

Casein Kinase II Phosphorylation of the Yeast Phospholipid Synthesis Transcription Factor Opi1p*

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The transcription factor Opi1p regulates phospholipid synthesis in the yeast *Saccharomyces cerevisiae* by repressing the expression of several UAS_{INO}-containing genes (e.g. *INO1*). Opi1p repressor activity is most active in inositol-supplemented cells. Regulation of Opi1p repressor activity is mediated by multiple phosphorylations catalyzed by protein kinases A and C. In this work, we showed that Opi1p was also phosphorylated by casein kinase II. Using purified maltose-binding protein-Opi1p as a substrate, casein kinase II activity was dose- and time-dependent and dependent on the concentrations of maltose-binding protein-Opi1p ($K_m = 25 \mu\text{g/ml}$) and ATP ($K_m = 7 \mu\text{M}$). Of three mutations (S10A, S38A, and S239A) in putative phosphorylation sites, only the S10A mutation affected Opi1p phosphorylation. That Ser¹⁰ was a specific target of casein kinase II was confirmed by the loss of a phosphopeptide in the S10A mutant protein. The S10A mutation did not affect phosphorylation of Opi1p by either protein kinase A or protein kinase C. Likewise, phosphorylation of Opi1p by casein kinase II was not affected by mutations in protein kinase A (S31A and S251A) and protein kinase C (S26A) phosphorylation sites. Expression of the *OPI1*^{S10A} allele in an *opi1Δ* mutant attenuated (2-fold) the repressive effect of Opi1p on *INO1* expression, and this effect was only observed when cells were grown in the absence of inositol. These data supported the conclusion that casein kinase II phosphorylation at Ser¹⁰ played a role in stimulating the repression of *INO1* when Opi1p was not in its most active state (i.e. in inositol-deprived cells).

The synthesis of phospholipids in the yeast *Saccharomyces cerevisiae* is coordinately regulated through genetic and biochemical mechanisms (1–4). Factors that control phospholipid synthesis include the supplementation of inositol, zinc, and carbon source (1–7). Of these nutrients, inositol has been extensively characterized for its role in the regulation of phospholipid synthesis (1, 3–6). Inositol is an essential nutrient for mammalian cells, which is synthesized in yeast via the *INO1*-encoded inositol-3-phosphate synthase (8). It is an essential precursor for the synthesis of phosphatidylinositol and other inositol-containing lipids in yeast and in mammalian cells (6, 9–15). *INO1* and genes (e.g. *CDS1*, *CHO1/PSS1*, *PSD1*, *CHO2/PEM1*, *OPI3/PEM2*, *CK11*, and *CPT1*) that code for enzymes responsible for the synthesis of phosphatidylcholine are maximally expressed when inositol is absent from the growth medium (1, 3–6). This regulation involves the positive transcription factors Ino2p (16) and Ino4p (17) and a UAS_{INO}² cis-acting element (5,

18–21) in the promoters of the coregulated genes (1, 3, 4, 22). The UAS_{INO} element contains a consensus-binding site (5'-CANNTG-3') for an Ino2p-Ino4p heterodimer, which is required for maximum expression of the UAS_{INO}-containing genes (1, 3, 4, 23–25). Expression of these genes is repressed when inositol is supplemented to the growth medium (1, 3–6). The inositol-mediated repression of these genes requires the ongoing synthesis of phosphatidylcholine (26, 27) and is enhanced by the addition of choline to the growth medium (1, 3–6). The negative transcription factor Opi1p is required for the repression of the UAS_{INO}-containing genes (28, 29).

Based on genetic and biochemical data (3, 4), a model for the inositol-mediated repression of the UAS_{INO}-containing genes has been proposed (30). According to the model, Opi1p is associated with the ER through interactions with the integral membrane protein Scs2p (31) and with PA (30) when cells are grown without inositol. Upon inositol supplementation, the levels of PA decrease because of its utilization in the synthesis of phosphatidylinositol (30, 32). The decrease in PA results in the loss of Opi1p association with the ER, followed by its translocation into the nucleus (30). Opi1p mediates repression of the coregulated phospholipid biosynthetic genes through the UAS_{INO} element (33), but not by direct interaction (34). Instead, Opi1p represses transcriptional activation by binding to DNA-bound Ino2p (35). In addition, the global repressor Sin3p interacts with Opi1p (35), and this interaction plays a role in Opi1p repressor function (34). Opi1p also functions to control expression of the UAS_{INO}-containing genes when cells are grown in the absence of inositol (1, 3–5). This conclusion is based on the fact that *opi1Δ* mutants exhibit elevated expression of the UAS_{INO}-containing genes (1, 3–6) and excrete inositol (28) because of overexpressed levels of the *INO1*-encoded inositol-3-phosphate synthase when cells are grown without inositol (8, 23, 36, 37).

The Opi1p transcription factor is phosphorylated on multiple serine residues (38, 39). Protein kinase A (39) and protein kinase C (38) are involved in this phosphorylation. Ser³¹ and Ser²⁵¹ are major phosphorylation sites for protein kinase A (39), whereas Ser²⁶ is a major protein kinase C phosphorylation site (38) (see Fig. 1). Phosphorylation of Opi1p at Ser³¹ and Ser²⁵¹ mediates the stimulation of the negative regulatory function of Opi1p (39), whereas phosphorylation at Ser²⁶ attenuates its negative regulatory function (38). The regulation of Opi1p function by phosphorylation via protein kinases A and C occurs in cells grown in the absence or presence of inositol (38, 39). In the present work, we examined the hypothesis that Opi1p was also a target of casein kinase II (protein kinase casein kinase 2) phosphorylation. Casein kinase II is a highly conserved serine/threonine protein kinase that is ubiquitous in eukaryotic organisms and is essential for cell viability in *S. cerevisiae* (40–42). The enzyme is composed of two catalytic and two regulatory subunits encoded by the *CKA1*, *CKA2*, *CKB1*, and *CKB2* genes, respectively (43–46). Of three potential phosphorylation sites (see Fig. 1), Ser¹⁰ was identified as a major site. We also showed that phosphorylation of Ser¹⁰ played a role in stimulating Opi1p function in

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² The abbreviations used are: UAS, upstream activating sequence; ER, endoplasmic reticulum; PA, phosphatidate; MBP, maltose-binding protein; HA, hemagglutinin; PVDF, polyvinylidene difluoride.

