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# IN02, A Positive Regulator of Lipid Biosynthesis, Is Essential for the Formation of Inducible Membranes in Yeast

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Expression of the 180-kDa canine ribosome receptor in *Saccharomyces cerevisiae* leads to the accumulation of ER-like membranes. Gene expression patterns in strains expressing various forms of p180, each of which gives rise to unique membrane morphologies, were surveyed by microarray analysis. Several genes whose products regulate phospholipid biosynthesis were determined by Northern blotting to be differentially expressed in all strains that undergo membrane proliferation. Of these, the *INO2* gene product was found to be essential for formation of p180-inducible membranes. Expression of p180 in *ino2*Δ cells failed to give rise to the p180-induced membrane proliferation seen in wild-type cells, whereas p180 expression in *ino4*Δ cells gave rise to membranes indistinguishable from wild type. Thus, Ino2p is required for the formation of p180-induced membranes and, in this case, appears to be functional in the absence of its putative binding partner, Ino4p.

## INTRODUCTION

Biological membranes that enclose the organelles of eukaryotic organisms are composed of a lipid bilayer and integral proteins that reside within it. Although the structure and function of various cellular membranes have been extensively characterized, how they assemble in response to certain stimuli is poorly understood. The endoplasmic recticulum (ER), a prominent feature of actively secreting cells, is the site of translocation and initial processing of secretory proteins in eukaryotes. Developmentally regulated ER biogenesis occurs in cells of specialized mammalian tissues, such as pancreas and liver (Dallner *et al.*, 1966a, 1966b) as well as during the antigen-induced maturation of B lymphocytes into plasma cells (Chen-Kiang, 1995).

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Abbreviations used: bHLH, basic helix-loop-helix; PrI, propidium iodide.

Simplified systems for studying ER biogenesis in *Saccharomyces cerevisiae* have been recently described. Membrane proliferation has been observed in cells that express high levels of certain integral ER membrane proteins such as the yeast HMG-CoA reductase isozymes, Hmg1p and Hmg2p (Wright *et al.*, 1988; Koning *et al.*, 1996), cytochrome P450 (Schunck *et al.*, 1991), and various domains of the mammalian ribosome receptor, p180 (Wanker *et al.*, 1995; Becker *et al.*, 1999). ER-like membrane morphologies arise from the overexpression of the peroxisomal integral membrane protein, Pex15p (Elgersma *et al.*, 1997). In addition, a yeast strain harboring a temperature-sensitive allele of the Golgi membrane protein, Yip1p, has also been shown to accumulate of ER-like membranes (Yang *et al.*, 1998).

Several obvious questions arise. Is there a common mechanism that leads to the proliferation of intracellular membranes in these situations? If so, which gene products participate? How is the process regulated, and what are the regulatory elements? It is the purpose of the work described here to begin to address these issues.

The lipid component of the yeast ER membrane consists largely of phosphatidylcholine and phosphatidylinositol (Jakovcic *et al.*, 1971). The rate-limiting step in inositol phospholipid biosynthesis is carried out by the *INO1* gene prod-

uct. The transcription of *INO1* and other phospholipid biosynthetic enzymes have been well characterized in yeast. Many of these genes are regulated by the intracellular concentration of free inositol and choline (see Greenberg and Lopes, 1996; Henry and Patton-Vogt, 1998 for review). When inositol and choline levels are low, a transcription factor complex composed of the basic helix-loop-helix (bHLH) proteins, Ino2p and Ino4p, activates the expression of many genes encoding phospholipid, fatty acid, and sterol biosynthetic enzymes. Ino2p and Ino4p form a functional heterodimer that binds to a conserved upstream activating sequence ($UAS_{INO}$) residing in the promoters of these genes (Lopes *et al.*, 1991; Ambroziak and Henry, 1994; Nikoloff and Henry, 1994; Koipally *et al.*, 1996). Ino2p has been shown to contain transactivation domains (Schwank *et al.*, 1995), whereas Ino4p is required for the binding of the complex to $UAS_{INO}$.

Genes involved in lipid biosynthesis are also negatively regulated by a subset of genes. Of these, the *OPI1* gene product represses the transcription of *INO1* and other phospholipid biosynthetic genes in response to inositol and choline (Lai and McGraw, 1994; Ashburner and Lopes, 1995a, 1995b). However, there is evidence that Opi1p may not be responsible for the transmission of the signal that leads to repression of *INO1* in response to phospholipid precursors (Graves and Henry, 2000). Overproduction of Opi1p was shown to render wild-type yeast auxotrophic for inositol, supporting the notion that it is a negative regulator of phospholipid biosynthesis (Wagner *et al.*, 1999).

Genes involved in phospholipid biosynthesis might be differentially expressed in systems where ER biogenesis is accelerated. To identify genes whose levels of expression change during stimulated membrane production, microarray analysis was performed using mRNA isolated from cells expressing the canine ribosome receptor (p180) or specific regions of it known to produce membrane proliferation in yeast. The canine ribosome receptor is an integral membrane protein of the endoplasmic reticulum (ER) consisting of three distinct regions based on its amino acid sequence (Wanker *et al.*, 1995). Briefly, full-length p180 (FL) consists of an amino terminal membrane-anchoring domain, a basic region consisting of 54 tandem decapeptide repeats involved in ribosome binding and a C-terminal predicted coiled-coil domain of unknown function (Langley *et al.*, 1998). Expression of FL results in the proliferation of rough membranes evenly spaced throughout the cytoplasm. Expression of the ΔCT construct, which lacks the C-terminal domain, gives rise to closely packed rough membranes. Expression of the ΔNT construct, lacking the ribosome-binding domain, leads to the proliferation of smooth, evenly spaced membranes 80–100 nm apart. When the membrane-anchoring (MA) region alone was expressed, proliferation of "karmellae" or stacks of closely packed, smooth perinuclear membranes was observed.

Pilot studies using microarray analysis suggested that several genes involved in phospholipid biosynthesis are differentially expressed in all strains where membrane proliferation was induced. Among them were key transcription factors involved in the regulation of phospholipid metabolism. Of these, *INO2* mRNA was upregulated, and the transcripts of *INO4* and *OPI1* were downregulated. The results presented here show that Ino2p is essential for the process of stimulated ER biogenesis in yeast, irrespective of the membrane protein expressed. Actions that ameliorate the viability of strains deleted for Ino2p, such as added inositol and choline, do not restore the ability to proliferate membranes. Moreover, other putative regulatory proteins, such as Ino4p, do not appear to be essential to the process.

