Yeast Nem1-Spo7 Protein Phosphatase Activity on Pah1 Phosphatidate Phosphatase Is Specific for the Pho85-Pho80 Protein Kinase Phosphorylation Sites*  

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Background: Pah1 phosphatidate phosphatase translocates to the membrane through its dephosphorylation by the membrane-associated Nem1-Spo7 phosphatase complex.

Results: Nem1-Spo7 phosphatase was characterized for its enzymological, kinetic, and regulatory properties with phosphorylated forms of Pah1.

Conclusion: Nem1-Spo7 phosphatase exhibits the highest specificity for Pah1 phosphorylated by the Pho85-Pho80 protein kinase complex.

Significance: The Nem1-Spo7-mediated dephosphorylation regulates the function of Pah1 in lipid metabolism.

Pah1 is the phosphatidate phosphatase in the yeast Saccharomyces cerevisiae that produces diacylglycerol for triacylglycerol synthesis and concurrently controls the levels of phosphatidate used for phospholipid synthesis. Phosphorylation and dephosphorylation of Pah1 regulate its subcellular location and phosphatidate phosphatase activity. Compared with its phosphorylation by multiple protein kinases, Pah1 is dephosphorylated by a protein phosphatase complex consisting of Nem1 (catalytic subunit) and Spo7 (regulatory subunit). In this work, we characterized the Nem1-Spo7 phosphatase complex for its enzymological, kinetic, and regulatory properties with phosphorylated Pah1. The dephosphorylation of Pah1 by Nem1-Spo7 phosphatase resulted in the stimulation (6-fold) of phosphatidate phosphatase activity. For Pah1 phosphorylated by the Pho85-Pho80 kinase complex, maximum Nem1-Spo7 phosphatase activity was dependent on the concentrations of Pah1 phosphorylated by Pho85-Pho80, Cdc28-cyclin B, PKA, and PKC with \( k_{cat} \) and \( K_m \) values of 0.29 s\(^{-1} \) and 81 nM, 0.11 s\(^{-1} \) and 127 nM, 0.10 s\(^{-1} \) and 46 nM, and 0.02 s\(^{-1} \) and 38 nM, respectively. Its specificity constant \( (k_{cat}/K_m) \) for Pah1 phosphorylated by Pho85-Pho80 was 1.6-, 4-, and 6-fold higher, respectively, than that phosphorylated by PKA, Cdc28-cyclin B, and PKC.

Pah1 PAP\(^2\) (1), which catalyzes the Mg\(^{2+}\)-dependent dephosphorylation of PA to form DAG and P\(_1\) (Fig. 1A), has emerged as one of the most highly regulated enzymes of lipid metabolism in the yeast Saccharomyces cerevisiae (3–5). The PAP reaction of Pah1 is governed by a conserved DXDX(T/V) catalytic motif within its haloacid dehalogenase-like domain and a conserved glycine residue within its NLIP domain (1, 6) (Fig. 1B). By the nature of its reaction, PAP affects the levels of PA and DAG, thereby controlling the PA-derived membrane phospholipids and the DAG-derived storage lipid TAG (7, 8). The DAG generated by the PAP reaction may also be used to synthesize phosphatidylcholine and phosphatidylethanolamine via the Kennedy pathway, which is primarily used when yeast cells are supplemented with choline and ethanolamine, respectively (7, 8). The physiological relevance of Pah1 is exemplified by the pah1Δ phenotypes that correlate with the increased level of PA (e.g. derepression of phospholipid synthesis gene expression and the abnormal nuclear/ER membrane expansion) and the decreased levels of DAG and TAG (e.g. susceptibility to fatty acid-induced lipotoxicity and defects in lipid droplet formation) (1, 6, 9–11). The lack of Pah1 also causes defects in cell wall integrity (12, 13) and vacuole homeostasis (14), classic hallmarks of apoptosis (10), temperature sensitivity (1, 9, 15), and the inability to grow on non-fermentable carbon sources (1). The complementation of pah1Δ phenotypes by the loss of Dgk1 DAG kinase (10, 11, 16) indicates that an imbalance in the cellular levels of PA and DAG is the molecular basis for the deleterious physiological defects (5).

Pah1 is conserved in higher eukaryotic organisms, including humans (e.g. lipins 1–3) (1, 17–24). In fact, defects in mammalian lipin PAP enzymes underlie several metabolic disorders.

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2 The abbreviations used are: PAP, phosphatidate phosphatase; PA, phosphatidate; DAG, diacylglycerol; TAG, triacylglycerol; ER, endoplasmic reticulum; Nem1, nuclear envelope morphology protein 1; Spo7, sporulation-specific protein 7; CTDNEP1, C-terminal domain nuclear envelope phosphatase 1; NEP1-R1, nuclear envelope phosphatase 1-regulatory subunit 1; 7A, alanine mutations of Pah1 at Ser-110, Ser-114, Ser-168, Ser-602, Thr-723, Ser-744, and Ser-748.
that include lipodystrophy and insulin resistance, peripheral neuropathy, rhabdomyolysis, and inflammation (18, 25–36). As in yeast (5), these “lipinopathies” appear to be rooted, at least in part, in the molecular function of PAP to control the levels of PA and DAG (27, 37, 38).

