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Phosphorylation of the Yeast Phospholipid Synthesis Regulatory Protein Opi1p by Protein Kinase C*

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Opi1p is a negative regulator of expression of phospholipid-synthesizing enzymes in the yeast *Saccharomyces cerevisiae*. In this work, we examined the phosphorylation of Opi1p by protein kinase C. Using a purified maltose-binding protein-Opi1p fusion protein as a substrate, protein kinase C activity was time- and dose-dependent, and dependent on the concentrations of Opi1p and ATP. Protein kinase C phosphorylated Opi1p on a serine residue. The Opi1p synthetic peptide GVLKQSCRQK, which contained a protein kinase C sequence motif at Ser^{26}, was a substrate for protein kinase C. Phosphorylation of a purified S26A mutant maltose-binding protein-Opi1p fusion protein by the kinase was reduced when compared with the wild-type protein. A major phosphopeptide present in purified wild-type Opi1p was absent from the purified S26A mutant protein. *In vivo* labeling experiments showed that the phosphorylation of Opi1p was physiologically relevant, and that the extent of phosphorylation of the S26A mutant protein was reduced by 50% when compared with the wild-type protein. The physiological consequence of the phosphorylation of Opi1p at Ser^{26} was examined by measuring the effect of the S26A mutation on the expression of the phospholipid synthesis gene *INO1*. The β-galactosidase activity driven by an *INO1-CYC-lacI′Z* reporter gene in *opi1*Δ mutant cells expressing the S26A mutant Opi1p was about 50% lower than that of cells expressing the wild-type Opi1p protein. These data supported the conclusion that phosphorylation of Opi1p at Ser^{26} mediated the attenuation of the negative regulatory function of Opi1p on the expression of the *INO1* gene.

Saccharomyces cerevisiae serves as a model eukaryote to study the regulation of phospholipid synthesis (1–6). PC,[1] PE, PI, and PS are the major phospholipids found in the membranes of this yeast (1, 2). Mitochondrial membranes also contain phosphatidylglycerol and cardiolipin (1, 2). PC is produced by two alternative pathways: the CDP-DG pathway and the CDP-choline pathway (1–6) (Fig. 1). Wild-type cells primarily synthesize PC by the CDP-DG pathway when they are grown in the absence of exogenous choline (1–6). However, the CDP-choline pathway contributes to PC synthesis under this growth condition (5, 7). The PC synthesized via the CDP-DG pathway is constantly hydrolyzed to free choline and PA by the reaction catalyzed by phospholipase D (7). The free choline is incorporated back into PC via the CDP-choline pathway and the PA is incorporated back into phospholipids (*e.g.* PI) via CDP-DG (7) (Fig. 1). The CDP-choline pathway is essential for mutants defective in CDP-DG pathway enzymes. Mutants defective in the synthesis of PS, PE, or PC require choline for growth in order to synthesize PC via the CDP-choline pathway (1–6). Mutants defective in the synthesis of PS and PE can also synthesize PC if they are supplemented with ethanolamine (1–6). The ethanolamine is utilized in the CDP-ethanolamine pathway for the synthesis of PE, which is then methylated to form PC in the CDP-DG pathway (1–6) (Fig. 1).

A number of factors regulate phospholipid synthesis in *S. cerevisiae*. These include water-soluble phospholipid precursors, nucleotides, lipids, and growth phase (1–6). Phospholipid synthesis regulation is complex and occurs by genetic and biochemical mechanisms. The water-soluble phospholipid precursor of PI, namely inositol (Fig. 1), plays a major role in the genetic regulation of phospholipid synthesis (1, 2, 4–6). Enzymes in the CDP-DG (*e.g.* encoded by the *CDS1*, *CHO1*/*PSS*, *PSD1*, *CHO2*/*PEM1*, and *OPI3*/*PEM2*) and CDP-choline (*e.g.* encoded by *CKI1* and *CPT1*) pathways, as well as inositol-1-phosphate synthase (encoded by *INO1*), are repressed by inositol supplementation (1, 2, 4–6). Repression by inositol is enhanced by the inclusion of choline in the growth medium (1, 2, 4–6). The regulation by inositol supplementation involves the transcriptional regulatory proteins Ino2p, Ino4p, and Opi1p (1, 2, 4–6). Regulation of the co-regulated enzymes by inositol is mediated by a UAS_{INO} *cis*-acting element (1, 8, 9) present in the promoters of their structural genes (1, 2, 4–6). The UAS_{INO} element contains the binding site for an Ino2p/Ino4p heterodimer, which is necessary for maximum expression of the co-regulated UAS_{INO}-containing genes (4–6, 10, 11). Repression of the co-regulated genes requires Opi1p (12, 13). Opi1p contains a leucine zipper and two glutamine-rich domains (13). These motifs, which are generally associated with DNA-binding proteins (13), are indispensable for Opi1p repressor function (14). Opi1p mediates its negative regulatory role through the UAS_{INO} element (15). However, it does not interact with the UAS_{INO} element directly or with Ino2p or Ino4p (14, 16). The mechanism of Opi1p activity is not yet fully understood.

To gain further insight into the transcriptional regulation of phospholipid synthesis genes from *S. cerevisiae*, we initiated studies to examine the posttranslational modification of the

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[1] The abbreviations used are: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PA, phosphatidate; CDP-DG, CDP-diacylglycerol; DG, diacylglycerol; PCR, polymerase chain reaction; kb, kilobase(s); MBP, maltose-binding protein; SC, synthetic complete; UAS, upstream activating sequence; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

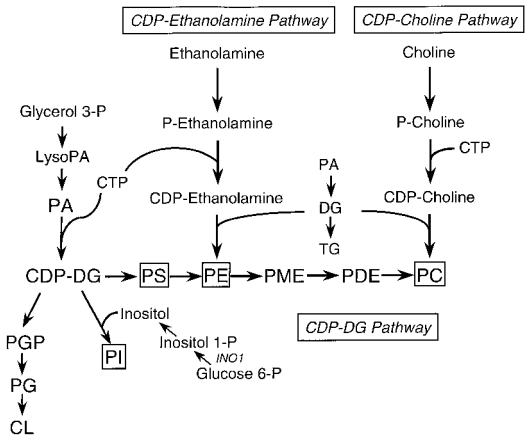

FIG. 1. **Pathways for the synthesis of phospholipids in *S. cerevisiae*.** The pathways shown for phospholipid synthesis include the relevant steps discussed in the text. The major phospholipids (PC, PE, PI, and PS) are indicated by *boxes*. The CDP-DG, CDP-choline, and CDP-ethanolamine pathways are indicated. The *INO1*-encoded inositol-1-P synthase reaction is indicated in the figure. A more comprehensive description of these pathways that includes additional steps can be found elsewhere (2, 5). *PME*, phosphatidylmonomethylethanolamine; *PDE*, phosphatidyldimethylethanolamine; *TG*, triacylglycerol; *PGP*, phosphatidylglycerophosphate; *PG*, phosphatidylglycerol; *CL*, cardiolipin; other abbreviations are defined in Footnote 1.

Opi1p regulatory protein by phosphorylation via protein kinase C. Phosphorylation is a mechanism by which the function of a regulatory protein may be controlled (17, 18). Protein kinase C is a transducer of lipid second messengers (19–21) and plays a central role in the regulation of a host of cellular functions, including cell growth and proliferation (22–24). In this study, we demonstrated that Opi1p was phosphorylated by protein kinase C, and that a major site of phosphorylation was Ser^{26}. Moreover, a S26A mutant Opi1p protein caused reduced *INO1* expression. Elimination of functional Opi1p by deletion of the *OPI1* gene results in higher levels of *INO1* expression, indicating that Opi1p functions as a negative regulator. Since a mutation reducing phosphorylation of Opi1p has an effect opposite of an *opi1*Δ mutation (*i.e.* reduction in *INO1* expression *versus* deletion), we hypothesize that phosphorylation at Ser^{26} functions to inactivate Opi1p and attenuate its activity.