## MATERIALS AND METHODS

### Strains and Expression Plasmids

*S. cerevisiae* strains used in this study were as follows: SEY6210 (*MATα leu2-3112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9*; Wilsbach and Payne, 1993), J51-5c (*MATα ura3-52 lys2-801 ade2-101 trp1-Δ901 ptl1-1*; Toyn *et al.*, 1988), W303 (*MAT**a** ura3-1 leu2-3112 trp1-1 ade2-1 his3-11,15 can1-100*), JAG1 (*MAT**a** his3 leu2 trp1 ura3 opi1Δ::LEU2*), JAG2 (*MAT**a** his3 leu2 trp1 ura3 ino2Δ::TRP1*), JAG4 (*MAT**a** his3 leu2 trp1 ura3 ino4Δ::LEU2*). JAG1, 2, and 4 were obtained from Susan Henry (Carnegie Mellon University) and constructed from the parental strain W303 (Graves and Henry, 2000).

p180 plasmids used for microarray analysis and Northern blotting were constructed in the pYEX-BX plasmid containing the *CUP1* promoter (Amrad Biotech, Victoria, Australia). Cloning and plasmid transformation were carried out as described by Becker *et al.* (1999), and the constructs used are diagrammed in the Appendix. The ΔCT-GFP construct was created by amplifying an EGFP fragment (Patterson *et al.*, 1997) using *Acc*III- and *Sal*I-modified primers: (5′AAGGAGTCCGGAGTAAAGGAGAAGAACTT 3′, 5′GATCTCGGGCCCGTCGACCTACAATTCGTCGTG 3′). The GFP fragment was inserted into the YEX–BX vector containing full-length p180 cut at *Acc*III and *Sal*I sites, creating a C-terminal truncation of p180. Expression of copper-inducible p180 plasmids was carried out in 2% dextrose (Fisher Scientific, Pittsburgh, PA), 0.17% Difco yeast nitrogen base without amino acids and ammonium sulfate (Fisher Scientific), and 5% ammonium sulfate (Fisher Scientific) plus 0.5 mM copper sulfate. The concentration of inositol from yeast nitrogen base is 2 mg/l or 11 μM. Amino acids were supplemented (without uracil and leucine) as described by Guthrie and Fink (1991). After 5 h growth in copper, yeast cells were harvested and used for further study. Plasmids containing HMG1 and HMG2-GFP fusions were obtained from Robin Wright (University of Washington), transformed into W303 and JAG2 strains, and expressed under conditions described by Koning *et al.* (1996).

### RNA Isolation and Northern Blotting

RNA isolation was performed by the method of Hollingsworth *et al.* (1990). Total RNA (5 μg) was separated on a 1.2% formamide-containing agarose gel (Maniatis *et al.*, 1982) and transferred to MagnaGraph nylon membrane (Osmonics, Westborough, MA). Probes were generated from PCR using primer pairs listed below and using yeast genomic DNA as a template: *PGK1*, 5′-AACGTCCCATTGGACGGTAA-3′ and 5′-TCTTGTCAGCAACCTTGGCA-3′; *INO2*, 5′-ATGCAACAAGCAACTGGGAA-3′ and 5′-TTCATGGAAGCGTTGGAAGA-3′; *INO4*, 5′-TGACGAACGATATTAAGGAGATAC-3′ and 5′-TCACTGACCACTCTGTCCATCA-3′; *OPI1*, 5′-TGTCTGAAAATCAACGTTTAGGA-3′ and 5′ CAACAAGGTCCTGTAAACACGA-3′. Quantitative Northern blotting was performed using ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

### Microarray Analysis

p180-plasmids in strain SEY6210 were induced for 5 h, and RNA was harvested as described above. For microarrays, cDNA synthesis, labeling, hybridization, and scanning were performed as described by Lipshutz *et al.* (1999) and Lockhart and Winzeler (2000). Genes involved in phospholipid biosynthesis were categorized according to lists provided by the Yeast Proteome Database (Costanzo *et al.*, 2000, 2001).

### *$DiOC_6$ and Propidium Iodide Staining*

Yeast cells were grown under desired conditions in liquid culture to mid log phase. For $DiOC_6$ (3,3′-dihexyloxacarbocyanineiodide) staining, ~1 $OD_{600}$ of cells were harvested and resuspended in 1 ml TE buffer. $DiOC_6$ (Molecular Probes, Eugene, OR) was resuspended to 1 mg/ml in ethanol. One microliter $DiOC_6$ stock solution was added to the yeast cell suspension. Cells were analyzed immediately. Propidium iodide (PrI) was purchased from Molecular Probes. Staining was carried out as described by Deere *et al.* (1998).

### *Flow Cytometry*

Approximately 30,000 yeast cells were acquired per sample using a FACScan flow cytometer (Becton-Dickinson, Mountain View, CA). The detection threshold was set in the FSC channel just below the lowest detectable level of the yeast suspension with the lowest intensity. GFP-expressing and $DiOC_6$-stained cells were detected in channel FL-1. PrI-stained cells were detected in channel FL-2. Analysis was carried out using CELLQuest software (Becton-Dickinson).

### *Fluorescence Microscopy*

$DiOC_6$-stained and GFP-expressing cells were visualized with a Nikon DIOPHOT 200 (Garden City, NY) inverted microscope with 60× and 100× objective lenses and fluorescent filters with 488-nm excitation wavelength. Images were collected using Inovision Isee software (Raleigh, NC).

### *Electron Microscopy*

Yeast cells were fixed by immersion in 2.5% glutaraldehyde in 100 mM sodium cacodylate (pH 7.4) by addition of double-strength fixative to cells in suspension. The cells were pelleted and left in fixative for 24 h. They were embedded in Spurr low viscosity resin (EMS, Fort Washington, PA) using a modification of a previously published method (Kaiser and Schekman, 1990).

The cells were transferred to 15-ml plastic falcon tubes in 10 ml of water. All exposures to processing solutions were performed in at least a 10 ml volume. Cells were washed in three changes of water and then resuspended in 10 ml of 1% aqueous osmium tetroxide. The closed tubes were exposed to microwaves for 40 s and then left for 3 h. The microwave processor used (Ted Pella Inc, Redland, CA) was calibrated and operated as previously described (Giberson and Demaree, 1999) and equipped with recycling water load, in the form of a "cold spot," as supplied by the manufacturer. All microwave exposures were performed using the processor set to deliver full power. Cells were washed again in water and resuspended in 70% methanol saturated with uranyl acetate. The cells were exposed to microwaves for 40 s and then left overnight at 4°C.

The cells were washed with five changes of 70% methanol and dehydrated through graded acetone series, starting at 70%. Each dehydration step consisted of a 40-s exposure to microwaves with 10 min on a stirring wheel.

Infiltration in resin consisted of a 15-min exposure to microwaves followed by an overnight incubation in a 1:1 mixture of Spurr resin and acetone. The yeast were resuspended in the resin/acetone mixture and left on a mixing wheel. The following day, the yeast were resuspended in 10 ml of fresh resin, exposed to microwaves for 15 min, and left mixing for 2 h. The cells were embedded in fresh resin that was polymerized at 60°C.

Thin sections were mounted onto coated metal specimen grids and photographed in a CM120 BioTwin TEM (FEI-Philips, Hillboro, OR) operating at 80 kV. Negative film, developed in liquid developer, was digitalized using a flat-bed scanner and the images were manipulated to adjust contrast and brightness with Adobe PhotoShop (Adobe Systems, San Jose, CA).