TABLE 1
Strains and plasmids used in this work

Strain or plasmid	Genotype or relevant characteristics	Source or Ref.
<i>E. coli</i> DH5 α	F ⁻ , ϕ 80dlacZ Δ M15, Δ (lacZYA-argF)U169, <i>deoR</i> , <i>recA1</i> , <i>endA1</i> , <i>hsdR17</i> (r _k ⁻ m _k ⁺), <i>phoA</i> , <i>supE44</i> , λ ⁻ <i>thi-1</i> , <i>gyrA96</i> , <i>relA1</i>	Ref. 48
<i>S. cerevisiae</i> WCG4 SH1100 MC13	<i>MATa his3-11,15 leu 2-3,112 ura3,5</i> <i>opi1Δ::kanMx</i> derivative of WCG4 <i>MATα can1 ino1-13 lys2</i>	Ref. 71 Ref. 38 Ref. 49
Plasmids		
pMAL-c2	<i>E. coli</i> vector with an inducible <i>malE</i> gene used for MBP fusion protein expression	Ref. 72
pMAL-OPI1	<i>OPI1</i> coding sequence cloned into the EcoR1/BamH1 site of pMAL-c2	Ref. 38
pMAL-OPI1 ^{S10A}	<i>OPI1</i> ^{S10A} derivative of pMAL-OPI1	This work
pMAL-OPI1 ^{S38A}	<i>OPI1</i> ^{S38A} derivative of pMAL-OPI1	This work
pMAL-OPI1 ^{S239A}	<i>OPI1</i> ^{S239A} derivative of pMAL-OPI1	This work
pRS415	Single-copy <i>E. coli</i> /yeast shuttle vector containing the <i>LEU2</i> gene	Ref. 73
YEp351	Multiplicity <i>E. coli</i> /yeast shuttle vector containing the <i>LEU2</i> gene	Ref. 74
pJH354	<i>OPI1</i> gene ligated into the SacI/HindIII site of YEp351	Ref. 29
pSA1	HA sequence inserted into pJH354 after the ATG start codon in the <i>OPI1</i> gene	Ref. 38
pSA3	HA-tagged <i>OPI1</i> gene from pSA1 ligated into the SacI/HindIII site of pRS415	Ref. 38
pYC1	HA-tagged <i>OPI1</i> ^{S10A} derivative of pSA3	This work
pYC2	HA-tagged <i>OPI1</i> ^{S10A} from pYC1 ligated into the SacI/HindIII site of YEp351	This work
pJH359	<i>P_{INO1}-lacZ</i> reporter gene construct containing the <i>URA3</i> gene	Ref. 19

cells grown without inositol. This is the first report of the casein kinase II phosphorylation of a membrane phospholipid synthesis transcription factor in yeast.

EXPERIMENTAL PROCEDURES

Materials—All of the chemicals were reagent grade. The growth media were obtained from Difco Laboratories. New England Biolabs was the source of MBP, amylose affinity chromatography resin, recombinant Vent DNA polymerase, restriction endonucleases, and modifying enzymes. DNA gel extraction and plasmid DNA purification kits were purchased from Qiagen. The oligonucleotides were synthesized at Genosys Biotechnologies, Inc. Stratagene was the source of the QuikChange site-directed mutagenesis kit. The Yeast Maker yeast transformation system was from Clontech. The radiochemicals were purchased from PerkinElmer Life Sciences. Aprotinin, benzamide, bovine serum albumin, leupeptin, Nonidet P-40, *O*-nitrophenyl β -D-galactopyranoside, pepstatin, phenylmethylsulfonyl fluoride, phosphoamino acids, and polyvinylpyrrolidone were purchased from Sigma. Bio-Rad was the source of DNA size ladders, electrophoresis reagents, immunochemical reagents, isopropyl β -D-thiogalactoside, molecular mass protein standards, and protein assay reagents. Mouse monoclonal anti-HA antibodies (12CA5) and goat anti-mouse IgG alkaline phosphatase conjugates were from Roche Applied Science and Pierce, respectively. Anti-phosphoglycerate kinase antibodies were from Molecular Probes. Casein kinase II was purchased from New England Biolabs. Protein kinase A catalytic subunit and protein kinase C were purchased from Promega. Lipids were obtained from Avanti Polar Lipids. Protein A-Sepharose CL-4B beads, Hybond-P PVDF paper, and the enhanced chemifluorescence Western blotting detection kit were purchased from Amersham Biosciences. Cellulose thin layer glass plates were from EM Science. Scintillation counting supplies and acrylamide solutions were purchased from National Diagnostics.

Strains, Plasmids, and Growth Conditions—The strains and plasmids used in this work are listed in Table 1. *Escherichia coli* strain DH5 α was used for the propagation of plasmids and for the production of MBP-Opi1p fusion proteins. The cells were grown in LB medium (1% tryptone, 0.5% yeast extract, 1% NaCl, pH 7.4) at 37 °C. Ampicillin (100 μ g/ml) was added to cultures of DH5 α cells that carried plasmids. For the expression of MBP-Opi1p fusion proteins, cultures (250 ml) were grown to the exponential phase ($A_{600\text{ nm}} = 0.5$) at 37 °C, and the cells

were harvested by centrifugation at 5,000 \times g for 5 min and resuspended in fresh media containing 0.6 mM isopropyl β -D-thiogalactoside. After incubation for 3 h at 30 °C, the induced cells were harvested by centrifugation at 5,000 \times g for 5 min, washed with cold 10 mM Tris-HCl buffer (pH 7.4) containing 0.2 M NaCl, 10 mM 2-mercaptoethanol, and 1 mM EDTA, and then stored at -70 °C. The induction was carried out at 30 °C to reduce the degradation of the fusion proteins. The methods for yeast growth were performed as described previously (47, 48). Yeast cultures were grown in YEPD medium (1% yeast extract, 2% peptone, 2% glucose) or in complete synthetic medium (49) containing 2% glucose at 30 °C. For selection of cells bearing plasmids, appropriate amino acids were omitted from the growth medium. 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.

DNA Manipulations, Amplification of DNA by PCR, and DNA Sequencing—Standard methods were used to prepare genomic and plasmid DNA, to digest DNA with restriction enzymes, and to ligate DNA (48). Transformation of *E. coli* (48) and yeast (50, 51) was performed as described previously. PCRs were optimized according to the methods described by Innis and Gelfand (52). DNA sequencing reactions were performed by the dideoxy method using Taq polymerase (48) and analyzed by automated DNA sequencing.

Construction of Plasmids—Plasmid pMAL-OPI1 containing the *malE*-*OPI1* fusion gene (38) was used for the expression of MBP-Opi1p fusion protein. The codons for Ser¹⁰, Ser³⁸, and Ser²³⁹ in *OPI1* were changed to alanine codons. The *OPI1*^{S10A}, *OPI1*^{S38A}, and *OPI1*^{S239A} mutations were constructed by PCR with a QuikChange site-directed mutagenesis kit using appropriate primers and plasmid pMAL-OPI1 as the template. The correct mutations in the *OPI1* alleles were confirmed by DNA sequencing. Plasmid pSA3 is a single-copy plasmid that contains the *OPI1* gene with the sequence for an HA epitope tag inserted after the start codon (38). Plasmid pYC1 that bears the *HA-OPI1*^{S10A} was derived from plasmid pSA3 by site-directed mutagenesis. Plasmid pYC2 was constructed by subcloning *HA-OPI1*^{S10A} from pYC1 into the SacI/HindIII site of plasmid YEp351.

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 amylose-agarose affinity chromatography as described previously (38).

Casein Kinase II Phosphorylation of Yeast *Opi1p*

Phosphorylation Reactions—Phosphorylation reactions were measured for 10 min at 30 °C in a total volume of 25 μ l. The reaction mixture for casein kinase II contained 50 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 10 mM dithiothreitol, 50 μ M [γ -³²P]ATP (5,000 cpm/pmol), 100 μ g/ml MBP-Opi1p, and 10 units of casein kinase II. The reaction mixture for protein kinase A contained 50 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 50 μ M [γ -³²P]ATP (10,000 cpm/pmol), 50 μ g/ml MBP-Opi1p, and 5 units of protein kinase A. The reaction mixture for protein kinase C contained 50 mM Tris-HCl buffer (pH 8.0), 10 mM MgCl₂, 10 mM 2-mercaptoethanol, 0.375 mM EDTA, 0.375 mM EGTA, 1.7 mM CaCl₂, 20 μ M diacylglycerol, 50 μ M phosphatidylserine, 20 μ M [γ -³²P]ATP (10,000 cpm/pmol), 10 μ g/ml MBP-Opi1p, and 25 units of protein kinase C. At the end of the phosphorylation reactions, the samples were treated with 2 \times Laemmli's sample buffer (53), followed by SDS-PAGE. SDS-polyacrylamide gels were dried, and the phosphorylated proteins were subjected to phosphorimaging analysis. The relative amounts of phosphate incorporated into MBP-Opi1p were quantified using ImageQuant software. A unit of protein kinase activity was defined as the amount of enzyme that catalyzed the formation of 1 pmol of phosphorylated product/min.