Pah1 is regulated by genetic and biochemical mechanisms. The expression of PAH1 is controlled on a transcriptional level by growth phase and nutrient supplementation (39, 40), whereas on a biochemical level, PAP activity is governed by membrane phospholipids and sphingoid bases (41, 42), phosphorylation and dephosphorylation (9, 44–49), and proteasome-mediated degradation (50). Of the biochemical regulations, phosphorylation and dephosphorylation have the greatest impact on the physiological functions of Pah1 (4, 5). Pah1 is primarily found in the cytosol as an inactive phosphoprotein but becomes active through its translocation to the nuclear/ER membrane, where its substrate PA resides (9, 44–48) (Fig. 1A). The membrane translocation of Pah1 occurs via its dephosphorylation by the membrane-associated protein phosphatase complex Nem1 (catalytic subunit)-Spo7 (regulatory subunit) (9, 44–47, 51–53) (Fig. 1A). The interaction with Nem1-Spo7 occurs via the acidic tail of Pah1 (53), and the dephosphorylated enzyme interacts with the membrane via its amphipathic helix (52). Phosphorylation and dephosphorylation of Pah1 also regulate its PAP activity as well as its degradation by proteasomes (44, 46, 47, 49, 50).

The phosphatase activity of Nem1, like that of Pah1, is dependent on the DXDX(T/V) catalytic motif (9, 51); both enzymes in the phosphatase cascade are members of the haloacid dehalogenase superfamily (54, 55). Spo7, which binds to the catalytic domain of Nem1, is essential for the phosphatase activity of the complex (9, 51). Because Nem1-Spo7 is required for Pah1 PAP function, cells lacking either subunit (e.g. nem1Δ or spo7Δ) of the phosphatase complex display many of the same phenotypes (e.g. derepression of phospholipid synthesis genes, aberrant expansion of the nuclear/ER membrane, decreased TAG content and lipid droplet formation, and temperature sensitivity) exhibited by pah1Δ mutant cells (9, 39, 45–47, 49, 51, 56). Nevertheless, the expression of phosphorylation-deficient forms of Pah1 complements nem1Δ mutant phenotypes because they allow for membrane association and enzyme activation without the need for dephosphorylation by the Nem1-Spo7 phosphatase (44–46, 48, 52).

Like Pah1, the yeast Nem1-Spo7 phosphatase is conserved in higher eukaryotes. The orthologous complex in humans, which is composed of CTDNEP1 (catalytic subunit formerly known as Dullard) and NEP1-R1 (regulatory subunit formerly known as TMEM188), complements the yeast nem1Δ spo7Δ mutant with respect to membrane expansion, TAG content, and lipid droplet formation (56). Moreover, when expressed in human cells, the CTDNEP1-NEP1-R1 complex, but not the catalytic subunit alone, dephosphorylates lipin-1 and lipin-2 PAP isoforms (56).

Although the physiological importance of the Nem1-Spo7 phosphatase is well established, little is known about its enzymological, kinetic, and regulatory properties. Progress in this area has been limited by the availability of Pah1 phosphorylated by defined protein kinases. In this work, we took advantage of the protein kinase-Pah1 target site relationships (Fig. 1B) and prepared specifically phosphorylated forms of Pah1. By characterizing the mode of action and specificity of Nem1-Spo7, we advanced the understanding of the protein phosphatase complex that plays a crucial role in the regulation of Pah1 PAP.

**EXPERIMENTAL PROCEDURES**

**Materials**—Difco was the source of growth media. Plasmid DNA purification kits and nickel-nitritriacetic acid-agarose resin were from Qiagen. Carrier DNA for yeast transformation was from Clontech. Reagents for electrophoresis, Western blotting, protein determination, DNA size ladders, and molecular mass protein standards were purchased from Bio-Rad. Phos-tag™ AAL-107 was obtained from Wako Pure Chemical Industries. Radiochemicals and scintillation counting supplies were obtained from PerkinElmer Life Sciences and National Diagnostics, respectively. IgG-Sepharose, Q-Sepharose, PVDF membrane, and the enhanced chemifluorescence Western blotting detection kit were from GE Healthcare. Amicon ultracentrifugal filters were purchased from EMD Millipore. Thermo Scientific was the source of alkaline phosphatase-conjugated goat anti-rabbit IgG antibodies. PKA catalytic subunit and conventional PKC were from Promega. New England Bio- labs was the source of cyclin-dependent kinase-cyclin B Protease inhibitors, Triton X-100, and bovine serum albumin were obtained from Sigma-Aldrich. Lipids were purchased from Avanti Polar Lipids. All other chemicals were reagent grade.