### *$^{35}S$ Labeling and Immunoprecipitation of Carboxypeptidase Y*

Yeast cultures were grown to stationary phase in synthetic medium, with the appropriate amino acid supplements, and with or without inositol for *ino2*Δ strains. Cells were diluted to 0.2 OD/ml and grown to $OD_{600}$ = 1–2, harvested by centrifugation, and washed twice with synthetic complete media, pH 5.7. Cells were resuspended at 2 $OD_{600}$/ml in synthetic complete media plus BSA at 1 mg/ml and $\alpha_2$-macroglobulin at 10 mg/ml. After preincubation for 15 min at the desired temperature, 50 μCi of $^{35}$S-cysteine/methionine was added per 1 $OD_{600}$, and cells were incubated for 10 min shaking at 37°C (for *ptl1* and wt strains) of 30°C (for *ino2*Δ strains). Cells were chased by adding 1/10 vol 3 mg/ml methionine and 3 mg/ml cysteine dissolved in 2% yeast extract. Aliquots (250 μl) of cells were removed at the desired time points to tubes on ice containing 2.5 μl 1 M NaF and 2.5 μl $NaN_3$.

Cells were pelleted and supernatant was removed. The cells were spheroplasted with 10 μg/ml oxyliticase in 100 μl spheroplast buffer (50 mM Tris-Cl, pH 7.4, 1.4 M sorbitol, and 5 mM $MgCl_2$) plus 4 mM β-ME and 10 mM $NaN_3$. Cells were incubated at 30°C for 30 min and pelleted for 6 min at 3000 rpm, and the supernatant was removed. The cells were resuspended in 100 μl 2% SDS and incubated at 100°C for 3 min. PT (1× PBS, 1% TX-100), 0.9 ml, was added to cell lysate. The lysates were precleared with 50 μl 10% *Staphylococcus aureus* cells on ice for 15 min and then pelleted 15 min at full-speed in a microcentrifuge. The supernatant was removed to a new tube with carboxypeptidase Y (CPY) antisera (Greg Payne, UCLA) and Protein A sepharose was added (25 μl of 20% solution). The tubes were rotated overnight at 4°C. Samples were pelleted and washed twice with 0.5 ml of each: PTS (1× PBS, 1% TX-100, 0.1% SDS), urea wash (2 M urea, 0.1 M Tris-HCl, pH 7.5, 1% TX-100, 2 M NaCl), and Tris/NaCl wash (10 mM Tris-HCl, pH 6.8, 10 mM NaCl). After washing, pellets were resuspended in 25 μl 1× Laemmli sample buffer, heated for 3 min at 100°C, and resolved on an 8% SDS-PAGE.

## RESULTS

### *Quantification of p180-induced Membrane Proliferation: Increased Appearance of Lipid Bilayers*

The expression of different regions of canine p180 in yeast gives rise to rough or smooth ER-like membranes as previously documented primarily by electron microscopy as well as biochemically through measurement of increase in membrane lipid on a per cell basis (Wanker *et al.*, 1995). To demonstrate that the membranes observed resulted from the synthesis of new membranes and not from the incorporation of proteins into preexisting membranes, total membrane quantification was carried out. Here, the lipophilic fluorescent dye, $DiOC_6$, was used to quantify increases in intracellular membrane content in living cells upon expression of the ΔCT construct of p180 in yeast. $DiOC_6$ has been used previously to assess and quantify membrane proliferations that arise from overexpression of the HMG-CoA reductase isoforms, Hmg1p and Hmg2p (Koning *et al.*, 1996; Parrish *et al.*, 1995).

ΔCT and vector-expressing cells were incubated with $DiOC_6$, and fluorescence was quantified by flow cytometry. Calculations based on the mean fluorescence value per cell revealed that ΔCT-expressing cells absorb ~1.6 times as much of the lipophilic dye compared with vector-expressing cells (Figure 1A). However, it should be noted that this represents a minimum value because the absorbance of

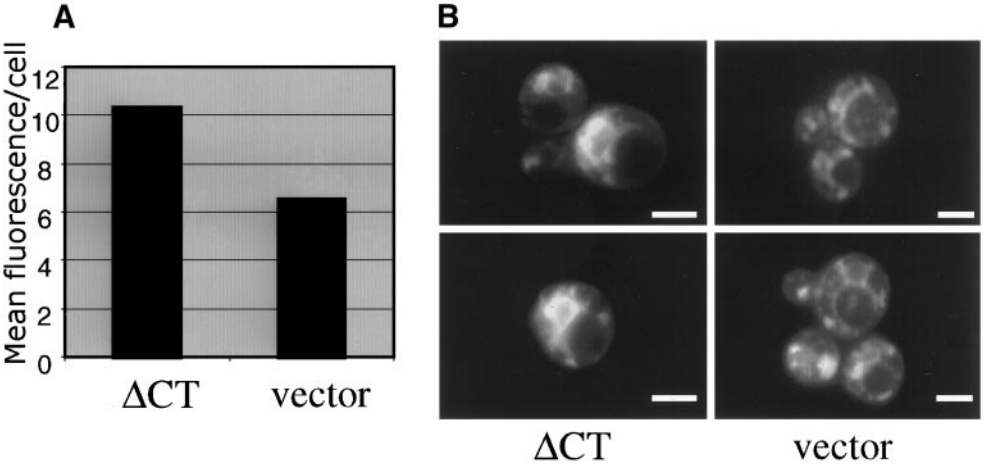


**Figure 1.** Membrane proliferation quantified by the incorporation of the lipophilic dye, $DiOC_6$. ΔCT-YEX– and YEX-BX (vector)–expressing cells were induced for 5 h with 0.5 mM $CuSO_4$. Cells were harvested and resuspended to 1 $OD_{600}$/ml in TE buffer. Cells were treated with $DiOC_6$ to a final concentration of 1.0 μg/ml. Cells were subjected to flow cytometry analysis and the mean fluorescence value per cell was plotted in A. $DiOC_6$-stained cells viewed by fluorescence microscopy (B). Bar, 1.0 μm.

$DiOC_6$ is not limited to ER and nuclear membranes, and thus the quantification of membranes by $DiOC_6$ staining is more accurately a representation of total cellular membranes (e.g., mitochondria, Golgi, and vacuole). To confirm that the increase in $DiOC_6$ absorption resulted from an increase in ER membrane content, ΔCT- and vector-expressing cells stained with $DiOC_6$ were analyzed by fluorescence microscopy (Figure 1B). Cells expressing ΔCT were visualized as having bright asymmetric rings emanating from the nucleus. In contrast, control cells showed only dim perinuclear and other membrane staining. These observations corroborate the morphological changes previously seen in electron micrographs and establish increased lipid content due to p180-induced membrane proliferation.

### *Genes Involved in Lipid Metabolism are Differentially Expressed in p180-Expressing Cells*

To determine if induction of specific genes is associated with p180-stimulated membrane proliferation, microarray analysis was performed. RNA isolated from strains expressing four different forms of p180 (ΔCT, ΔNT, FL, and MA) as well as a vector-transformed control was used to prepare probes. A pilot study revealed that several genes involved in lipid biosynthesis may be differentially expressed in strains expressing all forms of p180 compared with the vector transformed control. This single set of microarray data, collected under the conditions described, enabled the selection of genes of interest chosen for further investigation. In each of these cases, accurate changes in mRNA levels were established by quantitative Northern analysis. Interestingly, *INO2* mRNA, which encodes a bHLH transcription factor required for the derepression of phospholipid biosynthetic genes was found by array analysis and confirmed by Northern blotting to be upregulated in all p180-expressing strains (Figure 2). In contrast, *OPI1* mRNA, which encodes a transcriptional repressor of phospholipid biosynthesis, was downregulated (Figure 2). Microarray analysis also revealed *INO4* mRNA to be downregulated (Figure 2). This was unexpected because Ino2p and Ino4p have been shown to form a functional heterodimer that activates transcription of phospholipid biosynthetic genes upon inositol starvation (Lopes and Henry, 1991). In addition, *INO4* has not been shown previously to be regulated at the transcriptional level (Ashburner and Lopes, 1995b; Robinson and Lopes, 2000a). Other genes involved in lipid biosynthesis found to be upregulated by Northern blotting, included those encoding inositol-1-phosphate (*INO1*), glycerol-3-kinase (*GUT1*), and a gene required for inositol prototrophy (*SCS3*; our unpublished results).

