The Opi1p transcription factor plays a negative regulatory role in the expression of UASINO-containing genes involved in phospholipid synthesis in the yeast Saccharomyces cerevisiae. The phosphorylation of Opi1p by protein kinase A (cAMP-dependent protein kinase) was examined in this work. Using a maltose-binding protein-Opi1p fusion protein as a substrate, protein kinase A activity was time- and dose-dependent and dependent on the concentrations of Opi1p and ATP. Protein kinase A phosphorylated Opi1p on multiple serine residues. The synthetic peptides SCRQKSQPSE and SQVRESLLNL containing the protein kinase A motif for Ser31 and Ser251, respectively, within Opi1p were substrates for protein kinase A. Phosphorylation of S31A and S251A mutant maltose-binding protein-Opi1p fusion proteins by protein kinase A was reduced when compared with the wild type protein, and phosphopeptides present in wild type Opi1p were absent from the S31A and S251A mutant proteins. In vivo labeling experiments showed that the extent of phosphorylation of the S31A and S251A mutant proteins was reduced when compared with the wild type protein. The physiological consequence of the phosphorylation of Opi1p at Ser31 and Ser251 was examined by measuring the effects of the S31A and S251A mutations on the expression of the UASINO-containing gene INO1. The β-galactosidase activity driven by an INO1-CYC-lacZ reporter gene in opi1Δ mutant cells expressing the S31A and S251A mutant Opi1p proteins was elevated 42 and 35%, respectively, in the absence of inositol and 55 and 52%, respectively, in the presence of inositol when compared with cells expressing wild type Opi1p. These data supported the conclusion that phosphorylation of Opi1p at Ser31 and Ser251 mediated the stimulation of the negative regulatory function of Opi1p on the expression of the INO1 gene.

Phospholipids are essential molecules that contribute to the structure and function of membranes, and indeed, the synthesis of phospholipids is a major activity in which cells engage throughout growth. The yeast Saccharomyces cerevisiae serves as a model eukaryotic organism to study the regulation of phospholipid synthesis (1–6). Almost all of the structural and regulatory genes involved in phospholipid synthesis have been cloned and characterized, and mutations in these genes have been isolated (1–8). Moreover, several of the phospholipid synthetic enzymes have been purified and characterized (1–6). The characterization of the wild type and mutant genes, as well as the gene products encoded by these alleles, has significantly advanced understanding of phospholipid synthesis. The regulation of phospholipid synthesis is complex and occurs by both genetic (e.g. transcriptional) and biochemical (e.g. phosphorylation) mechanisms (1–6, 9, 10).

The expression of genes encoding enzymes responsible for the synthesis of phosphatidylinositol (e.g. INO1) and phosphatidylcholine (e.g. CDS1, CHO1/PS1, PDS1, CHO2/PEM1, OPI3/PEM2, CKI1, and CPT1), the two most abundant and essential phospholipids in S. cerevisiae, is regulated by inositol (1, 2, 4–6). These genes are maximally expressed when inositol is absent from the growth medium and repressed when inositol is supplemented to the growth medium. Repression by inositol supplementation is enhanced by the inclusion of choline in the growth medium (1, 2, 4–6). Inositol-mediated regulation involves the transcriptional regulatory proteins Ino2p, Ino4p, and Opi1p (1, 2, 4–6, 9). Ino2p and Ino4p (11) and Ino4p (12) are positive transcription factors, whereas Opi1p (13) is a negative transcription factor. Regulation of phospholipid synthesis by inositol is mediated by a UASINO (inositol/choline-responsive) cis-acting element (1, 14–17) present in the promoters of the structural genes that code for phospholipid synthesis enzymes (1, 2, 4–6, 18). The UASINO element contains the binding site for an Ino2p-Ino4p heterodimer, which is required for maximum expression of the co-regulated UASINO-containing genes (4–6, 19–21). Repression of the co-regulated phospholipid synthesis genes depends on Opi1p (13, 22).