Phosphoamino Acid Analysis and Phosphopeptide Mapping—³²P-Labeled MBP-Opi1p in SDS-polyacrylamide gel slices was subjected to acid hydrolysis with 6 N HCl as described previously (54). Hydrolysates were dried *in vacuo* and applied to 0.1-mm cellulose thin layer chromatography plates with standard phosphoamino acids (2.5 μ g of phosphoserine, 2.5 μ g of phosphothreonine, and 5 μ g of phosphotyrosine). Phosphoamino acids were separated by two-dimensional electrophoresis (55). Following electrophoresis, the plates were dried and subjected to phosphorimaging analysis. Standard phosphoamino acids were visualized by spraying the plate with 0.25% ninhydrin in acetone. ³²P-Labeled MBP-Opi1p in PVDF membrane slices was subjected to digestion with L-1-tosylamido-2-phenylethyl chloromethyl ketone-trypsin and two-dimensional peptide mapping analysis (56). Electrophoresis (1% ammonium bicarbonate buffer at 1000 volts for 45 min) and ascending chromatography (*n*-butyl alcohol/glacial acetic acid/pyridine/water, 15:10:3:12 for 9 h) were performed on cellulose thin layer glass plates. The dried plates were then subjected to phosphorimaging analysis.

Preparations of Yeast Cell Extracts and Protein Determination—Exponential phase yeast cells were harvested by centrifugation and disrupted with glass beads (0.5-mm diameter) using a Biospec Products Mini-Bead Beater-8 as described previously (37). The cell disruption buffer contained 50 mM Tris-maleate (pH 7.0), 1 mM EDTA, 0.3 M sucrose, 10 mM 2-mercaptoethanol, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, and 5 μ g/ml each of aprotinin, leupeptin, and pepstatin. Glass beads and cell debris were removed by centrifugation at 1,500 \times g for 5 min. The supernatant was used as the cell extract. Protein concentration was estimated by the method of Bradford (57) using bovine serum albumin as the standard.

β -Galactosidase Assay— β -Galactosidase activity was measured in cell extracts at 25 °C by following the conversion of *O*-nitrophenyl β -D-galactopyranoside to *O*-nitrophenol (molar extinction coefficient of 3,500 M⁻¹ cm⁻¹) at 410 nm on a recording spectrophotometer (58). The reaction mixture contained 100 mM sodium phosphate buffer (pH 7.0), 3 mM *O*-nitrophenyl β -D-galactopyranoside, 1 mM MgCl₂, 100 mM 2-mercaptoethanol, and enzyme protein in a total volume of 0.1 ml. The enzyme reactions were linear with time and protein concentration. The average standard deviation of the enzyme assays (performed in triplicate) was \pm 5%. A unit of enzymatic activity was defined as the amount of enzyme that catalyzed the formation of 1 μ mol of product/min.

SDS-PAGE and Immunoblot Analysis—SDS-PAGE (53) and immunoblotting (59) using PVDF paper were performed as described previ-

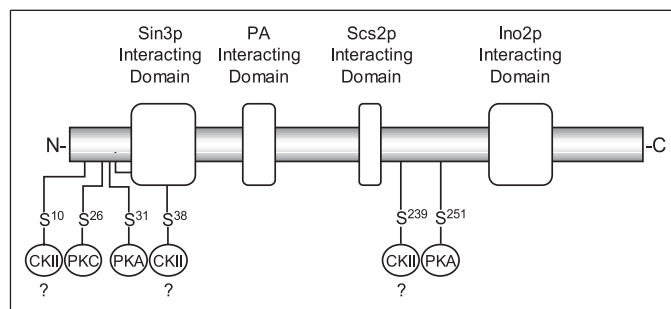


FIGURE 1. **Domain structure of *Opi1p***. The diagram shows the domains in *Opi1p* that interact with Sin3p (residues 45–106), PA (residues 109–138), Scs2p (residues 193–204), and Ino2p (residues 321–380) and the target sites for protein kinase A (PKA) and protein kinase C (PKC). Putative casein kinase II target sites are indicated by question marks.

ously. Mouse monoclonal anti-HA antibodies (12CA5) were used at a final protein concentration of 0.8 μ g/ml as primary antibody, and goat anti-mouse Ig-G-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 chemifluorescence Western blotting detection kit as described by the manufacturer. Immunoblot analysis of phosphoglycerate kinase was performed as a loading control. Anti-phosphoglycerate kinase antibodies were used at a final concentration of 2 μ g/ml. The images were acquired by fluorimaging analysis. Immunoblotting signals were in the linear range of detectability.

***In Vivo* Labeling and Analysis of HA-tagged *Opi1p* Proteins**—*opi1 Δ* mutant cells bearing multicopy plasmids containing the HA-tagged wild type and S10A mutant *OPI1* alleles were grown to exponential phase and then labeled with ³²P_i (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 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 5 μ g/ml aprotinin, 5 μ g/ml leupeptin, 5 μ g/ml pepstatin) and phosphatase inhibitors (10 mM NaF, 5 mM β -glycerophosphate, 1 mM sodium vanadate). The HA-tagged *Opi1p* proteins were immunoprecipitated from cell extracts (0.5 mg of protein) using 4 μ g of anti-HA antibodies in 0.5 ml of radioimmune immunoprecipitation buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) (60). Protein-antibody complexes were collected by centrifugation following incubation with 0.1 ml of protein A-Sepharose CL-4B beads (10% suspension, w/v). The HA-tagged *Opi1p* proteins were dissociated from protein-antibody complexes (60), subjected to SDS-PAGE, and transferred to PVDF paper. The relative amounts of the ³²P-labeled proteins were quantified using ImageQuant software after phosphorimaging analysis.

Analysis of Data—Kinetic data were analyzed according to the Michaelis-Menten and Hill equations using the EZ-FIT enzyme kinetic model fitting program (61). Statistical analyses were performed with SigmaPlot software.

RESULTS

Phosphorylation of *Opi1p* by Casein Kinase II—Analysis of the *Opi1p* sequence using the NetPhosK 2.0 server (www.cbs.dtu.dk/services/NetPhosK/) (62) indicated potential phosphorylation sites at serine residues for casein kinase II (Fig. 1). Accordingly, we questioned whether *Opi1p* was a substrate for casein kinase II. The human casein kinase II holoenzyme expressed and purified from *E. coli* was used as the source of enzyme. For *in vitro* phosphorylation studies, we utilized MBP-Opi1p fusion protein purified from *E. coli* (Fig. 2) (38). The MBP is used to facilitate isolation of *Opi1p* for defined phosphorylation studies (38, 39).

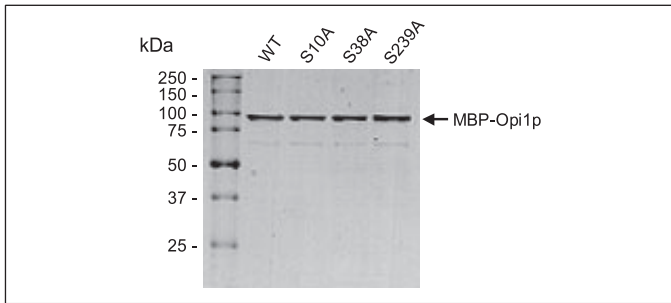


FIGURE 2. SDS-PAGE of purified wild type and S10A, S38A, and S239A mutant MBP-Opi1p fusion proteins. Wild type (WT) and S10A, S38A, and S239A mutant MBP-Opi1p proteins were expressed in *E. coli* and purified by amylose-agarose affinity chromatography. The purified proteins were subjected to SDS-PAGE and stained with Coomassie Blue. The positions of the protein molecular mass standards and the MBP-Opi1p fusion protein (~95 kDa) are indicated in the figure. The protein between the 75- and 50-kDa standards is a proteolysis product of MBP-Opi1p.

