Control of Phospholipid Synthesis by Phosphorylation of the Yeast Lipin Pah1p/Smp2p Mg$^{2+}$-dependent Phosphatidate Phosphatase*

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Phosphorylation of the conserved lipin Pah1p/Smp2p in Saccharomyces cerevisiae was previously shown to control transcription of phospholipid biosynthetic genes and nuclear structure by regulating the amount of membrane present at the nuclear envelope (Santos-Rosa, H., Leung, J., Grimsey, N., Peak-Chew, S., and Siniossoglou, S. (2005) EMBO J. 24, 1931–1941). A recent report identified Pah1p as a Mg$^{2+}$-dependent phosphatidate (PA) phosphatase that regulates de novo lipid synthesis (Han G.-S., Wu, W. I., and Carman, G. M. (2006) J. Biol. Chem. 281, 9210–9218). In this work we use a combination of mass spectrometry and systematic mutagenesis to identify seven Ser/Thr-Pro motifs within Pah1p that are phosphorylated in vivo. We show that phosphorylation on these sites is required for the efficient transcriptional derepression of key enzymes involved in phospholipid biosynthesis. The phosphorylation-deficient Pah1p exhibits higher PA phosphatase-specific activity than the wild-type Pah1p, indicating that phosphorylation of Pah1p controls PA production. Opi1p is a transcriptional repressor of phospholipid biosynthetic genes, responding to PA levels. Genetic analysis suggests that Pah1p regulates transcription of these genes through both Opi1p-dependent and -independent mechanisms. We also provide evidence that derepression of phospholipid biosynthetic genes is not sufficient to induce the nuclear membrane expansion shown in the pah1Δ cells.

Over the years there has been significant progress in understanding the mechanisms by which proteins are targeted and assembled into the various intracellular compartments. Despite this, little is still known about how eukaryotic cells regulate the growth of membrane-bound organelles. Homeostatic mechanisms must be in place to ensure that organelles grow in size or in number before cell division. Similar mechanisms must operate during development when certain organelles undergo dramatic morphological changes to perform their specialized function in differentiated tissues (1, 2).

A defining organelle of eukaryotic cells is the nucleus. The intranuclear compartment is delimited by the nuclear envelope and consists of a double lipid bilayer, the outer and the inner nuclear membrane (3). The outer nuclear membrane is physically and functionally linked to the endoplasmic reticulum (ER), whereas the inner nuclear membrane faces the nucleoplasm and in metazoans is covered by the nuclear lamina. Dynamic changes in the structure of the nuclear envelope are essential for the proper execution of nuclear division in all eukaryotes. In metazoan cells the nuclear envelope breaks down during mitosis, whereas yeast undergo “closed mitosis,” where the spindle separates the chromosomes within the confines of an intact nucleus that partitions between mother and daughter cell (4).

How the membrane is targeted and incorporated into the nuclear envelope and how the nucleus expands to accommodate changes in chromatin condensation and content during the cell cycle are fascinating but still unanswered questions. Cell-free assays suggest that nuclear membrane expansion and nuclear growth takes place via homotypic fusion of vesicles with the outer nuclear membrane (3, 5–7). It is not clear however whether nuclear growth in vivo depends on vesicle fusion. Because the outer nuclear membrane is continuous with the ER, the major site of phospholipid biosynthesis in eukaryotic cells, an alternative possibility is that nuclear growth results from lateral flow of ER membranes into the nuclear envelope.

Phospholipid homeostasis in yeast is regulated primarily by the concentration of the essential phospholipid precursor inositol (8, 9). The presence of inositol in the medium promotes biosynthesis of PI at the expense of PA. As PA levels fall, the PA-binding protein Opi1p translocates from the ER into the nucleus (10) where it represses transcription of many genes encoding phospholipid, fatty acid, and sterol biosynthetic enzymes (8, 9). Conversely, low levels of inositol result in dere-
expression of transcription by allowing a complex consisting of the basic helix-loop-helix proteins Ino2p and Ino4p to induce transcription of the phospholipid biosynthetic genes that contain a UASINO element (called also ICRE) (11–13). Because phospholipids are key structural components of intracellular membrane compartments, their biosynthesis must be also coordinated with the cellular needs for organellar biogenesis. Phospholipid biosynthesis in yeast is transcriptionally induced in response to the need for more ER membrane during the unfolded protein response (14), indicating that lipid metabolism is coordinated with ER growth. In mammals, enforced expression of the transcription factor activated during the unfolded protein response increases the activity of enzymes involved in PC biosynthesis and causes expansion of the ER (15).

Using genetic approaches in the yeast Saccharomyces cerevisiae, we have previously identified a network of three genes that regulate nuclear membrane growth: SPO7, NEM1, and PAH1 (SMP2) (16, 17). Nem1p and Spo7p form a transmembrane phosphatase complex that localizes to the nuclear membrane and ER and dephosphorylates Pah1p (Smp2p)3 (17). Deletion of PAH1 or loss of its dephosphorylated form by deletion of NEM1–SPO7 causes transcriptional up-regulation of ER enzymes involved in phospholipid biosynthesis, proliferation of the nuclear membrane, and a massive expansion of the nucleus (17). Deletion ofINO2 or overexpression ofOPI1 restores normal nuclear morphology in nem1Δ, spo7Δ, and pah1Δ (smp2Δ)3 mutants, suggesting that de novo phospholipid biosynthesis is necessary for nuclear growth (17). The mechanism by which Pah1p controls nuclear membrane biogenesis and the role of phosphorylation in this process is not understood. However, a recent study reported that Pah1p is a Mg2+-dependent PA phosphatase enzyme and that the pah1Δ mutants display a 2-fold increase of intracellular PA levels and reduced amounts of DAG and its derivative, triacylglycerol (18). The pah1Δ mutant also shows a decrease in PC and increases in PE and PI, indicating that Pah1p plays a role in phospholipid synthesis (18). Pah1p contains a conserved N-lipin domain of unknown function (19) and a HAD-like domain with a DXDXT motif found in a superfamily of Mg2+-dependent phosphatases (18, 20, 21).

