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The Membrane Proteins, Spt23p and Mga2p, Play Distinct Roles in the Activation of *Saccharomyces cerevisiae OLE1* Gene Expression

FATTY ACID-MEDIATED REGULATION OF Mga2p ACTIVITY IS INDEPENDENT OF ITS PROTEOLYTIC PROCESSING INTO A SOLUBLE TRANSCRIPTION ACTIVATOR*

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The *Saccharomyces OLE1* gene encodes the Δ-9 fatty acid desaturase, an enzyme that converts saturated fatty acyl-CoAs into *cis*-Δ-9 unsaturated fatty acids. *OLE1* gene expression is regulated by unsaturated fatty acids, which repress transcription and destabilize the *OLE1* mRNA. Expression of *OLE1* is activated by N-terminal proteolytic fragments of two homologous endoplasmic reticulum membrane proteins, Spt23p and Mga2p. Disruption of either gene does not significantly affect cell growth or fatty acid metabolism; cells that contain null alleles of both genes, however, are unsaturated fatty acid auxotrophs. An analysis of *spt23*Δ and *mga2*Δ strains shows that Spt23p and Mga2p differentially activate and regulate *OLE1* transcription. In glucose-grown cells, both genes activate transcription to similar levels of activity. Expressed alone, Mga2p induces high levels of *OLE1* transcription in cells exposed to cobalt or grown in glycerol-containing medium. Spt23p expressed alone activates *OLE1* transcription to levels similar to those in wild type cells. *OLE1* expression is strongly repressed by unsaturated fatty acids in *spt23*Δ or *mga2*Δ cells, under all growth conditions. To test if *OLE1* expression is controlled by fatty acids at the level of membrane proteolysis, soluble N-terminal fragments of Spt23p and Mga2p that lack their membrane-spanning regions (Δtm) were expressed under the control of their native promoters in *spt23*Δ;*mga2*Δ cells. Under those conditions, Mga2pΔtm acts as a powerful transcription activator that is strongly repressed by unsaturated fatty acids. By comparison, the Spt23pΔtm polypeptide weakly activates transcription and shows little regulation by unsaturated fatty acids. Co-expression of the two soluble fragments results in activation to levels observed with the Mga2pΔtm protein alone. The fatty acid repression of transcription under those conditions is attenuated by Spt23Δtm, however, suggesting that the two proteins may interact to modulate *OLE1* gene expression.

The regulation of lipid metabolic enzymes is an essential process that affects growth, development, and many genetic diseases. Because of the importance of lipids to numerous cellular functions, eukaryotes have developed complex mechanisms for regulating lipid biosynthetic activity and membrane lipid composition.

The *Saccharomyces OLE1*[1] gene is a central enzyme in cellular lipid metabolism. It encodes a Δ-9 fatty acid desaturase, an intrinsic membrane enzyme that converts saturated fatty acyl-CoA substrates to monounsaturated fatty acid species by an oxygen-dependent mechanism (1–3). The unsaturated fatty acid products of Ole1p compose 75–80% of the fatty acyl groups in membrane lipids, and the regulation of its activity plays a dominant role in governing the composition and physical properties of membranes in growing cells. Expression of the *OLE1* gene is regulated by a number of physiological and nutritional controls, including nutrient fatty acids (3–6) and molecular oxygen (7–9). We have shown previously that unsaturated fatty acids exhibit strong repressive effects on *OLE1* expression at the levels of transcription (6) and mRNA stability (5). Fatty acid-mediated repression of transcription is mediated through at least two elements in the *OLE1* promoter. The fatty acid regulated (FAR) sequences (6) act as a primary transcription activation region under aerobic conditions. It can activate and confer fatty acid-mediated repression on a foreign gene when placed upstream of the *Saccharomyces CYC1* basal promoter elements. We have also identified a second low oxygen response element (LORE) that is positioned ~200 bases downstream from the FAR element (7). That element acts with the FAR element to strongly activate transcription in response to hypoxic conditions and on induction by cobalt, which is thought to interfere with an unidentified oxygen sensor. Tandem copies of the LORE element that are placed in a heterologous promoter exhibit hypoxic induced transcription activation and can be repressed by unsaturated fatty acids (7, 8).

Recent studies (10, 11) have identified regulatory circuits that employ intrinsic membrane proteins that act as lipid sensors. These control gene activity by a process that involves regulated intramembrane proteolysis (10, 11), which involves the proteolytic release of a fragment of an integral membrane

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[1] The abbreviations used are: *OLE1*, gene encoding Ole1p, an intrinsic membrane bound Δ-9 fatty acid desaturase; ER, endoplasmic reticulum; PCR, polymerase chain reaction; SREBP, sterol regulatory element binding protein; *ole1*Δ, null (gene disrupted) allele of the *OLE1* gene; *SPT23*, gene encoding the ER membrane protein Spt23p; *spt23*Δ, null (gene disrupted) allele of the *SPT23* gene; *MGA2*, gene encoding the ER membrane protein Mga2p; *mga2*Δ, null (gene disrupted) allele of the *MGA2* gene; FAR (fatty acid regulated) upstream activation sequences of *OLE1* promoter; LORE, low oxygen response element activation sequences of the *OLE1* promoter.

TABLE I
S. cerevisiae strains used in this study

Strain	Genotype	Source
DTY10A	*MATα, leu2-3,leu2-112,can1-100,ura-3-1,ade2-1,his3-11,his3-15(TRP1$^+$)*	This laboratory
DTY11A	*MATα, leu2-3,leu2-112,can1-100,ura-3-1,ade2-1,trp1-1(HIS3$^+$)*	This laboratory
1-1D	*MATα, leu2-3,leu2-112,can1-100,ura-3-1,ade2-1,his3-11,his3-15(TRP1+),* mga2Δ::LEU2;spt23Δ::LEU2	This laboratory
1-3D	*MATα, leu2-3,leu2-112,can1-100,ura-3-1,ade2-1,trp1-1,his3-11,his3-15, mga2Δ::LEU2;spt23Δ::LEU2*	This laboratory
1-8A	*MATα, leu2-3,leu2-112,can1-100,ura-3-1,ade2-1,his3-11,his3-15(TRP1+), mga2Δ::LEU2;spt23Δ::LEU2*	This laboratory
BY4741	*MATα, his3, leu2, met15, ura3*	ResGen
5968	*MATα, his3, leu2, met15, ura3, mga2::kanMAX4*	ResGen
4869	*MATα, his3, leu2, met15, ura3, spt23::kanMAX4*	ResGen
mga2Δ	*MATα, leu2-3,leu2-112,can1-100,ura-3-1,ade2-1,trp1-1(HIS3$^+$), mga2Δ::LEU2,(SPT23$^+$)*	This laboratory
*spt23*Δ	*MATα, leu2-3,leu2-112,can1-100,ura-3-1,ade2-1,trp1-1(HIS3$^+$), spt23Δ::LEU2*	This laboratory

protein that can serve as a transcription factor. The most well studied example is that of the sterol regulatory element-binding protein (SREBP), which is an integral endoplasmic reticulum (ER) membrane protein. Under conditions of sterol depletion, SREBP is cleaved by two membrane-bound proteases, releasing a soluble N-terminal polypeptide that contains DNA binding and transcription activation domains that participate in the activation of a number of lipid metabolic genes (10).

