Multiple Endoplasmic Reticulum-to-Nucleus Signaling Pathways Coordinate Phospholipid Metabolism with Gene Expression by Distinct Mechanisms

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In many organisms the coordinated synthesis of membrane lipids is controlled by feedback systems that regulate the transcription of target genes. However, a complete description of the transcriptional changes that accompany the remodeling of membrane phospholipids has not been reported. To identify metabolic signaling networks that coordinate phospholipid metabolism with gene expression, we profiled the sequential and temporal changes in genome-wide expression that accompany alterations in phospholipid metabolism induced by inositol supplementation in yeast. This analysis identified six distinct expression responses, which included phospholipid biosynthetic genes regulated by Opi1p, endoplasmic reticulum (ER) luminal protein folding chaperone and oxidoreductase genes regulated by the unfolded protein response pathway, lipid-remodeling genes regulated by Mga2p, as well as genes involved in ribosome biogenesis, cytosolic stress response, and purine and amino acid metabolism. We also report that the unfolded protein response pathway is rapidly inactivated by inositol supplementation and demonstrate that the response of the unfolded protein response pathway to inositol is separable from the response mediated by Opi1p. These data indicate that altering phospholipid metabolism produces signals that are relayed through numerous distinct ER-to-nucleus signaling pathways and, thereby, produce an integrated transcriptional response. We propose that these signals are generated in the ER by increased flux through the pathway of phosphatidylinositol synthesis.

The endoplasmic reticulum (ER) is a dynamic organelle that responds to environmental and developmental cues by regulating the levels of lipids and proteins required for the biogenesis and maintenance of membrane-bound compartments. It is the site of the synthesis and turnover of a major fraction of the lipid components that comprise the entire endomembrane system (1, 2), including phosphatidylinositol (PI), which is the precursor of the essential glycosylphosphatidylinositol lipids, sphingolipids, and phosphoinositides, as well as the soluble inositol polyphosphates (reviewed in Refs. 3–5). A variety of feedback-control systems have been described in animals and fungi that allow cells to monitor and adjust membrane constituents to their proper stoichiometry. For example, the sterol regulatory-element-binding protein-Scap pathway, which senses sterol levels in the ER, regulates the transcription of genes required for sterol biosynthesis (reviewed in Ref. 6), whereas the unfolded protein response (UPR) pathway, which senses secretory stress, regulates the expression of genes that are required for ER homeostasis (reviewed in Ref. 7). However, our understanding of how cells sense the ER membrane environment, then integrate and transmit these signals to the nucleus is incomplete.

The model eukaryote, Saccharomyces cerevisiae, adjusts its membrane lipid composition according to the availability of the soluble phospholipid precursors, inositol and choline (8–13). The addition of inositol to yeast cells starved for inositol induces a rapid and profound change in the pattern and synthesis of membrane phospholipids (11–13) and inositol-containing sphingolipids (14). PI levels increase up to 6-fold, while CDP-diacylglycerol and phosphatidic acid (PA), the immediate precursors to PI, are consumed. These changes in membrane lipid composition are sensed by Opi1p (12), which is localized to the ER through its association with the conserved vesicle-associated-membrane protein-associated protein, Scs2p (15), and PA (12). Upon addition of inositol, Opi1p rapidly dissociates from the ER, translocates to the nucleus, and represses the expression of the structural genes required for phospholipid biosynthesis (12, 16). The regulated expression of these structural genes requires both Ino2p and Ino4p, which bind as a heterodimer to the UASINO element present in the promoters of Opi1p target genes (17). Opi1p appears to mediate the repression of UASINO-containing genes through a direct interaction with Ino2p (18, 19).

In a previous study, we showed that the transcription of INO1, the most highly regulated of the Ino2p-Ino4p target genes (16), is rapidly repressed by Opi1p when inositol is added to the growth medium (12). In the present study, we profiled the temporal changes in global gene expression after the addition of inositol to the growth medium of dividing yeast cells. The goal...
of this experiment was to identify both the sequence and timing of every gene whose expression level responds to the rapid and profound changes in phospholipid metabolism that are induced by inositol supplementation (13). By comparing the identified patterns of gene expression with the measured ongoing and induced changes in phospholipid metabolism under identical growth conditions in a parallel study (13), we reasoned that we would identify metabolic signaling networks, originating at the site of membrane synthesis, that coordinate phospholipid metabolism with gene expression.

Our findings indicate that multiple distinct signals, produced by the rapid changes in phospholipid metabolism induced by inositol supplementation, emanate from the ER and directly control the expression of genes representing at least six distinct transcriptional profiles. These include phospholipid biosynthetic genes regulated by Opi1p, ER luminal protein folding chaperone and oxidoreductase genes regulated by the UPR pathway, and lipid-remodeling genes regulated by Mga2p, an ER-localized transcription factor that is activated by proteolytic cleavage from the membrane. Within a similar time frame following the exposure to inositol, numerous genes involved in ribosome biogenesis are transiently up-regulated. Simultaneously, cytosolic stress response genes are down-regulated, suggesting that growth in the absence of inositol is a stress condition that is alleviated by the addition of inositol. We also report that the UPR pathway is rapidly inactivated by inositol supplementation. Moreover, our results demonstrate that altering membrane lipid composition has acute effects on global gene expression networks and suggest that the interplay between phospholipid metabolism with gene expression.