In this paper we address the role of phosphorylation in the regulation of Pah1p function. We find that Pah1p is phosphorylated on seven residues matching the minimal Cdk consensus. A mutant containing a phosphorylation-deficient Pah1p exhibits reduced induction of phospholipid biosynthetic genes in response to inositol depletion and shows a strong inositol auxotrophy when overexpressed. The same mutant exhibits increased Mg2+-dependent PA phosphatase activity, suggesting that the phosphorylation could lead to accumulation of PA in vivo and, therefore, impact on the transcriptional program through Opi1p. Genetic analysis shows that removal ofPAH1 andOPI1 has an additive effect on the transcriptional derepression, suggesting that Pah1p may have an additional OPI1-independent role in the regulation of phospholipid biosynthetic genes.

**EXPERIMENTAL PROCEDURES**

_Yeast Strains, Media, and Growth Conditions—_Yeast strains used in this study are listed in Table 1. Knock-out ofOPI1 in the RS453 strain andPAH1 andOPI1 in the W303 strain were done by homologous recombination using the appropriatepah1::TRP1 orop1::HIS3 constructs. Yeast cells were grown in YEPD or in synthetic medium containing 2% glucose lacking the appropriate amino acids for plasmid selection. GAL1/10-dependent overexpression was performed by changing the carbon source of early log phase cells from 2% raffinose to 2% galactose. MET25-dependent overexpression was performed by transferring cells from synthetic medium supplemented with 1 mM methionine (repressive conditions) to medium lacking methionine (inducing conditions). To assay growth of yeast cells in media lacking inositol, synthetic medium was prepared using yeast nitrogen base lacking inositol (Bio101).

_Plasmids and Construction of Fusion Genes—_Plasmids used in this study are listed in Table 2. All phosphorylation alleles described in the paper, includingPAH1-7P andPAH1-NC7P, were created by PCR-mediated mutagenesis and verified by DNA sequencing. ThePAH1, PAH1-7P, PAH1-NC7P, OPI1, andHMG1 genes were overexpressed using theGAL1/10 promoter. TheRFP-PLUS was constructed by fusing the last codon of the monomeric RFP with the second codon ofPLUS1. Express-

### Table 1

| Strain | Genotype | Reference/source |
|-------|----------|-----------------|
| RS453 | MATa ade2-1 his3-11,15 ura3-52 leu2-3,112 trp1-1 | 47 |
| SS1002 | MATa ade2-1 his3-11,15 ura3-1 leu2-3,112 trp1-1 can1-100 | 48 |
| W303 | MATa ade2-1 his3-11,15 ura3-1 leu2-3,112 trp1-1 | 16 |
| SS1026 | SRS3 nem1::HIS3 | 16 |
| SS1010 | SRS3 nem1::HIS3 spo7::HIS3 | 16 |
| SS1034 | SRS3 pah1::TRP1 + YCplac111-LEU2-PAH1-PTE | 17 |
| SS1037 | SRS3 nem1::HIS3 spo7::HIS3 pah1::TRP1 + YCplac111-LEU2-SMP2-PTE | 17 |
| SS1400 | SRS3 pah1::TRP1 + pRS313-HIS3-PAH1-PTE + YCplac111-LEU2-GAL110-NEM1 + Yep552-URA3-GAL110-SP07 | This study |
| SS1401 | SRS3 pah1::TRP1 + pRS313-HIS3-PAH1-PTE + YCplac111-LEU2 + YCplac33-URA3 | This study |
| SS1402 | SRS3 pah1::TRP1 + YCplac111-LEU2-PAH1 | This study |
| SS1403 | SRS3 pah1::TRP1 + YCplac111-LEU2-PAH1-7P | This study |
| SS1209 | SRS3 op1::HIS3 | This study |
| SS1121 | SRS3 op1::HIS3 pah1::TRP1 | This study |
| SS1224 | W303 op1::HIS3 | This study |
| SS1226 | W303 pah1::TRP1 | This study |
| SS1230 | W303 op1::HIS3 pah1::TRP1 | This study |

3 SMP2 has been renamedPAH1 (18). We, therefore, now use the new namesPAH1, Pah1p, andpah1Δ instead of SMP2, Smp2p, andsmp2Δ.
sion of the fusion gene, cloned into Ycplac111-LEU2, was driven by the NOP1 promoter and the PLIS1 terminator.

**Mass Spectrometric Analysis of Phospho-Pah1p**—All electrospray tandem mass spectrometry (ESI-MS/MS) were carried out on an API QSTAR Pulsar i hybrid quadrupole-time of flight instrument (MDS Sciex, Ontario, Canada). Matrix-assisted laser desorption ionization (MALDI) time-of-flight MS was performed using a Voyager-DE STR (PerSeptive Biosystems, Framingham, MA) mass spectrometer. The protein bands of interest were excised from a Coomassie Blue-stained gel, ground in a mortar and pestle prechilled with liquid nitrogen. The ground cell powder was then thawed on ice. Affinity purifications of protein A fusions was performed as described before (24). Native elution of the untagged Pah1p protein was achieved by following the release of water-soluble radiolabeled ATP as described by Siniossoglou et al. (16). Native elution of the untagged Pah1p protein was achieved by following the release of water-soluble radiolabeled ATP as described by Siniossoglou et al. (16).