To determine if the differentially expressed genes profiled above play any role in the formation of p180-induced membranes, genetic and biochemical analyses were carried out.

### *Regulators of Phospholipid Biosynthesis in Yeast: The Roles of INO2, INO4, and OPI1 in the Formation of Inducible Membranes*

The observation that transcript levels of positive and negative regulators of lipid biosynthesis were differentially ex-

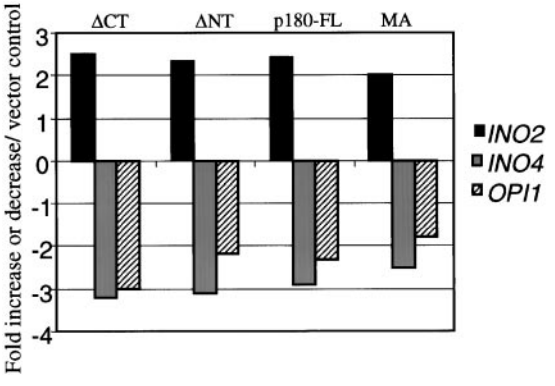


**Figure 2.** Northern blot quantification of *INO2, INO4,* and *OPI1* upon p180 induction. Yeast strains were induced to express various forms of p180 (ΔCT, ΔNT, p180-FL, MA) and a vector control for 5 h. RNA was harvested, and 5 μg RNA was loaded per lane. Blots were probed with radiolabeled fragments of *INO2, INO4, OPI1,* and *PGK1.* Quantified band intensities were normalized to *PGK1* for loading control. Values obtained for RNA from p180-expressing strains were compared with vector control strains. Black bars, *INO2;* gray bars, *INO4;* striped bars *OPI1.*

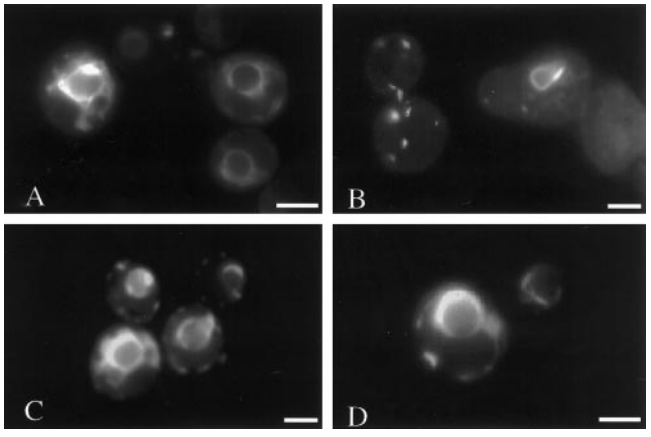


**Figure 3.** Cells deleted for *INO2* fail to undergo p180-induced membrane proliferation. Cells carrying the ΔCT-GFP-YEX plasmid were induced for 5 h with 0.5 mM $CuSO_4$. Cells were resuspended to 1 $OD_{600}$/ml in TE buffer. Seven microliters of cell suspension was placed on a glass slide and viewed by fluorescence microscopy. Wild-type strain W303 expressing ΔCT-GFP exhibited perinuclear fluorescent patterns (A). Cells deleted for *INO2* (JAG2) exhibited aberrant membrane structures (B). Cells deleted for *INO4* (JAG4) gave rise to membranes similar to wild type (C). Cells deleted for *OPI1* contained bright, dense perinuclear structures (D). Bar, 1.0 μm.

pressed in p180-expressing strains suggests that regulation of *INO2*, *INO4,* and *OPI1* may be important for the formation of p180-induced membranes. A ΔCT-GFP fusion construct was expressed in wild-type, *ino2*Δ, *ino4*Δ, and *opi1*Δ backgrounds (Figure 3) to observe if strains lacking these genes are affected in membrane proliferation.

### *INO2, but not INO4, Is Required for p180-induced Membrane Proliferation*

In wild-type cells, both the expression of ΔCT-GFP and $DiOC_6$-stained ΔCT-expressing cells gave rise similar proliferated membrane morphologies (cf. Figures 1A and 3A). However, *ino2*Δ cells expressing ΔCT-GFP failed to accumulate p180-induced membranes (Figure 3B). Instead, these cells included shrunken perinuclear structures and punctate fluorescent spots. ΔCT-expressing cells deleted for *INO4*, which encodes the putative binding partner for Ino2p, were indistinguishable from wild-type cells (cf. Figure 3, C and A). This observation is intriguing because evidence to date has linked both Ino2p and Ino4p to derepression of phospholipid biosynthetic genes (Lopes and Henry, 1991; Ashburner and Lopes, 1995b). Both proteins are required for the formation of a complex that binds to the $UAS_{INO}$ of *INO1* and other phospholipid biosynthetic genes (Ambroziak and Henry, 1994; Schwank *et al.*, 1995).

### *p180-induced Membrane Proliferation Appears Enhanced in opi1Δ Cells*

To determine the role of Opi1p, a negative regulator of phospholipid biosynthesis, in p180-induced membrane biogenesis, we expressed ΔCT-GFP in *opi1*Δ cells. It is unclear how Opi1p represses phospholipid biosynthesis, but it has been shown to repress *INO1* transcription in the presence of inositol (Lai and McGraw, 1994; Ashburner and Lopes, 1995a, 1995b; Henry and Patton-Vogt, 1998). This result is consistent with the downregulation of *OPI1* mRNA in p180-expressing cells. Figure 3D shows that *opi1*Δ cells expressing ΔCT-GFP accumulate thick, bright perinuclear rings. Because it is difficult to quantify the degree of membrane accumulation by fluorescence microscopy, we subjected *opi1*Δ and the above-mentioned strains expressing ΔCT-GFP to flow cytometry analysis.

Fluorescence-activated flow cytometry is an efficient and rapid method of gauging the amount of fluorescence per cell for a population expressing a fluorescent marker. Here, flow cytometry was used as a measure of membrane accumulation in cells expressing ΔCT fused to GFP. This proved to be a valid measure of membrane accumulation as the mean fluorescence value per cell of ΔCT-GFP–expressing cells was approximately twice that of MA-GFP–expressing cells (unpublished observations). This difference mirrors the relative amounts of membranes visualized in ΔCT- versus MA-expressing cells by electron microscopy (Becker *et al.*, 1999).