Experimental Procedures

Strains and Plasmids—The wild-type strain BY4742 (MATa, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0) derived from S288C (20) was used in all microarray experiments. The isogenic hac1Δ strain (MATa, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, hac1Δ::KanMX) was purchased from Research Genetics (now Invitrogen). Individual strains containing single disruptions in Elo1, Mga2, Nte1, Spt23, Plb2, Tip1, or Yor175c, in which the entire targeted ORF was replaced with the HIS3 marker, were constructed by PCR-mediated gene replacement using the pFA6a-His3MX6 template (kind gift of M. Longtine) as described previously (21) in the wild-type strain BY4742. All strains were maintained on YPD plates (1% yeast extract, 2% bactopeptone, 2% glucose, and 2% agar). The hac1Δ strain was transformed with the centromeric YCP-HAC1Δintron plasmid (pHAC1', kind gift of K. Mori) and maintained on leucine dropout medium. The wild-type and isogenic mga2Δ strains were transformed with the pAM6 plasmid (kind gift of C. Martin), containing a tandem repeat LORE-CYC1-lacZ fusion (22), and maintained on uracil dropout medium. The mga2Δ strain was also co-transformed with the pAM6 plasmid and pAM31 centromeric plasmid (kind gift of C. Martin), containing MGA2 lacking codons for the C-terminal 172 amino acids (23), and maintained on uracil and leucine double dropout medium.

Cell Growth—Cells were grown in chemically defined synthetic growth media for at least twelve generations without inositol containing 1 mM choline-Cl at 30 °C to mid-logarithmic growth phase exactly as previously described (16). myo-Inositol was then added to the growing culture at a final concentration of 75 μM. Samples were harvested by filtration immediately prior to the addition of inositol and at 5-, 15-, 30-, 60-, and 120-min intervals following the addition of inositol. Harvested cells were flash frozen and stored at −80 °C.

RNA Isolation and Microarray Analysis—Total RNA was obtained from cells by the high temperature acid phenol method, and mRNA was isolated using oligo(dT) cellulose as previously described (16). mRNA from cells collected immediately prior to the addition of inositol was used as a common reference. Cy5- and Cy3-labeled cDNA probes were synthesized from mRNA collected at each time point, combined with fluorescently labeled cDNA probes from the reference sample, and hybridized to Corning CMT Yeast-S288c Gene Arrays (version 1.32, Corning, Inc.) containing 6,135 unique Saccharomyces cerevisiae ORFs as described previously (16). Hybridized microarray slides were simultaneously scanned with lasers at 532- and 635-nm bandwidths using a GenePix 4000B array scanner (Axon Instruments, Inc.). Each experiment was performed in quadruplicate.

Statistical Analysis—To ensure that dye-specific biases were not introduced, a dye-swap design was performed for each time point/reference pair. Image analysis for each array was processed using the GenePix Pro 4.0 (Axon Instruments, Inc.) software package, which produces fluorescence intensity pairs (R and G) for each gene. After image acquisition, individual data spots on each microarray were visually inspected for size, signal-to-noise ratio, background level, and uniformity. Using these quality control criteria, ~14% of the spots for each quadruplicate set of experiments were discarded because of poor spot quality, a conventional practice for microarray data analysis (24). As a result, we obtained 5991 unique ORFs for our subsequent analysis. Normalization for each slide was conducted as follows: let \( M = \log_2(R/G) \), and \( A = 0.5\log_2(RG) \).

The log ratio \( M \) is dependent upon the overall spot intensity \( A \) (25). This intensity-dependent trend for each replicate was fitted using the PROC LOESS function (26) in SAS. Next, the log ratio values (\( M \)) were normalized by subtracting the trend values. For each ORF, in which at least three high quality replicates were available at each time point, a Student \( t \) test was performed on normalized \( M \) using the null hypothesis of no change in expression (\( i.e. \) normalization to \( M = 0 \)). The \( t \) statistic was computed for each ORF at each time point, and 1455 ORFs were selected with a \( p \) value of \( \leq 0.025 \) for at least one of the five time points. From this set of ORFs, 712 unique genes were selected whose absolute normalized \( M \) values exhibited \( \approx 0.5 \)-fold change in at least a single time point. For these genes, a B-spline projection was performed followed by principle component analysis for the projected values, which captures the variance in a dataset (27). The principle components were then subjected to hierarchical clustering, a technique for finding gene clusters based on co-expression (28), and 28 clusters were...
formed. When used sequentially, principle component analysis and hierarchical clustering are a powerful statistical method for identifying regulated patterns in microarray time-course data (29). Because a common reference sample was used, the absolute abundance for eight representative mRNAs (ACT1, ELO1, FAA4, HAC1, INO1, KAR2, OLE1, and TCM1) over the time course was verified by quantitative Northern blot analysis. These data obtained by Northern blotting agreed with the ratios obtained by the microarray analysis; therefore, changes in the ratios measured by microarray analysis should reliably reflect actual changes in mRNA levels at each time point.