**Affinity Purifications of Protein A Fusions**—Cells were grown to \( A_{600} = 1 \), washed once with water, resuspended in a 1:1 ratio with water, and injected directly into liquid nitrogen. Frozen cell pastes were ground in a mortar and pestle prechilled with liquid nitrogen. The ground cell powder was then thawed on ice. Affinity purifications of protein A fusions was performed as described by Siniossoglou et al. (16). Native elution of the untagged Pah1p protein was achieved by following the release of water-soluble radiolabeled ATP as described by Siniossoglou et al. (16).

**PA Phosphatase Assays**—Mg\(^{2+}\)-dependent PA phosphatase activity was measured by following the release of water-soluble \(^{32}\)P from chloroform-soluble \(^{32}\)P-labeled ATP (10,000 cpm/nmol) as

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**Table 2: Plasmids used in this study**

| Plasmid       | Description                          | Reference/source |
|---------------|--------------------------------------|------------------|
| Ycplac111-PAH1| PAH1 into CEN/LEU2 vector            | 17               |
| Ycplac111-PAH1-PKA| PAH1-PKA into CEN/LEU2 vector         | 17               |
| Ycplac111-PAH1-PKA| PAH1-PKA with the following mutations (7P): S110A/S114A/ S168A/S602A/T723A/S744A/S748A cloned into CEN/LEU2 vector | This study |
| Ycplac111-PAH1-NC7-PKA| PAH1-PKA with the following mutations (NC7-7P): S773A/S774A/ S800A/S805A/S810A/S814A/S818A cloned into CEN/LEU2 vector | This study |
| Ycplac111-PAH1-P7| PAH1-P7 cloned into CEN/LEU2 vector | This study |
| YEpplac181-GAL1-10-PAH1| PAH1 under the control of GAL1/10 promoter, cloned into 2-µ/LEU2 vector | This study |
| YEpplac181-GAL1-10-PAH1-PKA| PAH1-PKA under the control of GAL1/10 promoter, cloned into 2-µ/LEU2 vector | This study |
| YEpplac181-GAL1-10-PAH1-NCK-7-PKA| PAH1-NCK-7P under the control of GAL1/10 promoter, cloned into 2-µ/LEU2 vector | This study |
| YEpplac181-GAL1-10-OP1| OP1 under the control of GAL1/10 promoter, cloned into 2-µ/LEU2 vector | This study |
| YEpplac181-GAL1-10-PAH1-PKA| PAH1-PKA under the control of GAL1/10 promoter, cloned into 2-µ/LEU2 vector | This study |
| YEpplac181-GAL1-10-PAH1-PKA| PAH1-PKA under the control of GAL1/10 promoter, cloned into 2-µ/LEU2 vector | This study |
| YEpplac181-GAL1-10-PAH1-NCK-7-PKA| PAH1-NCK-7P under the control of GAL1/10 promoter, cloned into 2-µ/LEU2 vector | This study |
| YEpplac33-RFP-PLIS1| RFP-PLIS1 under the control of the NOP1 promoter, cloned into CEN/URA3 vector | This study |
| PS470| INO2 under the control of the MET25 promoter into a 2-µ/TRP1 vector | 30               |
| PS471| INO4 under the control of the MET25 promoter into a 2-µ/HIS3 vector | 30               |
| YEpplac181-GAL1-10-HMG1| HMG1 under the control of the GAL1/10 promoter cloned into a 2-µ/LEU2 vector | This study |

**Liquid Chromatography-MS/MS**—The peptides of the in-gel digest peptide mixtures were separated first by micro-column pack with anion exchange resin into three fractions, then by nanoscale liquid chromatography (LC Packings, Amsterdam, Netherlands) on a reverse phase C18 column (150 × 0.075 mm inner diameter, flow rate 0.15 µl/min). The eluate was introduced directly into a Q-STAR hybrid tandem mass spectrometer. The spectra were searched against a NCBI non-redundant data base with MASCOT MS/MS Ions search. The identity and phosphorylation sites were confirmed by manual inspection of the fragmentation series.

**Nanospray-MS/MS**—A portion of the extracted tryptic peptide mixture was desalted and concentrated using a GELoader tip filled with Poros oligo R3 sorbent (Perseptive Biosystems). The bound peptides were eluted with 1 µl of 20% acetonitrile, 2.5% formic acid directly into a nanospray capillary and then introduced into a Q-STAR hybrid tandem mass spectrometer. Product ion scans were carried out in positive ion mode, and a MS survey scan for peptides from m/z 400 to 1500 were measured. Selected ions were fragmented by collision-induced dissociation with nitrogen in the collision cell, and the spectra of fragment ions produced were recorded in the time-of-flight mass analyzer.
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described by Carman and Lin (25). Enzyme reactions and kinetic analysis with purified Pah1p proteins from yeast were performed as described by Han et al. (18). Kinetic data and statistical analyses were performed as described by Han et al. (18).

**RNA Exmissions and Quantitative RT-PCR**—At least 10 absorbance units (1 A = 2.5 × 10^7 cells/ml) of an exponentially dividing culture (A_600 between 0.4 and 0.6) grown in the appropriate medium were spun down, washed, and then snap-frozen in liquid nitrogen. Total RNA was extracted using a hot phenol protocol (26). 400 μl of lysis buffer (50 mM sodium acetate, 10 mM EDTA, acetic acid buffered to pH 5), 600 μl of citrate-buffered phenol (pH 4.3), and 50 μl of 10% SDS were heated to 65 °C then added to the frozen pellet, which was then vigorously vortexed for 3 min. The sample was then cooled briefly on ice, and the organic and aqueous phases were separated by centrifugation at 16,100 × g for 10 min at 4 °C. The aqueous phase was then precipitated overnight with 0.1 volumes 3M sodium acetate (pH 5.3) and 2.5 volumes ethanol then re-suspended in nuclease-free water. mRNA was subsequently isolated from the sample using the Oligotex mRNA mini kit (Qiagen).