A second proteolytic activation mechanism of membrane proteins that controls *Saccharomyces cerevisiae OLE1* gene expression has been described recently (12–14). *SPT23* and *MGA2* are homologous genes that encode ER-resident membrane proteins. Disruption of either gene has little effect on the growth or production of unsaturated fatty acids, whereas disruption of both creates a synthetic auxotrophy for unsaturated fatty acids due to the loss of *OLE1* expression (12). Expression of N-terminal fragments of Spt23p and Mga2p further showed that those polypeptides, although they lack a functional DNA binding domain, serve as co-activators of *OLE1* transcription (12, 13). Membrane-bound Spt23p and Mga2p are apparently converted to these soluble active forms *in vivo* via a ubiquitin-mediated mechanism that involves the cleavage of a soluble 90-kDa N-terminal fragment from its inactive 120-kDa membrane-bound precursor by the 26 S proteosome (14). Evidence from those studies suggests that Spt23p processing may be selectively regulated by unsaturated fatty acids. The unsaturated fatty acids 16:1, 18:2, and 18:3 inhibited processing of Spt23p to its active 90-kDa form, whereas exposure to 18:1 failed to block the proteolytic step. The lack of inhibition of Spt23p proteolysis by the fatty acid that has the highest melting temperature suggests that the regulation mechanism might detect changes in membrane "fluidity" caused by the incorporation of the fatty acids into membrane lipids.

We have determined recently (15) that Mga2p is essential for the hypoxic induction of *OLE1* gene expression and that it is a component of the LORE DNA binding complex under those conditions. Given our previous observations (3–6) that *OLE1* is regulated by a wide range of unsaturated fatty acids at the levels of transcription and mRNA stability, we wanted to determine whether the proteolytically processed forms of Spt23p and Mga2p also contribute to controlling *OLE1* gene expression. To investigate whether the inhibition of Spt23p processing (and presumably a similar effect associated with Mga2p) is the basis for the fatty acid-mediated regulation of transcription, we tested the ability of cells to regulate *OLE1* transcription when only soluble forms of the two proteins were expressed. In this paper, we demonstrate that Spt23p and Mga2p have distinct but overlapping functions with respect to the activation of *OLE1* transcription. Evidence is presented that *OLE1* expression is strongly repressed by unsaturated fatty acids when activated by a soluble form of Mga2p and that Spt23p may act either as an activator of *OLE1* transcription or as a negative modulator of Mga2p fatty acid-mediated regulation.

EXPERIMENTAL PROCEDURES

Strains and Growth Media—S. cerevisiae strains used in this study are shown in Table I. Plasmids constructed for this study are shown in Table II. Standard yeast genetics methods were used for construction of strains bearing the appropriate mutations or gene disruptions. Yeast cells were grown at 30 °C in SDt (synthetic dextrose tergitol) drop out medium as described previously (6, 16). All growth media used in liquid cultures contained 1% tergitol Nonidet P-40 to disperse fatty acid supplements. Tergitol-Nonidet P-40 is not derived from fatty acids or fatty alcohols and is apparently not metabolized by yeast. 3% glycerol was used as an alternative carbon source for glucose in synthetic dropout media designated as SGt. Tergitol was obtained from Sigma. Fatty acids were obtained from Nu Chek Prep (Elysian, MN). *Escherichia coli* DH5α competent cells were obtained from Life Technologies, Inc. Growth tests were performed by monitoring A_{600} of the cultures or by hemocytometer counting.

Cloning and Disruption of SPT23 and MGA2—Strains containing disrupted forms of the *SPT23* and *MGA2* genes derived from the DTY11A background were constructed by standard yeast molecular biological methods using the cloned native gene sequences. DNA fragments encoding the *SPT23* genes and *MGA2* genes used for the gene disruptions were cloned by polymerase chain reaction (PCR) using *S. cerevisiae* strain DTY10A genomic DNA as a template. Oligonucleotide primers that were used for the PCR cloning are shown in Table III. Gene-disrupted strains in cells derived from the BY4741 backgrounds were obtained from Research Genetics Corp. (Huntsville, AL).

The *SPT23* gene was isolated using the PCR primer pair SF and SR, which amplifies a fragment extending from bases −1003 to +2152 of the chromosomal gene. Amplified fragments were ligated into vector pCRscript SK+. To assemble the gene disruption construct, a fragment extending from residues −48 to +1351 was removed by restriction digestion with *Xba*I and *Spe*I. The remaining vector fragment containing flanking *SPT23* sequences was blunt-ended and ligated with a blunt-ended *Hin*dIII fragment derived from the *Saccharomyces LEU2* gene to create the vector pCRspt23Δ::LEU2. The chromosomal *SPT23* gene was disrupted by transformation of the DTY11A strain with a DNA fragment isolated from the vector by digestion with restriction enzymes *Pst*I and *Not*I.

The *MGA2* gene was cloned using a similar strategy using the PCR primer pair MF and MR, which amplified a fragment extending from nucleotides −242 to +2992 of the chromosomal DNA. The gene disruption construct was created by replacing an *Xba*I/*Hpa*I fragment with the *LEU2* gene to create vector pCRmga2Δ::LEU2 by the above procedure. The chromosomal *MGA2* gene was disrupted with a DNA fragment derived from the vector by digestion with the restriction enzymes *Pst*I and *Not*I.

Construction of the spt23Δ;mga2Δ Strains—Strains containing disrupted forms of both *spt23*Δ and *mga2*Δ genes were constructed by crossing strains containing a disruption of one of the two genes. The diploid parent strain was sporulated, and haploid progeny were screened by comparison of growth on agar medium containing 0.5 mM 16:1 and 0.5 mM 18:1 or no fatty acids. Candidate strains that did not grow on the fatty acid-free medium were verified as authentic *spt23Δ; mga2*Δ strains by PCR analysis of both genes.

TABLE II
Plasmids used in this study

Plasmid	Description	Source
pCRmga2Δ::LEU2	Contains a disrupted form of the *MGA2* gene in which an internal *Xba*I/*Hpa*I fragment is replaced by the *LEU2* gene	This study
pCRspt23Δ::LEU2	Contains a disrupted form of the *SPT23* gene in which an internal *Xba*/*Spe*1 fragment is replaced by the *LEU2* gene	This study
pRSmga2NΔtm	Contains a fragment of the *MGA2* gene that includes 904 bases of its promoter and the 784 N-terminal codons of its protein coding sequence. The protein coding sequence lacks the C-terminal 329 codons that include the membrane-spanning elements of the encoded protein	This study
pRSspt23NΔtm	Contains a fragment of the *SPT23* gene that includes 1499 bases of its promoter and the 792 N-terminal codons of its protein-coding sequence. The protein-coding sequence lacks the C-terminal 290 codons that include the membrane-spanning elements of the encoded protein.	This study
p62::−934	Contains 934 bases of the *OLE1* promoter region and its 27 N-terminal codons fused in frame to the *E. coli lacZ* gene	Ref. 6
pCTm111	Contains bases −576 → 466 of the OLE1 promoter in *lacZ* test vector pCTm	Ref. 6
p62::−934Δ88	Derived from vector p62::−934 by deletion of an 88-base fragment from −576 → −489	Ref. 6
pAM6	Contains a tandem (+) repeat of the LORE element derived from bases −347 to −328 of the *OLE*1 promoter in the CYC1 basal promoter-*lacZ* fusion vector pTBA30	Ref. 7
pAM4	Derived from p62::−932 by mutagenesis of nucleotide substitutions (C324T,T341A,A339G) in the LORE region	Ref. 7

TABLE III
PCR primers used in this study

Oligonucleotides are designated by their homology to target gene sequences and numbered with respect to the position of the 5′ end of the primer relative to the +1 A of the ATG start codon.