Enrichment for specific transcription factor gene associations in our dataset was determined utilizing the yeast genome-wide location analysis dataset of Harbison et al. (30). The number of promoter regions in the 712 significantly regulated genes that contained a bound transcription factor (X), using a p value threshold of 0.005, was counted. This number was compared with the total gene occupancy across the genome for the same transcription factor (Y). Any specific transcription factor with a ratio X/Y > 1 was considered to be enriched. From this analysis, 58 transcription factors out of a total of 203 yeast transcription factors for which the genomic occupancy was determined (30) were enriched in our dataset. Gene ontology term associations for the yeast genome were obtained from the Saccharomyces Genome Database (www.yeastgenome.org) in August, 2005. The list of UPR pathway target genes was obtained from Travers et al. (31). Promoter analysis was performed using RSA TOOLS (rsat.ulb.ac.be/rsat/) (32) using the LORE consensus WCYCAACAA (22) as the seed sequence.

Northern Blot Analysis—2 μg of total RNA or 250 ng of mRNA was fractionated on 1.1% glyoxal agarose gels and transferred to Nytran SuPerCharge nylon membranes (Schleicher and Schuell) using a turboblotter (Schleicher and Schuell) as described (33). Strand-specific 32P-labeled riboprobes were synthesized from linearized plasmids pH310-INO1 (34), pAB309A-TCM1 (35), pGEM-HAC1 (36), pSJ29-KAR2 (16), pSJ33-OLE1, pSJ34-ACT1, pSJ35-FAA4, pSJ36-lacZ, and pSJ37-act1 by in vitro transcription according to the manufacturer’s instructions (16) and hybridized to membranes in formamide hybridization buffer as described (16). Quantitation was performed by phosphorimaging on a STORM 860 PhosphorImager (Amersham Bio-}

**RESULTS**

Previously, we reported the steady-state genome-wide expression profiles of wild-type yeast cells grown for approximately twelve generations in various combinations of the phospholipid precursors inositol and choline (16). This earlier study provided a static snapshot of the global differences from gene expression in cells grown under conditions that are known to alter phospholipid metabolism (11–13). We found that the individual effects of inositol and choline on gene expression are distinct but that the combined effects of inositol and choline significantly increase the number of genes that respond to either inositol or choline alone. From this study we concluded that inositol plays the major role in mediating target gene expression due to its dramatic effects on phospholipid metabolism and that choline exaggerates this effect (13, 16).

In a recent study from our laboratory (13), we reported that the addition of inositol to cells grown in media lacking inositol, but containing choline, leads to a rapid and dramatic reprogramming of cellular lipid synthesis and turnover within a 30- to 60-min time frame. In the present study, we utilized microarray analysis to profile both the timing and ordering of genome-wide transcriptional changes following the addition of inositol in wild-type yeast cells. The time course and growth conditions were chosen to examine both the immediate and long term changes in gene expression and to allow a direct comparison to the metabolic profile of the changes in lipid metabolism induced by inositol addition (13). The complete dataset is available in Supplemental Data.

Overview of Gene Expression Profiles following Inositol Supplementation—We selected ORFs for analysis that showed a p value ≤ 0.025 over four replicates following normalization of our microarray data (described under “Experimental Procedures”). Using these criteria, we detected 712 genes that showed ≥0.5-fold change in expression in at least one time point over the course of the experiment. As we observed for specific phospholipids in our metabolic profiling experiments (12, 13), the transcriptional response to the addition of inositol was extremely rapid, with most genes showing a change in expression at early time points. Both transient and sustained changes in the patterns of gene expression were observed, revealing a dynamic transcriptional response following the addition of inositol (Fig. 1).

To identify regulated patterns of expression from our kinetic genome-wide analysis, each significantly regulated gene was grouped into 1 of 28 clusters showing a similar pattern of expression using the method of principle component analysis (27) followed by hierarchical clustering (28). Next, each cluster was examined for evidence of co-regulation using the four criteria described below. First, we utilized the comprehensive data set from the genome-wide location analysis study conducted by Harbison et al. (30), which identified the genomic binding sites for 203 transcriptional regulators. We compared their data set (30) with the set of genes that were identified in our present study and focused our analysis only on those transcription factors whose gene associations were significantly enriched in our dataset (see Supplemental Data for the list of enriched transcription factors). As expected, genes bound by Ino2p, Ino4p, and OpI1p were significantly enriched in our data set. Next, we examined for enrichment of gene ontology terms annotated in the Saccharomyces Genome Database within each cluster. Third, given that a set of UPR pathway target genes were detected in our previous microarray study (16), we inspected
Supercluster I (Ribosome Biogenesis)—The expression pattern of this supercluster, which consists of genes from a single cluster identified by our statistical analysis, showed an increase in expression that that peaked by 15 min and returned to basal level by the 120-min time point (Fig. 2A). This supercluster is enriched for genes involved in ribosomal large subunit assembly and maintenance, 20S pre-rRNA processing, RNA polymerase I transcription, 35S primary transcript processing, and rRNA modification and processing (Fig. 2B). The expression pattern for the set of genes involved in ribosome biogenesis is tightly clustered, suggesting an increased demand for ribosomes immediately following the addition of inositol. Approximately one-half of the genes in this supercluster are involved in ribosome biogenesis (Fig. 2C).