For quantitative RT-PCR analysis, cDNA was synthesized using Superscript III RNase H-reverse transcriptase (Invitrogen) and 18-mer oligo(dT) primers (Ambion). The resulting cDNA was analyzed by real-time PCR on an ABI prism 7700 (ABI) using a SYBR green mastermix (Applied Biosystems) and primers appropriately designed for the gene of interest (sequences available upon request). A standard curve of wild-type yeast genomic DNA of known concentration, serially diluted from 1:10 to 1:100,000 was used for relative quantification of each measurement. Each sample was run in duplicate. Each measurement was normalized by dividing it by the value of an invariantly expressed gene appropriate for the level of expression (RTG2 or ACT1), then a mean was taken of the two standardized measurements. An intergenic region in chromosome V was used as a control for DNA contamination.

**Microscopy**—To visualize the SEC63-GFP and RFP-PLIS1 fusions, cells were grown in the appropriate selective medium at 30 °C to early logarithmic phase and examined with a 63× Plan Apochromat objective on a Zeiss Axiovert 200M inverted microscope with an LSM 510 confocal laser scanning attachment.

**Antibodies**—The antibody used to detect protein A fusions was from DAKO (catalog no. Z0113). The MPM2 monoclonal antibody was from Upstate Biotechnology (catalog no. 05-368).

**RESULTS**

**Identification of Phosphorylation Sites on Pah1p**—To identify the amino acid residues phosphorylated in Pah1p, a Pah1p-PtA fusion expressed under the control of the endogenous PAH1 promoter from a centromeric plasmid was purified from extracts of pah1Δ nem1Δ spo7Δ cells by IgG-Sepharose chromatography. The purified protein was digested with trypsin and subjected to electrospray ionization liquid chromatography-MS/MS as described under “Experimental Procedures.” Using this approach, we identified four phosphorylation sites matching the minimal Ser/Thr-Pro motif phosphorylated by cyclin-dependent kinases (Cdk): Ser-168, Ser-602, Ser-744, Ser-748 (Fig. 1A and Table 3). The same sites were identified in parallel experiment where Pah1p-PtA was isolated from wild-type cells (data not shown). Thr-723, which fits the optimal Cdk consensus ((S/T)P[X(K/R)], was identified by MALDI as a possible phosphorylation site (Tables 4 and 5). We have previously shown that deletion of the NEM1-SPO7 phosphatase causes the appearance of a second Pah1p-PtA band of reduced electrophoretic mobility (Ref. 17; Fig. 1D). Substitution of Thr-723 or Ser-744 and Ser-748 with alanines abolished this mobility shift, suggesting that these phosphorylated residues are targeted by the Nem1p-Spo7p phosphatase complex in vivo.

During this analysis, we noticed that peptides from the N terminus of Pah1p were underrepresented after tryptic digestion, raising the possibility that there could be additional phosphorylated sites not detected by this approach. In fact, pull down of a mutant where all serines in the Ser/Thr-Pro sites sequenced by mass spectrometry were substituted by alanines (S168A/S602A/T723A/S744A/S748A, called Pah1p-5P) followed by immunoblotting with an anti-MPM2 antibody, revealed that Pah1p-5P was still phosphorylated (Fig. 1C). The MPM2 antibody specifically recognizes phospho-Ser-Thr-Pro motifs (27) and native Pah1p from yeast but not after dephosphorylation by the purified Nem1p-Spo7p complex (17). We, therefore, mutated the remaining candidate Ser/Thr-Pro sites, pulled down the corresponding protein A fusions, and analyzed them by MPM2 immunoblotting. As seen in Fig. 1C, mutations of Ser/Thr-Pro sites within the conserved HAD-like domain did not abolish phosphorylation, although one (Ser-475) fits the optimal Cdk1 consensus ((S/T)P[X(K/R)]. In contrast, mutation of two sites found next to the C-terminal end of the lipin domain (Ser-110 and Ser-114) combined with Pah1p-5P completely abolished phosphorylation (S110A/S114A/S602A/T723A/S744A/S748A, the mutant called Pah1p-7P; Fig. 1C). Taken together these data show that Pah1p is phosphorylated in vivo on seven Ser/Thr-Pro sites.

The search for phosphorylated sites and peptides in Pah1p by mass spectrometry revealed additional sites that do not fit the Cdk consensus (Tables 4 and 5). We found that Ser-773, located within the predicted nuclear localization signal of Pah1p, is phosphorylated in vivo. Two overlapping peptides close to the C-terminal end of Pah1p (residues 798–829) that contain multiple serines and threonines were found phosphorylated at three sites (see Tables 4 and 5). We created an allele where Ser-773, Ser-774 (a candidate site, see Table 4), and five candidate serine residues found within the C-terminal peptide were mutated to alanines (S773A/S774A/S800A/S805A/S810A/S814A/S818A, called PAH1-NCK-7P), tagged it with a protein A sequence, and transformed into the nem1Δ pah1Δ strain. As seen in Fig. 1D, unlike the PAH1-7P, the PAH1-NCK-7P allele does not significantly change the migration of Pah1p, suggesting that the residues contributing mostly to the mobility shift during the cell cycle are among the seven Ser/Thr-Pro sites.