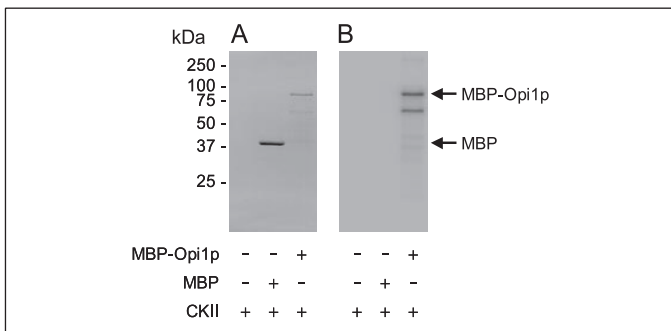


FIGURE 3. Phosphorylation of MBP-Opi1p by casein kinase II. Casein kinase II (CKII, 2 units) was incubated with 100 μM [γ - ^{32}P]ATP (5,000 cpm/pmol) and MBP-Opi1p (0.1 mg/ml) or MBP (0.1 mg/ml) for 10 min. Following the incubation, the samples were subjected to SDS-PAGE followed by Coomassie Blue staining (A) and phosphorimaging analysis (B). The positions of MBP-Opi1p, MBP, and molecular mass standards are indicated in the figure. The phosphorylated protein that is not labeled in the figure is a proteolysis product of MBP-Opi1p. The data shown are representative of three independent experiments.

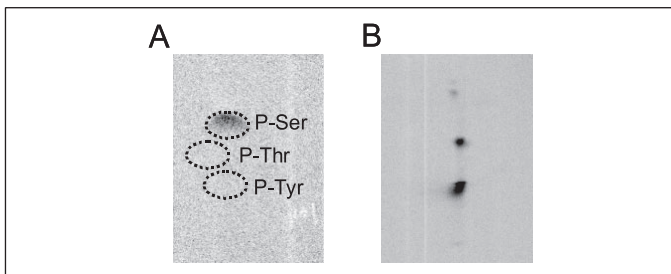


FIGURE 4. Phosphoamino acid and phosphopeptide mapping analyses of MBP-Opi1p phosphorylated by casein kinase II. MBP-Opi1p (0.1 mg/ml) was phosphorylated with 2.5 units of casein kinase II and 100 μM [γ - ^{32}P]ATP (5,000 cpm/pmol) for 30 min followed by SDS-PAGE and transfer to PVDF membrane. A, an SDS-polyacrylamide gel slice containing ^{32}P -labeled MBP-Opi1p was hydrolyzed with 6N HCl for 90 min at 110 $^{\circ}\text{C}$, and the hydrolysate was separated by two-dimensional electrophoresis. The positions of the standard phosphoamino acids phosphoserine (P-Ser), phosphothreonine (P-Thr), and phosphotyrosine (P-Tyr) are indicated in the figure. B, a PVDF membrane slice containing ^{32}P -labeled MBP-Opi1p was digested with trypsin. The resulting peptides were separated on cellulose thin layer plates by electrophoresis (from left to right) in the first dimension and by chromatography (from bottom to top) in the second dimension. The data shown in the two panels are representative of two independent experiments.

The MBP-Opi1p fusion protein was incubated with the casein kinase II in the presence of [γ - ^{32}P]ATP, and the phosphorylation of MBP-Opi1p was monitored by the incorporation of the radioactive γ phosphate into the protein. Phosphorimaging analysis of a dried SDS-polyacrylamide gel showed that MBP-Opi1p was phosphorylated by casein kinase II (Fig. 3B). The position of ^{32}P -labeled MBP-Opi1p on the gel was con-

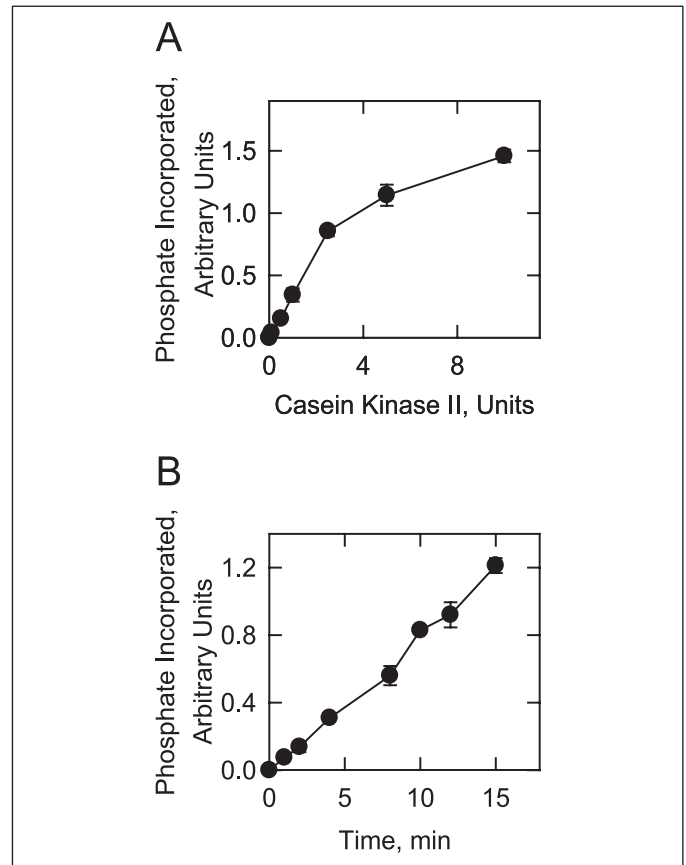


FIGURE 5. Dose- and time-dependent phosphorylation of MBP-Opi1p by casein kinase II. A, MBP-Opi1p (0.1 mg/ml) was incubated with [γ - ^{32}P]ATP (100 μM) and the indicated amounts of casein kinase II for 10 min. B, MBP-Opi1p (0.1 mg/ml) was incubated with 2.5 units of casein kinase II and [γ - ^{32}P]ATP (100 μM) for the indicated time intervals. Following the phosphorylation reactions, the samples were subjected to SDS-PAGE. The SDS-polyacrylamide gels were dried, and the phosphorylated proteins were subjected to phosphorimaging analysis. The relative amounts of phosphate incorporated into MBP-Opi1p were quantified using ImageQuant software. The values reported were determined from triplicate determinations \pm S.D.

firmed by Coomassie Blue staining (Fig. 3A). MBP was not phosphorylated by casein kinase II, and thus phosphorylation of MBP-Opi1p was specific to Opi1p (Fig. 3B).

Phosphoamino acid analysis of the ^{32}P -labeled protein showed that casein kinase II phosphorylated Opi1p at a serine residue (Fig. 4A). In addition, proteolytic digestion of the ^{32}P -labeled protein with trypsin followed by phosphopeptide mapping analysis showed two major phosphopeptides (Fig. 4B). Taken together, these results indicated that Opi1p was phosphorylated on multiple serine residues by casein kinase II.