Supercluster II (Amino Acid and Purine Metabolism)—The profile of expression of genes in this supercluster, which includes genes from a single cluster, showed a steady but gradual increase of expression throughout the time course (Fig. 3A). One quarter of the genes within this supercluster comprises genes that were shown by Harbison et al. (30) to contain bound Gcn4p or Bas1p transcription factors (Fig. 3, B and C). Gcn4p is a transcription factor that activates the transcription of amino acid biosynthetic genes in response to amino acid starvation (37). Gcn4p has also been shown to play a role in activating a subset of UPR target genes (38); however, none of the Gcn4p target genes found in this supercluster are known to be regulated by the UPR pathway. Bas1p is a transcription factor involved in regulating basal and induced expression of genes of the purine and histidine biosynthesis pathways (39, 40). Specifically, genes involved in the synthesis of adenine, histidine, and arginine are enriched in this supercluster.

Supercluster III (Stress Response)—Genes within this supercluster, which is composed of two gene clusters detected in our statistical analysis, were rapidly repressed and returned to basal expression level by 120 min (Fig. 4A). Over one-quarter of these genes that made up this supercluster are genes involved in stress responses, including ten genes in the cytoplasmic heat shock protein family of protein folding chaperones, HSC82, HSP30, HSP42, HSP78, HSP82, HSP104, SIS1, SSA1, SSA2, and STT1, and genes regulated by heat shock transcription factor (41) (Fig. 4, B and C). In addition, genes bound by both Oaf1p-Pip2p (30), transcription factors that regulate genes involved in peroxisome formation (42, 43), were enriched in this cluster (Fig. 4, B and C).

Supercluster IV (Ino2p-Ino4p Targets, UPR Pathway, and Ty Element)—Genes from five individual clusters identified in our statistical analysis were grouped to form this supercluster (Fig. 5A), which showed a rapid repression of gene expression levels that was sustained throughout the time course of the experiment. Three sets of genes were highly enriched in this supercluster, including genes containing bound Ino2p and Ino4p transcription factors, genes regulated by the UPR pathway, and Ty element retrotransposon genes (Fig. 5B).

Within the set of Ino2p-Ino4p targets, comprising 15% of the total number of genes in this supercluster (Fig. 5C), are genes that were previously identified as having the UASINo promoter element that are negatively regulated by inositol (16). Among this group are genes encoding enzymes for phospholipid bio-
synthesis (ACC1, CDS1, CHO1, CKII, CPT1, FAS1, FAS2, INO1, OPI3, PDI1, and PSD2), transcriptional regulators (INO1 and OPI1), and the inositol (ITR1) and choline (HMN1) transporters (Fig. 5B). Of this set of Ino2p-Ino4p target genes, only INO1 had previously been shown to exhibit the rapid kinetics for repression shown here (12). The present study is the first report to show that the entire set of Ino2p-Ino4p target genes are co-regulated temporally, and thus are likely to respond to the same signal, namely, PA levels directly sensed by Opi1p (12).

Interestingly, approximately 20% of the genes in this supercluster were previously shown to be regulated by the UPR pathway (31) and include all of the canonical UPR target genes, including EUG1, KAR2, PDI1, ERO1, LHS1, and SCJ1 (Fig. 5B). The expression profile of the UPR target genes was similar to the profile of many of the Ino2p-Ino4p target genes, except for a short delay in the repression kinetics at the beginning of the time course (Fig. 5A), suggesting that the UPR pathway is rapidly shut off upon the addition of inositol (see below).

In addition, 16 genes involved in Ty element transposition were also detected within this supercluster (Fig. 5B). We previously reported that the steady-state expression of Ty2 element TYA GAG ORFs are significantly repressed in the presence of inositol in the growth media (16). Supercluster IV also includes genes of the Ty1 element retrotransposon family, but Ty1 and
Ty2 element genes show different expression profiles at steady state (see Fig. 7 below) (16).

Supercluster V (Mga2p-regulated)—The expression profile for this supercluster, containing genes from two individual clusters, showed a dramatic increase in expression of genes that peaked by the 15-min time point, returned to basal level by the 30-min time point, and were slightly repressed by 60 min (Fig. 6A). This supercluster included genes implicated in lipid remodeling, including ATFI, encoding an alcohol acetyltransferase (22, 44); PLB2, encoding a phospholipase B (45); FAA4, encoding a fatty acyl-CoA synthetase (46); IZH1, IZH2, and IZH4, encoding a family that potentially alters membrane sterol content (47); OLE1, encoding the sole Δ9 fatty acid desaturase in S. cerevisiae (48); TIP1, encoding a potential lipase (49); and YOR175C, which is homologous to membrane-bound O-acetyltransferase genes (50) (Fig. 6B). Comparison of this set of genes to the genome-wide location analysis dataset from Harbison et al. (30) did not reveal enrichment for any specific transcription factor. However, upstream promoter analysis revealed that three-quarters of the genes within this supercluster contained the low oxygen-responsive element (LORE) (Fig. 6C), suggesting a common means of regulation. The transcription factor Mga2p has previously been shown to function through this element (23, 51). The mechanism for this regulation was investigated further (see below).

Most Genes Achieve Steady-state Expression Levels by 120 Min after Inositol Addition—Next we asked whether the expression profile of all genes following the addition of inositol had reached their steady-state level of expression within the 2-h time course. To this end we compared the expression level of each gene at the 120-min time point with its expression level after 12 generations of continuous growth in the presence of inositol obtained from our previous microarray analysis (16). This comparison showed that approximately 90% of the genes reached their steadystate expression level by 120 min following the addition of inositol (Fig. 7), suggesting that most changes in gene expression resulting from the addition of inositol are rapid and sustained until steady state. From this analysis, we concluded that the design of our current time-course microarray study effectively captured the full range of the dynamic transcriptional changes induced by the addition of inositol.