Deletion of NEM1 increases phosphorylation on the Ser/Thr-Pro sites, indicating that the Nem1p-Spo7p complex is
required in vivo for their dephosphorylation (Fig. 1C). To address more directly which sites are dephosphorylated by Nem1p-Spo7p, we first incubated hyperphosphorylated Pah1p purified from nem1Δ spo7Δ cells with either Nem1pPtA-Spo7p complex on IgG-Sepharose beads or control beads followed by SDS-PAGE and mass spectrometric analysis of Pah1p. All peptides analyzed from the fraction with Nem1pPtA-Spo7p lacked the peaks corresponding to the sequenced phospho residues, except a C-terminal peptide containing the Ser-773 (Fig. 2A). Moreover, a Pah1p-PtA fusion affinity purified from an pah1Δ strain overexpressing Nem1p and Spo7p lacked all phosphorylated peptides (Fig. 2B), showing that the Nem1p-Spo7p complex can dephosphorylate in vivo all phospho residues on Pah1p.

**Phosphorylation of Pah1p Regulates Expression of INO1 and OPI3**—Deletion of the NEM1-SPO7 genes derepresses transcription of INO1 and OPI3, the enzymes catalyzing the rate-

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**TABLE 3**

| m/z value* | Massa | Sequence | Phosphorylated residue | Method |
|------------|-------|----------|------------------------|--------|
| 902.4 (3+1) | 2704.13 | NSETTGLSpTSTTTPTPP (OH) DVEER | Ser-168 | Nano-ESI |
| 719.3 (2+)  | 1636.6 | TNTSMVPgSQPNR | Ser-602 | ESI-LC-MS/MS |
| 838.4 (2+1) | 1674.8 | QYLELLGpSPLapskk | Ser-773 | ESI-LC-MS/MS |
| 597.3 (3+1) | 1788.9 | RApSSAATIS1DEEFKk | Ser-773 | ESI-LC-MS/MS |

*a* Indicates double- and triple-charged ions, respectively.

Molecular mass of the phospho-form of peptide.
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Table 4: Phosphopeptides identified by linear mode MALDI-MS from Fe(III)-nitrilotriacetic acid IMAC enrichment

| MH+ IMAC | MH+ AP | Sequence | Number of phosphorylated residues |
|----------|--------|----------|----------------------------------|
| 1674.45  | 1514.33| QYLELGSPFAFPK | 2                               |
| 1740.26  | 1580.30| RASSAAATSIDKEFK | 2                               |
| 1866.96  | 1708.50| RASSAAATSIDKEFK | 2                               |
| 2103.94  | 1943.92| IDVSNDVHSLSNDTSESR | 2                              |
| 2259.97  | 2099.87| IDVSNDVHSLSNDTSESR | 2                              |
| 2432.11  | 2286.91| NSETTGLSPTESTSTTTTPP (OH) DSVEER | 2                         |
| 3340.01  | 3100.05| IDVSNDVHSLSNDTSESR | 3                              |
| 3791.26  | 3630.95| NVSGSTNNNEVLAASSDVENASDLVGSHSSGSTPNK | 2                         |
| 3871.82  | 3711.95| NVSGSTNNNEVLAASSDVENASDLVGSHSSGSTPNK | 3                         |

a Protonated molecular mass of the phospho-form of peptide.

Table 5: Phosphopeptides identified by reflectron mode MALDI-MS from Phos Select beads enrichment

| MH+ IMAC | MH+ AP | Sequence | No. of phosphorylated residues |
|----------|--------|----------|--------------------------------|
| 1437.6142 | 1357.6771 | TNTSMVPSPNPR | 1                          |
| 1453.6133 | 1373.6526 | TNTSM (U) VPSPNPR | 1                         |
| 1661.7277 | 1581.8531 | RASSAAATSIDKEFK | 1                          |
| 1789.8920 | 1709.9478 | RASSAAATSIDKEFK | 1                          |
| 2024.8356 | 1944.9265 | IDVSNDVHSLSNDTSESR | 1                         |
| 2181.0210 | 2100.9732 | IDVSNDVHSLSNDTSESR | 1                         |
| 2452.1948 | 2372.1848 | IVSKIDVSNVHLGSNDTSESR | 1                     |
| 2608.2430 | 2528.2985 | IVSKIDVSNVHLGSNDTSESR | 1                     |
| 2705.2529 | 2625.2586 | NSETTGLSPTESTSTTTTPP (OH) DSVEER | 1                     |
| 2889.3761 | 2809.3719 | TNTSM (U) VPSPNRTLDNFDSETGFR | 1                     |
| 3037.4349 | 2937.4515 | TNTSM (U) VPSPNRTLDNFDSETGFR | 1                     |

a Protonated molecular mass of the phospho-form of peptide.