**Strains, Plasmids, and Growth Conditions**—The strains and plasmids used in this study are listed in Table 1. The *Escherichia coli*
coli strain DH5α was used for the propagation of plasmids. E. coli strain BL21(DE3)pLysS bearing plasmid pGHis13 was used to express His6-tagged Pah1, and the strain BL21(DE3) bearing plasmids EB1164 and EB1076 was used to express the His-tagged Pho85 and Pho80. The bacterial cells were grown at 37 °C in LB medium (1% tryptone, 0.5% yeast extract, 1% NaCl, pH 7.0), and for the selection of cells carrying plasmids, the growth medium was supplemented with antibiotics (ampicillin, 100 μg/ml; chloramphenicol, 34 μg/ml; kanamycin, 50 μg/ml). The expressions of His6-tagged Pah1, His6-tagged Pho85, and Pho80 were induced with 1 mM isopropyl β-D-thiogalactoside. The S. cerevisiae strain RS453 bearing the plasmids YCplac111-GAL1/10-NEM1-PtA and pRS313-GAL1/10-SPO7 was used for the expressions of protein A-tagged Nem1 and Spo7. Yeast cells were generally grown at 30 °C in synthetic complete medium containing 2% glucose. Appropriate amino acids were omitted from the growth medium to select for cells carrying specific plasmids (57). For the expressions of protein A-tagged Nem1 and Spo7, cells were first grown to the exponential phase in synthetic complete medium with 2% raffinose as the carbon source and then induced for 8 h by the addition of 2% galactose. Cell numbers in liquid cultures were determined spectrophotometrically at an absorbance of 600 nm.

**Purification of Enzymes**—All steps were performed at 4 °C. The protein A-tagged Nem1-Spo7 complex was purified from *S. cerevisiae* by IgG-Sepharose affinity chromatography as described by Siniossoglou et al. (58) with minor modifications. Here, the cell extract was prepared by lysis with glass beads using a Biospec Products Mini Bead Beater-16 (59). Also, the phosphatase complex was eluted from IgG-Sepharose with 50 mM glycine-HCl (pH 3.0) because it could not be dissociated from the resin by treatment with tobacco etch virus protease. The acid-eluted enzyme complex was neutralized with 1 M Tris-HCl (pH 8.0) buffer, and the final preparation contained 160 mM Tris-HCl (pH 8.0), 0.02% Triton X-100, and 20% glycerol. The endogenous phosphorylated wild type and 7A (e.g. alanine mutations of Pah1 at Ser-110, Ser-114, Ser-168, Ser-242, Thr-723, Ser-744, and Ser-748) mutant forms of Pah1 were purified to near homogeneity from *S. cerevisiae* as described previously (44). His6-tagged Pah1 PAP expressed in *E. coli* was purified by affinity chromatography with nickel-nitriotriacetic acid-agarose (1, 60). A major contaminant (~70 kDa) in the affinity-purified enzyme was removed by ion exchange chromatography with Q-Sepharose. Briefly, the affinity-purified enzyme preparation was diluted in 10 volumes of 20 mM Tris-HCl (pH 7.5), 7 mM 2-mercaptoethanol, and 10% glycerol to reduce the concentrations of NaCl and imidazole, and was then applied to a 1-ml Q-Sepharose column equilibrated with the dilution buffer. The column was washed with 30 ml of equilibration buffer containing 250 mM NaCl to remove the major contaminating protein; Pah1 was then eluted with buffer containing 500 mM NaCl. The His6-tagged Pho85-Pho80 protein kinase complex expressed in *E. coli* was purified by nickel-nitritotrlicetic acid-agarose affinity chromatography as described by Jeffery et al. (61). As described previously (46), this complex was essentially homogeneous. The protein content of enzymes in solution was estimated by the method of Bradford (62) or by ImageQuant analysis of Coomassie Blue-stained SDS-polyacrylamide gels using bovine serum albumin as a standard.

**Preparation of 32P-Labeled Pah1**—Pah1 was phosphorylated with 100 μM [γ-32P]ATP (5,000–10,000 cpm/pmol) by Pho85-Pho80 (46), Cdc28-cyclin B (45), PKA (47), or PKC (49) for 2 h at 30 °C as described previously. The stoichiometry of each reaction was determined to confirm the maximum extent of phosphorylation (45–47, 49). 32P-Labeled Pah1 was purified by Q-Sepharose chromatography (see above) to eliminate the interference of nucleotides (ATP and ADP) and protein kinase in subsequent Nem1-Spo7 phosphatase assays. The salt concentration of purified phosphoenzyme preparations was reduced by dilution in 10 volumes of 20 mM Tris-HCl (pH 7.0), 7 mM 2-mercaptoethanol, and 10% glycerol; the diluted phosphoenzyme was then concentrated with an Amicon ultracentrifugal filter unit. The phosphoenzyme concentration was determined as described above. The yield of purified 32P-labeled Pah1 was typically 40–70%.

**SDS-PAGE and Western Blot Analysis**—SDS-PAGE (63) was routinely performed with either 8 or 10% slab gels. Phos-tag® AAL-107 (50 μM) and MnCl2 (100 μM) were added to SDS-polyacrylamide gels (5%) for analysis of the Pah1 dephosphorylation. Western blotting (64, 65) with PVDF membrane was performed by standard protocols. Rabbit anti-Pah1 antibodies (45) and alkaline phosphatase-conjugated goat anti-rabbit IgG antibodies were used at a concentration of 1 μg/ml and at a dilution of 1:5,000, respectively. Immune complexes were detected using the enhanced chemifluorescence Western blot detection kit. Fluorography was used to acquire images from Western blots, and the signal intensity of the image was analyzed using ImageQuant software. Signals were in the linear range of detectability.