Opi1p contains a leucine zipper and two glutamine-rich domains (13) (Fig. 1) that are required for Opi1p repressor activity (23). Opi1p mediates its negative regulatory activity through the UASINO element (24) but not by direct interaction (23). Instead, in vitro data indicate that Opi1p interacts with DNA-bound Ino2p within the leucine zipper domain of Opi1p (25). In addition, the global repressor Sin3p interacts with the N-terminal region of Opi1p (25) (Fig. 1). Studies using mutant alleles of INO2, INO4, OPI1, and SIN3 support a model whereby these interactions play a role in the expression of UASINO-containing genes in vivo (25–29).

Phosphorylation is a mechanism by which the activity of a regulatory protein may be controlled (30–35), and indeed in vivo labeling experiments have shown that Opi1p is a phosphoprotein (36). Some of this phosphorylation is due to protein kinase C, and Ser26 has been identified as a major site of phosphorylation.
phosphorylation by this protein kinase (36) (Fig. 1). Phosphorylation of Ser251 attenuates the negative regulatory activity of Opi1p on the expression of the UASINO-containing INO1 gene (36). In this work, we demonstrated that Opi1p was phosphorylated by protein kinase A, the principal mediator of signals transmitted through the Ras-CAMP pathway in S. cerevisiae (37, 38). Ser211 and Ser251 were identified as major sites of protein kinase A phosphorylation, and phosphorylation of these sites played a role in stimulating Opi1p activity.

EXPERIMENTAL PROCEDURES

Materials—All chemicals were reagent grade. Growth medium supplies were purchased from Difco Laboratories. Restriction endonucleases, modifying enzymes, recombinant Vent DNA polymerase, amylase affinity chromatography resin, MBP, and anti-MBP antibodies were purchased from New England Biolabs. The plasmid DNA purification and DNA gel extraction kits were purchased from Qiagen. The oligonucleotides were prepared by Genosys Biotechnologies, Inc. The QuickChange site-directed mutagenesis kit was purchased from Stratagene. The Yeast Maker yeast transformation system was from Clontech. The DNA size ladder used for agarose gel electrophoresis was from Invitrogen. Radiochemicals were purchased from PerkinElmer Life Sciences. Phosphocellulose filters were purchased from Pierce. Phenylmethylsulfonyl fluoride, bovine serum albumin, histone, benzamidine, aprotinin, leupeptin, pepstatin, Nonidet P-40, polyvinylpyrrolidone (40 kDa), phosphoamino acids, and O-nitrophenyl-

Methods for yeast growth were performed as described previously (40, 41). Yeast cultures were grown in YEPD medium (1% yeast extract, 2% peptone, 2% glucose) or in SC medium containing 2% glucose at 30 °C. For selection of cells bearing plasmids, appropriate amino acids were omitted from SC medium. The cell numbers in liquid media were determined spectrophotometrically at an absorbance of 600 nm. The media were supplemented with 2% agar for growth on plates. The inositol excretion phenotype was examined on SC medium plates (minus inositol) by using growth of the inositol auxotrophic mutant ino1-42 as described previously (43).

DNA Manipulations, Amplification of DNA by PCR, and DNA Sequencing—Preparation of plasmid DNA, restriction enzyme digestions, and DNA ligations were performed using standard protocols (41). Transformation of yeast (44) and E. coli (41) were performed as described previously. Amplification of DNA by PCR was optimized as described previously (45). DNA sequencing reactions were performed by the dyeexchange method using Vent (exo) polymerase (41).

Construction of Plasmids—Plasmid pMAL-OPI1 containing a malE-OPI1 fusion gene (36) was used for the expression of MBP-Opi1p fusion protein. The codons for Ser31 and Ser251 in Opi1p were changed to alanine codons by site-directed mutagenesis. The OPL1S31A (primers, 5' -CAATCAGGACAGAAAGGGCACTTCAGACGA-3' and 5' -GAGCTCTCAGAAGGGTCCCTCCTCGAGATTG-3') and OPI1S251A (primers, 5' -CACAGATCTCAGGTTGGGAGACACACATTAC-3' and 5' -GCCATTTGAGATTCTTGTCAAGAGTCTCC-3') mutations were constructed by PCR with a QuickChange site-directed mutagenesis kit using plasmid pMAL-OPI1 as the template. The lowercase letters in the primers refer to sequences used for the mutations. Clones containing the wild type and mutant OPI1 coding sequence were identified by restriction enzyme analysis. DNA sequencing of the wild type and mutant genes confirmed that the constructs were in frame with the malE gene and did not possess additional mutations. Plasmid pSA3 is a single-copy plasmid that contains the OPI1 gene with sequences for a HA epitope tag inserted after the start codon (36). Plasmids pSA5 and pSA6, which bear the HA-OPI1S31A and HA-OPI1S251A mutations, respectively, were derived from plasmid pSA3 after site-directed mutagenesis using the primers described above. Plasmid pSA1 is a multicyclopolymer that contains the OPI1 gene with sequences for a HA epitope tag inserted after the start codon (36). Plasmids pSA7 and pSA8 were constructed by subcloning HA-OPI1S31A and HA-OPI1S251A from pSA5 and pSA6, respectively, into the SacI/HindIII sites of plasmid YEp351. These plasmid constructions were confirmed by DNA sequencing.