FIGURE 2. Identification of phosphorylation sites on Pah1p dephosphorylated by the Nem1p-Spo7 complex. A, hyperphosphorylated Pah1p, purified from a culture of pah1Δ nem1Δ spo7Δ expressing Pah1p-PtA by IgG-Sepharose chromatography and tobacco etch virus protease digested to remove the PtA tag was incubated with IgG-Sepharose beads with or without Nem1p-PtA-Spo7 complex. Reactions were resolved by 7% SDS-PAGE followed by Coomassie staining, and the Pah1p bands were excised from gels and subjected to liquid chromatography-MS/MS analysis as described under “Experimental Procedures.” B, Pah1p-PtA purified from cells overexpressing the Nem1p-Spo7 complex using the galactose promoter or the control vectors was incubated with IgG-Sepharose beads with or without Nem1p-PtA-Spo7 complex. Reactions were resolved by 7% SDS-PAGE followed by Coomassie staining, and the Pah1p-PtA bands were excised from gels and subjected to nanoelectrospray mass spectrometry. In both A and B, the positions of phosphoserine residues identified by mass spectrometry within the two Pah1p samples are highlighted by asterisks (Ser/Thr-Pro sites) or triangles (non-Ser/Thr-Pro sites).

limiting steps in the production of PI and PC respectively (17). Thus, dephosphorylation of Pah1p seems to be required for normal repression of INO1 and OPI3. A prediction of this model would be that in a pah1Δ mutant that cannot be phosphorylated, INO1, OPI3, and possibly other phospholipid biosynthetic genes would be repressed to a greater extent than in a wild-type yeast strain. To test this model, we analyzed the transcription of INO1 and OPI3 in the yeast strain where the seven Ser/Thr-Pro sites were mutated to alanines (PAH1-7P). A pah1Δ strain expressing wild-type PAH1 or PAH1-7P from centromeric vectors was grown in the presence of inositol and transferred to media lacking inositol, and the levels of INO1 and OPI3 transcripts were followed by quantitative real-time RT-PCR. As seen in Fig. 3A, the PAH1–7P mutant exhibits a delay in the induction and reaches lower mRNA levels for both the INO1 and OPI3 genes. In a parallel experiment, a mutant where both the Ser/Thr-Pro and the C-terminal non-Cdk site mutations were combined together exhibited the same levels of INO1 and OPI3 transcripts as the PAH1–7P strain (data not shown). Overexpression of PAH1-7P using the GAL1/10 promoter increases the inhibitory effect of wild-type PAH1 and causes a tight inositol auxotrophy comparable with that induced by the overexpression of the repressor OPI1 (Fig. 3B). This is consistent with a role of phosphorylation in promoting the derepression of inositol-regulated genes. On the other hand, expression of the PAH1-NCK-7P from the galactose promoter did not cause any growth inhibition on media lacking inositol when compared with cells expressing wild-type PAH1 (Fig. 3B). Taken together, these data indicate that phosphorylation of Pah1p on the Ser/Thr-Pro sites regulates the expression of phospholipid biosynthetic enzymes.

Phosphorylation of Pah1p Regulates Its PA Phosphatase Activity—A recent report showed that Pah1p has Mg2+-dependent PA phosphatase activity and that knock-out of PAH1
deficient mutant exhibited a 2-fold increase in the $V_{max}$, whereas the $K_m$ and Hill number for the PA substrate are not significantly affected (Fig. 4C). Taken together these results indicate that phosphorylation of Pah1p on the Ser/Thr-Pro sites inhibits its PA phosphatase activity.

Cooperative Effects of Opi1p and Pah1p on Transcriptional Repression—Because the Pah1p-7P mutant reduces PA levels and PA is required to relieve repression by Opi1p, then Pah1p-7P should no longer be toxic in an $opi1\Delta$ background. Indeed, deletion of $OPI1$ rescues the toxicity caused by the overexpression of $PAH1$-7P on media lacking inositol (Fig. 5), confirming that the inositol auxotrophy is caused by the inability of the cells to induce the phospholipid biosynthetic machinery during inositol starvation. These data support a model where the Pah1p-mediated repression of lipid biosynthetic genes is exerted through Opi1p. To further test this model, we used quantitative real-time RT-PCR to compare mRNA levels of the $INO1$ gene in the $pah1\Delta$ $opi1\Delta$ double mutant with those measured in the respective isogenic single mutants and wild-type strain. As seen in Fig. 6A and consistent...
Derepression of Phospholipid Biosynthesis Is Not Sufficient for Nuclear Membrane Growth—Given the link between nuclear membrane growth, PA production, and transcriptional derepression of phospholipid biosynthetic genes, we asked whether nuclear membrane expansion could be caused by induction of phospholipid biosynthesis at the ER. Excess PA in the \( \text{pah1}\) /H9004 mutant would be expected to result in removal of Opi1p from the promoters of the phospholipid biosynthetic genes (10, 18). We, therefore, asked whether constitutive derepression of UASINO genes in an \( \text{opi1}\) /H9004 strain would cause similar nuclear defects as those observed in the \( \text{pah1}\) /H9004 cells (Fig. 7B). As seen in Fig. 7C, \( \text{opi1}\Delta \) cells co-expressing RFP-\( \text{PUS1}\) (to label the nucleus) and \( \text{SEC63}\)-GFP (to label the nuclear/ER membrane) show normal nuclear and ER membrane structure when compared with the wild-type cells.

To induce membrane production in a different way, we next overexpressed the \( \text{INO2}\) and \( \text{INO4}\) genes, whose products form a complex that binds the UASINO element and activates transcription of phospholipid biosynthetic genes (11–13). Interestingly, in contrast to the \( \text{opi1}\Δ \) cells, overexpression of \( \text{INO2}\) and \( \text{INO4}\) using the inducible \( \text{MET25}\) promoter (30) resulted in large cells exhibiting very slow growth. In many cells the \( \text{SEC63}\)-GFP staining was irregular and occasionally accumulated in cytoplasmic structures, suggesting that proper ER structure was perturbed (Fig. 7D). Nuclei, however, did not resemble the expanded and irregularly shaped nuclei of \( \text{pah1}\Δ \) cells, indicating again that up-regulation of membrane biogenesis in the ER is not sufficient for nuclear expansion (Fig. 7D). Consistent with this, induction of karmellae, perinuclear ER membrane stacks produced by the overexpression of the enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase (\( \text{HMG1}\) (31)), did not result in nuclear membrane expansion and growth (Fig. 7E). Taken together, these data provide evidence that, although phospholipid production is necessary for nuclear membrane proliferation in the \( \text{pah1}\Delta \) cells (17), on its own it is not sufficient to trigger nuclear expansion.