Using MBP-Opi1p as a substrate, casein kinase II activity was dependent on the amount of the protein kinase (Fig. 5A) and on the time of the phosphorylation reaction (Fig. 5B). Casein kinase II activity was dependent on the concentration of MBP-Opi1p in a manner consistent with positive cooperative kinetics (Fig. 6A). Analysis of the data according to the Hill equation yielded a Hill number and a K_m value for MBP-Opi1p of 1.5 and 25 $\mu\text{g}/\text{ml}$, respectively. The dependence of casein kinase II activity on ATP followed typical saturation kinetics with respect to ATP (Fig. 6B). Analysis of the data according to the Michaelis-Menten equation yielded a K_m for ATP of 7 μM . The stoichiometry of the phosphorylation of MBP-Opi1p by casein kinase II was determined by calculating the amount of radioactive phosphate incorporated into the fusion protein after the kinase reaction was carried out to comple-

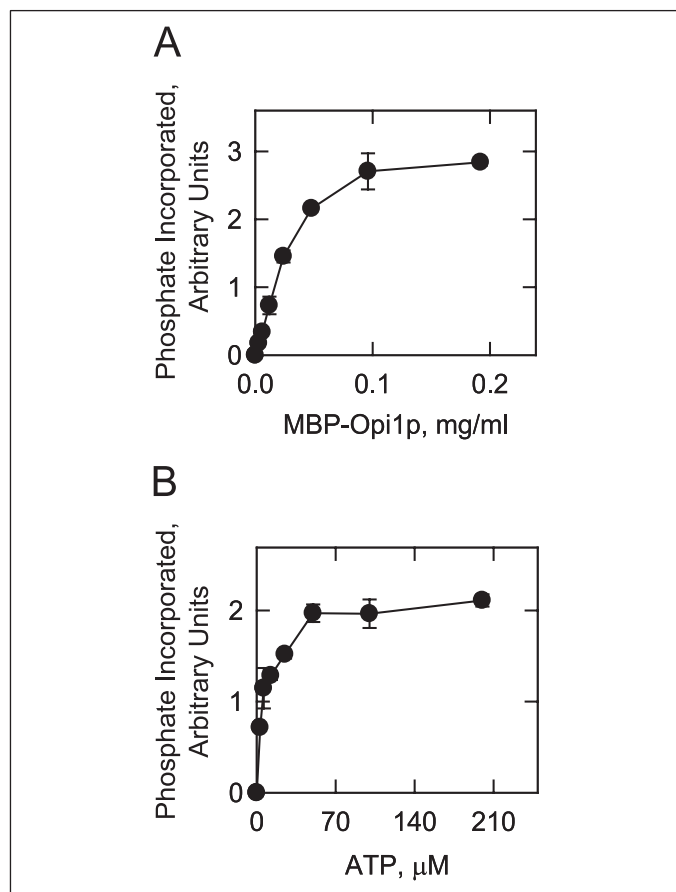


FIGURE 6. Dependence of casein kinase II activity on the concentrations of MBP-Opi1p and ATP. *A*, casein kinase II (2.5 units) was incubated with [γ - 32 P]ATP (100 μ M) and the indicated concentrations of MBP-Opi1p for 10 min. *B*, casein kinase II (2.5 units) was incubated with MBP-Opi1p (0.1 mg/ml) and the indicated concentrations of [γ - 32 P]ATP for 10 min. Following the phosphorylation reactions, the samples were subjected to SDS-PAGE. The SDS-polyacrylamide gels were dried, and the phosphorylated proteins were subjected to phosphorimaging analysis. The relative amounts of phosphate incorporated into MBP-Opi1p were quantified using ImageQuant software. The values reported were determined from triplicate determinations \pm S.D.

tion. At the point of maximum phosphorylation, casein kinase II catalyzed the incorporation of 0.2 mol of phosphate/mol of MBP-Opi1p.

Effects of S10A, S38A, and S239A Mutations on the Phosphorylation of Opi1p—Ser¹⁰, Ser³⁸, and Ser²³⁹ in the Opi1p sequence are putative target sites for casein kinase II phosphorylation (Fig. 1). The codons for these serine residues in *OPI1* were changed to alanine codons by site-directed mutagenesis. The *OPI1*^{S10A}, *OPI1*^{S38A}, and *OPI1*^{S239A} alleles were expressed in *E. coli* as MBP-Opi1p fusion proteins and purified to near homogeneity by amylose-agarose affinity chromatography (Fig. 2). To examine whether Ser¹⁰, Ser³⁸, and Ser²³⁹ were sites of phosphorylation, the S10A, S38A, and S239A mutant proteins were phosphorylated with casein kinase II followed by trypsin digestion and two-dimensional phosphopeptide mapping analysis. The phosphopeptide labeled 1 in the phosphopeptide map of the wild type MBP-Opi1p (Fig. 7A) was absent in the map of the S10A mutant MBP-Opi1p (Fig. 7B). This indicated that Ser¹⁰ was contained in phosphopeptide 1 and thus a target for casein kinase II phosphorylation. All of the phosphopeptides from the wild type protein were present in the maps of the S38A and S239A mutant proteins (Fig. 7). The identities of the amino acid residues contained in the phosphopeptides labeled 2 remain to be determined.

The effect of the S10A mutation on the phosphorylation state of Opi1p *in vivo* was examined using an HA-tagged version of the protein. Previous work has established that HA-Opi1p is functional *in vivo* (38).

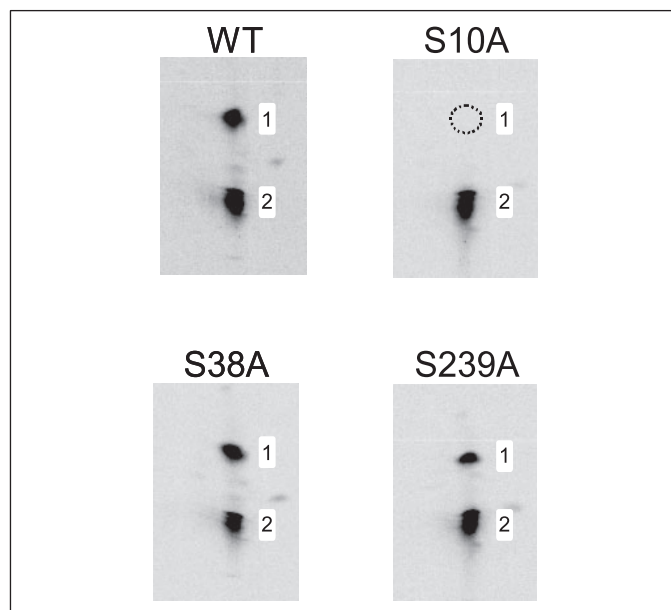
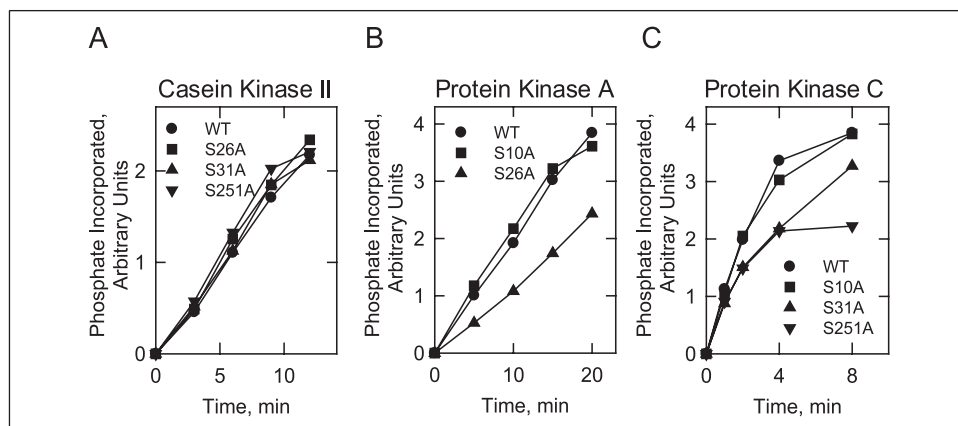


FIGURE 7. Effects of the S10A, S38A, and S239A mutations on the phosphopeptide map of MBP-Opi1p phosphorylated by casein kinase II. Wild type (WT) MBP-Opi1p and S10A, S38A, and S239A mutant MBP-Opi1p proteins were phosphorylated with casein kinase II and 100 μ M [γ - 32 P]ATP (5,000 cpm/pmol) for 30 min followed by SDS-PAGE and transfer to PVDF membrane. PVDF membrane slices containing the 32 P-labeled MBP-Opi1p proteins were digested with trypsin. The resulting peptides were separated on cellulose thin layer plates by electrophoresis (from left to right) in the first dimension and by chromatography (from bottom to top) in the second dimension. The position of the phosphopeptide (labeled 1) that was absent in the S10A mutant MBP-Opi1p protein that was present in the wild type protein is indicated in *B*. The data shown are representative of two independent experiments.