Purification of Wild Type and Mutant MBP-Opi1p Fusion Proteins from E. coli—Wild type and mutant MBP-Opi1p fusion proteins were purified from E. coli by disruption of cells with a French press followed by amylase-agarase affinity chromatography as described by Sreenivas et al. (36).

Phosphorylation of MBP-Opi1p and Synthetic Peptides with Protein Kinases A and C—The phosphorylation reactions were measured for 10 min at 30 °C in a total volume of 40 μl. The indicated concentrations of MBP-Opi1p or synthetic peptides were phosphorylated with protein kinase A in a reaction mixture that contained 50 mM Tris-HCl (pH 7.5), 10 mM MgCl2, 0.2 mM [γ-32P]ATP (5,000 cpm/pmol), and protein kinase A (0.2 unit/ml). MBP-Opi1p (0.1 mg/ml) was phosphorylated with protein kinase C in a reaction mixture that contained 50 mM Tris-HCl buffer (pH 8.0), 10 mM MgCl2, 10 mM 1-mercaptoethanol, 0.375 mM EDTA, 0.375 mM EGTA, 1.7 mM CaCl2, 20 μM diacetyl, 50 μM phosphatidylinerine, 50 μM [γ-32P]ATP (5,000 cpm/pmol), and protein kinase C (1 unit/ml). Samples containing 32P-labeled MBP-Opi1p were treated with an equal volume of 2× Laemmli’s sample buffer (46), boiled, and separated by SDS-polyacrylamide gel electrophoresis and transferred to PVDF paper, and visualized by phosphorimaging. The extent of phosphorylation was analyzed using ImageQuant software. Phosphorylation signals were in the linear range of detectability. The reactions containing synthetic peptides were terminated by spotting an aliquot of the reaction mixture onto phosphocellulose filters. The filters were washed...
with 75 mM phosphoric acid and subjected to scintillation counting. The phosphorylation reactions were performed in triplicate. A unit of protein kinase A activity was defined as the amount of enzyme that catalyzed the formation of 1 nmol of product/min.