**Enzyme Assays**—All enzyme assays were conducted in triplicate at 30 °C. Nem1-Spo7 phosphatase activity was routinely measured by following the release of 32P, from [32P]Pah1. The reaction mixture contained 100 mM sodium acetate (pH 5.0), 10 mM MgCl2, 0.25 mM Triton X-100, 1 mM DTT, 0.25 μM phosphorylated Pah1, and the indicated amounts of Nem1-Spo7 in a total volume of 50 μl. Following a 10-min incubation, the reaction was terminated by the addition of 0.5 ml of trichloroacetic acid (20% final concentration) and 0.2 ml of bovine serum albumin (0.4 mg/ml final concentration); samples were cooled on ice for 15 min to allow precipitation of proteins. The mixture was centrifuged for 20 min at 15,000 × g to separate the reaction product 32P from the substrate, and a 0.2-ml aliquot of the supernatant was measured for radioactivity by scintillation counting. The radioactivity detected from the reaction product was at least 2,000 cpm above that (~50 cpm) from the reaction lacking the Nem1-Spo7 phosphatase. The amount of phosphate produced in the reaction was calculated on the basis of the specific activity of the [γ-32P]ATP used to prepare [32P]Pah1. A unit of Nem1-Spo7 phosphatase activity was defined as the amount of enzyme that catalyzed the formation of 1 nmol of phosphate/min. PAP activity was measured by following the formation of water-soluble 32P, from chloroform-soluble [32P]PA (10,000–15,000 cpm/nmol) as described by Carman and Lin (66). The reaction mixture contained 50 mM Tris-HCl (pH 7.5), 1 mM MgCl2, 0.2 mM PA, 2 mM Triton X-100, and...
Nem1-Spo7 Dephosphorylation of Pah1 Phosphatidate Phosphatase

FIGURE 1. Model for the dephosphorylation of Pah1 by Nem1-Spo7 and sites of phosphorylation by Pho85-Pho80, Cdc28-cyclin B, PKA, and PKC. A, Pah1 in the cytosol is phosphorylated (P) on multiple Ser/Thr residues by protein kinases. The phosphorylated enzyme is recruited to the nuclear/ER membrane for its dephosphorylation by Nem1-Spo7 phosphatase complex. The dephosphorylation membrane-associated Pah1 catalyzes the dephosphorylation of PA to produce DAG that is acylated to TAG, B, domain structure of Pah1 showing the positions of the amphipathic helix (AH) required for interaction with the membrane (52), the NLIP domain containing the conserved glycine (G), the haloacid dehalogenase (HAD)-like domain containing the DxD(T,V) catalytic motif, the acidic tail (AT) required for interaction with Nem1-Spo7 (53), and the serine (S) and threonine (T) residues that are phosphorylated by Pho85-Pho80 (46), Cdc28-cyclin B (45), PKA (47), and PKC (49).

and enzyme protein in a total volume of 100 μl. A unit of PAP activity was defined as μmol/min. The [32P]-labeled PA used for the assay was enzymatically synthesized from DAG and [γ-32P]ATP using E. coli DAG kinase (66). All enzyme reactions were linear with time and protein concentration.

Analyses of Data—Statistical analyses were performed with SigmaPlot software. The p values of <0.05 were taken as a significant difference. The Enzyme Kinetics module of the SigmaPlot software was used to analyze kinetic data.

RESULTS

Dephosphorylation of Pah1 by the Nem1-Spo7 Phosphatase and Its Stimulatory Effect on PAP Activity—Purified preparations of the wild type and 7A mutant forms of Pah1, which are endogenously phosphorylated in S. cerevisiae (44), were used as substrates for the Nem1-Spo7 phosphatase. SDS-PAGE analysis indicated that the phosphatase complex preparation used in our experiments was highly purified (Fig. 2A). Quantification of the purified complex by SDS-PAGE analysis indicated a stoichiometry of Nem1 to Spo7 of 1:1. Phos-tag SDS-PAGE (67) was used to separate the phosphorylated and unphosphorylated/ dephosphorylated forms of Pah1. The analysis of the wild type Pah1 and its 7A mutant indicated that they were heterogeneously phosphorylated (Fig. 2B). For the wild type enzyme, this heterogeneity was shown as a very diffuse signal in the Phos-tag gel. Because Pah1–7A contains alanine mutations at the seven sites phosphorylated by the Pho85-Pho80 protein kinase (Fig. 1B), its phosphorylated forms migrated faster than the phosphorylated forms of the wild type enzyme. Upon incubation with the purified Nem1-Spo7 phosphatase, both the wild type and 7A mutant enzymes migrated at the same position as discrete protein bands (Fig. 2B). As described previously (44), the PAP activity of the endogenously phosphorylated 7A mutant enzyme was 3.9-fold higher than that of the endogenously phosphorylated wild type enzyme (Fig. 2C). This result confirms that Pah1 PAP activity is regulated by phosphorylation at the seven sites targeted by the Pho85-Pho80 protein kinase (46). The dephosphorylations of the wild type and 7A mutant enzymes resulted in the stimulations of PAP activity by 6- and 1.7-fold, respectively (Fig. 2C). This result confirmed for the first time that the Nem1-Spo7 phosphatase has a direct stimulatory effect on Pah1 PAP activity. That the PAP activity of the 7A mutant was also stimulated by Nem1-Spo7 phosphatase indicated the inhibitory effect of phosphorylation by other protein kinase(s) (e.g. PKA (47)). The PAP activity of the 7A mutant treated with Nem1-Spo7 phosphatase was only 20% higher than the activity of the wild type enzyme treated with the phosphatase (Fig. 2C), indicating that the extent of dephosphorylation for both forms of Pah1 was about the same.