The HA-tagged *OPI1*^{S10A} allele was expressed in *opi1* Δ mutant cells to eliminate the effect of Opi1p encoded by the genomic wild type copy of the *OPI1* gene. A multicopy plasmid was used to increase the expression of Opi1p to facilitate isolation of the phosphorylated form of the protein from cell extracts. As described previously for wild type HA-Opi1p (38), immunoblot analysis showed that the S10A mutant HA-Opi1p migrated on SDS-polyacrylamide gels with a molecular mass of 50 kDa. Moreover, the S10A mutation did not affect the levels of HA-Opi1p. Cells expressing the wild type and S10A mutant HA-Opi1p proteins were labeled with 32 P_i followed by the immunoprecipitation from cell extracts with anti-HA antibodies. Analysis of the immunoprecipitates indicated that the S10A mutation did not have a significant effect on the extent of Opi1p phosphorylation *in vivo* (data not shown).

Hierarchical Phosphorylation of Opi1p by Protein Kinases A and C—Ser³¹ and Ser²⁵¹ are target sites for protein kinase A (39), whereas Ser²⁶ is a target site for protein kinase C (38). We examined the effects of S26A, S31A, and S251A mutations on the phosphorylation of Opi1p by casein kinase II using purified MBP-Opi1p fusion proteins as substrates. Neither the protein kinase A nor the protein kinase C phosphorylation site mutations affected the time-dependent phosphorylation of Opi1p by casein kinase II (Fig. 8A). We also examined the effects of the casein kinase II phosphorylation site mutation (S10A) on Opi1p phosphorylation by protein kinase A and by protein kinase C. The casein kinase II phosphorylation site mutation did not affect the time-dependent phosphorylations of Opi1p by protein kinases A (Fig. 8B) and C (Fig. 8C). However, the S26A protein kinase C phosphorylation site mutation inhibited the phosphorylation of Opi1p by protein kinase A by 37% (Fig. 8B). Likewise, the S31A and S251A protein kinase A phosphorylation site mutations inhibited the phosphorylation of Opi1p by protein kinase C by 15 and 42%, respectively (Fig. 8C).

FIGURE 8. Effects of casein kinase II (S10A), protein kinase A (S31A and S251A), and protein kinase C (S26A) phosphorylation site mutations on phosphorylation of MBP-Opi1p. Wild type and the indicated mutant MBP-Opi1p proteins were phosphorylated with casein kinase II (A), protein kinase A (B), or protein kinase C (C) and [γ - 32 P]ATP for the indicated time intervals. Following the phosphorylation reactions, the samples were subjected to SDS-PAGE. The SDS-polyacrylamide gels were dried, and the phosphorylated proteins were subjected to phosphorimaging analysis. The relative amounts of phosphate incorporated into MBP-Opi1p were quantified using ImageQuant software. The data are the averages of two independent experiments. *WT*, wild type.



Effect of the S10A Mutation on Opi1p Function In Vivo—The *INO1* gene, which encodes inositol-3-phosphate synthase (8, 36, 63), is the most highly regulated UAS_{INO} -containing gene in *S. cerevisiae* (1, 3, 4). Opi1p mediates the repression of *INO1* when wild type cells are supplemented with inositol, whereas the *opi1Δ* mutation results in elevated expression of *INO1* regardless of the presence of inositol (Fig. 9) (8, 23, 36, 37). To address the physiological consequence of casein kinase II phosphorylation of Opi1p at Ser¹⁰, we examined the effect of the S10A mutation on the expression of *INO1* in cells grown with and without inositol. This analysis was facilitated by use of a P_{INO1} -*lacZ* reporter gene where the expression of β -galactosidase activity is dependent on transcription driven by sequences in the *INO1* promoter (19). β -Galactosidase activity was measured in *opi1Δ* mutant cells bearing the HA-tagged wild type *OPI1* and *OPI1*^{S10A} alleles present on single copy plasmids. Immunoblot analysis using anti-HA antibodies showed that these alleles were expressed at similar levels in *opi1Δ* mutant cells. As described previously (38), the expression of *INO1* was suppressed in *opi1Δ* mutant cells grown in the absence and presence of inositol by the wild type *OPI1* gene (Fig. 9). In cells grown without inositol, the *OPI1*^{S10A} allele also suppressed *INO1* expression in *opi1Δ* mutant cells but not to the same extent as that of the wild type allele (Fig. 9). The β -galactosidase activity in cells bearing the *OPI1*^{S10A} allele was 2-fold greater when compared with cells bearing the wild type *OPI1* allele (Fig. 9). In contrast, the suppression of *INO1* expression by *OPI1* in the *opi1Δ* mutant was not affected by the S10A mutation in cells supplemented with inositol (Fig. 9).

DISCUSSION

The transcription factor Opi1p, which plays a major role in the regulation of membrane phospholipid synthesis in *S. cerevisiae* (1, 3–5, 64), is phosphorylated on multiple serine residues (38, 39). Phosphorylations of Ser³¹ and Ser²⁵¹ and of Ser²⁶ by protein kinases A (39) and C (38), respectively, contribute to the overall phosphorylation of the protein (38, 39). In this work, we addressed the hypothesis that Opi1p is also phosphorylated by casein kinase II. This highly conserved serine/threonine protein kinase is essential to cell growth through phosphorylation of proteins involved in gene expression, growth control, signal transduction, and cell cycle progression (40, 65, 66). Studies using the purified MBP-Opi1p fusion protein showed that Opi1p was indeed a *bona fide* substrate for casein kinase II *in vitro*. The phosphorylation of Opi1p was dependent on the amount of casein kinase II and on the time of the reaction. In addition, the phosphorylation reaction was dependent on the concentrations of MBP-Opi1p and ATP. That Opi1p was phosphorylated on multiple serine residues by casein kinase II was demonstrated by phosphopeptide mapping analysis.