**Protein Kinase A Phosphorylation of Yeast Opi1p**

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype or relevant characteristics</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli DH5α</td>
<td>F− Δ800ΔlacZΔM15 ΔlacZYA-argF/ΔU169 deoR recA1 endA1 hsdR17 (rK− mK+) phoA supE44 thi-1 gyrA96 relA1</td>
<td>Ref. 41</td>
</tr>
<tr>
<td>S. cerevisiae</td>
<td></td>
<td>Ref. 42</td>
</tr>
<tr>
<td>WCG4</td>
<td>MATa his3-11.15 leu 2-3, 112 ura3-5</td>
<td>Ref. 35</td>
</tr>
<tr>
<td>SH1100</td>
<td>opi1Δ::kanMX derivative of WCG4</td>
<td>Ref. 36</td>
</tr>
<tr>
<td>MC13</td>
<td>MATa can1 ino1-13 lys2</td>
<td>Ref. 42</td>
</tr>
<tr>
<td>Plasmids</td>
<td></td>
<td>Ref. 43</td>
</tr>
<tr>
<td>pMAL-c2</td>
<td>E. coli vector with an inducible malE gene used for fusion protein expression</td>
<td>Ref. 44</td>
</tr>
<tr>
<td>pMAL-OPI1</td>
<td>OPI1 coding sequence cloned into the EcoRI/BamHI site of pMAL-c2</td>
<td>Ref. 36</td>
</tr>
<tr>
<td>pMAL-OPI1&lt;sup&gt;313A&lt;/sup&gt;</td>
<td>OPI1&lt;sup&gt;313A&lt;/sup&gt; derivative of pMAL-OPI1</td>
<td>This work</td>
</tr>
<tr>
<td>pMAL-OPI1&lt;sup&gt;251A&lt;/sup&gt;</td>
<td>OPI1&lt;sup&gt;251A&lt;/sup&gt; derivative of pMAL-OPI1</td>
<td>This work</td>
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<td>pSB415</td>
<td>Single-copy E. coli/yeast shuttle vector containing the LEU2 gene</td>
<td>Ref. 68</td>
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<tr>
<td>Yepl351</td>
<td>Multicopy E. coli/yeast shuttle vector containing the LEU2 gene</td>
<td>Ref. 69</td>
</tr>
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<td>pJH354</td>
<td>OPI1 gene ligated into the SacI/HindIII site of Yepl351</td>
<td>Ref. 13</td>
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<td>HA sequence inserted into pJH354 after the ATG start codon in the OPI1 gene</td>
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<td>This work</td>
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<td>HA-tagged OPI1&lt;sup&gt;251A&lt;/sup&gt; derivative of pSaA</td>
<td>This work</td>
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<td>pSaA</td>
<td>HA-tagged OPI1&lt;sup&gt;251A&lt;/sup&gt; gene from pSaA ligated into the SacI/HindIII site of Yepl351</td>
<td>This work</td>
</tr>
<tr>
<td>pJH359</td>
<td>INO1-CYC1:: LacZ reporter construct containing the URA3 gene</td>
<td>Ref. 15</td>
</tr>
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</table>

**TABLE I**

**Strains and plasmids used in this work**

**SDS-Polyacrylamide Gel Electrophoresis and Immunoblot Analysis**—SDS-polyacrylamide gel electrophoresis (46) and immunoblotting (52) using PVDF paper were performed as described previously. Mouse monoclonal anti-HA antibodies (12CA5) were used at a final protein concentration of 0.8 μg/ml as a primary antibody, and goat anti-mouse IgG-alkaline phosphatase conjugate was used as a secondary antibody at a dilution of 1:5,000. The HA-tagged Opi1p proteins were detected on immunoblots using the enhanced chemiluminescence Western blotting detection kit as described by the manufacturer, and the images were acquired by fluorimaging analysis. The relative densities of the protein bands were analyzed using ImageQuant software. Immunoblotting signals were in the linear range of detectability.

**In Vivo Labeling of HA-tagged Opi1p Proteins**—The cells (opi1Δ mutant) bearing multicopy plasmids containing the HA-tagged wild type and S31A and S251A mutant OPI1 alleles were used to examine the phosphorylation of Opi1p in vivo. Exponential phase cells grown in SC medium containing 75 μM inositol were labeled with <sup>32</sup>P (0.25 mCi/mL) for 3 h. Following the incubation, the labeled cells were harvested by centrifugation, washed, and disrupted with glass beads in 50 mM Tris·HCl (pH 7.4) containing protease (0.5 μg of protein) and phosphatase (10 mM NaF, 5 mM β-glycerophosphate, 1 mM sodium vanadate) inhibitors. The HA-tagged Opi1p proteins were immunoprecipitated from cell extracts (0.5 mg of protein) using 4 μg of anti-HA antibodies in 0.5 ml of radioimmunoprecipitation buffer (50 mM Tris·HCl (pH 8.0), 150 mM NaCl, 0.1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) (53). The HA-tagged Opi1p proteins were dissociated from enzyme-antibody complexes (53), subjected to SDS-polyacrylamide gel electrophoresis, and transferred to PVDF paper. The relative amounts of the <sup>32</sup>P-labeled proteins were quantified using ImageQuant software after phosphorimaging analysis.

**Analysis of Data**—The kinetic data were analyzed according to the Michaelis-Menten equation using the EZ-FIT enzyme kinetic modeling program (54). Statistical analyses, including the t test for significance, were performed with SigmaPlot 5.0 software.