However, several interesting sets of genes were detected in the remaining 10% of genes that were not expressed at their steady-state level after 2 h (Fig. 7). First, although the vast majority of Ino2p-Ino4p target genes achieved their steady-state expression level by the 120-

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FIGURE 4. Gene expression profile of supercluster III (stress response). A, expression ratio plots of individual genes from two identified clusters (described under “Experimental Procedures”) showing similar patterns of expression are shown. Those genes classified as “Response to Stress” (teal) were annotated according to gene ontology terms found in the Saccharomyces Genome Database or genes regulated by heat shock transcription factor (41). Oaf1p-Pip2p target genes (aqua) were shown by Harbison et al. (30) to contain bound Oaf1p and Pip2p transcription factors. Genes that do not fit these annotations are labeled in gray. B, expression ratios of individual genes in “Response to Stress” or Oaf1p-Pip2p target gene categories. C, the relative number of genes present in categories “Response to Stress” (teal), Oaf1p-Pip2p target genes (aqua), and other gene annotations (gray).

A

B

C

Cluster 13

Cluster 26

Min

Er

Nucleus

Signal

Induced

Inositol
VTC3, and VTC4, were significantly down-regulated in the presence of inositol (16). However, in the present study, these genes were not significantly repressed by 120 min following the addition of inositol (Fig. 7). The zinc-regulated transcription factor gene, ZAPI, is also repressed at steady state but not at 120 min (Fig. 7). On the other hand, two zinc transporter genes, ZRT1 and ZRT2, were highly up-regulated at the 120-min time point but showed little differential expression by steady state (Fig. 7). Both phosphate and zinc are important nutrients that play important roles in regulating phospholipid metabolism (57–63). Taken together, these results suggest that long term adaptation to growth in the presence of inositol may require reprogramming of a number of regulatory circuits that sense nutrient availability.

The steady-state expression levels of Ty2 element transcripts are significantly repressed at steady state in cells grown in the presence of inositol and choline, whereas Ty1 element transcript levels were not significantly altered in cells grown in the absence or presence of either precursor (16). In the present study, we detected significant repression of seven Ty1 element genes at the 120-min time point (Fig. 7). However, these genes are expressed at basal levels or are slightly up-regulated at steady state (16). Thus, both the Ty1 and Ty2 element retrotransposon families are initially repressed upon the addition of inositol, but regulation of the long term expression levels of each set of genes presumably respond to different signals.

The UPR Pathway Is Rapidly Inactivated upon the Addition of Inositol—Yeast cells grown in the absence of inositol induce the UPR pathway (36, 64, 65), an ER-to-nucleus signaling pathway that is activated in response to secretory stress (reviewed in Ref. 7). Activation of Ire1p, an ER-localized transmembrane protein that senses elevated levels of unfolded luminal ER proteins (66, 67), triggers the UPR response after its activation by oligomerization and autophosphorylation (68–71). Activated Ire1p subsequently catalyzes the unconventional splicing of the HAC1u (uninduced HAC1) transcript (72), relieving a translation block of this mRNA (73). Spliced HAC1i transcript (induced HAC1) is efficiently translated into active Hac1p transcription factor, which activates the transcription of target genes, including ER-luminal protein folding chaperones and oxidoreductases.
min, the level of spliced HAC1 mRNA was reduced by ~1.5-fold and was virtually undetectable by 15 min (Fig. 8A). The rate of disappearance of the spliced HAC1i transcript is consistent with the kinetics of repression that was measured for KAR2 and other UPR pathway target genes (Figs. 5 and 8A, top panel). Given that only the spliced HAC1i is efficiently transcribed, and the measured half-life of the Hac1p transcription factor is only 1–2 min (80, 81), these data demonstrate that the UPR pathway is rapidly and completely inactivated upon the addition of inositol to yeast cells starved for inositol. To our knowledge this is the first reported example of conditions that rapidly deactivate the UPR pathway in any organism.

The UPR Pathway Does Not Directly Control the Expression of Ino2p-Ino4p Target Genes—Activation of the UPR pathway is thought to boost ER secretory capacity not only by increasing the abundance of essential ER luminal protein folding components but also by activating the synthesis of membrane phospholipids (82). For example, overexpression of the mammalian homolog of Hac1p, XBP1, in B-lymphocytes results in increased phosphatidylcholine biosynthesis (83). In yeast, it has been proposed that activation of the UPR pathway is required for the activation of INO1 and other co-regulated phospholipid biosynthetic genes (31, 65, 84). These studies suggested that Hac1p negatively regulates the activity of the Opi1p repressor (65, 84). For example, Brickner et al. (84) suggested that Hac1p may participate in sequestering Opi1p in the ER under conditions of UPR activation, allowing the efficient transcription of Ino2p-Ino4p target genes. Thus, the regulation of UPR pathway and Ino2p-Ino4p target genes may be directly coupled. Consistent with this model, we observed that both UPR pathway and Ino2p-Ino4p target genes are repressed with similar kinetics (Figs. 6 and 8A).