EXPERIMENTAL PROCEDURES

Materials—All the chemicals were reagent-grade. Growth medium supplies were purchased from Difco Laboratories. Restriction endonucleases, modifying enzymes, recombinant Vent DNA polymerase, pMAL-c2 vector (25), amylose-agarose affinity chromatography resin, MBP, anti-MBP antibodies, and factor Xa protease were purchased from New England Biolabs. Genosys Biotechnologies, Inc. prepared the oligonucleotides used for PCR and DNA sequencing. The QuikChange site-directed mutagenesis kit was purchased from Stratagene. Phenylmethylsulfonyl fluoride, bovine serum albumin, histone, benzamidine, aprotinin, leupeptin, pepstatin, standard phosphoamino acids, nitrocellulose paper, and *O*-nitrophenyl β-D-galactopyranoside were purchased from Sigma. CNBr was obtained from Aldrich. Radiochemicals were purchased from PerkinElmer Life Sciences. Phosphocellulose filters were purchased from Pierce. Protein assay reagents, electrophoretic reagents, immunochemical reagents, and isopropyl-β-D-thiogalactoside were purchased from Bio-Rad. Mouse monoclonal anti-HA antibodies (12CA5) were from Roche Molecular Biochemicals. Purified rat brain protein kinase C was purchased from Promega. *PKC1*-encoded ZZ-tagged protein kinase C was partially purified from *S. cerevisiae* (26). Protein A-Sepharose, polyvinylidene difluoride membrane, and the enhanced chemifluorescence Western blotting detection kit were purchased from Amersham Pharmacia Biotech. PS and DG were purchased from Avanti Polar Lipids. Cellulose thin layer sheets were from EM Science. Scintillation counting supplies and acrylamide solutions were purchased from National Diagnostics. Peptides GVLKQSCRQK, GVLKQACRQK, ILDRVSNKII, MSIESKKRLV, and ANKQLSDKIS were synthesized and purified commercially by Bio-Synthesis, Inc.

Strains, Plasmids, Oligonucleotides, and Growth Conditions—The strains and plasmids, and oligonucleotides used in this work are listed in Tables I and II, respectively. *Escherichia coli* strain DH5α was used for the propagation of plasmids and the production of MBP-Opi1p fusion protein directed by the inducible pMAL-c2-OPI1 plasmids. Cells were grown in LB medium (1% tryptone, 0.5% yeast extract, 1% NaCl, pH 7.4) at 37 °C. Ampicillin (150 μg/ml) was added to cultures of DH5α-carrying plasmids. For the production of MBP-Opi1p fusion proteins, cultures (250 ml) were grown to the exponential phase ($A_{600\ nm}$ = 0.4–0.6) at 37 °C, harvested, and resuspended in fresh medium containing 0.6 mM isopropyl-β-D-thiogalactoside. After incubation for 3 h at 30 °C, cells were harvested, washed with 10 mM Tris-HCl buffer (pH 7.4) containing 0.2 M NaCl, 10 mM 2-mercaptoethanol, and 1 mM Na_2EDTA, and then frozen at −70 °C. The induction was carried out at 30 °C to reduce the degradation of the fusion proteins. Methods for yeast growth were performed as described previously (27, 28). Yeast cultures were grown in YEPD medium (1% yeast extract, 2% peptone, 2% glucose) or in synthetic complete (SC) medium minus inositol (29) containing 2% glucose at 30 °C. For selection of cells bearing plasmids, appropriate amino acids were omitted from SC medium. Cell numbers in liquid media were determined spectrophotometrically at an absorbance of 600 nm. Media were supplemented with 2% agar for growth on plates. An *opi1*Δ mutant was constructed in strain WCG4 (30) using an *opi1*Δ::kanMx deletion cassette. The kanMX module, flanked by *OPI1* coding sequences, was amplified by PCR (primers: OPI-S1 and OPI-S2) using plasmid pFA6aKanMX4 (31) as the template. The *opi1*Δ::kanMx deletion cassette was transformed into WCG4 to delete the chromosomal copy of the *OPI1* gene by the one-step gene replacement technique (32). Transformants were selected on YEPD plates containing Geneticin (G418) (31). Deletion of the chromosomal copy of the *OPI1* gene was confirmed by PCR (primers: OPI-A1 and OPI-A2) using genomic DNA isolated from the transformed colonies. One of the *opi1*Δ mutants that we isolated was designated strain SH1100. This *opi1*Δ mutant exhibited the inositol excretion phenotype that was characteristic of other *opi1* mutants (12). The inositol excretion phenotype was examined on SC medium plates (minus inositol) by using growth of the inositol auxotrophic mutant strain MC13 (*ino1*) (29) as described by McGee *et al.* (33).

DNA Manipulations, Amplification of DNA by PCR, Site-directed Mutagenesis, and DNA Sequencing—Plasmid DNA preparation, restriction enzyme digestion, and DNA ligations were performed by standard methods (28). Transformation of yeast (34) and *E. coli* (28) were performed as described previously. Conditions for the amplification of DNA by PCR were optimized as described previously (35). Site-directed mutagenesis was performed by PCR using the QuikChange site-directed mutagenesis kit. DNA sequencing reactions were performed by the dideoxy method using *Taq* polymerase (28).

Construction of Plasmids—Plasmid pMAL-OPI1 contains a *malE-OPI1* fusion gene, which was used for the expression of the MBP-Opi1p fusion protein. The *OPI1* coding sequence was amplified by PCR (primers: VMB-1 and JAG-20) using wild-type yeast genomic DNA as template. The PCR amplification of the *OPI1* gene resulted in the generation of *Eco*RI and *Bam*HI restriction sites at the 5′ and 3′ ends, respectively. The PCR product was digested with *Eco*RI and *Bam*HI and cloned into the *Eco*RI/*Bam*HI site of plasmid pMAL-c2 to form plasmid pMAL-OPI1. The codons for Ser^{26}, Ser^{60}, Ser^{135}, and Ser^{154} in Opi1p were changed to alanine codons by site-directed mutagenesis. The $OPI1^{S26A}$ (primers: ASR-1 and ASR-2), $OPI1^{S60A}$ (primers: ASR-10 and ASR-11), $OPI1^{S135A}$ (primers: ASR-5 and ASR-6), and $OPI1^{S154A}$ (primers: ASR-7 and ASR-8) mutations were constructed by PCR with the QuikChange site-directed mutagenesis kit using plasmid pMAL-OPI1 as the template. Clones containing the wild-type and mutant *OPI1* coding sequence were identified by restriction 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 pSA1 is a multicopy plasmid that contains the *OPI1* gene with sequences for a HA epitope tag inserted after the start codon. A fragment (0.68 kb) containing the *OPI1* promoter, the start codon, and the HA epitope sequence was amplified by PCR (primers: ASR-12 and ASR-16) using pJH354 as the template. Plasmid pJH354 is a multicopy plasmid based derived from YEp351 that contains the wild-type *OPI1* gene (13). Another fragment (0.7 kb) containing the HA epitope sequence and a portion of the *OPI1* coding sequence was amplified by PCR (primers: ASR-14 and ASR-17) using plasmid pJH354 as template. The two PCR products were mixed, annealed, and extended. The overlap-extended *HA-OPI1* 1.46-kb DNA fragment was then amplified by PCR (primers: ASR-12 and ASR-17). The 1.46-kb PCR product of the *HA-OPI1* DNA fragment was digested with *Sac*I/*Bgl*II and cloned