Primer	Description
MF	5′-CTGATGTTACCCTAGAAATGTCG-3′
MR	5′-GATGCTGACTGCTCCACAATACTC-3′
SF	5′-CAGCGTAACTTCATCTAGCCAGTC-3′
SR	5′-GCTGCCAGATGTAATAGAGTTCTACC3′
MGApro-F	5′-CCAAATGTTCTTCCTTGCCAAG-3′
MGA2rev2	5′-CCG**CTCGAG**AATACTTCTCTTGCAGTTAATCCGTTG-3′
SPTpro-F	5′-CTTGCGTGCATGCACGTACACAC-3′
SPT23rev1	5′-CACT**CTCGAG**TGAATCACTGCCAACAATCCTTTTAGC-3′

Construction of SPT23Δtm and MGA2Δtm Expression Plasmids—To construct plasmids that express the ~90-kDa soluble forms of Spt23p and Mga2p under control of their native promoters, DNA fragments were amplified from their respective genes with oligonucleotide pairs MGApro-F/MGA2rev2 and SPTpro-F/SPT23rev1. Each amplified gene fragment was cloned into pCR-Script SK+ to yield plasmids pCRmga2NΔtm and pCRspt23NΔtm. Fragments containing the promoter and protein-coding sequences for each gene were then isolated by *Not*I and *Xho*I digestion and inserted into the centromeric plasmid pRS413 (Stratagene) yielding plasmids pRSmga2NΔtm and pRSspt23NΔtm.

β-Galactosidase Assays—β-Galactosidase activities of cells containing the *lacZ* reporter plasmids were performed as described previously (6). Cell densities for the assays were determined by measurement at A_{600}, and corrections for light scattering were made at A_{550}. At least three independent transformants were assayed for each experiment, and β-galactosidase activities are the result of at least three independent experiments. Each experimental assay was performed in duplicate.

Fatty Acid Repression/Derepression Studies—Cells containing *OLE1* promoter::*lacZ* reporter genes were tested according to protocols published previously (3, 6) that measure induction of reporter gene activity from the fatty acid-repressed state. Cells were pre-grown in the appropriate SDt dropout medium supplemented with 1% tergitol and 1 mM 18:2. Those cultures were used to inoculate (at 1/10 volume) fresh fatty acid-free medium or medium containing 1 mM 18:2, metal ions, or carbon source. Cells were then incubated for 6–24 h and harvested by centrifugation for analysis of β-galactosidase activity.

RNA Isolation, Quantitative Northern Blot Analysis, and Fatty Acid Analysis—RNA was isolated for Northern blot analysis and quantification by PhosphorImaging as described previously (5). Fatty acid methyl esters were extracted from cell pellets derived from strains grown on fatty acid-free medium and were first washed with cold water. Strains grown on fatty acid-supplemented media were first washed with 1% tergitol in deionized water and then cold water. Methyl esters were analyzed by gas chromatography as described previously (17).

RESULTS

Effects of Disruption of the SPT23 and MGA2 Genes on Growth and Fatty Acid Desaturase Activity—To examine the independent roles of the *SPT23* and *MGA2* genes on *OLE1* expression, we tested the effects on growth and cellular fatty acid composition in strains that contained disrupted forms of the two genes (Table IV). Although there were reduced levels of 16:1 in the gene disrupted strains, these were compensated for by increases in 18:1, so that all strains contained >68% total (16:1 + 18:1) unsaturated fatty acids. This indicates that disruption of either gene does not produce strong effects on overall *OLE1* expression under normal laboratory growth conditions. A *spt23*Δ;*mga2*Δ strain containing null alleles in both genes, however, exhibited a strict requirement for unsaturated fatty acids on liquid minimal medium, which supports the previous observations (12) that *SPT23* and *MGA2* have overlapping functions that are essential for *OLE1* gene expression.

Previous studies (12) using a strain that contained a disrupted *MGA2* gene and a temperature-sensitive allele of *SPT23* showed that very high levels of unsaturated fatty acids (~50%) persist in cellular lipids 15 h after a shift from permissive to restrictive temperatures. This suggested that high basal levels of *OLE1* expression might occur in the absence of both proteins. To determine whether *OLE1* continues to be expressed under those conditions, we examined the effects of fatty acid deprivation on the growth and relative desaturase activity of wild type and *spt23*Δ;*mga2*Δ cells that contained disrupted forms of both genes (Fig. 1, *A* and *B*). To monitor the *in vivo* activity of the desaturase, the *spt23*Δ;*mga2*Δ and wild type strains were initially grown on medium containing linoleic acid (18:2). Under those conditions,

TABLE IV

Fatty acid composition of strains that contain disrupted alleles of SPT23 and MGA2

Cells were grown to mid-logarithmic phase at 30 °C in fatty acid-free SDt medium. Cells were harvested and washed by centrifugation and subjected to HCl methanolysis as described under "Experimental Procedures." Data represent the results of analysis of lipids from three independent cultures of each strain ± S.D.

Strain	Fatty acid								
	14:0	14:1	16:0	16:1	18:0	18:1	26:0	26:0-OH	16:1+18:1
DTY11A	2.42 ± 0.01	1.68 ± 0.35	16.78 ± 2.70	41.59 ± 8.71	4.23 ± 0.96	29.39 ± 4.23	1.02 ± 0.67	2.89 ± 0.51	70.98
mga2Δ;SPT23	2.62 ± 0.27	2.30 ± 0.39	17.36 ± 0.34	36.54 ± 1.53	4.53 ± 0.08	32.21 ± 1.61	1.39 ± 0.14	3.04 ± 0.09	68.75
spt23Δ;MGA2	1.57 ± 0.18	1.80 ± 1.97	14.40 ± 1.3	33.62 ± 0.45	4.86 ± 0.66	39.12 ± 0.85	1.48 ± 0.05	3.16 ± 0.18	72.74