**RESULTS**

**Phosphorylation of Opi1p by Protein Kinase A in Vitro**—We examined the hypothesis that Opi1p was a substrate for protein kinase A in vitro. To facilitate well-defined studies, purified MBP-Opi1p was utilized as a protein kinase A substrate. Protein kinase A catalytic subunit from bovine heart was used as the source of kinase enzyme. This kinase is structurally and functionally similar to the S. cerevisiae protein kinase A catalytic subunit (55). To determine whether Opi1p was a target for phosphorylation by protein kinase A, we examined whether the kinase catalyzed the incorporation of the γ phosphate of <sup>32</sup>P-labeled ATP into MBP-Opi1p. After the phosphorylation reaction, the samples were subjected to SDS-polyacrylamide gel electrophoresis and transfer to PVDF paper. Phosphorimaging
analysis of the PVDF paper showed that Opi1p was a substrate for protein kinase A (Fig. 2, lane 3). The position of 32P-labeled MBP-Opi1p on the PVDF paper was confirmed by immunoblot analysis using antibodies to MBP and to Opi1p. The MBP itself was not a substrate for protein kinase A (Fig. 2, lane 2). The autophosphorylation of protein kinase A is also shown in Fig. 2. Immunoblot analysis with anti-MBP antibodies showed that the phosphorylated protein that was not labeled in lane 3 of Fig. 2 is a proteolysis product of MBP-Opi1p. The dependence of protein kinase A activity on MBP-Opi1p and on ATP was examined. Protein kinase A followed saturation kinetics with respect to MBP-Opi1p (Fig. 3A) and with respect to ATP (Fig. 3B). An analysis of the data according to the Michaelis-Menten equation yielded $K_m$ values for MBP-Opi1p and ATP of 70 $\mu$g/ml and 75 $\mu$M, respectively. Under standard phosphorylation conditions, protein kinase A activity was linear with time and with kinase protein concentration.

**Phosphoamino Acid Analysis and Two-dimensional Phosphopeptide Mapping of MBP-Opi1p Phosphorylated by Protein Kinase A**—Protein kinase A (56–58) is a serine/threonine-specific protein kinase. To examine which amino acid residue(s) of Opi1p was a target for phosphorylation, MBP-Opi1p was phosphorylated with protein kinase A, and the 32P-labeled fusion protein was subjected to phosphoamino acid analysis. Protein kinase A phosphorylated Opi1p on a serine residue (Fig. 4). 32P-Labeled MBP-Opi1p was also subjected to digestion with 1-1-tosylamido-2-phenylethyl chloromethyl ketone-trypsin followed by thin layer electrophoresis and chromatographic analysis. The protease digestion yielded several phosphopeptides (Fig. 5A). This indicated that Opi1p was phosphorylated on multiple serine residues by protein kinase A. Some of the peptides shown in the map also resulted from incomplete proteolysis of the MBP-Opi1p (see below).

Previous studies have shown that Opi1p is phosphorylated by protein kinase C at Ser26 as well as at other unidentified sites (36). We questioned whether sites phosphorylated by protein kinase C were the same as the sites phosphorylated by protein kinase A. To address this question, purified MBP-Opi1p was phosphorylated with protein kinase C, and the 32P-labeled protein was subjected to two-dimensional phosphopeptide mapping analysis. The phosphopeptide map of the protein kinase C-phosphorylated MBP-Opi1p fusion protein

**Fig. 2. Phosphorylation of MBP-Opi1p by protein kinase A.** MBP-Opi1p (0.1 mg/ml) was incubated with protein kinase A (0.2 unit/ml) and 0.2 mM [γ-32P]ATP (5,000 cpm/pmol) for 10 min. Following the incubation, the samples were subjected to SDS-polyacrylamide gel electrophoresis, immunoblot analysis, and phosphorimaging. Lane 1 did not contain MBP-Opi1p. Lane 2 contained MBP (0.1 mg/ml) instead of MBP-Opi1p. The positions of MBP-Opi1p, MBP, protein kinase A, and molecular mass standards are indicated in the figure. The phosphorylated protein that was not labeled in the figure is a proteolysis product of MBP-Opi1p. The data shown are representative of two independent experiments.