TABLE I
Strains and plasmids used in this work

Strain or plasmid	Genotype or relevant characteristics	Source or ref.
E. coli		
DH5α	F′ ϕ80d*lac*ZΔM15 Δ(*lacZYA-arg*F)U169 *deo*R *rec*A1 *end*A1 *hsd*R17(r_k^- m_k^+) *pho*A *sup*E44 λ$^-$ *thi-1 gyr*A96 *rel*A1	(28)
S. cerevisiae		
WCG4	*MAT***a** *leu2–3, 112 his3–11, 15 ura3,5*	(30)
SH1100	*opi1*Δ::kanMx derivative of WCG4	This work
MC13	*MATα ino1–13 lys2 can1*	(29)
Plasmids		
pMAL-c2	*E. coli* vector with an inducible *malE* gene used for fusion protein expression	(25)
pMAL-OPI1	*OPI1* coding sequence cloned into the *Eco*RI/*Bam*HI site of pMAL-c2	This work
pMAL-OPI1^{S26A}	*OPI1*^{S26A} derivative of pMAL-OPI1	This work
pMAL-OPI1^{S60A}	*OPI1*^{S60A} derivative of pMAL-OPI1	This work
pMAL-OPI1^{S135A}	*OPI1*^{S135A} derivative of pMAL-OPI1	This work
pMAL-OPI1^{S154A}	*OPI1*^{S154A} derivative of pMAL-OPI1	This work
pRS415	Single-copy *E. coli*/yeast shuttle vector containing the *LEU2* gene	(62)
YEp351	Multicopy *E. coli*/yeast shuttle vector containing the *LEU2* gene	(63)
pJH354	*OPI1* gene ligated into the *Sac*I/*Hin*dIII site of YEp351	(13)
pSA1	HA sequence inserted into pJH354 after the ATG start codon in the *OPI1* gene	This work
pSA2	*OPI1*^{S26A} derived of pSA1	This work
pSA3	HA-tagged *OPI1* gene from pSA1 ligated into the *Sac*I/*Hin*dIII site of pRS415	This work
pSA4	HA-tagged *OPI1*^{S26A} gene from pSA2 ligated into the *Sac*I/*Hin*dIII site of pRS415	This work
pJH359	*INO1-CYC1lac1′Z* reporter construct containing the *URA*3 gene	(9)

TABLE II
Primers used in this work

Oligonucleotide	Sequence
OPI-S1	GCGTGTGTATCAGGACAGTGTTTTTAACGAAGATACTAGTCATTGCGTACGCTGCAGGTCGAC
OPI-S2	TATTATTACTGGTGGTAATGCATGAAAGACCTCAATCTGTCTCGGATCGATGAATTCGAGCTCG
OPI-A1	CATTGATTTCGAGATTCCG
OPI-A2	TGGCCAGTTGTGAAGAGTT
VMB-1	GGCGAATTCATGTCTGAAAATCAACTG
JAG-20	GGTGGATCCTTAGTCCTTGCTATCCAC
ASR-1	GGGTGTTGAAACAAGTAGCAGACAGAAGTCG
ASR-2	CGACTTCTGTCAGCATGCTTCTTTCAACACCC
ASR-10	TTTTGGATCGCGTAGCTAACAAAATTATC
ASR-11	GATAATTTTGTTAGCTACGCGATCCAAAA
ASR-5	GTCCATCGAGGCTAAGAAGAGGC
ASR-6	GCCTCTTCTTAGCCTCGATGGAC
ASR-7	GCTGGCCAATAAGCAGCTAGCTGATAAAATCTCG
ASR-8	CGAGATTTTATCAGCTAGCTGCTTATTGGCCAGC
ASR-12	CGAATTCGAGCTCACAGTACAACAGCGACG
ASR-16	AGCGTAGTCTGGGACGTCGTATGGGTACATCAATGACTAGTATCTTCGTTAAAAACACTGTCCTG
ASR-14	TACCCATACGACGTCCCAGACTACGCTTCTGAAAATCAACGTTTAGGATTATCAGAGG
ASR-17	GAAGACTTTCCCGAACCTGAGATCTTGCGGGCTC

into the same restriction site in pJH354 to form plasmid pSA1. Plasmid pSA2, which bears the *HA-OPI1*^{S26A} mutation, was derived from plasmid pSA1 after site-directed mutagenesis by PCR (primers: ASR1 and ASR2). Plasmids pSA3 and pSA4 are single-copy plasmids that contain the wild-type *HA-OPI1* gene and the *HA-OPI1*^{S26A} mutation, respectively. The *HA-OPI1* gene from pSA1 and the *HA-OPI1*^{S26A} mutant from pSA2 were cloned into the *Sac*I/*Hin*dIII site of plasmid pRS415 to form plasmids pSA3 and pSA4, respectively. These plasmid constructions were confirmed by DNA sequencing.

Purification of MBP-Opi1p Fusion Proteins—All steps were performed at 4 °C. Frozen *E. coli* cells containing the induced MBP-Opi1p fusion proteins were suspended in 10 mM Tris-HCl buffer (pH 7.4) containing 0.2 M NaCl, 10 mM 2-mercaptoethanol, 1 mM Na_2EDTA, and 1 mM phenylmethylsulfonyl fluoride. Cells were disrupted with a French press at 10,000 p.s.i. The extract was then centrifuged at 20,000 × *g* for 30 min to remove unbroken cells and cell debris. The supernatant (~70 mg of protein) was applied to an amylose-agarose affinity chromatography column (0.6 × 7 cm) equilibrated with 10 mM Tris-HCl buffer (pH 7.4) containing 0.2 M NaCl, 10 mM 2-mercaptoethanol, 1 mM Na_2EDTA, and 1 mM phenylmethylsulfonyl fluoride. The column was washed with 10 column volumes of the same buffer to remove unbound protein from the column. The MBP-Opi1p fusion proteins were eluted from the column with chromatography buffer containing 10 mM maltose as described in the instructions provided by the manufacturer.

Preparations of Yeast Cell Extracts, Assay of β-Galactosidase Activity, and Immunoblot Analysis—Yeast cells were disrupted with glass beads (36) in 50 mM Tris-maleate buffer (pH 7.0) containing 1 mM Na_2EDTA, 0.3 M sucrose, 10 mM 2-mercaptoethanol, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, and 5 μg/ml each of aprotinin, leupeptin, and pepstatin. Glass beads and unbroken cells were removed by centrifugation at 1,500 × *g* for 10 min. The supernatant (cell extract) was used for the assay of β-galactosidase activity and immunoblot analysis of HA-tagged Opi1p proteins. β-Galactosidase activity was measured 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 (37). The reaction mixture contained 100 mM sodium phosphate buffer (pH 7.0), 3 mM *O*-nitrophenyl β-D-galactopyranoside, 1 mM $MgCl_2$, 100 mM 2-mercaptoethanol, and enzyme protein in a total volume of 0.1 ml. The average standard deviation of the enzyme assays (performed in triplicate) was ±5%. The enzyme reactions were linear with time and protein concentration. A unit of enzymatic activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol of product/min unless otherwise indicated. Specific activity was defined as units/mg of protein. Protein concentration was determined by the method of Bradford (38) using bovine serum albumin as the standard. Statistical analyses were performed with SigmaPlot 5.0 software. SDS-polyacrylamide gel electrophoresis (39) and immunoblotting (40) using polyvinylidene difluoride membranes were performed as described previously. Anti-Opi1p antibodies (41) and anti-HA antibodies were used at a dilution of 1:500. Proteins were detected on immunoblots using the enhanced chemifluorescence Western blotting detection kit as described by the manufacturer and acquired by fluorimaging analysis. Immunoblot signals were in the linear range of detectability.

In Vivo Labeling of HA-tagged Opi1p Proteins—Cells (*opi1*Δ mutant)

bearing multicopy plasmids containing the HA-tagged wild-type and S26A mutant *OPI1* alleles were used to examine the phosphorylation of Opi1p *in vivo*. Exponential phase cells grown in SC medium were labeled with $^{32}P_i$ (0.25 mCi/ml) for 3 h. Following the incubation, the labeled cells were harvested by centrifugation, washed, and disrupted in 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and protease and phosphatase inhibitors (radioimmune immunoprecipitation buffer) (42). The protease and phosphatase inhibitors included 0.5 mM phenylmethylsulfonyl fluoride, 1 mM benzamide, 5 μg/ml aprotinin, 5 μg/ml leupeptin, and 5 μg/ml pepstatin; and 10 mM NaF, 5 mM β-glycerophosphate, and 1 mM sodium vanadate, respectively. The HA-tagged Opi1p proteins were immunoprecipitated from cell lysates (0.5 mg of protein) using 4 μg of anti-HA antibodies in 0.5 ml of radioimmune immunoprecipitation buffer (42). The HA-tagged Opi1p proteins were dissociated from enzyme-antibody complexes (42), subjected to SDS-polyacrylamide gel electrophoresis, and transferred to polyvinylidene difluoride membranes. The ^{32}P-labeled proteins were visualized and quantified by phosphorimaging analysis.