Nem1-Spo7 Phosphatase Dephosphorylates Pah1 Phosphorylated by the Pho85-Pho80 Kinase—To utilize a more defined substrate for the Nem1-Spo7 phosphatase, highly purified unphosphorylated Pah1 expressed in E. coli was radioactively phosphorylated (Fig. 3). Of the protein kinases known to phos-
Nem1-Spo7 Dephosphorylation of Pah1 Phosphatidate Phosphatase

FIGURE 3. Nem1-Spo7 phosphatase dephosphorylates Pah1 phosphorylated by Pho85-Pho80. A, His$_6$-tagged Pah1 expressed and purified from E. coli was separated by SDS-PAGE and stained with Coomassie Brilliant Blue R-250. The positions of the molecular mass standards are indicated. B, E. coli-expressed recombinant Pah1 was phosphorylated with [γ-32P]ATP by Pho85-Pho80 protein kinase. The phosphorylated enzyme was purified and then incubated with the Nem1-Spo7 phosphatase for the indicated time intervals. The Pah1 samples were subjected to Phos-tag SDS-PAGE, transferred to the PVDF membrane, and analyzed by Western blotting using anti-Pah1 antibodies (left) and phosphorimaging analysis (right). The phosphorylated (p-Pah1) and dephosphorylated (Pah1) forms of the enzyme are indicated. U, unphosphorylated Pah1 isolated from E. coli. The immunoblot and phosphor image are representative of three independent experiments.

Enzymological Properties of Nem1-Spo7 Phosphatase—The enzymological properties of the Nem1-Spo7 phosphatase were characterized with radioactively phosphorylated Pah1. The phosphate generated from the enzyme reaction was measured after the removal of Pah1 (both phosphorylated and dephosphorylated forms) by trichloroacetic acid precipitation. This assay was more accurate and convenient to quantify the product formation when compared with Phos-tag SDS-PAGE analysis. The effect of pH on Nem1-Spo7 phosphatase activity was examined with various buffers ranging from pH 4.5 to 7.5. Nem1-Spo7 showed maximum activity at pH 5.0, which was 7-fold higher than the activity at pH 7.5 (Fig. 4B). The phosphatase activity required the divalent cation Mg$^{2+}$ (Fig. 4B). To a lesser extent (~25%), its requirement was substituted by Mn$^{2+}$ at lower concentrations. In combination with Mg$^{2+}$, Triton X-100 stimulated Nem1-Spo7 phosphatase activity by 2.6-fold (Fig. 4, C and D). Triton X-100 was the only detergent tested for eliciting the optimal activity of the enzyme. The alkylation agent N-ethylmaleimide exhibited a 50% inhibition on the phosphatase activity at the concentration of 20 mM (Fig. 5A); in the absence of N-ethylmaleimide, the addition of 0.5 mM DTT (sulphydryl group protective reagent) stimulated activity by 42% (Fig. 5B). Under optimal assay conditions where less than 5% of the substrate was converted to product, the Nem1-Spo7 phosphatase activity followed zero order kinetics with the reaction being linear with respect to time and enzyme concentration (Fig. 6).

Effects of Temperature on Nem1-Spo7 Phosphatase Activity and Stability—The effects of temperature were examined on Nem1-Spo7 phosphatase activity and stability. Maximum
Following the incubation, the samples were cooled on ice to incubation for 10 min at temperatures ranging from 0 to 60 °C. Spo7 was determined by measuring its enzyme activity after the release of $^{32}$P from $[^{32}P]$Pah1 phosphorylated by Pho85-Pho80. The enzyme activity was measured in the absence and presence of the indicated concentrations of N-ethylmaleimide (A) or DTT (B). The data shown are means ± S.D. (error bars) from triplicate enzyme determinations. Error bars are hidden behind some symbols.

![FIGURE 5. Effects of N-ethylmaleimide and DTT on Nem1-Spo7 phosphatase activity.](image)

Nem1-Spo7 phosphatase activity was measured by following the release of $^{32}$P, from $[^{32}P]$Pah1 phosphorylated by Pho85-Pho80. The enzyme activity was measured at the indicated temperatures for 10 min in a temperature-controlled water bath. The curve drawn was a result of a least squares analysis of the data. The data yielded the activation energy of 8.4 kcal/mol for the enzyme. The enzyme activity was measured for the indicated time intervals (A) or for 10 min with the indicated amounts of Nem1-Spo7 (B). The data shown are means ± S.D. (error bars) from triplicate enzyme determinations. Error bars are hidden behind some symbols.