**Fig. 3. Dependence of protein kinase A activity on the concentrations of MBP-Opi1p and ATP.** A, protein kinase A (0.2 unit/ml) and 0.2 mM [γ-32P]ATP (5,000 cpm/pmol) were incubated with the indicated concentrations of MBP-Opi1p for 10 min. B, protein kinase A (0.2 unit/ml) and MBP-Opi1 (0.1 mg/ml) were incubated with the indicated concentrations of [γ-32P]ATP for 10 min. Following the phosphorylation incubations, the samples were subjected to SDS-polyacrylamide gel electrophoresis, transferred to PVDF paper, and subjected to phosphorimaging analysis. The data shown are representative of two independent experiments.

**Fig. 4. Phosphoamino acid analysis of MBP-Opi1p phosphorylated by protein kinase A.** MBP-Opi1p (0.1 mg/ml) was phosphorylated with protein kinase A (0.2 unit/ml) and 0.2 mM [γ-32P]ATP (5,000 cpm/pmol) for 10 min. Following the phosphorylation incubation, the sample was subjected to SDS-polyacrylamide gel electrophoresis. Gel slices containing 32P-labeled MBP-Opi1p were subjected to phosphoamino acid analysis. The positions of the carrier standard phosphoamino acids are indicated in the figure. The data shown are representative of two independent experiments. P-Ser, phosphoserine; P-Thr, phosphothreonine; P-Tyr, phosphotyrosine.
SQVRESLLNL peptides, which contain sequences for Ser31 peptides to serve as substrates for protein kinase A was examined. The deduced protein sequence of Opi1p revealed that the protein has five potential phosphorylation sites (Ser31, Ser60, Thr141, Thr183, and Ser251) within a protein kinase A sequence motif. Five peptides containing the potential target sites were synthesized based on the deduced protein sequence of Opi1p. The ability of these peptides to serve as substrates for protein kinase A was examined. Of the five peptides, the SCRQKSLPSE and SQVRESLLNL peptides, which contain sequences for Ser31 and Ser251, were substrates for protein kinase A (Fig. 6). None of the other potential sites proved to be targets for protein kinase A phosphorylation by this assay.

Effect of the S31A and S251A Mutations on the Phosphorylation of Opi1p by Protein Kinase A in Vitro—Protein kinase A sequence motif are substrates for protein kinase A. Protein kinase A activity was measured as a function of the concentration of the indicated synthetic peptides. The values reported are the averages of three separate experiments ± S.D.

![Phosphopeptide mapping analysis of wild type and mutant MBP-Opi1p fusion proteins phosphorylated by protein kinase A.](Image 182 to 314)

![Protein Kinase A Phosphorylation of Yeast Opi1p](Image 354 to 526)

![SDS-polyacrylamide gel electrophoresis of purified S31A and S251A mutant MBP-Opi1p fusion proteins.](Image 382 to 498)

![Effects of the S31A and S251A mutations on the phosphorylation of MBP-Opi1p by protein kinase A.](Image 750 to 988)
Protein Kinase A Phosphorylation of Yeast Opi1p

The levels of the wild type and mutant Opi1p on the immunoblots were essentially the same, indicating that the mutations did not affect the expression of the protein. Moreover, the S31A and S251A mutant Opi1p proteins suppressed the inositol excretion phenotype of the opi1Δ mutant, indicating that OPI1S31A and OPI1S251A alleles were functional in vivo.

opi1Δ mutant cells bearing plasmids with the wild type and mutant OPI1H12 alleles were labeled with 32P, followed by the immunoprecipitation of the Opi1p proteins from cell extracts with anti-HA antibodies. SDS-polyacrylamide gel electrophoresis of the immunoprecipitates, transfer to PVDF paper, and phosphorimaging analysis showed that the mutations caused a decrease in the extent of Opi1p phosphorylated in vivo (Fig. 9B). The S31A and S251A mutations caused a decrease in the extent of phosphorylation of Opi1p by 60 and 70%, respectively. Immunoblot analysis showed that the wild type and mutant Opi1p proteins were present at similar amounts. In these experiments, the effects of the mutations on Opi1p phosphorylation were examined in cells grown in the presence of inositol.