Phosphorylation of MBP-Opi1p Fusion Proteins and Synthetic Peptides with Protein Kinase C—Phosphorylation reactions were measured for the indicated time intervals at 30 °C in a total volume of 40 μl. The indicated concentrations of MBP-Opi1p fusion proteins were incubated with 50 mM Tris-HCl buffer (pH 8.0), 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 0.375 mM EDTA, 0.375 mM EGTA, 1.7 mM $CaCl_2$, 20 μM DG, 50 μM PS, 50 μM [γ-^{32}P]ATP (4 μCi/nmol), and the indicated concentrations of protein kinase C for the indicated time intervals. At the end of the phosphorylation reactions, samples were treated with 2× Laemmli's sample buffer (39) followed by SDS-polyacrylamide gel electrophoresis, transfer to nitrocellulose paper, and visualized by phosphorimaging. The extent of phosphorylation was analyzed using ImageQuant software. Phosphorylation signals were in the linear range of detectability. Protein kinase C activity was measured using synthetic peptides containing putative phosphorylation sites based on the deduced protein sequence of Opi1p. The indicated concentrations of Opi1p synthetic peptides were phosphorylated with protein kinase C and [γ-^{32}P]ATP (4 μCi/nmol) for 10 min at 30 °C. Loading samples onto phosphocellulose filter paper terminated reactions. The filters were washed with 75 mM phosphoric acid and subjected to scintillation counting. A unit of protein kinase C activity using the Opi1p synthetic peptide GVLKQSCRQK was defined as the amount of enzyme that catalyzed the formation of 1 nmol of product/min. The reactions were performed in triplicate with an average standard deviation of ±3%.

Phosphoamino Acid and Phosphopeptide Map Analyses—MBP-Opi1p fusion protein was phosphorylated with protein kinase C and [γ-^{32}P]ATP for 10 min and then subjected to SDS-polyacrylamide gel electrophoresis. Gel slices containing ^{32}P-labeled MBP-Opi1p were treated with 50 mM ammonium bicarbonate (pH 8.0) and 0.1% SDS at 37 °C for 30 h to elute the enzyme. Bovine serum albumin (50 μg) was added to the samples as carrier protein, and trichloroacetic acid was added to a final concentration of 20%. After incubation for 30 min at 4 °C, protein precipitates were collected by centrifugation. Proteins were washed three times with cold acetone and dried *in vacuo*. Samples were then subjected to acid hydrolysis with 6 N HCl at 100 °C for 4 h. The hydrolysates were dried *in vacuo* and applied to 0.1-mm cellulose thin layer chromatography plates with 2.5 μg of phosphoserine, 2.5 μg of phosphothreonine, and 5 μg of phosphotyrosine as carrier phosphoamino acids in water. Phosphoamino acids were separated by two-dimensional electrophoresis (43). Following electrophoresis, the plates were dried, sprayed with 0.25% ninhydrin in acetone to visualize carrier phosphoamino acids, and subjected to phosphorimaging analysis.

Phosphopeptide map analysis was performed on ^{32}P-labeled MPB-Opi1p fusion following CNBr digestion (44). Phosphorylated proteins were subjected to SDS-polyacrylamide gel electrophoresis, followed by transfer to nitrocellulose membrane. The MBP-Opi1p fusion protein was excised and digested with 0.2 ml of CNBr (100 mg/ml) in 70% formic acid for 1.5 h (44). The mixture was centrifuged, and the supernatant was collected and dried *in vacuo*. Samples were then suspended in 0.5 ml of deionized water, dried *in vacuo*, and suspended in 20 μl of SDS-polyacrylamide gel electrophoresis buffer. Peptides were separated by SDS-polyacrylamide gel electrophoresis using a 24% slab gel as described previously (44), and phosphopeptides were identified by phosphorimaging.

RESULTS

Phosphorylation of Opi1p by Protein Kinase C in Vitro—We examined the hypothesis that Opi1p was a substrate for protein kinase C. To facilitate well defined studies *in vitro*, Opi1p was expressed in *E. coli* as an MBP-Opi1p fusion protein and purified to near homogeneity by amylose-agarose affinity chromatography (Fig. 2). The size of the fusion protein (~95 kDa) was consistent with the combined sizes of MBP and Opi1p. Immunoblot analysis with anti-MBP and anti-Opi1p antibodies confirmed the identity of MBP and Opi1p, respectively, in the fusion protein. When the fusion protein was cleaved with factor Xa, the released Opi1p protein was degraded by the protease. Accordingly, the MBP-Opi1p fusion protein was utilized as a protein kinase C substrate in our studies. Phosphorylation of the MBP-Opi1p fusion was routinely examined with a commercial preparation of purified rat brain protein kinase C. This enzyme preparation contains a mixture of the α, β, and γ isoforms of the enzyme. We used rat brain protein kinase C in our studies because *S. cerevisiae* protein kinase C (45, 46) has catalytic properties characteristic of the α, β, and γ isoforms of the rat brain enzyme (19, 47), and phosphorylated the MBP-Opi1p fusion protein with the same efficiency as a partially purified preparation of yeast protein kinase C.

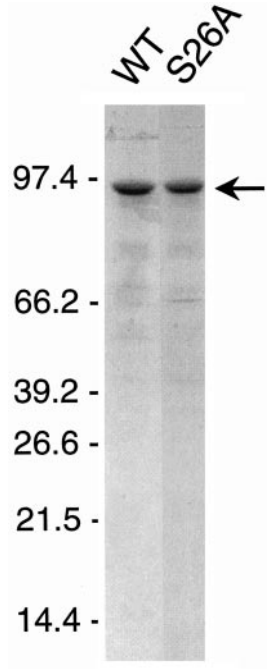

FIG. 2. **SDS-polyacrylamide gel electrophoresis of purified wild-type and S26A mutant MBP-Opi1p fusion proteins.** Wild-type (*WT*) and S26A mutant MBP-Opi1p proteins were expressed in *E. coli* and purified by amylose-agarose affinity chromatography. The purified proteins were subjected to SDS-polyacrylamide gel electrophoresis and stained with Coomassie Blue. The protein molecular mass standards were phosphorylase *b* (97.4 kDa), bovine serum albumin (66.2 kDa), aldolase (39.2 kDa), triosephosphate isomerase (26.6 kDa), soybean trypsin inhibitor (21.5 kDa), and lysozyme (14.4). The positions of the protein molecular mass standards and the MBP-Opi1p proteins are indicated in the figure.

To determine if Opi1p was a target for phosphorylation by protein kinase C, we examined whether protein kinase C catalyzed the incorporation of the γ phosphate of ^{32}P-labeled ATP into purified MBP-Opi1p fusion protein. After the phosphorylation reaction, samples were subjected to SDS-polyacrylamide gel electrophoresis and transfer to nitrocellulose paper. Phosphorimaging analysis of the nitrocellulose paper showed that Opi1p was a substrate for protein kinase C (Fig. 3). The MBP itself was not a substrate for protein kinase C. The position of ^{32}P-labeled MBP-Opi1p on the nitrocellulose paper was confirmed by immunoblot analysis. The phosphorylation of Opi1p was dependent on the time of the reaction (Fig. 3*A*) and the

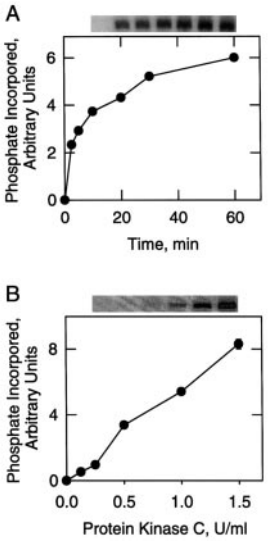

FIG. 3. **Dose- and time-dependent phosphorylation of purified MBP-Opi1p fusion protein by protein kinase C.** *Panel A*, purified MBP-Opi1p fusion protein (32 μg/ml) was incubated with protein kinase C (1 unit/ml) and [γ-^{32}P]ATP for the indicated time intervals. *Panel B*, purified MBP-Opi1p fusion protein (32 μg/ml) was incubated with the indicated amounts (unit = nmol/min) of protein kinase C and [γ-^{32}P]ATP for 30 min. Following the phosphorylation incubations, samples were subjected to SDS-polyacrylamide gel electrophoresis, transfer to nitrocellulose paper, and phosphorimaging analysis. Portions of the images showing the phosphorylation of MBP-Opi1p fusion protein are shown. The data shown are representative of two independent experiments.

