Protein kinase C mediates the phosphorylation of the Nem1-Spo7 protein phosphatase complex in yeast

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Running title: PKC phosphorylation of the Nem1-Spo7 phosphatase complex

The Nem1-Spo7 complex in the yeast Saccharomyces cerevisiae is a protein phosphatase required for the nuclear/endoplasmic reticulum membrane localization of Pah1, a phosphatidate phosphatase that produces diacylglycerol for triacylglycerol synthesis at the expense of phospholipid synthesis. In a previous study (Su, W.-M., Han, G.-S., Dey, P., and Carman, G. M. (2018) J. Biol. Chem. 293, 15801-15814), we show the protein phosphatase is subject to phosphorylation by protein kinase A (PKA). Here, we demonstrate that Nem1-Spo7 is regulated through its phosphorylation by protein kinase C (PKC), which plays multiple roles including the regulation of lipid synthesis and cell wall integrity. Phosphorylation analyses of Nem1-Spo7 and its synthetic peptides indicate that both subunits of the complex are bona fide PKC substrates. Site-directed mutagenesis of NEM1 and SPO7, coupled with phosphopeptide mapping and immunoblotting with a phosphoserine-specific PKC substrate antibody, revealed that Ser-201 in Nem1 and Ser-22/Ser-28 in Spo7 are major PKC target sites of phosphorylation. Activity analysis of mutant Nem1-Spo7 complexes indicates that the PKC phosphorylation of Nem1 exerts a stimulatory effect, but the phosphorylation of Spo7 has no effect. Lipid-labeling analysis of cells expressing the phosphorylation-deficient alleles of NEM1 and SPO7 indicate that the stimulation of the Nem1-Spo7 activity has the effect of increasing triacylglycerol synthesis. Prephosphorylation of Nem1-Spo7 by PKC inhibits the PKA phosphorylation of Nem1, whereas prephosphorylation of the phosphatase complex by PKA inhibits the PKC phosphorylation of Spo7. Collectively, this work advances the understanding of the Nem1-Spo7 regulation by phosphorylation and its impact on lipid synthesis.

The PAH1-encoded PA^2 phosphatase (Pah1) (1), which catalyzes the dephosphorylation of PA to yield DAG (2), has emerged as a key regulatory enzyme in yeast (3) that controls the bifurcation of PA between DAG and CDP-DAG (3-7) (Fig. 1A). Elevated PA phosphatase activity is associated with the production of DAG that is used for the synthesis of the storage lipid TAG, whereas reduced enzyme activity is associated with the production of CDP-DAG that is used for the synthesis of membrane phospholipids (3-7). In yeast, the PA-derived synthesis of membrane phospholipids predominates over the synthesis of TAG during logarithmic growth, but as cells enter the stationary phase of growth, the PA is primarily partitioned into TAG (3, 5-7). The Pah1PA phosphatase is the primary regulator of this metabolic switch (3, 5-7).

Loss of PA phosphatase activity, as exemplified by the pah1Δ mutation, has devastating effects on lipid metabolism and cell physiology (7). Lack of DAG production reduces TAG synthesis and lipid droplet formation (8). Consequently, fatty acids accumulate (1), rendering the mutant cells susceptible to fatty acid-induced lipotoxicity (1,
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PA accumulates at the nuclear/ER membrane (10), which drives the synthesis of membrane phospholipids via CDP-DAG by mass action and by the PA/Opi1-mediated derepression of phospholipid synthesis genes (1, 9, 11-13). The notable pah1Δ phenotype is an aberrant nuclear morphology with the expansion of the nuclear/ER membrane (11). Moreover, the mutant cells have fragmented vacuoles (14) and a weakened cell wall (15, 16). They are also hypersensitive to hydrogen peroxide (17), do not grow on glycerol (non-fermentable carbon source) (1, 18), are sensitive to heat (1, 11, 19, 20) and cold (21), exhibit a defect in autophagy induction after TORC1 inactivation (22), have a shortened chronological life span (17), and exhibit apoptotic cell death in the stationary phase (9).