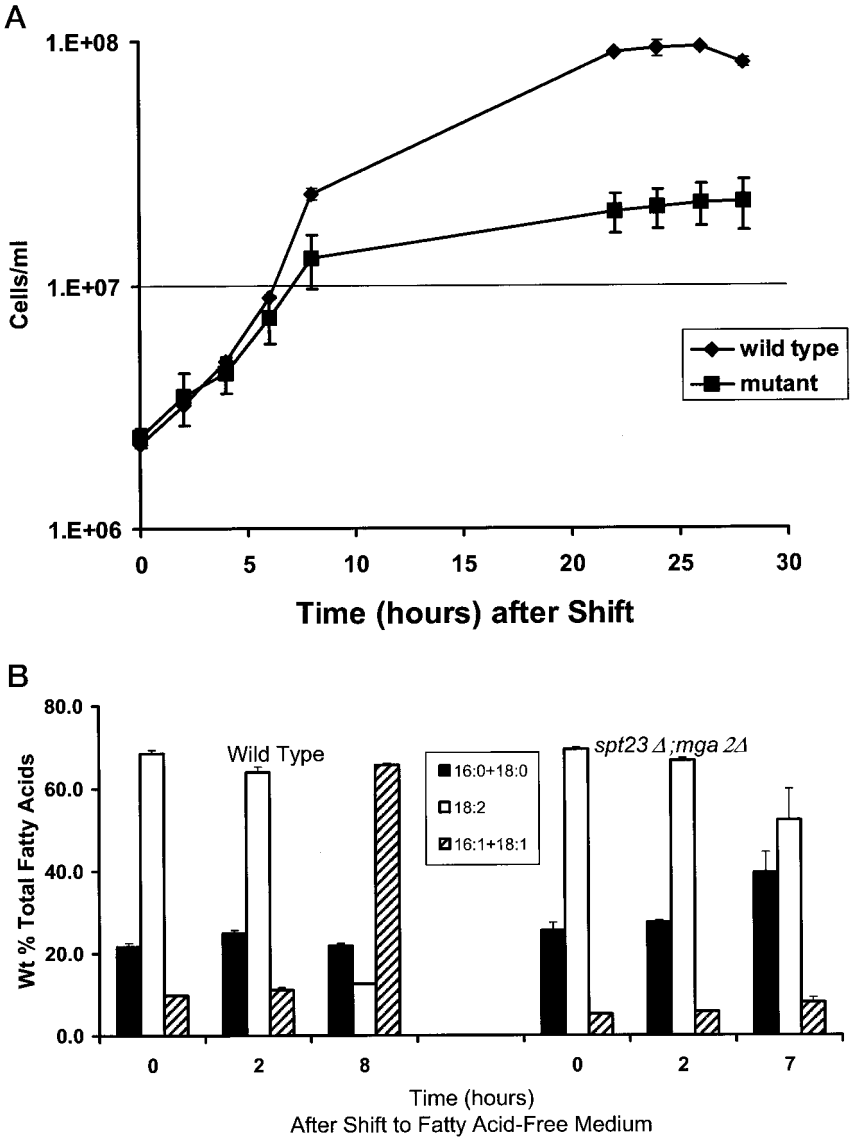

FIG. 1. **Growth and fatty acid compositions of wild type and *spt23Δ;mga2Δ* cells following a shift from 1 mM 18:2-supplemented SDt medium to fatty acid-free medium.** *A,* cells were grown to logarithmic phase at 30 °C on SDt medium containing 1 mM 18:2. At 0 h, cells were harvested and washed 1× with fatty acid-free SDt medium and then resuspended in SDt medium without fatty acids. Growth was monitored by A_{600}. Data shown represents average cell densities for wild type (2 experiments) and 3 independently isolated *spt23Δ;mga2Δ* strains ± S.D. *B,* levels of total C_{16-18} saturated fatty acids (*black bars*), 18:2 (*open bars*), and total C_{16-18} monounsaturated (*striped bars*) in wild type and *spt23Δ;mga2Δ* cells pregrown on 1 mM 18:2 and at 0, 2, and 8 h after transfer to fatty acid-free (NFA) medium.

wild type desaturase activity is repressed, and over multiple generations, the endogenous 16:1 and 18:1 products of the enzyme are replaced in cellular lipids by the exogenous 18:2. Cells were then transferred to fatty acid-free medium to follow the induction of Ole1p-dependent desaturase activity.

When the *spt23Δ;mga2Δ* cells were transferred to fatty acid-free medium, growth proceeded at wild type rates for ~2 generations before slowing, and stationary growth occurred ~3.5 generations after the transfer. In both strains the most abundant fatty acids at the time of transfer were 18:2 (~70 weight %) and the saturated species 16:0 and 18:0 (~20 weight %) (Fig. 1*B*). Very low cumulative levels of 16:1 and 18:1 were found in the wild type (~7%) and *spt23Δ;mga2Δ* (~5%) strains, suggesting that *OLE1* might be expressed at low basal levels, even under fatty acid-repressed conditions. The wild type cells exhibited the previously observed pattern of *OLE1* induction after transfer to fatty acid-free medium. Eight hours after the transfer, levels of the assimilated 18:2 dropped ~5-fold, and the 16:1 and 18:1 products of the induced desaturase increased to greater than 60% of the total fatty acid mass. Under the same conditions, the *spt23Δ;mga2Δ* cells showed no significant increases in 16:1 and 18:1 levels, indicating that *OLE1* expression was not induced by fatty acid depletion significantly above the previously observed basal levels. At the same time, there was a striking increase in saturated fatty acids and a reduction in 18:2 in the *spt23Δ;mga2Δ* strain, which would be expected if saturated fatty acid synthesis continued in the absence of induced desaturase activity.

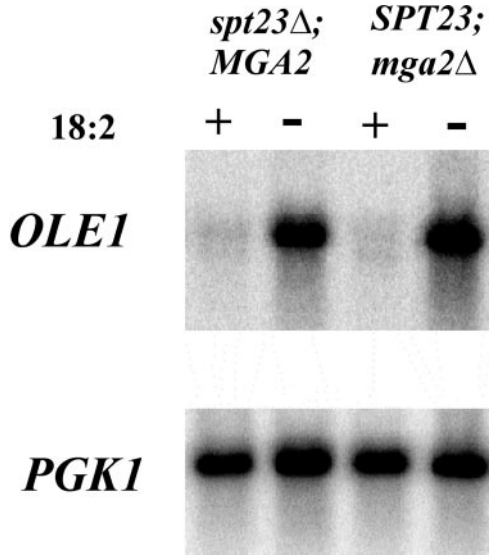

FIG. 2. **Quantitative Northern blot hybridization of *OLE1* mRNA in *spt23*Δ and *mga2*Δ cells exposed to 1 mM 18:2 and 2 h after transfer to fatty acid-free medium.** *spt23*Δ;*MGA2* and *SPT23*; *mga2*Δ cells were pre-grown on 1 mM 18:2 to a cell density of less than 1×10^7 cells/ml. Aliquots of the culture were then washed as described under "Experimental Procedures" and transferred to fresh medium containing 1 mM 18:2 or fatty acid-free medium. Total RNA was isolated 2 h after the transfer and subjected to Northern blot analysis. Blots were probed with radiolabeled *OLE1* DNA and then stripped and reprobed with DNA complementary to the *Saccharomyces PGK1* gene. Images are derived from PhosphorImaging.

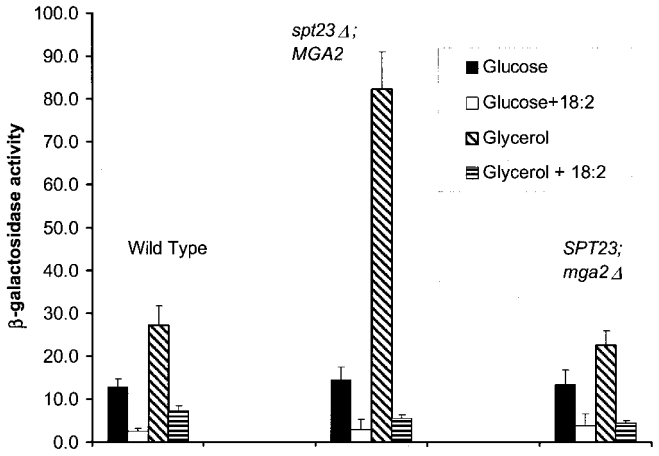

FIG. 3. **Gene activation and 18:2-mediated repression of *OLE1* promoter::*lacZ* reporter activity in wild type, *spt23*Δ, and *mga2*Δ cells grown on glucose or glycerol.** Cells containing the p62 plasmid were grown for at least 6 generations to mid-logarithmic growth phase in synthetic medium containing either 2% glucose + 1 mM 18:2 or 3% glycerol + 1 mM 18:2 to repress reporter gene activity and to reduce residual β-galactosidase activity. Cultures were harvested at a density of $<2 \times 10^7$/ml, washed with the corresponding fatty acid-free medium, and then transferred to fresh medium with no fatty acids or with 1 mM 18:2. Assays were performed 6 h after the transfer. Data represents the average of three independent transformants performed in triplicate.