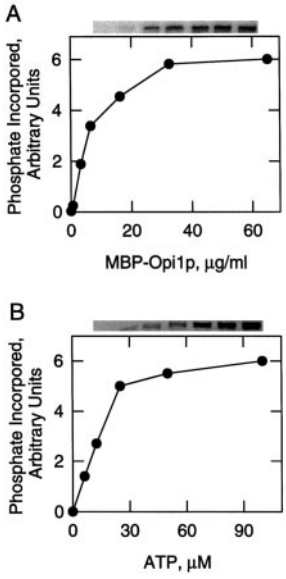

FIG. 4. **Dependence of protein kinase C activity on the concentrations of MBP-Opi1p fusion protein and ATP.** *Panel A*, protein kinase C (1 unit/ml) and [γ-^{32}P]ATP were incubated with the indicated concentrations of purified MBP-Opi1p fusion protein for 10 min. *Panel B*, protein kinase C (1 unit/ml) and MBP-Opi1 fusion protein (32 μg/ml) were incubated with the indicated concentrations of [γ-^{32}P]ATP for 10 min. Following the phosphorylation incubations, samples were subjected to SDS-polyacrylamide gel electrophoresis, transfer to nitrocellulose paper, and phosphorimaging analysis. Portions of the images showing the phosphorylation of MBP-Opi1p fusion protein are shown. The data shown are representative of two independent experiments.

concentration of protein kinase C (Fig. 3*B*). In addition, protein kinase C activity followed typical saturation kinetics with respect to MBP-Opi1p (Fig. 4*A*) and with respect to ATP (Fig. 4*B*). An analysis of the data according to the Michaelis-Menten equation using the EZ-FIT enzyme kinetic model fitting program (48) yielded K_m values for MBP-Opi1p and ATP of 8 μg/ml and 18 μM, respectively.

Protein kinase C is a serine/threonine-specific protein kinase (19). To examine which amino acid residue(s) of Opi1p was a target for phosphorylation, MBP-Opi1p was phosphorylated with protein kinase C, and the ^{32}P-labeled fusion protein was subjected to phosphoamino acid analysis. Protein kinase C primarily phosphorylated Opi1p on a serine residue (Fig. 5).

Identification of Ser^{26} as a Major Protein Kinase C Phosphorylation Site in Opi1p—Examination of the deduced sequence of Opi1p revealed that the protein has four potential serine phosphorylation sites (Ser^{26}, Ser^{60}, Ser^{135}, and Ser^{154}) within a protein kinase C sequence motif. Four peptides, GVLKQSCRQK, ILDRVSNKII, MSIESKKRLV, and ANKQLSDKIS containing the potential serine target sites within the protein kinase C sequence motif were synthesized based on the deduced protein sequence of Opi1p. We examined whether these peptides (1 mM) served as substrates for protein kinase C *in vitro*. Of the four peptides, only the GVLKQSCRQK peptide, which contains the sequence with Ser^{26}, was a substrate for protein kinase C. The dependence of protein kinase C activity on the GVLKQSCRQK peptide followed saturation kinetics (Fig. 6) with a K_m value for the peptide of 0.18 mM. The peptide GVLKQACRQK, which corresponds to a S26A mutation, did not serve as a substrate for protein kinase C (Fig. 6).

An *OPI1*^{S26A} allele was constructed by site-directed mutagenesis and expressed as an MBP-Opi1p fusion protein in *E. coli*. The S26A mutant MBP-Opi1p fusion protein was purified to near homogeneity by amylose-agarose affinity chromatography (Fig. 2) and examined for its ability to be phosphorylated by protein kinase C *in vitro*. The S26A mutant MBP-Opi1p

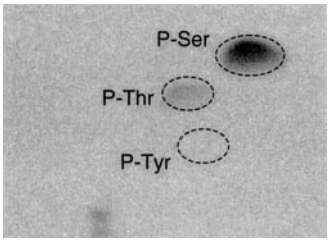

FIG. 5. **Phosphoamino acid analysis of MBP-Opi1p fusion protein phosphorylated *in vitro* by protein kinase C.** MBP-Opi1p fusion protein (32 μg/ml) was phosphorylated with PKC using [γ-^{32}P]ATP for 10 min. SDS-polyacrylamide gel slices containing ^{32}P-labeled MBP-Opi1p fusion protein were subjected to phosphoamino acid analysis. The positions of the carrier standard phosphoamino acids are indicated in the figure. *P-Ser*, phosphoserine; *P-Thr*, phosphothreonine; *P-Tyr*, phosphotyrosine. The data shown are representative of two independent experiments.

fusion protein was phosphorylated by protein kinase C in a time-dependent (Fig. 7*A*) and dose-dependent (Fig. 7*B*) manner. However, the extent of phosphorylation of the S26A mutant MBP-Opi1p fusion protein was reduced in comparison with the wild-type fusion protein (Fig. 7). We next examined the effect of the S26A mutation on the phosphopeptide map of the MBP-Opi1p fusion protein. The wild-type and S26A fusion proteins were phosphorylated with protein kinase C and ^{32}P-labeled ATP. The protein kinase C-phosphorylated wild-type and S26A mutant MBP-Opi1p fusion proteins were then subjected to CNBr cleavage. The resulting peptides were analyzed on a 24% low bis-Tricine SDS gel and visualized by phosphorimaging. The S26A mutation resulted in the loss of the major phosphopeptide present in the wild-type protein (Fig. 8). *OPI1*^{S60A}, *OPI1*^{S135A}, and *OPI1*^{S154A} alleles were also constructed, expressed in *E. coli* as MBP-Opi1p fusion proteins, and purified by amylose-agarose affinity chromatography. The S60A, S135A, and S154A mutations did not affect the ability of protein kinase C to phosphorylate Opi1p, and the phosphopep-

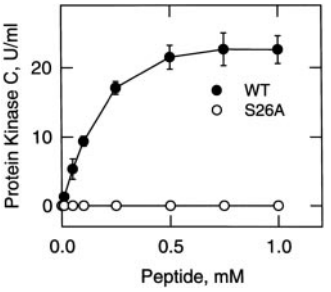

FIG. 6. **Opi1p synthetic peptide containing a protein kinase C sequence motif is a substrate for protein kinase C.** Protein kinase C activity was measured as a function of the concentration of the Opi1p wild-type (*WT*) (GVLK**QSCR**QK) and S26A mutant (GVLK**QACR**QK) synthetic peptides (protein kinase C motif is indicated by bold letters). The values reported were the average of three separate experiments ± S.D.