**DISCUSSION**

Nuclear membrane remodeling is essential for the dynamic changes that take place in the yeast nuclear envelope during cell division. A possible function for \( \text{PAH1}\) in regulating nuclear membrane growth is to maintain the dynamic balance of PA production and membrane expansion. Derepression of phospholipid biosynthesis allows for the increased production of PA, which in turn activates the transcription of genes involved in membrane biogenesis. However, the results presented here suggest that the mere induction of phospholipid biosynthesis is not sufficient to trigger nuclear expansion. This implies that additional factors, possibly involving the coordination of multiple signaling pathways, are required for the proper regulation of nuclear membrane growth. Further studies are needed to elucidate the molecular mechanisms underlying this process.

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**FIGURE 4.** Effect of phosphorylation on Ser/Thr-Pro sites on the PA phosphatase activity of Pah1p. A, SDS-PAGE of the purified untagged Pah1p and Pah1p-7P from wild-type yeast cells, stained with Coomassie Blue. The positions of the molecular mass standards are indicated. B, the Mg\(^{2+}\)-dependent PA phosphatase activity of the purified Pah1p and Pah1p-7P proteins was measured with the indicated protein contents. The values shown were determined from triplicate enzyme determinations (errors fall within the size of the circles). C, effect of PA surface concentration on Pah1p- and Pah1p-7P-dependent PA phosphatase activity. The indicated purified Pah1p and Pah1p-7P proteins from yeast were assayed for Mg\(^{2+}\)-dependent PA phosphatase activity at the indicated surface concentrations (mol %) of PA. The molar concentration was held constant at 0.2 mM. The values shown were determined from triplicate enzyme determinations ± S.D. Lower panel, the kinetic parameters for the Pah1p and Pah1p-7P samples analyzed in B and C are shown.
Regulation of Pah1p Function by Phosphorylation

structure has been first suggested by studies in fission yeast showing that Ned1p, the Schizosaccharomyces pombe orthologue of Pah1p, interacts with an essential nucleoporin and exhibits genetic interactions with factors involved in nucleocytoplasmic transport and chromosome segregation (32). More recently, Pah1p was shown to be the substrate of the Nem1p-Spo7p complex, a transmembrane phosphatase complex that localizes to the nuclear and ER membrane and interacts genetically with components of the nuclear pore complex (16, 17). Mutations in PAH1, NEM1, and SPO7 cause derepression of phospholipid biosynthetic genes and massive nuclear membrane proliferation that leads to nuclear growth (17). The recent demonstration by Han et al. (18) that Pah1p is a Mg$^{2+}$-dependent PA phosphatase controlling biosynthetic pools of PA and the major phospholipids PC, PE, and PI levels provides a intriguing link between lipid biosynthesis and nuclear structure.

The fact that nem1Δ spo7Δ cells display the same phenotypic defects as pah1Δ cells suggests that phosphorylation acts as a switch that inactivates Pah1p function. In this paper we use a combination of systematic mutagenesis and mass spectrometry and show that Pah1p is phosphorylated on multiple Ser/Thr-Pro sites in vivo. Consistent with this, different genome-wide screens have recently identified Pah1p as a possible substrate of Cdc28p (33, 34) and Pho85p (34, 35), two Cdks in budding yeast. Alanine substitution of the C-terminal Cdk phosphorylation sites alone completely inhibits the Nem1p-Spo7p-dependent mobility shift in SDS-PAGE. However, the Pah1p-5P mutant that carries these C-terminal substitutions still gets phosphorylated on the remaining sites, indicating that phosphorylation can take place independently on the different Cdk phosphorylation sites of the protein. Interestingly, Pah1p is phosphorylated at the C-terminal junctions of both of its conserved domains (Ser-110 for the N-terminal lipin domain and Ser-602 for the C-terminal HAD-like domain), raising the possibility that this phosphorylation could regulate the activities of these domains. Although the precise mechanism is not known, the fact that phosphorylation affects the $V_{\text{max}}$ without affecting the $K_m$ or the Hill number for the PA substrate suggests that phosphorylation decreases catalytic turnover without affecting significantly association with PA. It should be noted that the function of the phosphorylation of Pah1p seems to be conserved in evolution, as Ned1p, the fission yeast orthologue of Pah1p, is a phosphoprotein (32), and mammalian Lipins, which are putative orthologues of Pah1p (19), are phosphorylated on Cdk phosphorylation sites in nocodazole-arrested HeLa cells.4

The fact that Pah1p is phosphorylated on additional, non-Cdk sites indicates that the regulation of Pah1p function by phosphorylation is very complex. The role of this phosphorylation is not known, but the fact that the PAH1-7P- allele does not affect the inositol response pathway and does not increase the inhibitory effect of the PAH1-7P suggests that it does not overlap with the function of the Ser/Thr-Pro-dependent phosphorylation in transcription and PA production. Consistent with this, we found that phosphorylation on the C-terminal non-Cdk sites in vivo is independent of the phosphorylation on the seven Cdk sites.5 Interestingly, a recent phospho-site array screening identified Pah1p as a putative target of the Dbf2p-Mob1p kinase complex (36), a component of the mitotic exit network (MEN) cascade that preferentially phosphorylates substrates with RXXS motifs (36). One sequenced phosphoserine (Ser-773) and one more candidate site within the C-terminal phosphopeptide (Fig. 1A) match the RXXS motif. Additional studies will be needed to examine whether Pah1p is in vivo a substrate of the Dbf2-Mob1 complex.