*OLE1 mRNA Levels in spt23*Δ *and mga2*Δ *Cells Are Regulated by Unsaturated Fatty Acids in a Manner Consistent with Wild Type Cells*—The small differences in unsaturated fatty acid levels in wild type, *spt23*Δ, and *mga2*Δ cells suggested that either gene could independently activate and regulate *OLE1* expression under normal laboratory growth conditions. This was confirmed by quantitative Northern blot experiments (Fig. 2) that showed that disruption of either gene did not affect *OLE1* steady state mRNA levels in either fatty acid-free or 18:2-supplemented growth medium containing glucose as a carbon source. Under the conditions used in the experiment, *OLE1* mRNA levels were strongly suppressed by exposure to the unsaturated fatty acid.

In Spt23p-deficient Cells, Mga2p Elicits a Strong OLE1 Transcription Activation Response to Glycerol and Cobalt—To test the roles of Spt23p and Mga2p on *OLE1* transcription, a *lacZ* reporter gene that was linked to 934 bases of the *OLE1* promoter was transformed into the *spt23*Δ and *mga2*Δ gene-disrupted strains. Cells were grown under conditions that we had observed previously (6, 7) to alter *OLE1* transcription activity, including repression by unsaturated fatty acids and activation in response to cobalt and the non-fermentable carbon source, glycerol.

The effects of the gene disruptions on reporter gene activity in cells grown on glucose and glycerol carbon sources are shown in Fig. 3. To prevent residual β-galactosidase activity from masking the effects of fatty acid repression, cells were initially grown for 12 h in the appropriate medium containing 1 mM 18:2. Aliquots were then washed with fatty acid-free medium and grown for 6 h prior to the assays in either fatty acid-free medium or medium containing 1 mM 18:2.

Wild type, *spt23*Δ, and *mga2*Δ mutant cells grown on glucose-containing medium showed no significant differences in reporter gene activity under derepressed or 18:2 repressed conditions. Under the conditions used in the experiment, the 18:2 repressed reporter gene activity to ~20% of the levels seen in derepressed cells. Distinct phenotypic differences were observed, however, in cells that were grown on glycerol. In the wild type and *mga2*Δ strains, transfer to glycerol medium without fatty acid resulted in a 1.5–2-fold induction in reporter gene activity that was repressed by 18:2 to levels similar to those in glucose-grown cells. Deletion of the *SPT23* gene, however, resulted in a striking 6-fold induction of reporter activity in fatty acid-free glycerol medium. Furthermore, the addition of 18:2 in the medium repressed the reporter gene activity to the same levels found in the wild type and mutant cells, resulting in a 20-fold increase in the range of transcription repression.

Previous studies (7, 9) have shown that *OLE1* expression is also strongly induced by cobalt. Again, Mga2p and Spt23p appear to play distinct roles in the induction response (Fig. 4, *a* and *b*). Exposure of logarithmic phase cells to 400 μM cobalt chloride for 6 h resulted in a 4–5-fold induction of reporter gene activity in wild type and *spt23*Δ;*MGA2* cultures. By contrast, cells containing the disrupted *MGA2* gene showed no significant response under the same conditions. Reporter activity in all of the strains exposed to cobalt was repressed by 18:2. Cells containing only the functional Mga2p showed the most dramatic range of repression (>20-fold) between fatty acid-free cultures and those exposed to 18:2.

An analysis of *OLE1* steady state mRNA levels (Fig. 4*b*) also illustrates the effects of Mga2p in the cobalt induction response. In wild type and *spt23*Δ;*MGA2* cells, which express Mga2p, there is an equivalent 5–6-fold increase in mRNA levels of cells grown on fatty acid-free medium. Although reporter gene activity is induced to higher levels in the *spt23*Δ cells, the resulting mRNA levels between that strain and wild type are equivalent, suggesting that post-transcriptional mechanisms might operate to maintain the transcripts at similar levels. By comparison, mRNA levels in *mga2*Δ cells, which express only Spt23p, show no inductive response to cobalt. Under the conditions of the experiment, mRNA levels in all three strains are repressed by exposure to 18:2, which correlates with reporter gene activities.

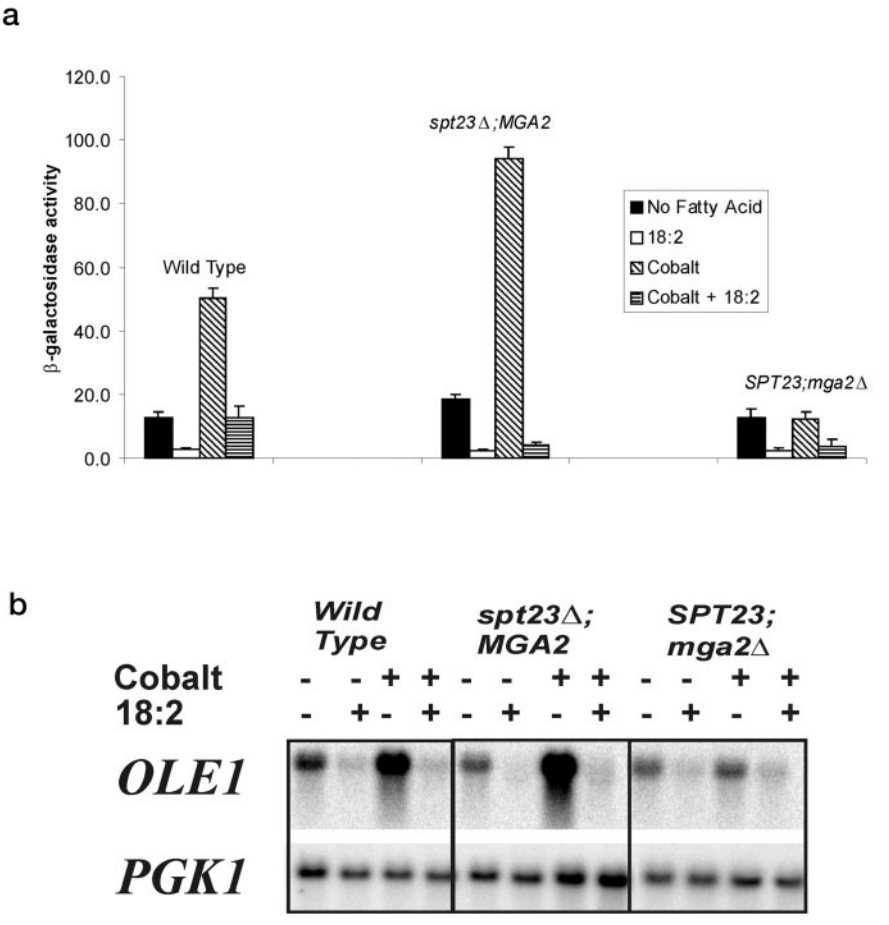

FIG. 4. **Gene activation and 18:2-mediated repression of *OLE1* promoter ::*lacZ* reporter activity in wild type, *spt23*Δ, and *mga2*Δ cells exposed to 400 μM cobalt.** *a,* p62 *lacZ* reporter gene activity. Cultures were pre-grown in glucose + 1 mM 18:2-supplemented media containing no cobalt or 400 μM cobalt as described in Fig. 3. Cells were harvested and washed with the corresponding fatty acid-free medium and then suspended in either fresh fatty acid-free or 1 mM 18:2-supplemented medium ± 400 μM cobalt chloride. Assays were performed 6 h after the transfer as described in Fig. 3. *b,* steady state *OLE1* mRNA levels. Cultures were pre-grown as described in *a*, and total cellular RNA was isolated for Northern blot analysis 3 h after the transfer to fresh medium.