Figure 4 shows the fluorescence profiles of wild-type and various knockout strains expressing ΔCT-GFP. This method is used to quantify the fluorescence levels of ΔCT-GFP–expressing strains on a per-cell basis. The autofluorescence of wild-type yeast cells is profiled in Figure 4A. Expression of ΔCT-GFP caused wild-type cells to accumulate membranes and a consequential shift in the fluorescence profile to the right indicating an increase in fluorescence (Figure 4B). Consistent with fluorescence microscopy, the flow cytometry profile of *ino2*Δ cells shifted back to the left, indicating a deficiency in membrane proliferation (Figure 4C). The profiles of *ino4*Δ and *opi1*Δ strains expressing ΔCT-GFP resembled that of the wild-type population (Figure 4, D and E). The mean fluorescence level per cell for the above profiles was plotted in Figure 4F. Cells harboring a deletion in *INO2* emitted approximately fivefold less fluorescence than wild-type cells expressing ΔCT-GFP. We conclude that *ino2*Δ cells are deficient in their ability to proliferate p180-induced membranes. To rule out the possibility that p180 expression was affected in an *ino2*Δ background, Northern blot analysis was performed on *ino2*Δ cells expressing ΔCT-GFP. The results confirmed normal levels of p180 expression (unpublished observations). In contrast to the *ino2*Δ strain, the mean fluorescence values of *ino4*Δ and *opi1*Δ strains expressing ΔCT-GFP were 1.5- and 1.2-fold higher, respectively, than wild type. An interesting pattern seems to emerge, at least in the case of lipid biosynthetic genes. Strains harboring deletions in genes whose transcript levels increase during induced membrane proliferation (*INO2*) failed to respond to p180 induction by proliferating membranes, whereas cells deleted for genes whose transcript levels fall (*INO4* and *OPI1*), appeared to produce increased levels of membranes. Visualization of membrane accumulation along with fluorescence quantification of ΔCT-GFP indicates that *INO2* is essential for the formation of p180-inducible membranes in yeast, whereas *INO4* is dispensable and that membrane accumulation is even enhanced in its absence.

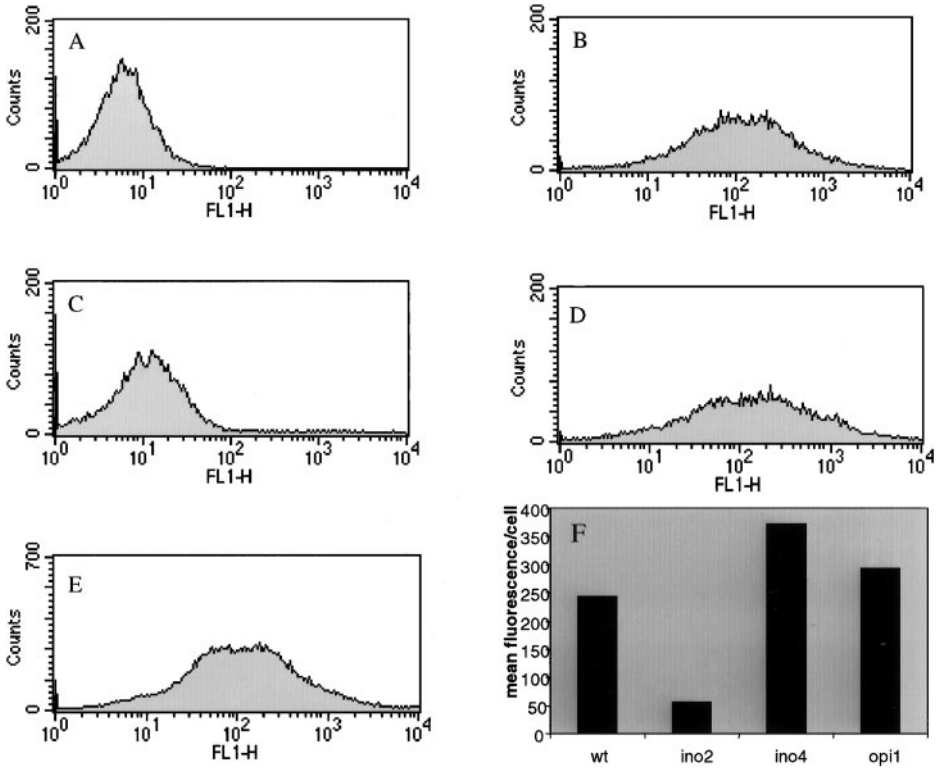


**Figure 4.** Quantification of membrane biogenesis: *ino2*Δ cells fail to accumulate membranes. Cells expressing ΔCT-GFP or vector were induced with 0.5 mM $CuSO_4$ for 5 h. Cells were resuspended to 1 $OD_{600}$/ml in TE buffer. Approximately 30,000 cells were acquired in a FACScan flow cytometer. The auto-fluorescence of yeast cells is documented in A. (B–D) Different cell populations expressing ΔCT-GFP: wild type (B), *ino2*Δ (C), *ino4*Δ (D), *opi1*Δ (E). The histogram in F compares the mean fluorescence value per cell for B–D.

### *ino2*Δ *Cells Are Compromised in the Proliferation of Karmellae*

To establish that *INO2* is required for the formation of membranes other than those that arise from p180 expression, GFP fusions of the HMG-CoA reductase isoforms, *HMG1* and *HMG2*, were expressed in *ino2*Δ cells. Increased production of Hmg1p gives rise to whorls of karmellae, or layers of smooth ER membranes that are contiguous with the nuclear membrane (Figure 5A), whereas elevated levels of Hmg2p cause the formation of short stacks of karmellae, with characteristics similar to those of peripheral ER (Koning *et al.*, 1996). Consistent with what was observed in *ino2*Δ cells expressing ΔCT-GFP, *HMG1*-GFP expression in this strain gave rise to similar aberrant membrane morphologies (Figure 5). Similar results were observed for *ino2*Δ cells expressing *HMG2*-GFP (unpublished results). As with p180-expressing cells, *INO4* was not required for the proliferation of karmellae. Cells deleted for *INO4* expressing *HMG1* or *HMG2* fused to GFP gave rise to karmellae indistinguishable from the karmellae of wild-type cells expressing these constructs (unpublished results). Based on these results, *INO2* appears to be essential for the formation of karmellae.

### *Inositol and Choline Do Not Restore Membrane Proliferation in ino2*Δ *Cells*

*INO2* and *INO4* were identified by complementation as suppressors of inositol auxotrophy (Culbertson and Henry, 1975; Donahue and Henry, 1981). The fact that *INO2* mutants are defective in inositol and choline phospholipid biosynthesis raised the possibility that the membrane proliferation defect in *ino2*Δ cells is due to the absence of inositol and choline for phospholipid biosynthesis. To address this issue, *ino2*Δ cells expressing ΔCT-GFP were grown in con-

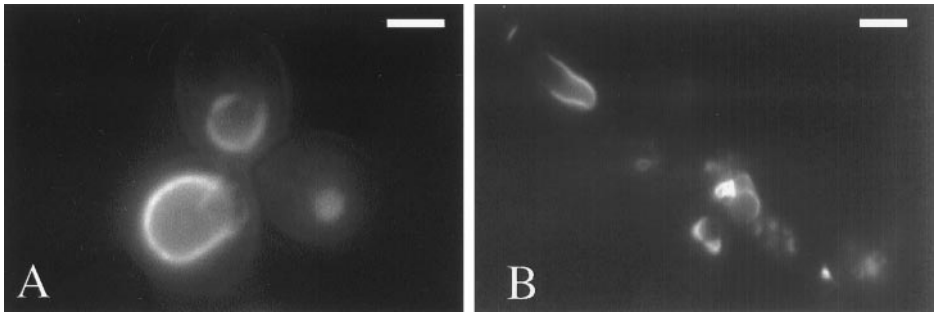


**Figure 5.** Cells deleted for *INO2* are compromised in the proliferation of karmellae. Hmg1-GFP was expressed in wild-type and *ino2*Δ cells under the control of the galactose promoter. Wild-type cells contained whorls of perinuclear membranes or karmellae (A). Cells deleted for *INO2* exhibited aberrant punctate membrane structures and elongated perinuclear morphology (B). Bar, 1.0 μm.