Effect of the S31A and S251A Mutations in Opi1p on the Regulation of INO1 Expression—Expression of several UASINO-containing genes, including INO1, is negatively regulated by Opi1p (1, 4–6). Indeed, opi1Δ mutant cells exhibit elevated expression of the INO1 gene in cells grown in the absence and presence of inositol (1, 4–6). The effect of the S31A and S251A mutations in Opi1p on INO1 expression was examined in opi1Δ mutant cells using an INO1-CYC1-lacZ reporter gene (15). In these experiments, we used HA-tagged wild type OPI1 and the OPI1S31A and OPI1S251A alleles in the single-copy plasmids pSA3, pSA5, and pSA6, respectively. Immunoblot analysis using anti-HA antibodies showed that these alleles were expressed at similar levels in opi1Δ mutant cells. The cells were grown in the absence of inositol to the exponential phase, the extracts were prepared, and β-galactosidase activity was measured. As described previously (36), the HA-tagged wild type OPI1 allele suppressed the elevated (9-fold) expression of the INO1 gene in opi1Δ mutant cells grown in the absence of inositol (Fig. 10A). The β-galactosidase activity in opi1Δ mutant cells bearing wild type Opi1p was similar to that found in cells with the chromosomal copy of the OPI1 gene (Fig. 10A). In contrast, the β-galactosidase activity in opi1Δ mutant cells bearing the S31A and S251A mutant Opi1p proteins was 42 and 35% higher (p < 0.001), respectively, than that found in opi1Δ mutant cells bearing the wild type Opi1p protein (Fig. 10A). Thus, under the derepressed condition (i.e. absence of inositol), the S31A and S251A mutations blunted the negative regulatory function of Opi1p.

INO1, as well as other UASINO-containing genes, is repressed by inositol supplementation (1, 4–6). As described previously (36), the β-galactosidase activity directed by the INO1-CYC1-lacZ reporter gene was not repressed by inositol supplementation in opi1Δ mutant cells (Fig. 10B). This defect in the regulation of INO1 was suppressed by the wild type HA-tagged OPI1 allele (Fig. 10B). Moreover, the INO1 gene was repressed (3.5-fold) by inositol supplementation in opi1Δ mutant cells bearing the wild type HA-tagged OPI1 allele (Fig. 10, compare A with B). The INO1 gene was also repressed by inositol in opi1Δ mutant cells bearing the HA-tagged OPI1S31A (2.7-fold) and OPI1S251A (2.6-fold) alleles (Fig. 10, compare A with B). However, the levels of β-galactosidase activity in opi1Δ mutant cells with the S31A and S251A mutant Opi1p proteins were 55 and 52% higher (p < 0.001), respectively, when compared with cells containing wild type Opi1p (Fig. 10B).

DISCUSSION

The Opi1p transcription factor plays a negative regulatory role in the expression of UASINO-containing genes involved in
were relative to the activity (0.1 \text{H9262}) in vitro mutant OPI1 ical and molecular approaches was used to identify protein on the regulatory activity of Opi1p. A combination of biochem-

 kinase A sequence motifs at Ser 31 and Ser 251, respectively, phosphorylation via protein kinase A. 

edge, this is the first report of the posttranslational modifica-

tion of Opi1p on Ser31 and Ser251 using HA-tagged versions of Opi1p expressed in \text{opi1A} mutant cells. The S31A and S251A

mutant HA-tagged proteins were expressed at the same levels as that of wild type Opi1p\text{A}. Moreover, the mutant HA-tagged proteins were functional \text{in vivo} as evidenced by the suppres-

sion of the characteristic inositol excretion phenotype (22) of the \text{opi1A} mutant. The S31A and S251A mutant HA-tagged proteins were phosphorylated \text{in vivo}, but the extent of their phosphorylation was reduced by 60 and 70\%, respectively, when compared with the wild type control protein. The effects of the phosphorylation site mutations on Opi1p regulatory activity were examined \text{in vivo} by the analysis of \text{INO1} expression using a sensitive \text{INO1-CYC1-lacZ} reporter gene assay. This analysis indicated that \text{INO1} expression reached higher dere-

pressed \text{i.e.} absence of inositol) levels in cells carrying the S31A (42\%) and S251A (35\%) mutations when compared with cells carrying wild type Opi1p. The mutations did not have a major effect on the inositol-mediated regulation of \text{INO1} ex-

pression (1, 2, 4–6). However, the inositol-repressed levels of \text{INO1} were elevated in cells carrying the S31A (55\%) and S251A (52\%) mutant forms of Opi1p when compared with the control. These results, together with the evidence that the extent of phosphorylation of the S31A and S251A mutant proteins were reduced when compared with wild type Opi1p, indicated that phosphorylation of Ser\text{31} and Ser\text{251} resulted in the stimulation of Opi1p repressor activity in cells grown in the absence and presence of inositol. The fact that the phosphoryl-

ation sites in question were targets for protein kinase A \text{in vitro} supported the conclusion that protein kinase A was involved in the stimulation of Opi1p repressor activity \text{in vivo}.