The Induction Responses to Cobalt and Glycerol Are Additive and Synergistic—We showed previously (7) that cobalt can induce *OLE1* transcription through the LORE. Given that glycerol requires oxygen-dependent respiration to be metabolized, we asked whether the strong activation of reporter gene activities in the *spt23*Δ cells by glycerol and cobalt might be caused by the same function. Fig. 5 shows that the addition of cobalt to cells grown on glycerol stimulates activity to 2–3-fold higher levels than glycerol alone in all three strains, suggesting that the two effects occur by different mechanisms. The *spt23*Δ; *MGA2* cells, which only express Mga2p, exhibit the highest levels of reporter gene activity under those conditions. In those cells, the activity induced by the combination of glycerol and cobalt is the approximate sum of the activities observed in cells that were separately induced. In the wild type and *SPT23; mga2*Δ cells, however, induction by the combined stimuli resulted in even higher activities than the sum of the individual responses. The combined glycerol- and cobalt-induced reporter activities in all the tested strains were also strongly repressed by exposure to 18:2. These results suggest that the glycerol and cobalt induction are triggered by separate mechanisms, although their apparent synergism suggests that these processes may be also linked to some common elements that activate transcription.

The Cobalt and Glycerol Responses Act through Different OLE1 Promoter Elements—The fatty acid-regulated components of the *OLE1* transcription unit include the FAR element, which is located ~580 bases upstream of the start codon (6), and a 30-base pair LORE element that is positioned ~200 bases downstream of the FAR region. The LORE region is a strong activator of transcription when cells are starved for oxygen (7) or exposed to cobalt. Although these two effects are

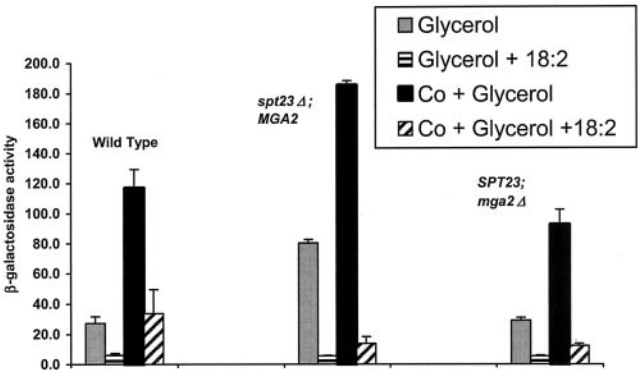

FIG. 5. **Gene activation and 18:2-mediated repression of *OLE1* promoter::*lacZ* reporter activity in wild type, *spt23*Δ, and *mga2*Δ cells exposed to 400 μM cobalt.** Cultures were pre-grown in glycerol + 1 mM 18:2-supplemented media containing no cobalt or 400 μM cobalt as described in Fig. 3. Cells were harvested and washed with the corresponding fatty acid-free medium and then suspended in either fresh fatty acid-free or 1 mM 18:2-supplemented medium. Assays were performed 6 h after the transfer as described in Fig. 3.

closely linked, we have shown recently (7) that point mutations within the LORE region can suppress the response to hypoxia without affecting induction by cobalt. Given the differential effects produced by glycerol and cobalt on gene activation, we theorized that the FAR and LORE elements might play specific roles in those responses and thus provide useful tools for investigations into Mga2p and Spt23p functions. To test this possibility, we examined the expression of *lacZ* reporter genes under control of the isolated FAR and LORE elements and reporter plasmids that contain mutations in those regions

TABLE V

β-Galactosidase activities from reporter genes containing mutant forms of OLE1 promoter elements in strain DTY11A under glucose, glycerol and cobalt-induced growth conditions

Cells were grown to a density of less than 2×10^7/ml and analyzed for β-galactosidase activity as described under "Experimental Procedures." Activity levels represent a minimum of three independent assays ± S.D.

Plasmid	Glucose	Glycerol	Glucose + 400 μM cobalt
pCTΔm111 (FAR)	1.7 ± 0.14	1.5 ± 0.2	4.0 ± 0.1
p62Δ88 (far$^-$)	1.2 ± 0.1	2.8 ± 0.3	5.7 ± 0.01
pAM4 (lore$^-$)	5.5 ± 0.4	5.9 ± 0.3	26.7 ± 2.0
pAM6 (LORE)	10.7 ± 1.0	10.2 ± 1.9	117 ± 2.9

within the 934 bases upstream of the *OLE1* start codon. The results of that study are shown in Table V.

Plasmids that contained the isolated FAR and LORE elements were not induced by glycerol but were activated by exposure to cobalt. The plasmid containing the LORE element showed the strongest response to cobalt (>10-fold over glucose-grown cells), whereas the plasmid containing the FAR element was activated slightly more than 2-fold. The two reporter plasmids that contain mutations within the context of the 934-base upstream region were also differentially induced by glycerol and cobalt. Plasmid p62::Δ88, which contains an 88-base pair deletion in the FAR element, is induced ~2.3-fold by glycerol and about 4.8-fold by cobalt. Plasmid pAM4, which contains a 3-base substitution in the LORE region that abolishes the hypoxia response but not the cobalt response (7) is not induced by glycerol but is induced ~5-fold by cobalt. The complete inactivation of the glycerol response caused by a 3-base substitution in the LORE element within the 934-base sequence, combined with the inability of the isolated LORE element to be activated by glycerol, indicates that elements contained within the LORE region are necessary, but not sufficient, for the glycerol induction response.

Taken together, these data show that the glycerol and cobalt responses are associated with different *OLE1* upstream activation sequences. These data further suggest that both responses act through multiple sites within the *OLE1* promoter, including some that have not yet been identified.

OLE1 Expression Activated by a Soluble Form of Mga2p Is Regulated by 18:2—Previous studies (14) indicated that the proteolytic processing of the membrane-bound forms of Spt23p and Mga2p to their soluble forms is essential for the expression of *OLE1*. That study showed that Spt23p processing could be inhibited by exposing cells to 16:1, 18:2, and 18:3 but not 18:1. This suggested that increased membrane fluidity, caused by the incorporation of fatty acids with lower melting temperatures into membrane lipids, might be the basis of the fatty acid-mediated regulation of *OLE1* expression.

To clarify the potential regulatory role of the proteolytic processing step on *OLE1* expression, we constructed genes that express soluble forms of Spt23p and Mga2p by deleting the regions that encode their C-terminal membrane-anchoring domains. Given the very low levels of expression by the chromosomal *SPT23* and *MGA2* genes, the truncated genes were placed under control of their native promoters in single copy, centromere-based vectors.