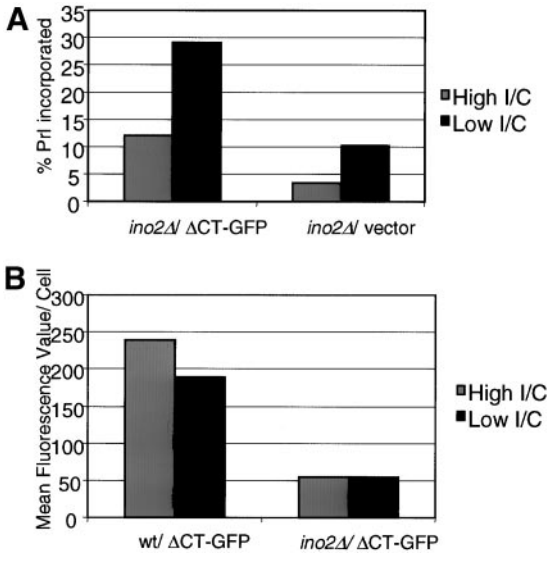


**Figure 6.** Addition of inositol and choline restores viability to *ino2*Δ cells. (A) PrI incorporation was assessed in *ino2*Δ cells expressing ΔCT or an empty vector. Cells were grown under conditions of low or high concentrations of inositol and choline. Cells were harvested, and PrI was added to a final concentration of 3 $\mu$g/1 $OD_{600}$. PrI-stained cells were acquired by a FACScan flow cytometer, and the percentage of PrI-stained cells was calculated. (B) Fluorescence quantification by flow cytometry of wild-type and *ino2*Δ cells expressing ΔCT-GFP. Gray bars, high I/C (75 $\mu$M inositol, 1 mM choline); black bars, low I/C (10 $\mu$M inositol, no choline).

ditions of low (10 $\mu$M inositol, no choline) or high (75 $\mu$M inositol, 1 mM choline) phospholipid precursors. High concentrations of inositol and choline allow *ino2*Δ cells to achieve growth levels comparable to wild type (Ashburner and Lopes, 1995b).

Here, viability of *ino2*Δ cells under conditions of high or low inositol and choline was ascertained by the incorporation of PrI. PrI can be used to assess yeast cell viability and membrane integrity because it will stain DNA of cells with porous membranes but not intact cells (Deere *et al.*, 1998). Figure 6A shows that the incorporation of PrI into *ino2*Δ cells was greatly reduced in cells grown in high inositol and choline. Approximately 30% of ΔCT-GFP–expressing *ino2*Δ cells grown in low inositol were PrI-positive, indicating that this population of cells was dead or had compromised membrane integrity. The fitness of this strain was improved during growth in high inositol and choline, reducing PrI-positive cells to ~10% of the population. The viability of vector-expressing *ino2*Δ cells also improved when grown in media containing high inositol and choline.

Although addition of inositol and choline restored the viability of *ino2*Δ cells expressing ΔCT-GFP, it failed to rescue the ability of these cells to proliferate membranes. Fluorescence microscopy revealed that these cells appeared nearly identical to cells grown in low inositol (unpublished results; see Figure 3B), and flow cytometry quantification showed no increase in fluorescence of *ino2*Δ cells supplemented with inositol and choline (Figure 6B). Electron microscopy demonstrated that, although the aberrant morphology of *ino2*Δ cells was improved when supplemented with inositol and choline, the ability to proliferate membranes was not restored (Figure 7). Cells deleted for *INO4* were capable of undergoing membrane proliferation during growth in low or high concentrations of inositol, although they exhibited abnormal morphology when grown without inositol and choline (Figure 7).

### *Protein Translocation Is Not Affected in ino2*Δ *Cells: A Functional Assay for Membrane Integrity*

Owing to the pivotal role played by Ino2p in phospholipid biosynthesis, the question arises as to the integrity of cellular membranes in Δ*ino2* cells. Strains deleted for *INO2* display a pleiotropic phenotype characterized by defects in nuclear segregation, bud formation and sporulation as well as an oversized morphology (Hammond *et al.*, 1993). This observation raised the possibility that deletion of *INO2* may cause a generalized membrane defect that prevents insertion of p180 and other ER membrane proteins into the ER, resulting in an inability of the cells to undergo membrane proliferation. To address the issue of ER membrane integrity in *ino2*Δ cells, a translocation assay was performed after the maturation of the endogenous yeast glycoprotein, CPY. Wild-type and *ino2*Δ cells were pulse-labeled, and CPY was immunoprecipitated from cells grown in the presence or absence of inositol. The *ptl1* strain described by Toyn *et al.* (1988) was used as a control for defective CPY translocation. *PTL1* is allelic to the *SEC63* gene of *S. cerevisiae*, which encodes an integral membrane protein that is required for the translocation of secretory proteins into the ER (Rothblatt *et al.*, 1989). As shown in Figure 8, CPY was initially observed largely as its glycosylated intermediate forms (p1, p2) for both wild-type and *ino2*Δ strains in the absence or presence of inositol. As expected, CPY was not translocated into the ER in the *ptl1* mutant and remained unmodified as prepro-CPY. After 15 min, the majority of p1 and p2 CPY was chased to its mature form in wild-type cells. Similarly, CPY immunoprecipitated from *ino2*Δ cells grown in the absence or presence of inositol appeared to be nearly completely processed to mature CPY after 15 min, whereas *ptl1* cells exhibited primarily unprocessed prepro-CPY. These data indicate that the ER membrane in *ino2*Δ cells is intact and functional for protein translocation, suggesting that the requirement for Ino2p in membrane proliferation is not due to compromised membrane integrity.