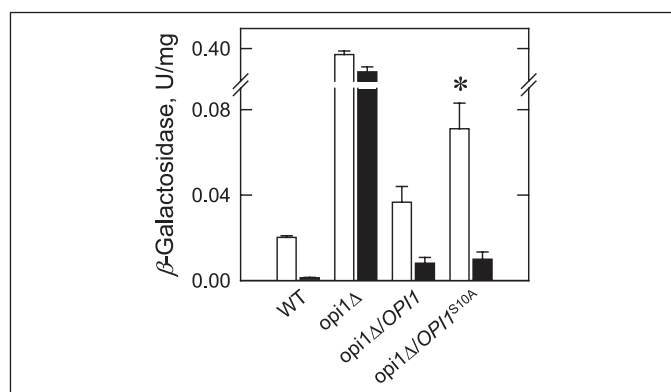


FIGURE 9. Effect of the S10A mutation in Opi1p on the expression of the *INO1* gene. Wild type (*WT*) cells, *opi1Δ* mutant cells, and *opi1Δ* mutant cells expressing either the wild type *OPI1* gene or the mutant *OPI1*^{S10A} gene from the single copy plasmids pSA3 and pYC1, respectively, were transformed with plasmid pJH359, which contains the P_{INO1} -*lacZ* reporter gene. The cells were grown in complete synthetic medium in the absence (white bars) and presence (black bars) of 75 μ M inositol. The cells were harvested at the exponential phase of growth; cell extracts were prepared and used for the measurement of β -galactosidase activity. The values reported were determined from triplicate determinations from four independent growth studies \pm S.D. The asterisk signifies $p < 0.001$.

We examined the hypothesis that Ser¹⁰, Ser³⁸, and Ser²³⁹ were casein kinase II phosphorylation sites through the analysis of serine to alanine mutations. Of the three mutations, only the S10A mutation affected Opi1p phosphorylation *in vitro*. Phosphopeptide mapping analysis of casein kinase II-phosphorylated Opi1p proteins showed that a major phosphopeptide present in the wild type Opi1p protein was absent from the S10A mutant protein. This confirmed that Ser¹⁰ was a specific target of casein kinase II. Although Opi1p was phosphorylated by casein kinase II at sites other than Ser¹⁰, the potential sites at Ser³⁸ and Ser²³⁹ were not targets because the S38A and S239A mutations did not affect the phosphopeptide map of the protein. The phosphorylation of Opi1p is consistent with the role casein kinase II plays in controlling cell growth (40).

Opi1p is phosphorylated on multiple residues (38, 39), and the loss of one phosphorylation site may not be expected to affect the overall phosphorylation state of the protein *in vivo*. Indeed, the S10A mutation did not have a significant effect on the extent of Opi1p phosphorylation. On the other hand, the overall phosphorylation state of Opi1p is reduced (~50%) by protein kinase A phosphorylation site (S31A and S251A) and protein kinase C phosphorylation site (S26A) mutations (38, 39). *In vitro*, the mutation (S26A) in the protein kinase C target site reduced phosphorylation of Opi1p by protein kinase A. Likewise, the mutations (S31A and S251A) in protein kinase A target sites reduced phosphorylation by protein kinase C. In contrast, the mutation (S10A) in the casein

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kinase II target site did not affect the *in vitro* phosphorylation by either protein kinase A or protein kinase C. Furthermore, the mutations in the protein kinase A or protein kinase C target sites did not affect phosphorylation of Opi1p by casein kinase II. These results indicated that phosphorylation by protein kinase A stimulated phosphorylation by protein kinase C and vice versa and that the phosphorylations by these kinases were independent of the phosphorylation by casein kinase II. The hierarchical phosphorylations (67) by protein kinases A and C may provide an explanation as to why the protein kinase A and protein kinase C phosphorylation site mutations affected the overall phosphorylation state of Opi1p *in vivo*, whereas the casein kinase II site mutation did not have a major effect on the overall phosphorylation state of the protein.

Opi1p plays a negative regulatory role in the expression of *INO1* and other UAS_{INO}-containing genes involved in the synthesis of membrane phospholipids in *S. cerevisiae* (1, 3–6, 32). Opi1p exerts its repressor activity by a mechanism that involves its translocation from the ER into the nucleus and interaction with the positive transcription factor Ino2p that exists in a complex with Ino4p bound to the promoters of UAS_{INO}-containing genes (30, 32, 35). Genetic and biochemical data indicate that the repressor activity of Opi1p is governed to a large extent by the concentration of PA, one of the molecules that Opi1p associates with at the ER (3, 4, 30). Reduction in PA concentration (e.g. in response to inositol supplementation) correlates with the Opi1p-mediated repression of UAS_{INO}-containing genes (3, 4, 30, 32). However, Opi1p has a repressive effect on the expression of UAS_{INO}-containing genes even when wild type cells are grown in absence of inositol (1, 3–5). This indicates that some population of Opi1p is always localized within the nucleus to interact with Ino2p.

As described previously (35, 39), the expression of the wild type *OPI1* gene in an *opi1Δ* mutant caused repression of *INO1* in cells grown in the absence or presence of inositol. Expression of the *OPI1*^{S10A} allele in the *opi1Δ* mutant attenuated (2-fold) the repressive effect of Opi1p on *INO1* expression. However, this effect was only observed when cells were grown in the absence of inositol. Thus, the phosphorylation at Ser¹⁰ by casein kinase II played a role in stimulating the repression of a UAS_{INO}-containing gene when Opi1p was not in its most active state (i.e. inositol-deprived cells). That Ser¹⁰ was a target for casein kinase II *in vitro* supported the conclusion that this kinase was involved in the stimulation of Opi1p repressor activity. Although casein kinase II has been found to be associated with the cytoplasm, it is primarily associated within the nucleus where it phosphorylates proteins to control transcription and cell growth (40, 68–70). One mechanism by which casein kinase II phosphorylation might stimulate Opi1p repressor activity is to facilitate Opi1p interaction with Ino2p in the nucleus. An alternative mechanism is that phosphorylation facilitates the dissociation of Opi1p from Scs2p and/or PA at the ER. Additional studies will be required to address these hypotheses.

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REFERENCES

- Greenberg, M. L., and Lopes, J. M. (1996) *Microbiol. Rev.* **60**, 1–20
- Carman, G. M., and Zeimet, G. M. (1996) *J. Biol. Chem.* **271**, 13293–13296
- Henry, S. A., and Patton-Vogt, J. L. (1998) *Prog. Nucleic Acids Res.* **61**, 133–179
- Carman, G. M., and Henry, S. A. (1999) *Prog. Lipid Res.* **38**, 361–399
- Carman, G. M., and Henry, S. A. (1989) *Annu. Rev. Biochem.* **58**, 635–669
- Paltauf, F., Kohlwein, S. D., and Henry, S. A. (1992) in *The Molecular and Cellular Biology of the Yeast Saccharomyces: Gene Expression* (Jones, E. W., Pringle, J. R., and Broach, J. R., eds) pp. 415–500, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