Plasmid Spt23pΔtm contains 1499 bases of the *SPT23* promoter and the N-terminal protein-coding sequence encoding amino acid residues 1–792. Plasmid Mga2pΔtm contains 904 bases of the *MGA2* promoter and encodes N-terminal residues 1–784 of the protein. Expression of either plasmid in a *spt23*Δ; *mga2*Δ strain repaired its unsaturated fatty requirement. This demonstrated that the soluble protein fragment from either gene could activate *OLE1* transcription. An examination of p62 *OLE1*::*lacZ* reporter gene activity in the same cells, however, showed striking differences in transcription activation levels induced by the truncated forms of Mga2p and Spt23p (Fig. 6). The soluble Spt23pΔtm protein activated the reporter gene in the *spt23*Δ;*mga2*Δ cells to levels similar (1.0–1.5-fold) to those seen in the wild type and *SPT23;mga2*Δ strains (Fig. 6, also *cf.* Fig. 3). Furthermore, the reporter gene activity induced by the truncated protein was only weakly repressed by 18:2 (~25%). This differed markedly from the strong repression observed in wild type and *SPT23;mga2*Δ strains that produce full-length Spt23p (Figs. 3 and 4).

Expression of the soluble Mga2Δtm protein, however, resulted in a 25-fold increase over the levels of reporter gene activity produced by its native form in wild type or *spt23*Δ*; MGA2* cells. Furthermore, that activity was very strongly repressed by 18:2.

Spt23pΔtm Attenuates the 18:2-Mediated Repression of Mga2pΔtm-dependent Transcription—The high level of activation in cells that express only the soluble form of Mga2pΔtm and the correspondingly high level of glycerol- or cobalt-induced activities in the *spt23*Δ cells that only express the full-length Mga2p (Figs. 3 and 4) suggested that Spt23p can suppress or attenuate Mga2p-dependent gene activation. To test the possibility that this suppression might occur after the proteolytic processing step, p62 reporter gene activity was measured in *spt23*Δ;*mga2*Δ cells that co-expressed the soluble forms of the two proteins. In fatty acid-free medium, reporter gene activity was found to be the same as when only the Mga2pΔtm protein was expressed, suggesting that Spt23pΔtm had no significant effect on the Mga2pΔtm-mediated activation (Fig. 6). The combined expression of both soluble proteins, however, suppressed the strong 18:2-mediated repression of the reporter activity, which remained at 75% of the levels found in fatty acid-free cultures. The resulting fatty acid "repressed" levels in the cells that co-expressed Spt23pΔtm and Mga2pΔtm were 6-fold greater than those found in wild type cells grown in fatty acid-free medium and >60-fold higher than the corresponding wild type fatty acid repressed activity.

Effect of Spt23Δtm and Mga2Δtm on Steady State mRNA Levels—Quantitative Northern blots of *OLE1* mRNA isolated from strains expressing the soluble Spt23Δtm and the Mga2Δtm proteins are shown in Fig. 7. As expected, mRNA levels in the *spt23*Δ;*mga2*Δ strain that contained the empty vector are severely reduced compared with the levels found in wild type cells. Expression of the truncated form of the Spt23Δtm protein in the gene-disrupted strain results in mRNA levels that are consistent with the above reporter gene activities. Transcript levels in those cells grown on fatty acid-free medium are nearly identical to those found in wild type cells and they remain at the same levels after release from fatty acid repression.

By contrast, mRNA levels in cells that express the soluble Mga2pΔtm protein are about 5-fold higher than those in the wild type cells, and they are strongly repressed by exposure to 18:2. *OLE1* transcript levels in cells that co-express both soluble proteins are ~7.5-fold greater than wild type cell levels when grown on fatty acid-free medium, and as predicted from the reporter gene assays, they are unaffected by exposure to 18:2.

DISCUSSION

The regulation of membrane fatty acid composition requires cells to respond to a complex set of stimuli. In addition to physiological controls that monitor the demand for membrane assembly, cells must detect and respond to changes of bilayer fluidity, the availability of nutrient lipids, and changes in the composition of intracellular fatty acid pools to regulate lipid

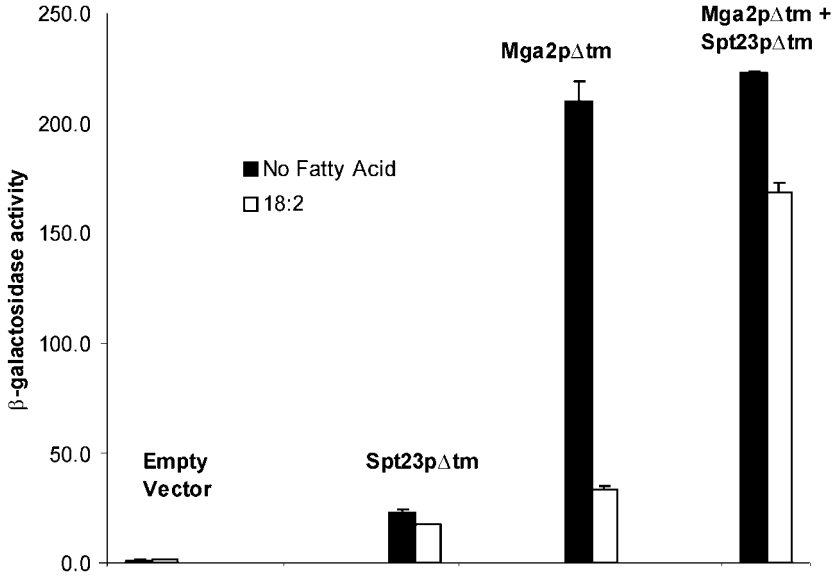

FIG. 6. **Gene activation and 18:2-mediated repression of *OLE1* promoter ::*lacZ* reporter activity in *spt23Δ; mga2Δ* cells expressing soluble, 90-kDa forms of Spt23p or Mga2p.** Cultures were pre-grown in glucose + 1 mM 18:2-supplemented media as described in Fig. 3. Cells were harvested and washed with the corresponding fatty acid-free medium and then suspended in either fresh fatty acid-free or 1 mM 18:2-supplemented medium for 6 h prior to assay.

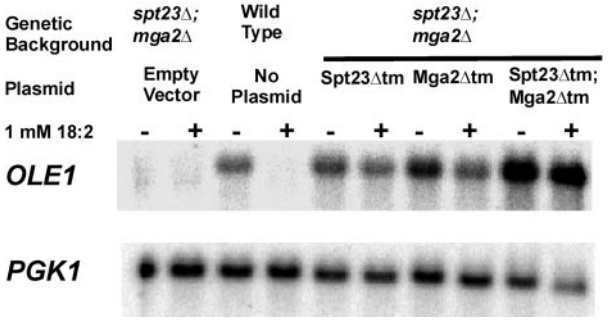

FIG. 7. **Regulation of *OLE1* mRNA levels in *spt23Δ;mga2Δ* cells expressing soluble, 90-kDa, forms of Spt23p and Mga2p.** Cultures were pre-grown in glucose + 1 mM 18:2-supplemented media as described in Fig. 3. Cells were harvested and washed with the corresponding fatty acid-free medium and then suspended in either fresh medium with no fatty acids or 1 mM 18:2. Total cellular RNA was isolated for Northern blot analysis 4 h after the transfer to fresh medium.

biosynthetic activity. Fatty acid desaturases represent important control points in these metabolic systems because their unsaturated products compose most (70–80%) of the acyl groups found in membrane lipids. Furthermore, manipulations of their activities can produce radical changes in the physical state of membrane lipid bilayers that, in turn, can affect numerous membrane-associated cellular functions.