## DISCUSSION

The work presented here defines an essential role for Ino2p, a transcriptional activator of phospholipid biosynthesis, in the formation of inducible membranes in *S. cerevisiae*. Yeast cells expressing p180 accumulated ER membranes as visualized and quantified by incorporation of the lipophilic dye, $DiOC_6$. Microarray analysis of strains expressing various forms of canine p180 revealed the differential expression of several transcripts whose products function in lipid biosynthetic pathways. Among the genes identified in this screen were those encoding the positive transcriptional regulators Ino2p and Ino4p as well as a negative regulator of phospholipid biosynthesis, Opi1p. Membrane accumulation was diminished in an *ino2*Δ strain expressing the ΔCT form of p180 compared with wild type. Strains deleted for *INO4* and *OPI1*

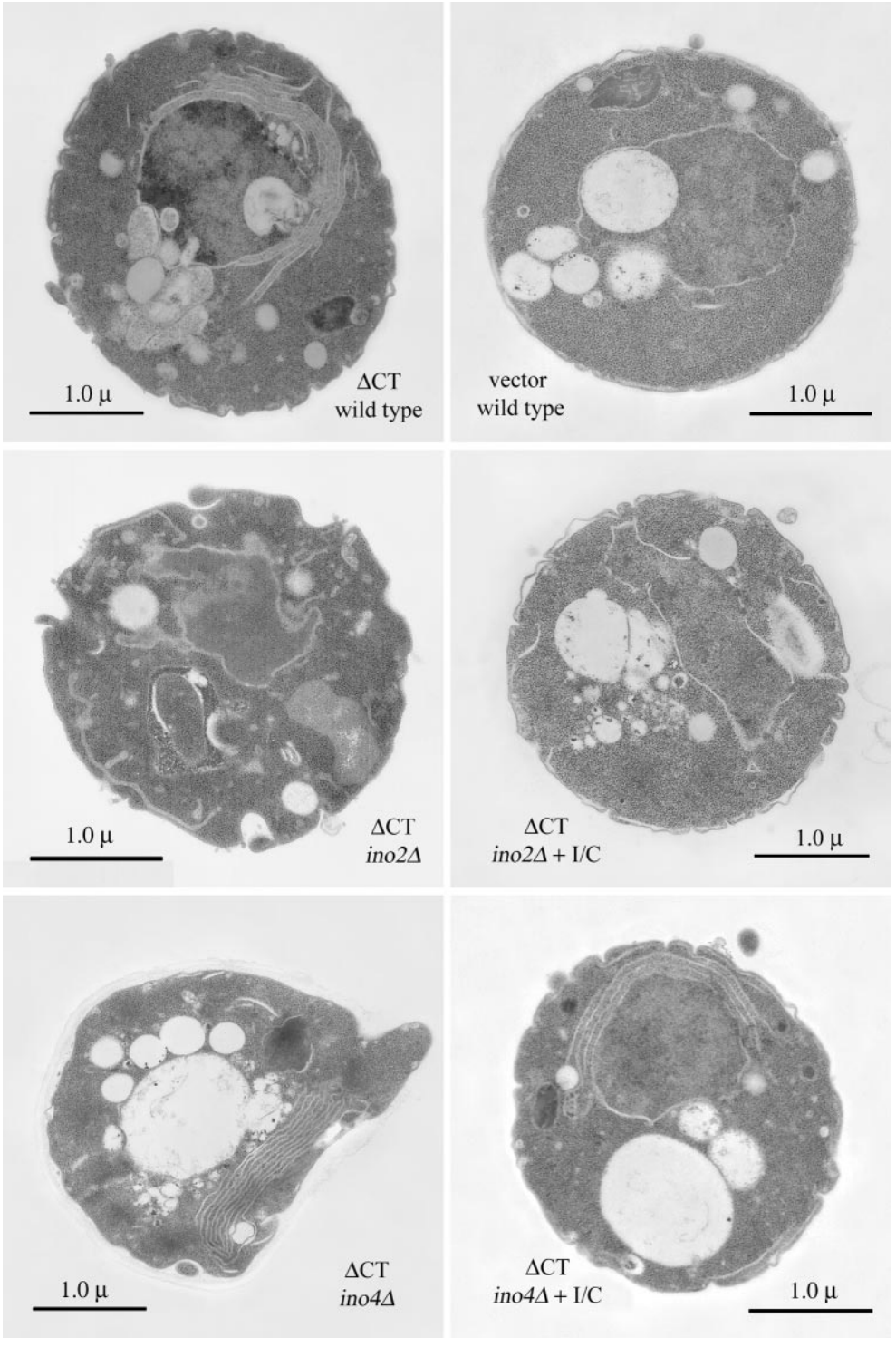


**Figure 7.** Addition of inositol and choline to *ino2*Δ cells expressing ΔCT does not restore membrane proliferation. Electron micrographs depict membrane morphologies of wild-type cells expressing ΔCT or vector (top panels). ΔCT-expressing cells contain perinuclear arrays of rough membranes. Cells deleted for *INO2* were not capable of membrane proliferation, and addition of inositol and choline failed to restore the ability to proliferate membranes (middle panels). Cells deleted for *INO4* expressing ΔCT were able to undergo membrane proliferation in the absence of inositol and choline (bottom panels). I/C, 75 μM inositol, 1 mM choline.

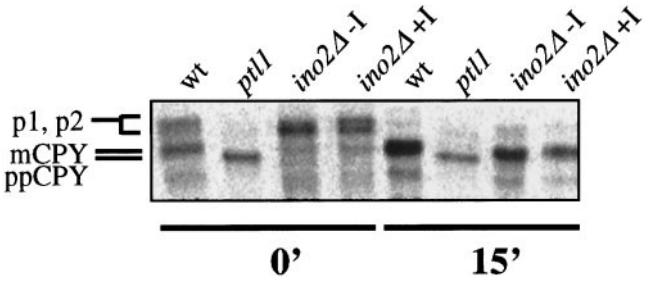


**Figure 8.** Translocation of CPY is normal in *ino2*Δ cells. A pulse-chase experiment of CPY shows the maturation of CPY in *ino2*Δ cells is similar to wild type. The mutant strain J51-5c (Toyn *et al.*, 1988), harboring a temperature-sensitive allele of *ptl1*, was used as a control for defective translocation. Cells were preincubated for 15 min at 37°C (wild type and *ptl1*) or 30°C (*ino2*Δ grown in the presence or absence of 75 μM inositol). Cells were pulse-labeled with $^{35}$S-methionine/cysteine for 10 min, chased with cold methionine/cysteine, and collected at the desired time points. Preparation of cell extracts and CPY immunoprecipitation was performed as described in MATERIALS AND METHODS. ppCPY: prepro-CPY; p1, p2: Golgi glycosylated CPY intermediates; mCPY: mature CPY. +I: 75 μM inositol.

were not compromised and appeared enhanced in their ability to proliferate membranes. Addition of inositol and choline to *ino2*Δ cells rescued viability but not the ability to proliferate membranes. Thus, our results establish a new role for Ino2p in membrane biogenesis that is distinct from its role with Ino4p in phospholipid biosynthesis.

Past work has implicated Ino2p, a member of the bHLH family of transcription factors, as a key regulator of phospholipid biosynthetic genes such as *INO1* in response to intracellular levels of inositol and choline. Ino2p was found to be present in a complex that binds to the $UAS_{INO}$ of the *INO1* promoter when inositol levels were limiting (Lopes and Henry, 1991; Nikoloff and Henry, 1994). Subsequent work identified Ino4p, another HLH protein, as essential for recruiting Ino2p to $UAS_{INO}$ (Ambroziak and Henry, 1994). The first six bases of the $UAS_{INO}$ consists of the consensus sequence, 5′-CANNTG-3′, recognized by the amphipathic helices of dimerized HLH domains (Ferre-D'Amare *et al.*, 1993; Ma *et al.*, 1994).