Protein kinase A activity is required for proper regulation of growth, progression through the cell cycle, and development in response to various nutrients (37, 38). The enzyme consists of two catalytic subunits (encoded by \text{TPK1}, \text{TPK2}, and \text{TPK3}) and two regulatory subunits (encoded by \text{BCY1}). Elevated \text{CAMP} levels, which are controlled by adenylate cyclase (encoded by \text{CYR1}) via the \text{Ras-CAMP} pathway, promote dissociation of subunits allowing the catalytic subunit to phosphorylate a variety of substrates (37, 38). Some of these substrates are enzymes responsible for the synthesis of phospholipids. For example, the activities of CTP synthetase (59, 60), choline kinase (61, 62), and phosphatidate phosphatase (63) are stim-

ulated by protein kinase A phosphorylation, whereas phosphatidylinerine synthase (64) activity is inhibited by phospho-

rylation. These enzymes play key regulatory roles in phospholipid synthesis (1, 2, 4–6). Studies using phosphoryla-

tion site mutants and mutants defective in the \text{Ras-CAMP} path-

way have shown that the phosphorylation of these enzymes by protein kinase A plays a role in regulating phospholipid syn-

thesis (62, 63, 65). Thus, in the short term, protein kinase A may regulate phospholipid synthesis by modulating the activity of enzymes that are already expressed in the cell, whereas in the long term protein kinase A may regulate phospholipid synthesis by controlling the expression of enzymes via the phosphorylation of the transcription factor Opi1p.

The effect of protein kinase A phosphorylation on Opi1p regulatory activity was opposite to that of protein kinase C. Protein kinase C phosphorylates Opi1p at Ser\text{35}, and this phosphorylation mediates the attenuation of Opi1p regulatory activity (36). Thus, signals transmitted through the \text{Ras-CAMP} and the protein kinase C signaling pathways appear to regu-

late expression of phospholipid synthesis \text{UAS}_{\text{INO1C}} containing genes by opposing mechanisms. The precise mechanism by which phosphorylation via protein kinases A and C mediates Opi1p regulatory activity is not yet known. Phosphorylation of a regulatory protein can control its cellular location, ability to bind DNA, or interaction with other proteins (30–35). The precise target of Opi1p has been an enigma (23, 27).

![Fig. 10. Effects of the S31A and S251A mutations in Opi1p on the expression of the \text{INO1} gene. Wild type cells (WT), \text{opi1A} mutant cells, and \text{opi1A} cells expressing either the wild type \text{OPI1} gene or the mutant \text{OPI1S31A} and \text{OPI1S251A} genes from the single-copy plasmids pS3A, pS5A, and pS6A, respectively, were transformed with pasmid pJH359, which contains the \text{INO1-CYC1-lacZ} reporter gene. The cells were grown in SC medium in the absence (A) and presence (B) of 75 \text{ mg/mL} inositol. The cells were harvested at the exponential phase of growth; the cell extracts were prepared and used for the measurement of \beta-ga-

lactosidase activity. The \beta-galactosidase activities presented in figure A and B were relative to the activity (0.1 \text{ mol/min/mg}) derived from \text{opi1A} mutant cells bearing the wild type \text{OPI1} gene. The values reported were determined from triplicate determinations from four independent growth studies ± S.D.](image)
the recent work of Wagner et al. (25) has shown that Opi1p interacts with the pleiotropic repressor Sin3p and with the phospholipid synthesis positive transcription factor Ino2p, and indeed these interactions may be physiologically relevant (25–29). The availability of the phosphorylation site mutants will permit further studies on the role of phosphorylation by protein kinases A and C on Opi1p interactions with Sin3p and Ino2p and allow us to understand its repressor function in regulating phospholipid synthesis.

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