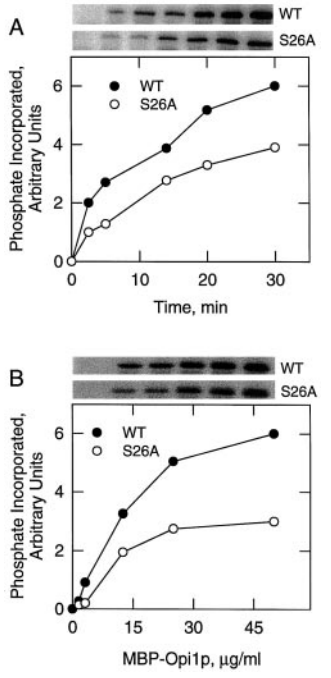

FIG. 7. **Effect of the S26A mutation on the phosphorylation of purified MBP-Opi1p fusion protein by protein kinase C.** *Panel A*, purified wild-type (*WT*) (32 μg/ml) and S26A mutant (32 μg/ml) MBP-Opi1p fusion proteins were incubated with protein kinase C (1 unit/ml) and [γ-^{32}P]ATP for the indicated time intervals. *Panel B*, protein kinase C (1 unit/ml) and [γ-^{32}P]ATP were incubated with the indicated concentrations of purified wild-type (*WT*) and S26A mutant MBP-Opi1p fusion proteins for 10 min. Following the phosphorylation incubations, samples were subjected to SDS-polyacrylamide gel electrophoresis, transfer to nitrocellulose paper, and phosphorimaging analysis. Portions of the images showing the phosphorylation of wild-type and S26A mutant MBP-Opi1p fusion proteins are shown. The data shown are representative of two independent experiments.

tide maps of these mutant proteins did not differ from that of the wild-type protein (data not shown).

Phosphorylation of Opi1p in Vivo—We addressed the question of whether Opi1p was phosphorylated *in vivo*. For these experiments, we used an epitope-tagged *OPI1* allele in the multicopy plasmid pSA1. In this plasmid, the sequence for HA was inserted into the N terminus of the *OPI1*-coding sequence. Plasmid pSA1 was expressed in an *opi1*Δ mutant to avoid interference from Opi1p encoded by the genomic wild-type copy of the *OPI1* gene. Immunoblot analysis showed that the epitope-tagged Opi1p protein was specifically recognized in *opi1*Δ mutant cells by anti-HA antibodies at the expected molecular mass of about 50 kDa (Fig. 9*A*). Moreover, the

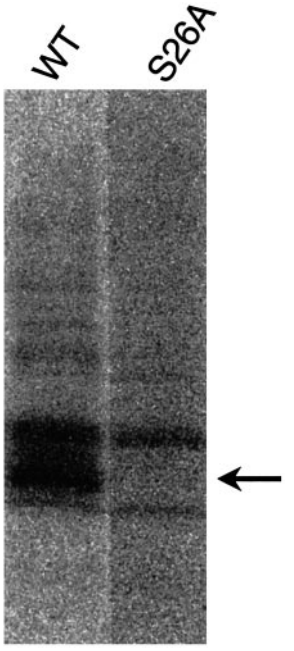

FIG. 8. **Effect of the S26A mutation on the phosphopeptide map of MBP-Opi1p fusion protein phosphorylated *in vitro* by protein kinase C.** Purified wild-type (*WT*) (32 μg/ml) and S26A mutant (32 μg/ml) MBP-Opi1p fusion proteins were incubated with protein kinase C (1 unit/ml) and [γ-^{32}P]ATP for 10 min. Following the phosphorylation incubations, samples were subjected to SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose paper. The labeled proteins were excised from the nitrocellulose paper, subjected to CNBr cleavage, analyzed on a 24% low bis-Tricine SDS gel, and visualized by phosphorimaging.

HA-tagged Opi1p suppressed the inositol excretion phenotype (12) of the *opi1*Δ mutant. This indicated that the epitope-tagged protein was functional *in vivo*. Cells bearing plasmid pSA1 were labeled with $^{32}P_i$ followed by the immunoprecipitation of the HA-tagged Opi1p from cell extracts with anti-HA antibodies. SDS-polyacrylamide gel electrophoresis of the immunoprecipitate, transfer to nitrocellulose paper, and phosphorimaging analysis revealed that Opi1p was phosphorylated *in vivo* (Fig. 9*B*).

The *in vitro* experiments indicated that Ser^{26} was a phosphorylation site for protein kinase C. We examined the hypothesis that this site was phosphorylated *in vivo*. A HA-tagged *OPI1*^{S26A} allele was constructed in the multicopy plasmid pSA2, and used for the expression of the S26A mutant Opi1p protein in *opi1*Δ mutant cells (Fig. 9*A*). The effect of the S26A mutation on the phosphorylation of Opi1p was examined by labeling cells with $^{32}P_i$. The S26A mutant Opi1p protein was phosphorylated *in vivo* (Fig. 9*B*). However, ImageQuant analysis of the phosphorylated protein revealed that the S26A mutation caused a 50% reduction in phosphorylation for Opi1p when compared with the wild-type control (Fig. 9*C*). Immunoblot analysis using anti-HA antibodies showed that the wild-type and S26A mutant Opi1p proteins were present at similar amounts in the immunoprecipitates.

The Effect of the S26A Mutation on Opi1p Function—The Opi1p protein is a transcriptional regulator that represses the expression of several phospholipid synthesis genes that are regulated by inositol supplementation (1, 4–6). An *opi1* mutant exhibits elevated expression of phospholipid synthesis enzymes, and the expression of these enzymes does not respond to inositol supplementation (1, 4–6). A characteristic property of *opi1* mutants is an inositol excretion phenotype (12), which is the result of the derepression of the *INO1* gene encoding inositol-1-phosphate synthase (10, 36, 49, 50). The effect of the

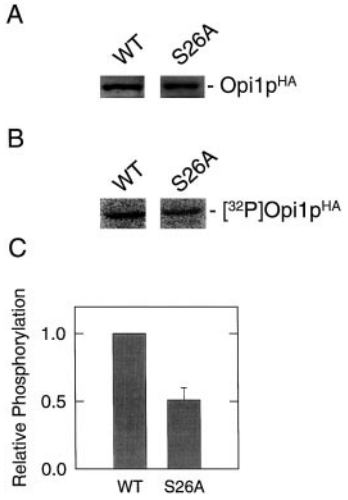

FIG. 9. **Effect of the S26A mutation on the phosphorylation of Opi1p *in vivo*.** Cultures (50 ml) of the *opi1*Δ mutant bearing the multicopy plasmid pSA1 with the HA-tagged *OPI1* gene or the plasmid pSA2 with the HA-tagged *OPI1*^{S26A} gene were grown in complete synthetic medium to the exponential phase of growth. *Panel A*, cells were harvested, cell extracts were prepared, and 25-μg samples were subjected to immunoblot analysis using a 1:500 dilution of anti-HA antibodies. *WT*, wild type. *Panel B*, cells were harvested, resuspended in 5 ml of fresh medium containing $^{32}P_i$ (0.25 mCi/ml), and incubated for 3 h. Following the incubation, the HA-tagged Opi1p proteins were immunoprecipitated from 500 μg of cell extract with anti-HA antibodies and then subjected to SDS-polyacrylamide gel electrophoresis and transfer to nitrocellulose paper. The ^{32}P-labeled Opi1p proteins were visualized by phosphorimaging analysis. *Panel C*, the relative density of the ^{32}P-labeled proteins of the data shown in *panel B* was quantified using ImageQuant software. The data shown are representative of three independent experiments.

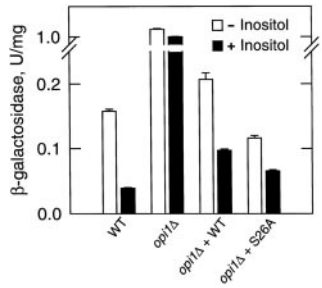

FIG. 10. **Effect of the S26A mutation in Opi1p on the expression of the *INO1* gene.** Mutant *opi1*Δ cells expressing either the wild-type (*WT*) *OPI1* gene or the mutant *OPI1*^{S26A} gene from the single copy plasmids pSA3 and pSA4, respectively, were transformed with plasmid pJH359, which contains the P_{INO1}-*lacZ* reporter gene. Cells were grown in complete synthetic medium in the absence and presence of 75 μM inositol. 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 three independent growth studies ± S.D.