The finding that the activity of the two homologous membrane proteins, Spt23p and Mga2p, is essential for *OLE1* gene activation raises several important questions about their functional roles in the regulation of the desaturase activity. Previous reports (13) have indicated that these proteins act as transcription co-factors. Although they do not have recognizable DNA binding domains, N-terminal elements of the proteins can activate transcription when they are fused to the DNA binding domain of the *GAL4* transcription factor (13). Furthermore, overexpression of Spt23p and Mga2p can suppress mutations that affect *SNF/SWI*-mediated transcription activation, leading to the proposal that they may do so by influencing chromatin accessibility (13).

The studies presented here indicate that under normal growth conditions in aerated glucose medium, Spt23p and Mga2p essentially perform overlapping functions. The independent expression of either gene produces almost equal levels of *OLE1* transcription activity and can maintain normal *OLE1* steady state mRNA levels under derepressed and unsaturated fatty acid-repressed conditions. Furthermore, disruption of either gene has little effect on the total unsaturated fatty acid levels in cellular lipids. Significant differences in Spt23p and Mga2p functions become evident, however, under conditions when *OLE1* transcription is induced above the glucose-grown basal levels by exposure to glycerol or to cobalt. The highest levels of induction to both stimuli were observed in cells that only express Mga2p, which appears to be the dominant transcription activator. Furthermore, the higher levels of *OLE1* induction by cobalt or glycerol in *spt23*Δ cells (in which Mga2p is independently expressed) compared with wild type cells (in which both proteins are expressed) suggest that Spt23p partially suppresses Mga2p activity under those conditions. The observation that the soluble form of Spt23p does not suppress Mga2p-dependent activation suggests that this effect could take place at the level of the membrane. This might occur through competition for Rsp5p (14), the ubiquitin ligase that activates the proteolytic conversion of Spt23p and Mga2p to their active, soluble forms.

The glycerol and the cobalt induction processes appear to act through different elements in the *OLE1* promoter. Although evidence presented here suggests that glycerol induction requires elements in the LORE region, it also appears to require additional promoter sequences that are distinct from the FAR and LORE elements (6, 7). Cobalt stimulates transcription activity through the LORE element and additionally induces activity through the FAR region. These observations suggest that both types of induction act through multiple but distinct sites in the *OLE1* promoter region. The observation that Mga2p associates with the LORE element under hypoxic conditions (15) also suggests that Mga2p and Spt23p may associate with additional DNA-binding complexes on the *OLE1* promoter and that it will be important to determine those interactions to define the functions of the two proteins.

The observation that proteolytic processing of Spt23p can be inhibited by certain unsaturated fatty acids offers a compelling mechanism for the fatty acid-mediated repression of *OLE1* transcription. If the proteolytic release of both proteins from the membrane were blocked by the exogenous fatty acids, then transcription activity could be effectively repressed by a mechanism that responds only to changes in the fatty acid composition of the surrounding membrane lipids. That type of control correlates with recently described (18–20) regulatory pathways that release other membrane-bound transcription factors through intramembrane proteolysis. These include the SREBP that is regulated by cholesterol and the *ATF6* and *IRE1* tran-

scription factors that are regulated by an unfolded protein response that is triggered by ER stress (18–20). Observations by Hoppe *et al.* (14) indicate that, although unsaturated fatty acids exert tight control over the processing of Spt23p, they do not have a strong effect on the proteolytic processing of Mga2p. Given the observations here that Mga2p acts as the primary activator of *OLE1* transcription in cobalt- and glycerol-induced cells and is strongly repressed by fatty acids suggests that a membrane fluidity-based mechanism may not be the sole determinant of that regulatory response.

To test further the hypothesis that *OLE1* repression occurs through a fluidity-regulated mechanism, we expressed soluble (90 kDa) forms of Spt23p and Mga2p in a strain containing disrupted alleles of both genes. Those experiments also revealed the striking differences in the functions of the two proteins and uncovered a second level of fatty acid-mediated control. Spt23pΔtm was found to activate transcription at a level similar to the basal activity found in wild type cells, but this was only weakly repressed (~25%) by exposure to 18:2. Mga2pΔtm, however, activated *OLE1* transcription at levels that were 20-fold greater than wild type cells and was strongly repressed by 18:2. The latter observation shows that fatty acid-mediated regulation of *OLE1* can occur independent of the proteolytic release of that protein from the membrane. Because Mga2p processing appears to be a stronger transcription activator, and its processing is not suppressed by unsaturated fatty acids in glucose grown cells, it is possible that the primary mechanism of fatty acid-mediated repression occurs downstream of Spt23p/Mga2p proteolytic processing.

The co-expression experiments involving the Spt23pΔtm and Mga2pΔtm suggest a mechanism by which the two proteolytically processed proteins might interact, either with each other or with a common set of proteins, to modulate transcription activity. Unlike the effects seen in cells that express the full-length proteins, Spt23pΔtm does not attenuate the high levels of transcription activation produced by Mga2pΔtm when the two proteins are co-expressed. It is particularly striking, however, that it exerts a strong suppressive effect on the fatty acid-mediated repression process. One possible mechanism for that effect might involve an unsaturated fatty acid (or fatty acyl-CoA)-binding protein that associates with the soluble forms of Mga2pΔtm and blocks its transcription activity. When the soluble form of Spt23p is co-expressed, it might compete for limited supplies of the fatty acid-binding regulator, thus preventing it from binding and suppressing Mga2p-dependent gene activation.

The observation that *OLE1* mRNA levels are higher when Spt23pΔtm and Mga2pΔtm are co-expressed than when Mga2pΔtm is expressed alone might be the result of several possible conditions. One is that the *OLE1* mRNA levels in cells that only express Mga2pΔtm may recover slowly following release from fatty acid repression and may not have reached the high steady state levels when analyzed. Alternatively, since *OLE1* mRNA levels are affected by both transcription activity and the rate of mRNA degradation (5), post-transcriptional control mechanisms, such as modulation of *OLE1* mRNA stability, might be influenced by the expression of the two soluble proteins, resulting in increased levels of transcript. We have recently observed that Mga2p is essential for the fatty acid-regulated stability of the *OLE1* transcript, suggesting that this protein might play a role in the functional linkage between transcription and mRNA stability.[2] This problem is currently under investigation.

Taken together, the experiments described here suggest that regulation of *SPT23*/*MGA2*-activated *OLE1* expression acts in multiple cellular locations that may include the nucleus as well as the ER membrane surface. It appears unlikely that membrane fluidity-dependent regulated proteolysis is the sole determinant of this process but rather that the control of *OLE1* expression occurs through several different mechanisms that allow desaturase activity to respond to the multiple metabolic and physiological demands for unsaturated fatty acids.

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The Membrane Proteins, Spt23p and Mga2p, Play Distinct Roles in the Activation of *Saccharomyces cerevisiae OLE1* Gene Expression: FATTY ACID-MEDIATED REGULATION OF Mga2p ACTIVITY IS INDEPENDENT OF ITS PROTEOLYTIC PROCESSING INTO A SOLUBLE TRANSCRIPTION ACTIVATOR

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