To address whether the UPR pathway directly participates in the rapid Ino2p-Ino4p target gene repression upon the addition of inositol, we measured the kinetics of INO1 repression after inositol supplementation under conditions when the UPR is constitutively activated. A hac1Δ strain was transformed with a plasmid containing a modified HAC1 gene, in which its intron sequence was deleted. This “intronless” HAC1 construct (pHAC1i) expresses the translationally active HAC1i mRNA, which continuously produces the Hac1p transcription factor even under non-ER stress conditions, thus constitutively activating the UPR. Using growth conditions identical to those used in the experiment with wild-type cells, inositol was added.
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A

KAR2

INO1

HAC1^u

HAC1^i

% spliced: 19 13 1 0 0 0

time (min) 0 5 15 30 60 120

B

KAR2

INO1

HAC1^i

time (min) 0 5 15 30 60

C

time (min) 0 20 40 60

INO1 fold repression

Intact UPR

Constitutive UPR

FIGURE 8. Kinetics of KAR2 and INO1 repression and HAC1 splicing and repression in cells with a wild-type or constitutively activated UPR pathway following inositol addition. A, wild-type cells were grown in the absence of inositol to mid-logarithmic growth phase, conditions that are known maximally derepress INO1 expression and activate the UPR pathway. KAR2, INO1, and HAC1 transcripts from wild-type cells immediately prior to and at indicated time points following the addition of inositol to a final concentration of 75 μM were analyzed by Northern blotting. The percent amount of HAC1 transcript present in its spliced form (HAC1^i), which indicates UPR pathway activation, was determined by phosphorimaging analysis. B, KAR2, INO1, and HAC1 transcripts from hac1Δ cells transformed with a single copy plasmid (pHAC1) expressing the active HAC1^i, which lacks the HAC1 intron sequences, were grown and analyzed as described in A. C, the fold repression of INO1 transcripts from panel A, containing a wild-type UPR pathway, and panel B, containing a constitutively active UPR pathway, were quantitated by phosphorimaging analysis.

to pHAC1^i transformants grown to mid-logarithmic phase in the absence of inositol, and KAR2, INO1, and HAC1 transcripts were measured at various time intervals by quantitative Northern blotting. As expected, KAR2 mRNA levels remained constant throughout the experiment, consistent with continuous UPR activation (Fig. 8B, top panel). Importantly, this result shows that addition of inositol cannot override the Hac1p-de-
the expression of FAA4, whose transcriptional regulation has not been reported, from wild-type and \( \text{mg}a2 \Delta \) strains after inositol addition by Northern blotting. Whereas the expression pattern of FAA4 in wild-type cells was similar to our microarray data (Fig. 9B, top panel), FAA4 mRNA levels from the \( \text{mg}a2 \Delta \) strain were unchanged throughout the time course of the experiment (Fig. 9B, bottom panel). These results demonstrate that the transient induction of FAA4 by inositol is controlled by Mga2p.

Previous work has shown that Mga2p activates transcription through the LORE element present in the promoter of OLE1 (51). Given that three-quarters of the genes present in supercluster V contain a LORE element (Fig. 6), we asked whether the inositol-mediated signal for the induction of these genes was mediated through this promoter element. To directly test this hypothesis, a wild-type strain transformed with a LORE reporter construct was analyzed for \( \text{lacZ} \) expression by quantitative Northern blot after inositol supplementation. The expression pattern of \( \text{lacZ} \) driven by the LORE element (Fig. 10A, top panel) was similar to the OLE1 and FAA4 expression profiles (Figs. 9 and 10B, top panel). The expression level of \( \text{lacZ} \) measured by Northern analysis was virtually identical to the \(-fold\) expression levels of OLE1 measured in the same blots (Fig. 10B, top panel). Following the addition of inositol, the expression levels of both \( \text{lacZ} \) and OLE1 were unchanged by 5 min, both transcripts showed a peak expression level increase of over 5-fold compared with basal expression by 15 min, were reduced to 2-fold by 30 min, and both were slightly repressed by 60 min, suggesting that the LORE element is the major transcriptional control element for the inositol-mediated response. Moreover, \( \text{mg}a2 \Delta \) strains showed no regulated expression from the LORE reporter (Fig. 10A, middle panel). Taken together, these results show that Mga2p regulates the expression of LORE-containing genes in supercluster V in response to inositol and expand the known number of Mga2p-regulated genes.

Importantly, the repression profiles of \( \text{INO}1 \) and \( \text{KAR}2 \) in response to inositol were unaffected in either the \( \text{spt}23 \Delta \) or \( \text{mg}a2 \Delta \) strains (data not shown), showing that neither transcription factor controls the inositol-mediated repression regulated by Opi1p or the UPR pathway.