S26A mutation in Opi1p on *INO1* expression was examined by scoring for the inositol excretion phenotype of *opi1*Δ mutant cells using growth of an *ino1* mutant on plates lacking inositol. Transformation of the *opi1*Δ mutant with a plasmid bearing the *OPI1*^{S26A} mutant allele suppressed the inositol excretion phenotype of the *opi1*Δ mutant (data not shown). Thus, the S26A mutation did not cause a defect in the negative regulatory function of Opi1p on *INO1* expression. Expression of *INO1* in *opi1*Δ mutant cells was also examined using an *INO1-CYC1-lacI′Z* reporter gene, a much more sensitive assay for assessing Opi1p function on *INO1* expression than the inositol excretion plate assay (9). Cells bearing the reporter gene were grown in complete synthetic medium minus inositol, cell extracts were prepared, and β-galactosidase activity was measured. Whereas inositol supplementation repressed (4-fold) the β-galactosidase activity in wild-type cells, this activity was not affected by inositol in *opi1*Δ mutant cells (Fig. 10) as described previously (15). Under repressing conditions (plus inositol), β-galactosidase activity in the *opi1*Δ mutant (1.2 units/mg) was 7.5-fold greater than the activity (0.16 unit/mg) in wild-type *OPI1* cells (Fig. 10). The elevated level of β-galactosidase activity was consistent with the inositol excretion phenotype of the *opi1*Δ mutant. These results are consistent with previous reports of the levels of *INO1* mRNA found in wild-type and *opi1*Δ mutant cells grown in the absence and presence of inositol (10, 13, 51).

The effect of the S26A mutation in Opi1p on the expression of *INO1* in *opi1*Δ mutant cells was examined using the reporter gene. For these experiments, we used HA-tagged *OPI1* and *OPI1*^{S26A} alleles in the single-copy plasmids pSA3 and pSA4, respectively. Immunoblot analysis using anti-HA antibodies showed that these alleles were expressed at similar levels in *opi1*Δ mutant cells. The β-galactosidase activity in *opi1*Δ mutant cells bearing the wild-type Opi1p protein was 0.2 unit/mg in cells grown under derepressing conditions (minus inositol) (Fig. 10). This level of activity was similar to that found in cells with the chromosomal copy of the *OPI1* gene except for a somewhat higher background of expression under repressing conditions (plus inositol). This effect has been observed previously in *opi1* mutants transformed with an *OPI1*-bearing plasmid.[2] This has been attributed to a background of elevated *INO1* expression coming from *opi1*Δ cells that have lost the plasmid.[2] In comparison, β-galactosidase activity in *opi1*Δ mutant cells bearing the S26A mutant Opi1p protein was nearly 50% lower than that found in *opi1*Δ mutant cells bearing the control Opi1p protein under fully derepressing conditions (minus inositol) (Fig. 10). The addition of inositol to *opi1*Δ mutant cells bearing the wild-type or the S26A mutant Opi1p proteins resulted in repression (~2-fold) of β-galactosidase activity in both cases (Fig. 10). As discussed above, in both cases the *opi1*Δ cells carrying a complementary wild-type *OPI1* or mutant *OPI1*^{S26A} allele expressed a higher background level of β-galactosidase under repressing conditions.

DISCUSSION

The Opi1p protein, encoded by the *OPI1* gene (13), plays a negative regulatory role in the expression of the *INO1* gene and other UAS_{INO}-containing genes involved in the synthesis of membrane phospholipids in *S. cerevisiae* (1, 2, 4–6). In this study, we addressed the posttranslational modification of Opi1p by phosphorylation via protein kinase C. Phosphorylation is a major mechanism by which a protein's function may be regulated (52). Phosphorylation of a regulatory protein can control its ability to bind DNA, its localization, or interaction with other proteins (17, 18, 53–56). Our analysis using a MBP-Opi1p fusion protein demonstrated that Opi1p was phosphorylated by protein kinase C *in vitro*. Protein kinase C primarily phosphorylated Opi1p on serine residues, and this reaction was time- and dose-dependent, and dependent on the concentrations of Opi1p and ATP. To our knowledge, this is the first report of the posttranslational modification of a phospholipid synthesis regulatory protein by phosphorylation via protein kinase C.

Computer analysis of the deduced amino acid sequence of the *OPI1*-encoded Opi1p revealed potential target sites for protein kinase C at Ser^{26}, Ser^{60}, Ser^{135}, and Ser^{154}. The peptide GV-LKQSCRQK, which contains the protein kinase C sequence motif at Ser^{26}, was shown to be a substrate for protein kinase C *in vitro*. We also demonstrated that the peptide GV-LKQACRQK, which corresponds to a S26A mutation, failed to

[2] J. Anthony Graves, personal communication.

serve as a specific substrate for protein kinase C, supporting the conclusion that a protein kinase C target sequence exists within the peptide GVLKQSCRQK. None of the other potential sites proved to be targets by this assay. An MBP-Opi1p fusion protein containing a S26A mutation behaved like the wild-type protein during purification, suggesting that the mutation did not have a significant effect on protein structure. Although protein kinase C phosphorylated the S26A mutant Opi1p protein, the extent of phosphorylation was reduced when compared with the wild-type protein. Moreover, peptide-mapping analysis of protein kinase C-phosphorylated Opi1p proteins showed that a major phosphopeptide present in the wild-type Opi1p protein was absent from the S26A mutant protein, thus confirming that this site is a specific target of protein kinase C. Although Opi1p was phosphorylated by protein kinase C at sites other than Ser^{26}, the potential sites at Ser^{60}, Ser^{135}, and Ser^{154} are apparently not the targets since the S60A, S135A, and S154A mutations did not affect the phosphorylation of Opi1p or the phosphopeptide map of the protein.

Opi1p was also phosphorylated *in vivo*, as demonstrated by immunoprecipitation of labeled HA-tagged Opi1p from labeled cells (Fig. 9). The degree of phosphorylation was reduced ~50% in *opi1*Δ cells transformed with the S26A mutant protein tagged with HA as compared with *opi1*Δ cells transformed with the wild-type HA-tagged protein. However, the *opi1*Δ cells carrying the S26A mutant did not exhibit the inositol excretion phenotype, suggesting that the mutation does not inactivate Opi1p with respect to its function as a transcriptional repressor of *INO1* and other UAS_{INO}-containing genes. Indeed, consistent with this result, analysis of *INO1* expression monitored using an *INO1-CYC1-lacI′Z* reporter gene indicated that *INO1* expression does not reach as high a derepressed level in cells carrying the S26A mutation when compared with cells transformed with the wild-type *OPI1* control. This result, together with the evidence that phosphorylation of the S26A mutant was reduced compared with the wild-type *OPI1* control, indicated that phosphorylation of Ser^{26} causes Opi1p to lose a portion of its activity as a negative regulator of *INO1*. This result implies that Opi1p must be partially inactivated *in vivo* in order for *INO1* to reach maximum derepression. Moreover, the fact that the phosphorylation site in question is a target for protein kinase C suggests that protein kinase C may be involved in the inactivation. If this is the case, the effect of protein kinase C activity on *INO1* expression would be positive (*i.e.* allowing full derepression) via a mechanism involving inactivation of a protein, Opi1p, involved in the mechanism of repression of *INO1* and other UAS_{INO}-containing genes. Preliminary data indicate that this is, indeed, the case since *pkc1*Δ mutants are inositol auxotrophs.[3] It is not likely that Opi1p functions by binding directly to the UAS_{INO} element in the co-regulated genes or by contacting either of the Ino2p and Ino4p activators (14, 16). The precise target of Opi1p is, as yet, unknown. Thus, the precise mechanism of this regulation must await identification of the target of Opi1p activity.

Protein kinase C is a lipid-dependent protein kinase required for *S. cerevisiae* cell cycle (19–21, 57, 58). In mammalian cells, protein kinase C plays a central role in the transduction of lipid second messengers generated by receptor-mediated hydrolysis of membrane phospholipids (59–61). The phosphorylation and regulation of Opi1p function by protein kinase C in *S. cerevisiae* may represent a mechanism by which lipid signal transduction pathways are coordinately regulated to phospholipid synthesis and cell growth.