Transcription of phospholipid biosynthetic genes that contain a UAS$_{INO}$ element is under the control of the activator Ino2p-Ino4p complex and the repressor Opi1p (8, 9). A number

4 N. Grimsey and S. Siniossoglou, unpublished data.
5 S. Peak-Chew and S. Siniossoglou, unpublished data.
of studies in *Saccharomyces cerevisiae* have established a genetic link between production of PA and the transcriptional regulation of UAS*INO* genes. The molecular basis of this link has been recently identified; reduced PA concentration in response to inositol supplementation, a key precursor of phospholipid production, causes translocation of Opi1p into the nucleus where it represses *INO1* and presumably other UAS*INO* phospholipid biosynthetic genes in cells expressing endogenous *PAH1*-7P is not enough to cause inositol auxotrophy, overexpression of *PAH1*-7P in wild-type cells does. Our data suggest that one mechanism contributing to this phenotype could be the depletion of PA from ER membranes and a constitutive repression of UAS*INO* genes by Opi1p. Consistent with this model, deletion of *OPI1* restores growth in GAL-*PAH1*-7P cells. Of course, overexpressed Pah1p-7P might compromise growth by additional mechanisms as well. For example, mislocalized Pah1p-7P in the overexpressing strain could affect viability by altering PA pools and lipid composition on other intracellular membranes.

If the function of Pah1p in the transcriptional regulation of UAS*INO* genes is upstream of Opi1p and depends exclusively on the Opi1p-mediated signaling pathway to the nucleus, then the *pah1Δ opi1Δ* mutant should display the same levels of derepression as the *opi1Δ* mutant. However, our data show that deletion of the two genes has a synthetic effect on the mRNA levels of both *INO1* and the *OPI3* genes, suggesting that Pah1p can also influence transcription in an Opi1p-independent manner. Consistent with this, high copy number suppressors of the inositol auxotrophy of the GAL-*PAH1*-7P strain fail to rescue the inositol auxotrophy of the GAL-*OPI1*. Thus, it is possible that there is a second factor-sensing PA and influencing transcription of phospholipid biosynthetic genes independently of Opi1p. Further studies are required to address these hypotheses.

The PA phosphatase reaction is the committed step for the synthesis of the storage lipid triacylglycerol, which is formed from PA via DAG (37, 38). The DAG generated in the PA phosphatase reaction is also utilized in the synthesis of the membrane phospholipids PC and PE via the Kennedy pathway. PA is used for the synthesis of membrane phospholipids via CDP-DAG. Thus, regulation of PA phosphatase activity may govern whether cells make storage lipids and phospholipids via DAG or phospholipids via CDP-DAG. In mammalian cells, PA phosphatase plays a role in lipid signaling as part of the phospholipase D-PA phosphatase pathway to generate DAG for activation of protein kinase C (39, 40). The importance of PA phosphatase in lipid signaling is further underscored because of its role in blunting the bioactive functions of PA, which include promotion of cell growth and proliferation, vesicular trafficking, secretion, and endocytosis (39 – 41).

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S. Siniossoglou, unpublished data.
The existing data so far indicate that phosphorylation of Pah1p regulates both production of PA and other phospholipids and nuclear membrane expansion. Does, then, modulation of phospholipid levels at the ER membrane control nuclear growth? One possibility could be that the two phenotypes are not related and are caused by different functions of Pah1p. However, mutations that inhibit de novo biosynthesis of phospholipids are epistatic on the phenotype of pah1Δ, nem1Δ, and spo7Δ, indicating that derepression of phospholipid production is necessary for nuclear growth (17). On the other hand, the data presented here suggest that overproduction of membrane alone is not sufficient for nuclear growth; excess synthesized membrane in cells over-expressing ino2-ino4 or hmg1 leads to ER membrane proliferation but not to nuclear membrane expansion, similar to that described in the pah1Δ, nem1Δ, and spo7Δ cells. How then does hyperphosphorylated Pah1p in the nem1Δ and spo7Δ mutants drive nuclear expansion? One possibility could be that localized production of PA or some metabolite closely linked to PA at the nuclear membrane rather than phospholipid production at the ER is the event that triggers nuclear membrane remodeling. Localized production of lipids on membranes, including PA and DAG, is thought to induce changes on organelle shape and morphology (41, 42). During sporulation of yeast cells, hydrolysis of PC to PA is essential for the production of the prospore membrane that originates from the nuclear envelope and eventually engulfs the haploid nuclei (43). Recent data suggest that it is the production of PA rather than the turnover of PC that is important for the biogenesis of the prospore membrane (44). Consistent with an important role for PA in nuclear structure, point mutations at the conserved phospho-acceptor motif of the HAD-like domain of Pah1p that are predicted to inactivate its PA phosphatase activity are enough to induce nuclear membrane proliferation. Alternatively, nuclear expansion might also depend on additional changes on the inner nuclear membrane that are regulated by Pah1p. A pool of Pah1p has been found in association with chromatin (17). Furthermore, Lipin1α, a mouse homologue of Pah1p, localizes into the nucleus of mature adipocytes (19, 45). Recently, Campbell et al. (46) showed that nuclear expansion in the nem1Δ and spo7Δ cells takes place at the nuclear membrane that is associated with the nucleolus, whereas the remaining nuclear membrane that associates with the bulk chromatin does not proliferate, suggesting the existence of a tethering mechanism that restricts nuclear growth. The additional possible functions of Pah1p in the nuclear membrane are currently under investigation.

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