- Carman, G. M. (2005) *Biochem. Soc. Trans.* **33**, 1150–1153
- Donahue, T. F., and Henry, S. A. (1981) *J. Biol. Chem.* **256**, 7077–7085
- White, M. J., Lopes, J. M., and Henry, S. A. (1991) *Adv. Microbiol. Physiol.* **32**, 1–51
- Lester, R. L., and Dickson, R. C. (1993) *Adv. Lipid Res.* **26**, 253–274
- Downes, C. P., and Macphee, C. H. (1990) *Eur. J. Biochem.* **193**, 1–18
- Divecha, N., and Irvine, R. F. (1995) *Cell* **80**, 269–278
- Dove, S. K., Cooke, F. T., Douglas, M. R., Sayers, L. G., Parker, P. J., and Michell, R. H. (1997) *Nature* **390**, 187–192
- Odom, A. R., Stahlberg, A., Wenthe, S. R., and York, J. D. (2000) *Science* **287**, 2026–2029
- Odorizzi, G., Babst, M., and Emr, S. D. (2000) *Trends Biochem. Sci.* **25**, 229–235
- Nikoloff, D. M., McGraw, P., and Henry, S. A. (1992) *Nucleic Acids Res.* **20**, 3253
- Hoshizaki, D. K., Hill, J. E., and Henry, S. A. (1990) *J. Biol. Chem.* **265**, 4736–4745
- Kodaki, T., Nikawa, J., Hosaka, K., and Yamashita, S. (1991) *J. Bacteriol.* **173**, 7992–7995
- Lopes, J. M., Hirsch, J. P., Chorgo, P. A., Schulze, K. L., and Henry, S. A. (1991) *Nucleic Acids Res.* **19**, 1687–1693
- Schuller, H. J., Hahn, A., Troster, F., Schutz, A., and Schweizer, E. (1992) *EMBO J.* **11**, 107–114
- Schuller, H. J., Richter, K., Hoffmann, B., Ebbert, R., and Schweizer, E. (1995) *FEBS Lett.* **370**, 149–152
- Ambroziak, J., and Henry, S. A. (1994) *J. Biol. Chem.* **269**, 15344–15349
- Hirsch, J. P., and Henry, S. A. (1986) *Mol. Cell. Biol.* **6**, 3320–3328
- Loewy, B. S., and Henry, S. A. (1984) *Mol. Cell. Biol.* **4**, 2479–2485
- Schwank, S., Ebbert, R., Rautenstrauss, K., Schweizer, E., and Schuller, H. J. (1995) *Nucleic Acids Res.* **23**, 230–237
- Gaynor, P. M., Gill, T., Toutenhoofd, S., Summers, E. F., McGraw, P., Homann, M. J., Henry, S. A., and Carman, G. M. (1991) *Biochim. Biophys. Acta* **1090**, 326–332
- Morash, S. C., McMaster, C. R., Hjelmsstad, R. H., and Bell, R. M. (1994) *J. Biol. Chem.* **269**, 28769–28776
- Greenberg, M., Reiner, B., and Henry, S. A. (1982) *Genetics* **100**, 19–33
- White, M. J., Hirsch, J. P., and Henry, S. A. (1991) *J. Biol. Chem.* **266**, 863–872
- Loewen, C. J. R., Gaspar, M. L., Jesch, S. A., Delon, C., Ktistakis, N. T., Henry, S. A., and Levine, T. P. (2004) *Science* **304**, 1644–1647
- Loewen, C. J., Roy, A., and Levine, T. P. (2003) *EMBO J.* **22**, 2025–2035
- Jesch, S. A., Zhao, X., Wells, M. T., and Henry, S. A. (2005) *J. Biol. Chem.* **280**, 9106–9118
- Bachhawat, N., Ouyang, Q., and Henry, S. A. (1995) *J. Biol. Chem.* **270**, 25087–25095
- Wagner, C., Blank, M., Strohmman, B., and Schüller, H. J. (1999) *Yeast* **15**, 843–854
- Wagner, C., Dietz, M., Wittmann, J., Albrecht, A., and Schuller, H. J. (2001) *Mol. Microbiol.* **41**, 155–166
- Klig, L. S., and Henry, S. A. (1984) *Proc. Natl. Acad. Sci. U. S. A.* **81**, 3816–3820
- Klig, L. S., Homann, M. J., Carman, G. M., and Henry, S. A. (1985) *J. Bacteriol.* **162**, 1135–1141
- Sreenivas, A., Villa-Garcia, M. J., Henry, S. A., and Carman, G. M. (2001) *J. Biol. Chem.* **276**, 29915–29923
- Sreenivas, A., and Carman, G. M. (2003) *J. Biol. Chem.* **278**, 20673–20680
- Glover, C. V., III (1998) *Prog. Nucleic Acids Res. Mol. Biol.* **59**, 95–133
- Litchfield, D. W. (2003) *Biochem. J.* **369**, 1–15
- Guerra, B., and Issinger, O. G. (1999) *Electrophoresis* **20**, 391–408
- Chen-Wu, J. L., Padmanabha, R., and Glover, C. V. (1988) *Mol. Cell. Biol.* **8**, 4981–4990
- Reed, J. C., Bidwai, A. P., and Glover, C. V. C. (1994) *J. Biol. Chem.* **269**, 18192–18200
- Bidwai, A. P., Reed, J. C., and Glover, C. V. (1995) *J. Biol. Chem.* **270**, 10395–10404
- Padmanabha, R., and Glover, C. V. C. (1987) *J. Biol. Chem.* **262**, 1829–1835
- Rose, M. D., Winston, F., and Heiter, P. (1990) *Methods in Yeast Genetics: A Laboratory Course Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
- Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
- Culbertson, M. R., and Henry, S. A. (1975) *Genetics* **80**, 23–40
- Ito, H., Yasuki, F., Murata, K., and Kimura, A. (1983) *J. Bacteriol.* **153**, 163–168
- Schiestl, R. H., and Gietz, R. D. (1989) *Curr. Genet.* **16**, 339–346
- Innis, M. A., and Gelfand, D. H. (1990) in *PCR Protocols: A Guide to Methods and Applications* (Innis, M. A., Gelfand, D. H., Sninsky, J. J., and White, T. J., eds) pp. 3–12, Academic Press, Inc., San Diego, CA
- Laemmli, U. K. (1970) *Nature* **227**, 680–685
- Yang, W.-L., and Carman, G. M. (1996) *J. Biol. Chem.* **271**, 28777–28783
- Boyle, W. J., Van der Geer, P., and Hunter, T. (1991) *Methods Enzymol.* **201**, 110–149
- MacDonald, J. I. S., and Kent, C. (1994) *J. Biol. Chem.* **269**, 10529–10537
- Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
- Craven, G. R., Steers, E., Jr., and Anfinson, C. B. (1965) *J. Biol. Chem.* **240**, 2468–2477
- Haid, A., and Suissa, M. (1983) *Methods Enzymol.* **96**, 192–205
- Harlow, E., and Lane, D. (1988) *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
- Perrella, F. (1988) *Anal. Biochem.* **174**, 437–447

62. Blom, N., Sicheritz-Ponten, T., Gupta, R., Gammeltoft, S., and Brunak, S. (2004) *Proteomics* **4**, 1633–1649
63. Dean-Johnson, M., and Henry, S. A. (1989) *J. Biol. Chem.* **264**, 1274–1283
64. Wu, W.-I., Lin, Y.-P., Wang, E., Merrill, A. H., Jr., and Carman, G. M. (1993) *J. Biol. Chem.* **268**, 13830–13837
65. Pinna, L. A. (2002) *J. Cell Sci.* **115**, 3873–3878
66. Meggio, F., and Pinna, L. A. (2003) *FASEB J.* **17**, 349–368
67. Roach, P. J. (1991) *J. Biol. Chem.* **266**, 14139–14142
68. Huh, W.-K., Falvo, J. V., Gerke, L. C., Carroll, A. S., Howson, R. W., Weissman, J. S., and O'Shea, E. K. (2003) *Nature* **425**, 686–691
69. Krek, W., Maridor, G., and Nigg, E. A. (1992) *J. Cell Biol.* **116**, 43–55
70. Chester, N., Yu, I. J., and Marshak, D. R. (1995) *J. Biol. Chem.* **270**, 7501–7514
71. Heinemeyer, W., Gruhler, A., Mohrle, V., Mahe, Y., and Wolf, D. H. (1993) *J. Biol. Chem.* **268**, 5115–5120
72. Maina, C. V., Riggs, P. D., Grandea III, A. G., Slatko, B. E., Moran, L. S., Tagliamonte, J. A., McReynolds, L. A., and di Guan, C. (1988) *Gene (Amst.)* **74**, 365–373
73. Sikorski, R. S., and Hieter, P. (1989) *Genetics* **122**, 19–27
74. Hill, J. E., Myers, A. M., Koerner, T. J., and Tzagoloff, A. (1986) *Yeast* **2**, 163–167