![FIGURE 6. Dependence of Nem1-Spo7 phosphatase activity on time and protein content.](image)

Nem1-Spo7 phosphatase activity was measured by following the release of $^{32}$P, from $[^{32}P]$Pah1 phosphorylated by Pho85-Pho80. The enzyme activity was measured for the indicated time intervals (A) or for 10 min with the indicated amounts of Nem1-Spo7 (B). The data shown are means ± S.D. (error bars) from triplicate enzyme determinations. Error bars are hidden behind some symbols.

![FIGURE 7. Effects of temperature on Nem1-Spo7 phosphatase activity and stability.](image)

Nem1-Spo7 phosphatase activity was measured by following the release of $^{32}$P, from $[^{32}P]$Pah1 phosphorylated by Pho85-Pho80. A, the enzyme activity was measured at the indicated temperatures for 10 min in a temperature-controlled water bath. B, the data in A from 10 to 40 °C were plotted as log Nem1-Spo7 phosphatase activity versus the reciprocal of the absolute temperature. The curve drawn was a result of a least squares analysis of the data. C, the enzyme samples were first incubated for 10 min at the indicated temperatures. After incubation, the samples were cooled in an ice bath to allow for renaturation and then assayed for phosphatase activity at 30 °C. About 75% of the activity was lost after incubation at 40 °C, and total inactivation occurred at 50 °C and higher temperatures (Fig. 7C). The purified Nem1-Spo7 phosphatase was completely stable for at least 2 years of storage at −80 °C and upon three cycles of freezing and thawing.

**Table 2** Effects of monovalent and divalent cations, phosphatase inhibitors, and nucleotides on Nem1-Spo7 phosphatase activity

<table>
<thead>
<tr>
<th>Compound</th>
<th>Nem1-Spo7 phosphatase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100 ± 4</td>
</tr>
<tr>
<td>+ 100 mM NaCl</td>
<td>79 ± 5</td>
</tr>
<tr>
<td>+ 100 mM KCl</td>
<td>68 ± 3</td>
</tr>
<tr>
<td>+ 1 mM CaCl$_2$</td>
<td>88 ± 4</td>
</tr>
<tr>
<td>+ 1 mM ZnCl$_2$</td>
<td>80 ± 3</td>
</tr>
<tr>
<td>+ 1 mM Na$_2$VO$_4$</td>
<td>3 ± 3</td>
</tr>
<tr>
<td>+ 10 mM NaF</td>
<td>58 ± 2</td>
</tr>
<tr>
<td>+ 10 mM imidazole</td>
<td>86 ± 2</td>
</tr>
<tr>
<td>+ 10 mM glycerophosphate</td>
<td>123 ± 7</td>
</tr>
<tr>
<td>+ 10 mM propanol</td>
<td>98 ± 3</td>
</tr>
<tr>
<td>+ 10 mM phenylglyoxal</td>
<td>63 ± 4</td>
</tr>
<tr>
<td>+ 1 mM ATP</td>
<td>114 ± 10</td>
</tr>
<tr>
<td>+ 1 mM CTP</td>
<td>113 ± 3</td>
</tr>
</tbody>
</table>

**Table 3** Effects of lipids on Nem1-Spo7 phosphatase activity

<table>
<thead>
<tr>
<th>Lipid</th>
<th>Nem1-Spo7 phosphatase activity %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100 ± 4</td>
</tr>
<tr>
<td>Neutral lipids</td>
<td></td>
</tr>
<tr>
<td>+ TAG</td>
<td>113 ± 19</td>
</tr>
<tr>
<td>+ DAG</td>
<td>103 ± 3</td>
</tr>
<tr>
<td>+ monooacylglycerol</td>
<td>69 ± 1</td>
</tr>
<tr>
<td>Phospholipids</td>
<td></td>
</tr>
<tr>
<td>+ phosphatidylcholine</td>
<td>105 ± 5</td>
</tr>
<tr>
<td>+ phosphatidylethanolamine</td>
<td>98 ± 3</td>
</tr>
<tr>
<td>+ phosphatidylnositol</td>
<td>89 ± 6</td>
</tr>
<tr>
<td>+ phosphatidylserine</td>
<td>80 ± 1</td>
</tr>
<tr>
<td>+ phosphatidylglycerol</td>
<td>74 ± 4</td>
</tr>
<tr>
<td>+ cardiolipid</td>
<td>82 ± 6</td>
</tr>
<tr>
<td>+ CDP-DAG</td>
<td>84 ± 6</td>
</tr>
<tr>
<td>Sphingolipids</td>
<td></td>
</tr>
<tr>
<td>+ ceramide</td>
<td>100 ± 3</td>
</tr>
<tr>
<td>+ phytosphingosine</td>
<td>100 ± 5</td>
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<tr>
<td>+ sphinganine</td>
<td>88 ± 4</td>
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<td>115 ± 6</td>
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<tr>
<td>+ sphingosine 1-phosphate</td>
<td>75 ± 6</td>
</tr>
<tr>
<td>+ sphingosine 1-phosphate</td>
<td>89 ± 8</td>
</tr>
</tbody>
</table>