[3] S. Henry, M. Ruiz-Noriega, and L. Nunez-Rodgriguez, unpublished data.

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REFERENCES

1. Carman, G. M., and Henry, S. A. (1989) *Annu. Rev. Biochem.* **58,** 635–669
2. 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
3. Carman, G. M., and Zeimetz, G. M. (1996) *J. Biol. Chem.* **271,** 13293–13296
4. Greenberg, M. L., and Lopes, J. M. (1996) *Microbiol. Rev.* **60,** 1–20
5. Henry, S. A., and Patton-Vogt, J. L. (1998) *Prog. Nucleic Acids Res.* **61,** 133–179
6. Carman, G. M., and Henry, S. A. (1999) *Prog. Lipid Res.* **38,** 361–399
7. Patton-Vogt, J. L., Griac, P., Sreenivas, A., Bruno, V., Dowd, S., Swede, M. J., and Henry, S. A. (1997) *J. Biol. Chem.* **272,** 20873–20883
8. Kodaki, T., Nikawa, J., Hosaka, K., and Yamashita, S. (1991) *J. Bacteriol.* **173,** 7992–7995
9. Lopes, J. M., Hirsch, J. P., Chorgo, P. A., Schulze, K. L., and Henry, S. A. (1991) *Nucleic Acids Res.* **19,** 1687–1693
10. Hirsch, J. P., and Henry, S. A. (1986) *Mol. Cell. Biol.* **6,** 3320–3328
11. Loewy, B. S., and Henry, S. A. (1984) *Mol. Cell. Biol.* **4,** 2479–2485
12. Greenberg, M., Reiner, B., and Henry, S. A. (1982) *Genetics* **100,** 19–33
13. White, M. J., Hirsch, J. P., and Henry, S. A. (1991) *J. Biol. Chem.* **266,** 863–872
14. Wagner, C., Blank, M., Strohmann, B., and Schüller, H. J. (1999) *Yeast* **15,** 843–854
15. Bachhawat, N., Ouyang, Q., and Henry, S. A. (1995) *J. Biol. Chem.* **270,** 25087–25095
16. Graves, J. A., and Henry, S. A. (2000) *Genetics* **154,** 1485–1495
17. Karin, M., and Hunter, T. (1995) *Curr. Biol.* **5,** 747–757
18. Calkhoven, C. F., and Ab, G. (1996) *Biochem. J.* **317,** 329–342
19. Nishizuka, Y. (1984) *Nature* **308,** 693–698
20. Nishizuka, Y. (1992) *Science* **258,** 607–614
21. Bell, R. M., and Burns, D. J. (1991) *J. Biol. Chem.* **266,** 4661–4664
22. Rosengurt, E., Rodriquez-Pena, A., Coombs, M., and Sinnet-Smith, J. (1984) *Proc. Natl. Acad. Sci. U. S. A.* **81,** 5748–5752
23. Kaibuchi, K., Takai, Y., and Nishizuka, Y. (1985) *J. Biol. Chem.* **260,** 1366–1369
24. Persons, D. A., Wilkison, W. O., Bell, R. M., and Finn, O. J. (1988) *Cell* **52,** 447–458
25. Maina, C. V., Riggs, P. D., Grandea, A. G., III, Slatko, B. E., Moran, L. S., Tagliamonte, J. A., McReynolds, L. A., and di Guan, C. (1988) *Gene* **74,** 365–373
26. Yang, W.-L., Bruno, M. E. C., and Carman, G. M. (1996) *J. Biol. Chem.* **271,** 11113–11119
27. 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
28. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual,* 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
29. Culbertson, M. R., and Henry, S. A. (1975) *Genetics* **80,** 23–40
30. Heinemeyer, W., Gruhler, A., Mohrle, V., Mahe, Y., and Wolf, D. H. (1993) *J. Biol. Chem.* **268,** 5115–5120
31. Wach, A., Brachat, A., Pohlmann, R., and Philippsen, P. (1994) *Yeast* **10,** 1793–1808
32. Rothstein, R. (1991) *Methods Enzymol.* **194,** 281–301
33. McGee, T. P., Skinner, H. B., and Bankaitis, V. A. (1994) *J. Bacteriol.* **176,** 6861–6868
34. Ito, H., Yasuki, F., Murata, K., and Kimura, A. (1983) *J. Bacteriol.* **153,** 163–168
35. 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
36. Klig, L. S., Homann, M. J., Carman, G. M., and Henry, S. A. (1985) *J. Bacteriol.* **162,** 1135–1141
37. Craven, G. R., Steers, E., Jr., and Anfinsen, C. B. (1965) *J. Biol. Chem.* **240,** 2468–2477
38. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254
39. Laemmli, U. K. (1970) *Nature* **227,** 680–685
40. Haid, A., and Suissa, M. (1983) *Methods Enzymol.* **96,** 192–205
41. Graves, J. A. (1996) *Analysis of the Role of the OPI1 Gene Product in the Negative Regulation of the Phospholipid Biosynthetic Pathway of Saccharomyces cerevisiae*, Ph.D. thesis, Carnegie Mellon University, Pittsburgh, PA
42. Harlow, E., and Lane, D. (1988) *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
43. Boyle, W. J., Van der Geer, P., and Hunter, T. (1991) *Methods Enzymol.* **201,** 110–149
44. Luo, K. X., Hurley, T. R., and Sefton, B. M. (1991) *Methods Enzymol.* **201,** 149–152
45. Ogita, K., Miyamoto, S., Koide, H., Iwai, T., Oka, M., Ando, K., Kishimoto, A., Ikeda, K., Fukami, Y., and Nishizuka, Y. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 5011–5015
46. Simon, A. J., Milner, Y., Saville, S. P., Dvir, A., Mochly-Rosen, D., and Orr, E. (1991) *Proc. R. Soc. Lond. B Biol. Sci.* **243,** 165–171
47. Nishizuka, Y. (1988) *Nature* **334,** 661–665
48. Perrella, F. (1988) *Anal. Biochem.* **174,** 437–447
49. Donahue, T. F., and Henry, S. A. (1981) *J. Biol. Chem.* **256,** 7077–7085
50. Klig, L. S., and Henry, S. A. (1984) *Proc. Natl. Acad. Sci. U. S. A.* **81,**

3816–3820
51. Jiranek, V., Graves, J. A., and Henry, S. A. (1998) *Microbiology* **144,** 2739–2748
52. Krebs, E. G., and Beavo, J. A. (1979) *Annu. Rev. Biochem.* **48,** 923–959
53. Komeili, A., and O'Shea, E. K. (1999) *Science* **284,** 977–980
54. Liu, C., Yang, Z., Yang, J., Xia, Z., and Ao, S. (2000) *J. Biol. Chem.* **275,** 31972–31978
55. Kaffman, A., Rank, N. M., O'Neill, E. M., Huang, L. S., and O'Shea, E. K. (1998) *Nature* **396,** 482–486
56. Hung, W., Olson, K. A., Breitkreutz, A., and Sadowski, I. (1997) *Eur. J. Biochem.* **245,** 241–251
57. Levin, D. E., Fields, F. O., Kunisawa, R., Bishop, J. M., and Thorner, J. (1990) *Cell* **62,** 213–224
58. Mellor, H., and Parker, P. J. (1998) *Biochem. J.* **332,** 281–292
59. Berridge, M. J. (1987) *Annu. Rev. Biochem.* **56,** 159–193
60. Exton, J. H. (1990) *J. Biol. Chem.* **265,** 1–4
61. Downes, C. P., and Macphee, C. H. (1990) *Eur. J. Biochem.* **193,** 1–18
62. Sikorski, R. S., and Hieter, P. (1989) *Genetics* **122,** 19–27
63. Hill, J. E., Myers, A. M., Koerner, T. J., and Tzagoloff, A. (1986) *Yeast* **2,** 163–167