Membrane-bound Mga2p Senses the Addition of Inositol and Operates through the LORE Element Present in Target Genes—To gain insight into how Mga2p senses the inositol-induced signal, we asked if membrane association of Mga2p is required for the transient expression of target genes. An \( \text{mg}a2 \Delta \) strain was transformed with an \( \text{MGA}2 \) construct lacking the codons for the C-terminal transmembrane domain. Using Northern blot analysis, this transformant, which expresses the soluble and active form of Mga2p, was tested for induction of LORE-\( \text{lacZ} \) expression by inositol. As shown in Fig. 10A, \( \text{lacZ} \) expression in the \( \text{MGA}2 \Delta \text{TMD} \) strain is constitutive (bottom panel), suggesting that the Mga2p\( \Delta \text{TMD} \) strain is continuously activating transcription through the LORE element. This is in agreement with results showing that Mga2p\( \Delta \text{TMD} \) acts as a powerful transcriptional activator (85). In addition, OLE1 was expressed at a basal level in the same transformant throughout the time course of the experiment (Fig. 10B, bottom panel). Importantly, neither OLE1 nor \( \text{lacZ} \) transcript levels in the \( \text{MGA}2 \Delta \text{TMD} \) strain were influenced by the addition of inositol. These results demonstrate that membrane association of Mga2p is necessary for the transient transcriptional activation of OLE1 and other co-regulated LORE genes in response to inositol, presumably by sensing the inositol-induced phospholipid metabolic changes in the ER.

Previous studies have shown that OLE1 expression, under the control of either full-length Mga2p or the constitutively active Mga2p\( \Delta \text{TMD} \), is strongly repressed by exogenously added unsaturated fatty acids (85), suggesting that Mga2p responds to multiple signals. Because the expression of many genes in supercluster V are repressed below basal level at the 60-min time point (Fig. 6), we hypothesized that a signal from fatty acid metabolism may be mediating this repression. However, in the \( \text{MGA}2 \Delta \text{TMD} \) strain both \( \text{lacZ} \) and OLE1 mRNA levels remain constant following the addition of inositol and are not repressed at the 60-min time point (Fig. 10, A and B). This result suggests that the repression of LORE-containing genes, following the addition of inositol, is not due to signals produced by exogenous unsaturated fatty acids.

Because supercluster V is highly enriched in genes involved in lipid remodeling, we asked whether the activity of specific genes in this set are required for the transient induction of LORE-containing genes. Deletion strains lacking genes found in supercluster V, including \( \text{TIP}1 \), which encodes a putative lipase, \( \text{YOR}175\text{C} \), which is homologous to membrane-bound \( \text{O-acetyltransferase} \) genes, and \( \text{PLB}2 \), which encodes a phospholipase B, were assayed for induction of OLE1 expression by inositol. In addition, two other strains lacking genes not found in supercluster V, including \( \text{NTE1} \), which encodes an ER-localized phospholipase B (94), and \( \text{ELO1} \), which encodes a fatty acid elongase (95), were analyzed. However, in single deletion strains lacking each of these genes, there was no effect on OLE1 regulation in response to inositol (data not shown), showing that the activity of these genes does not play in a direct role in

**FIGURE 9. Transient induction of OLE1 and FAA4 by inositol supplementation requires MGA2.** Yeast strains were grown to mid-logarithmic growth phase in the absence of inositol. RNA was extracted from yeast cells immediately prior to and following the addition of inositol at indicated time points. OLE1 (A) and FAA4 (B) transcript abundance at indicated times was analyzed by Northern blotting in both wild-type and cells lacking MGA2 (A and B) or cells lacking the MGA2 homolog, SPT23 (A). TCM1 (A) and ACT1 (B) transcript levels, whose expression is unaffected by inositol supplementation (Supplemental Data), served as loading controls.
mediating the expression of LORE-containing genes by inositol.

DISCUSSION

We have shown that the addition of inositol to the medium of logarithmically growing yeast cells triggers a cascade of changes in global gene expression. We identified six distinct transcriptional responses that are affected by inositol supplementation. These include genes involved in phospholipid biosynthesis, ER homeostasis, and lipid remodeling, suggesting a dynamic reprogramming of expression of genes involved in membrane biogenesis and homeostasis in response to signals derived from phospholipid metabolism. We also found rapid changes in the expression of genes not directly involved in lipid metabolism, including ribosome biogenesis, cytosolic protein folding chaperones, and purine and amino acid biosynthesis. In a recent parallel study carried out by our laboratory, we showed that addition of inositol results in a major and rapid reprogramming of phospholipid metabolism, which results in a profound remodeling of the membrane lipid composition (13). We propose that signals generated in the ER, induced by changes in the pattern and rate of phospholipid synthesis, lead to the dramatic alteration of global gene expression. A model for how these signals are integrated and sensed will be presented at the end of this section.

Signals Resulting from Inositol Metabolism Lead to the Rapid Inactivation of the UPR Pathway

—Recent studies have suggested a direct connection between the activation of the UPR pathway and increased phospholipid biosynthesis (65, 83, 84). The results reported in the present study show that the transcriptional responses of the UPR pathway and Ino2p-Ino4p target genes occur virtually simultaneously when inositol is added to the cells (Figs. 5 and 8), suggesting that the regulated expression of these two sets of genes is coupled. However, we present strong and compelling evidence that these two sets of genes are separately regulated. The phospholipid biosynthetic genes controlled by Opi1p are rapidly repressed by inositol even when the UPR pathway is constitutively activated (Fig. 8). These results are in agreement with other studies indicating that the UPR pathway is not directly involved in transcriptional regulation of Ino2p-Ino4p target genes (16, 36, 64).