The enzyme activity was observed at 40 °C; the activity was reduced at temperatures above this optimum (Fig. 7A). An Arrhenius plot of the data yielded the activation energy of 8.4 kcal/mol for the phosphatase reaction (Fig. 7B). The thermal stability of Nem1-Spo7 was determined by measuring its enzyme activity after incubation for 10 min at temperatures ranging from 0 to 60 °C. Following the incubation, the samples were cooled on ice to allow for renaturation and then assayed for phosphatase activity at 30 °C. About 75% of the activity was lost after incubation at 40 °C, and total inactivation occurred at 50 °C and higher temperatures (Fig. 7C). The purified Nem1-Spo7 phosphatase was completely stable for at least 2 years of storage at −80 °C and upon three cycles of freezing and thawing.

**Effectors of Nem1-Spo7 Phosphatase Activity**—Cations, phosphatase inhibitors, nucleotides, and lipids were examined for their effects on Nem1-Spo7 phosphatase activity (Tables 2 and 3). The rationale for examining some of these molecules was based on their roles in the regulation of Pah1 PAP activity (4). Monovalent and divalent cations exhibited a relatively small (20–30%) inhibitory effect on Nem1-Spo7 phosphatase activity. Of general phosphatase inhibitors, sodium vanadate (97%) was most potent, followed by sodium fluoride (42%) and phenylglyoxal (37%). Propranolol and the nucleotides ATP and...
when compared with the substrate phosphorylated by Cdc28-cyclin B. This result indicated that one or more of the four sites (i.e. Ser-110, Ser-114, Ser-168, and Ser-748) uniquely phosphorylated by Pho85-Pho80 has a positive effect on Nem1-Spo7 phosphatase activity. PKA and PKC also have three phosphorylation sites in common, namely Ser-677, Ser-773, and Ser-788 (Fig. 1B). Whereas the $K_m$ values of Nem1-Spo7 for the PKA- and PKC-phosphorylated substrates were similar, the $k_{cat}$ value for the PKA-phosphorylated substrate was 5-fold higher than the substrate phosphorylated by PKC. This result indicates that Ser-769, the unique PKC site, has a negative effect on the catalytic efficiency of the Nem1-Spo7 phosphatase, or the unique PKA sites (Ser-10 and Ser-774) have a positive effect on the phosphatase activity. The identification of the specific site(s) involved and how the site(s) regulate activity will require additional studies. The $K_m$ values for the PKA- and PKC-phosphorylated substrates were 2–3-fold lower than that of the substrates phosphorylated with Pho85-Pho80 or Cdc28-cyclin B. Overall, Pho85-Pho80-phosphorylated Pah1 was the preferred substrate for Nem1-Spo7 phosphatase; the specificity constant of this substrate was 1.6-, 4-, and 6-fold greater, respectively, than that of the substrates phosphorylated by PKA, Cdc28-cyclin B, and PKC (Table 4).

**DISCUSSION**

The Pah1 PAP enzyme has emerged as a key lipid homeostatic enzyme in the yeast *S. cerevisiae* (4, 5, 7, 8). Its phosphorylation and dephosphorylation play a major role in controlling its cellular location, enzymatic activity, and stability (9, 44–47, 49, 50, 52, 53). In particular, the dephosphorylation of Pah1 by Nem1-Spo7 phosphatase is crucial for its PAP activity on the nuclear/ER membrane. In this work, using the protein kinase-target site relationship confirmed in previous studies (Fig. 1B), we developed an assay system with the specific kinase-phosphorylated Pah1 as a substrate and characterized Nem1-Spo7 phosphatase with respect to its enzymological, kinetic, and regulatory properties.

The enzymological properties of Nem1-Spo7 phosphatase showed that its maximal activity depends on Mg$^{2+}$ ions and Triton X-100 at the pH optimum of 5.0. The Mg$^{2+}$ ion requirement, like that for PAP activity (1), is characteristic of the DXDX(T/V)-containing enzymes in the haloacid dehalogenase superfamily (54, 55). Triton X-100 is required to solubilize the phosphatase complex consisting of the integral membrane protein subunits in the nuclear/ER membrane (51). Compared with the alkaline pH of 7.5 for Pah1 PAP activity (1), the acidic pH of 5.0 is optimal for Nem1-Spo7 phosphatase activity. The different pH optima for these two enzymes are unexpected but are also shown for the human orthologs, namely the pH optimum of 5.5 for CTDNEP1 and pH optimum of 7.5 for lipin-1 PAP (17, 70). In *S. cerevisiae*, Pah1 PAP activity as monitored by TAG synthesis increases as cells progress into the stationary phase (39). During the growth transition, the intracellular pH is decreased from ~7.0 to ~5.5 (71). Thus, it is speculated that the acidic environment results in the activation of Nem1-Spo7 phosphatase activity and thereby an increase in Pah1 PAP activity. The inhibitory effect of N-ethylmaleimide suggests that the
sulhydryl group is required for the catalysis of Nem1-Spo7 phosphatase.