To date, Ino2p has only been known to function in phospholipid biosynthesis in conjunction with its putative binding partner, Ino4p. We have defined an essential role for Ino2p in the absence of Ino4p, where the absence of Ino4p appears to enhance membrane proliferation. We propose two models as to how Ino2p might function in membrane biogenesis in the absence of Ino4p: (1) Ino2p may bind to the $UAS_{INO}$ or other regulatory region by itself or (2) there may be an alternate binding partner or partners for Ino2p for the transcription of phospholipid biosynthetic genes in response to stimulated membrane biogenesis. We favor the second model, based on reports indicating that in vitro translated Ino2p is unable to bind the *INO1* promoter in the absence of Ino4p (Ambroziak and Henry, 1994) as well as studies using the yeast-two-hybrid system, suggesting that neither protein is capable of homodimerization (Schwank *et al.*, 1995).

Ino2p may form a heterodimer with another bHLH transcription factor. Mammalian proteins containing bHLH domains, such as Myc, Mad, Max, and Mxi, have the ability to form multiple heterodimer combinations (Amati and Land, 1994). The DNA-binding regions of Ino2p and Ino4p compared with the HLH-encoding regions of the mammalian Myc family of proteins revealed a high degree of similarity (Nikoloff *et al.*, 1992). In the case of Ino4p, there is some evidence for multiple partners. Yeast-two-hybrid analysis recently revealed interactions with four other known yeast bHLH proteins that have not been implicated in lipid biosynthesis: Pho4p, Rtg1p, Rtg3p, and Sgc1p (Robinson *et al.*, 2000). Ino4p has also been implicated in functioning independently of Ino2p in the synthesis of the sphingolipid biosynthetic enzyme, IPC synthase (Ko *et al.*, 1994). In this article we have presented evidence for Ino2p functioning independently of Ino4p, further indication that yeast HLH transcription factors can participate in multiple roles, possibly in multiple combinations, to regulate diverse biological processes.

The observation that addition of inositol and choline failed to rescue p180-induced membrane proliferation in *ino2*Δ cells raises the possibility that the assembly of lipid membranes is dependent on more proteins than merely those involved in inositol and choline phospholipid biosynthesis. Several genes involved in fatty acid and sterol biosynthesis as well as inositol transport have been reported as containing putative $UAS_{INO}$ elements in their promoters (Greenberg and Lopes, 1996). Functional analyses of many of these genes confirm a role for Ino2p and/or $UAS_{INO}$ in their activation (Chirala *et al.*, 1994; Koipally *et al.*, 1996; Grauslund *et al.*, 1999). Our microarray analysis of p180-expressing strains revealed several upregulated genes whose promoters contain known of putative $UAS_{INO}$ elements including *INO1, GUT1*, and *SCS3* (unpublished results). ΔCT-GFP–expressing strains harboring deletions in these genes accumulated membranes with abnormal morphologies as well as diminished membrane proliferation as quantified by flow cytometry (L. Block-Alper and D.I. Meyer, unpublished results). Although these membrane defects were not as severe as those observed for *ino2*Δ cells, it is possible that Ino2p may function to activate a subset of genes whose cumulative enzyme activities are necessary for membrane proliferation.

A recent observation suggests that phospholipid biogenesis is linked to ER perturbation. This has been demonstrated in cells that express high levels of certain ER membrane proteins as well as in cells that undergo an unfolded protein response (UPR) (Cox *et al.*, 1997). The UPR occurs when conditions disruptive to protein folding in the ER, such as the addition of reducing agents, trigger a signaling pathway from the ER that increases transcription of ER-localized chaperones such as *KAR2* and *PDI1* (Kohno *et al.*, 1993; see Chapman *et al.*, 1998 for review). The signal is transmitted through the ER transmembrane kinase, Ire1p, and cells that are deleted for *IRE1* cannot undergo a UPR (Cox *et al.*, 1993). Deletion of *IRE1* results in inositol auxotrophy, suggesting that the UPR and phospholipid biosynthesis may be linked (Nikawa and Yamashita, 1992). Moreover, wild-type cells that were induced to undergo a UPR had increased *INO1* transcription (Cox *et al.*, 1997). In addition, overexpression of HMG-CoA reductase, which triggers the proliferation of karmellae, impaired growth of *ire1*Δ cells, suggesting a block in membrane biogenesis, although

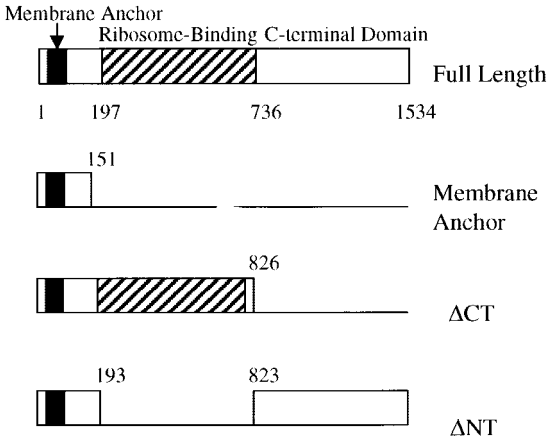


**Figure A1.** The p180 constructs used in this study. Full-length and various forms of p180 are shown. Numbers represent amino acid position; black boxes, hydrophobic amino acids predicted to span the ER membrane; and striped boxes, ribosome binding region.

membrane biogenesis per se was not assessed (Cox *et al.*, 1997).

However, others have shown that ER proliferation is not always linked to the UPR via Ire1p (Menzel *et al.*, 1997; Stroobants *et al.*, 1999). High levels of expression of cytochrome P450 were shown to result in accumulation of ER membranes and a concomitant upregulation of the ER chaperone, *KAR2*. In P450-expressing cells deleted for *IRE1*, membrane proliferation was still observed, although *KAR2* mRNA failed to be upregulated (Menzel *et al.*, 1997). In p180-expressing cells, increased levels of *KAR2* mRNA accompanied ER proliferation (Becker *et al.*, 1999). However, deletion analysis demonstrated Ire1p to dispensable for the production of membranes, as assessed by electron microscopy, as well as for the increased mRNA levels of *KAR2* (M. Hyde, L. Block-Alper, and D.I. Meyer, unpublished results). These findings suggest that there may be multiple mechanisms, Ire1p-dependent and -independent, for expanding the ER membrane and increasing its lumenal components.

The regulation of *INO2* is becoming increasingly complex. The *INO2* promoter contains a $UAS_{INO}$ element and has been shown to be autoregulated in response to levels of inositol and choline (Ashburner and Lopes, 1995a). In addition, *INO2* appears to be regulated at both the transcriptional and translational levels (Eiznhamer *et al.*, 2001). Transcription of *INO2* and *INO4* has also been reported as being regulated by the state of protein N-myristoylation (Cok *et al.*, 1998). In this article, we report that *INO2* mRNA is induced by expression of an integral ER membrane protein and that its gene product is essential for membrane proliferation. Induction of *INO2* in membrane proliferation appears to be independent of the cellular levels of phospholipid precursors, and p180-expression in wild-type cells failed to activate a $UAS_{INO}$-LacZ reporter construct (L. Block-Alper and D. I. Meyer, unpublished results). How then does the induction of ER membrane biogenesis lead to an increase in *INO2* mRNA? Is a signal sent from the ER that activates transcription of *INO2*? Is this signal mediated by a sensor in the ER membrane, such as the UPR is mediated by Ire1p? Further genetic and molecular analysis will help to uncover the mechanisms of *INO2* activation during inducible membrane biogenesis.

## APPENDIX

The constructs used for the cloning and plasmid transformation as described by Becker *et al.* (1999) are diagrammed in Figure A1.

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