Although the mechanism for activation of the UPR pathway in cells starved for inositol is unknown, we propose that activation of the UPR pathway is part of a generalized stress response that occurs when cells are starved for inositol. Wild-type cells grown in the absence of inositol exhibit a markedly reduced rate of PI synthesis, and a lower PI content relative to cells grown in the presence of inositol (12, 13), suggesting that reduced PI synthesis and/or content may result in UPR pathway activation. In addition, we also observed the transient down-regulation of genes involved in cytosolic protein folding chaperones regulated by heat shock transcription factor (41) (Fig. 4), suggesting
that a higher expression of this set of genes is also required in the absence of inositol.

One question is whether the UPR pathway is sensitive to PI levels in particular or to overall phospholipid metabolism in general. In mammalian cells, blocking de novo synthesis of phosphatidylcholine through the CDP-choline pathway does not lead to activation of the UPR pathway (96). Moreover, yeast cells grown in the absence of choline, which lowers overall cellular phosphatidylcholine levels (13), do not induce the UPR pathway (16). Taken together, these results suggest that activation of the UPR pathway is responsive to overall PI levels, which are known to affect the level and synthesis of other essential inositol-containing components, such as glycosylphosphatidylinositol lipids (97) and sphingolipids (14), and may also influence levels of phosphoinositides and inositol polyphosphates.

Two likely scenarios may explain the mechanism for the rapid inactivation of the UPR pathway following the addition of inositol. First, current models for UPR activation suggest that Ire1p senses the buildup of unfolded proteins either directly (98) or indirectly through Kar2p (99–101). Complete inositol starvation in an Ino− mutant, which is incapable of producing inositol, leads to a rapid inhibition of processing of Gas1p, a glycosylphosphatidylinositol-anchored protein, and its exclusion from COPII vesicles. Trafficking of other secreted proteins in the same Ino− mutant also exhibit reduced rates of trafficking under identical growth conditions (97). Moreover, wild-type cells starved of inositol do not make sufficient levels to support the rate of PI synthesis observed in cells supplemented with inositol (13). Thus, limitation of PI may cause a slowing of exit of secreted proteins from the ER leading to activation of the UPR pathway. Indeed, Sec− mutants blocked at various points in the secretory pathway also lead to activation of the UPR pathway (36). The addition of inositol may stimulate the rapid clearance of proteins from the ER, alleviating the need for UPR pathway activation. Alternatively, growth in the absence of inositol might lead to oligomerization and subsequent activation of Ire1p due to the lower PI content in the ER. Increasing PI levels might change the biophysical properties of the ER membrane to favor the monomeric, inactive state of Ire1p.

Potential Inositol-induced Signals That Activate Membrane-bound Mga2p—Previous studies have shown that growth of cells under hypoxic conditions stimulates Mga2p proteolytic processing and its subsequent transcriptional activation of OLE1 through the LORE promoter element (22, 23, 51). Our results suggest that the regulation of OLE1 and other LORE-containing genes in response to inositol occurs through a similar mechanism. Introduction of inositol might induce metabolic changes that produce a transient hypoxic state, thereby activating Mga2p proteolytic processing. For example, inositol might increase the demand for the oxygen-dependent fatty acid desaturation step catalyzed by Ole1p, thereby lowering local membrane oxygen levels, which in turn are sensed by Mga2p. This model is similar to the mechanism by which the Schizosaccharomyces pombe proteins Sre1 and Scp1 monitor oxygen-dependent sterol synthesis and mediate a hypoxic response (102, 103). On the other hand, it has been suggested that the hypoxic induction of OLE1 is coupled to respiratory chain function (104), suggesting that mitochondria produce reactive oxygen species that are sensed by Mga2p. However, we did not detect an increase in oxygen utilization after inositol addition (data not shown). Future research will focus on determining the molecular target for the activation of Mga2p by inositol.

Multiple Transcriptional Regulatory Networks Respond to Inositol-induced Metabolic Signals from the ER—Analysis of changes in gene expression accompanying inositol addition provides a powerful model for dissecting the interplay of lipid metabolism with major signaling networks in yeast cells. We propose that the dramatic changes in membrane lipid composition induced by inositol supplementation (13) produce signals in the ER that are responsible for the changes in global gene expression that we detected in the present study. Previously, we showed that a drop in PA levels induced by inositol is responsible for the regulation of Ino2p-Ino4p target genes through Opi1p (12). Mga2p may respond to an inositol-induced signal related to the consumption of molecular oxygen, whereas Ire1p may directly sense PI levels or indirectly detect the rate of secreted protein exit from the ER. Moreover, the secretory capacity of the ER may produce additional signals that influence the expression of genes found in supercluster I (Fig. 2). The expression of genes required for ribosome biogenesis is influenced by secretory function in a manner independent of the UPR pathway (105). Finally, the inositol polyphosphate multikinase, Arg82p, which is a component of the ArgR-Mcm1 complex, regulates many of the ARG genes found in supercluster II, through the inositol lipid kinase activity of this enzyme (106). Thus, increased flux through the phosphoinositide and soluble inositol polyphosphate pathway could play a role in regulating this set of genes. Given the current state of knowledge of the phospholipid metabolic pathways and availability of yeast mutants, it will be possible to dissect the relative inputs from various pathways to identify signals that regulate the global expression response. The next step will involve tests designed to probe how the various signals are integrated to achieve membrane homeostasis.

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