We initiated studies to examine regulatory effects of cellular components on Nem1-Spo7 phosphatase activity. Effector molecules (e.g. phospholipids, sphingoid bases, and nucleotides), which are known to modulate Pah1 PAP activity (41–43), did not have any effect on Nem1-Spo7 phosphatase activity. Thus, the molecules in the biochemical regulation of lipid synthesis are confined to the Pah1 component of the Nem1-Spo7/Pah1 phosphatase cascade. Like Pah1 PAP, Nem1-Spo7 phosphatase is subject to phosphorylation (72). Recently, Nem1 has been shown to be phosphorylated at Ser-195 and thereby activated for increased synthesis of TAG when yeast cells are treated with rapamycin, an inhibitor of the TORC1 kinase. Although a regulatory phosphorylation site is found in Nem1, the protein kinase responsible for its phosphorylation needs to be identified. The stimulatory effect of phosphorylation on Nem1 phosphatase contrasts with the inhibitory effect of phosphorylation on Pah1 PAP activity.

The highest specificity (\(k_{cat}/K_m\)) of Nem1-Spo7 phosphatase was shown for Pah1 phosphorylated by Pho85-Pho80, followed by PKA, Cdc28-cyclin B, and PKC. The difference in the Pah1 specificity correlates with the effects that each of the protein kinases has on the regulation of Pah1 function. In particular, the analyses of cells expressing Pah1 mutants deficient in the phosphorylation of the seven sites targeted by Pho85-Pho80 (e.g. 7A mutant) revealed the importance of the dephosphorylation of these sites in regulating Pah1 location, PAP activity, and stability (44–46, 48, 52). Alanine mutations of the three Cdc28-cyclin B sites exhibit only partial effects on Pah1 function, indicating that the dephosphorylation of all seven sites is needed for full regulation (45, 46). That the PKA-phosphorylated substrate yielded the second highest catalytic efficiency of Nem1-Spo7 phosphatase was consistent with studies showing that the phosphorylation/dephosphorylation of Ser-10 functions in conjunction with the phosphorylation/dephosphorylation of the seven Pho85-Pho80 sites (47). In contrast, alanine mutations of the sites targeted by PKC have no effect on the localization of Pah1 or its PAP activity but instead affect its stability in a manner opposite to that of the 7A mutations (49). The phosphorylation by PKC reduces Pah1 abundance, whereas the phosphorylation by Pho85-Pho80 stabilizes its abundance (45–47, 49). Thus, the low catalytic efficiency of Nem1-Spo7 phosphatase for the PKC-phosphorylated substrate is consistent with a regulatory mechanism that promotes Pah1 degradation (49). Interestingly, the PKC-phosphorylated substrate had the lowest \(K_m\) value for Nem1-Spo7 phosphatase activity, indicating that Nem1-Spo7 phosphatase activity.

When kinetic parameters are compared between Nem1-Spo7 and its human ortholog CTDNEP1, the \(K_m\) values for the kinase-specific Pah1 substrates were about 3 orders of magnitude lower than the \(K_m\) value of the truncated version of CTDNEP1 using a synthetic phosphopeptide substrate mimicking the insulin-dependent phosphorylation site (e.g. Ser-106) of lipin-1 (73). In addition, the \(k_{cat}/K_m\) values for Nem1-Spo7 phosphatase with phospho-Pah1 substrates were about 2 orders of magnitude higher than those of truncated CTDNEP1 using the phosphopeptide substrate (73). These differences may be a reflection of the substrates used (e.g. authentic phospho-Pah1 versus synthetic peptides) and/or the enzyme states (e.g. full-length Nem1-Spo7 versus truncated CTDNEP1 without NEP1-R1). Alternatively, these differences in the kinetic properties may simply indicate a difference between the orthologous enzymes, and the yeast Nem1-Spo7 phosphatase has a greater specificity for its substrate phospho-Pah1 when compared with its human counterpart phosphatase for its substrate phospholipin-1.

As discussed above, the loss of Pah1 or either subunit of the Nem1-Spo7 complex results in several deleterious phenotypes, indicating the importance of the Nem1-Spo7/Pah1 phosphatase cascade in lipid metabolism and cell physiology (1, 6, 9–11, 14, 15, 39, 45–47, 49, 51, 56). Nevertheless, the overexpression of the Nem1-Spo7 phosphatase or the hyperactive 7A mutant causes a lethal phenotype (9, 44), indicating that an excess level of PAP activity is also detrimental to cell physiology. We postulate that Nem1-Spo7 phosphatase plays a major role in limiting PAP activity by serving as a rate-limiting step in the phosphatase cascade. For example, the abundance of Nem1 (377 molecules/cell) is 10-fold less than that of Pah1 (3,910 molecules/cell) (74), and the turnover number (\(k_{cat}\)) of Nem1-Spo7 phosphatase (0.29 s\(^{-1}\)) is 80-fold lower than that of Pah1 PAP activity (23 s\(^{-1}\)). In addition, the differences in pH optima of Nem1-Spo7 phosphatase and Pah1 PAP in connection with changes in cellular pH during growth indicate an additional level of regulation mediated by the Nem1-Spo7 phosphatase.

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