. Med.* 61, 114–120.
- Roitelman, J., Bar-Nun, S., Inoue, S., and Simoni, R.D. (1991). Involvement of calcium in the mevalonate-accelerated degradation of 3-hydroxy-3-methylglutaryl-CoA reductase. *J. Biol. Chem.* 266, 16085–16091.
- Roitelman, J., Olender, E.H., Bar-Nun, S., Dunn, J.W.A., and Simoni, R.D. (1992). Immunological evidence for eight spans in the membrane domain of 3-hydroxy-3-methylglutaryl coenzyme A reductase: implications for enzyme degradation in the endoplasmic reticulum. *J. Cell Biol.* 5, 959–973.
- Rose, M.D., L.M. Mistra, and J.P. Vogel. (1989). KAR2, a karyogamy gene, is the yeast homolog of the mammalian BiP/GRP78 gene. *Cell* 57, 1211–1221.
- Schunck, W., Vogel, F., Gross, B., Kargel, E., Mauersberger, S., Kopke, K., Gengnagel, C., and Muller, H. (1991). Comparison of two cytochromes P-450 from *Candida maltosa*: primary structures, substrate specificities and effects of their expression in *Saccharomyces cerevisiae* on the proliferation of the endoplasmic reticulum. *Eur. J. Cell Biol.* 55, 336–345.
- Sekler, M.S., and Simoni, R.D. (1995). Mutation in the luminal part of the membrane domain of HMG-CoA reductase alters its regulated degradation. *Biochem. Biophys. Res. Commun.* 206, 186–193.
- Sengstag, C., Stirling, C., Schekman, R., and Rine, J. (1990). Genetic and biochemical evaluation of eukaryotic membrane protein topology: the polytopic structure of *S. cerevisiae* HMG-CoA reductase. *Mol. Cell. Biol.* 10, 672–680.
- Sherman, F., Fink, G.R., and Hicks, J.B. (1986). *Methods in Yeast Genetics*, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Sikorski, R.S., and Hieter, P. (1989). A uniform set of multipurpose shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *S. cerevisiae*. *Genetics* 122, 19–27.
- Skalnik, D.G., Narita, H., Kent, C., and Simoni, R.D. (1988). The membrane domain of 3-hydroxy-3-methylglutaryl CoA reductase confers endoplasmic reticulum localization and sterol-regulated degradation onto β -galactosidase. *J. Biol. Chem.* 263, 6836–6841.
- Spurr, A.R. (1969). A low-viscosity epoxy resin embedding medium for electron microscopy. *J. Ultrastruct. Res.* 26, 31–43.
- Taberero, L., Bochar, D.A., Rodwell, V.W., and Stauffacher, C.V. (1999). Substrate-induced closure of the flap domain in the ternary complex structures provides insights into the mechanism of catalysis by 3-hydroxy-3-methylglutaryl-CoA reductase. *Proc. Natl. Acad. Sci. USA* 96, 7167–7171.
- Takahashi, S., Kuzuyama, T., and Seto, H. (1999). Purification, characterization, and cloning of a eubacterial 3-hydroxy-3-methylglutaryl CoA reductase, a key enzyme involved in biosynthesis of terpenoids. *J. Bacteriol.* 181, 1256–1263.
- Tsuneoka, M., and Mekada, E. (1992). Degradation of a nuclear-localized protein in mammalian COS cells, using *Escherichia coli* beta-galactosidase as a model protein. *J. Biol. Chem.* 267, 9107–9111.
- Vergeres, G., Yen, T.S.B., Aggeler, J., Lausier, J., and Waskell, L. (1993). A model system for studying membrane biogenesis: overexpression of cytochrome b_5 in yeast results in marked proliferation of the intracellular membrane. *J. Cell Sci.* 106, 249–259.
- Wanker, E.E., Sun, Y., Savitz, A.J., and Meyer, D.I. (1995). Functional characterization of the 180-kD ribosome receptor in vivo. *J. Cell Biol.* 130, 29–39.
- Wilson, I.A., Niman, H.L., Houghten, R.A., Chersonson, A.R., Connolly, M.L., and Lerner, R.A. (1984). The structure of an antigenic determinant in a protein. *Cell* 37, 767–778.
- Wright, R., Basson, M., D'Ari, L., and Rine, J. (1988). Increased amounts of HMG-CoA reductase induce "karmellae": a proliferation of stacked membrane pairs surrounding the yeast nucleus. *J. Cell Biol.* 107, 101–114.
- Wright, R., and Rine, J. (1989). Transmission electron microscopy and immunocytochemical studies of yeast: analysis of HMG-CoA reductase overproduction by electron microscopy. *Methods Cell Biol.* 31, 473–512.
- Yamamoto, A., Masaki, R., and Tashiro, Y. (1996). Formation of crystalloid endoplasmic reticulum in COS cells upon overexpression of microsomal aldehyde dehydrogenase by cDNA transfection. *J. Cell Sci.* 109, 1727–1738.
- Zimmer, K.P., Matsuda, I., Matsuura, T., Mori, M., Colombo, J.P., Fahimi, H.D., Koch, H.G., Ullrich, K., and Harms, E. (1995). Ultrastructural, immunocytochemical and stereological investigation of hepatocytes in a patient with the mutation of the ornithine transcarbamylase gene. *Eur. J. Cell. Biol.* 67, 73–83.