As one might expect, the function of such an important regulatory enzyme is controlled by genetic and biochemical mechanisms, with perhaps, phosphorylation and dephosphorylation being the most important (3, 5-7). These posttranslational modifications control the location and stability of Pah1, as well as its PA phosphatase activity (23-33). In general, the phosphorylation on multiple sites, as mediated by several protein kinases (e.g., CKI, CKII, Cdc28, Pho85, PKA, and PKC), attenuates enzyme function by sequestering Pah1 in the cytosol apart from its membrane-associated substrate PA and by inhibiting the PA phosphatase activity. The dephosphorylation of Pah1, as mediated by the Nem1 (catalytic)-Spo7 (regulatory) protein phosphatase complex, has the opposite effects (23-30, 33). Paradoxically, the phosphorylation stabilizes Pah1 abundance, whereas the dephosphorylation promotes degradation via the 20S proteasome (32, 33). An exception to this situation is that phosphorylation by PKC, when not already phosphorylated by Pho85-Pho80, stimulates the 20S proteasome-mediated degradation of Pah1 (27).

The Nem1-Spo7 phosphatase complex (34) is a master regulator of Pah1 function; it is responsible for recruiting and dephosphorylating Pah1 at the ER membrane, and for stimulating PA phosphatase activity (23, 30, 31) (Fig. 1).

Given the function of Nem1-Spo7 to activate Pah1, it is not surprising that the nem1Δ and/or spo7Δ mutant exhibits the same phenotypes as that of the pah1Δ mutant (11, 12, 22, 34, 35). Whereas the phosphatase complex functions to dephosphorylate Pah1, both Nem1 and Spo7 are also subject to phosphorylation (36-40) (Fig. 1B). In the current work, we characterized the phosphorylation of Nem1-Spo7 complex by PKC, a PS/DAG-dependent protein kinase in yeast (39) that is required for cell cycle progression (41) and plays a role in regulating lipid synthesis (42-47) and in maintaining cell wall integrity (41, 48, 49). Ser-201 in Nem1 and Ser-22/Ser-28 in Spo7 were identified as the principal sites of phosphorylation. Lipid composition analysis of cells bearing phosphorylation site mutations indicated that PKC has a positive impact on fine-tuning TAG synthesis through the phosphorylation and stimulation of Nem1-Spo7 phosphatase activity.

Results

PKC phosphorylates Nem1 and Spo7

In the previous work, we demonstrated that S. cerevisiae PKC phosphorylated the E. coli-expressed Nem1-ATM and Spo7-ATM, and that these phosphorylations require PS and DAG (39). In the current work, we further characterized the PKC-mediated phosphorylations of Nem1 and Spo7 using full length proteins expressed and purified as a complex from S. cerevisiae. As described previously (40), we expressed Nem1 as a fusion protein tagged with protein A to facilitate its purification by affinity chromatography with IgG-Sepharose (34). The purified Nem1-Spo7 complex was nearly homogeneous and enzymatically active for the dephosphorylation Pah1 phosphorylated by Pho85-Pho80 (29). The Nem1-Spo7 complex was incubated with PKC and [γ-32P]ATP, followed by the separation of the phosphorylated products from ATP by SDS-PAGE. The phosphorylated proteins were transferred to a PVDF membrane, which was then subjected to phosphorimaging analysis. The phosphorimage shown in Fig. 2A (left) demonstrates that PKC phosphorylated both.
Nem1 and Spo7. The immunoblot analysis with the anti-Nem1 and anti-Spo7 antibodies confirmed that Nem1 and Spo7 are phosphorylated by PKC (Fig. 2A, right). The phosphoamino acid analysis of the $^{32}$P-labeled proteins showed that Nem1 and Spo7 are phosphorylated on both serine and threonine residues with serine being the major target of phosphorylation (Fig. 2B). The phosphopeptide mapping analysis showed one major phosphopeptide from $^{32}$P-labeled Nem1 and three major phosphopeptides from $^{32}$P-labeled Spo7 (Fig. 2C). The radioactive label in the Nem1 phosphopeptide was attributed to the phosphorylation of Ser-201, whereas the label in the Spo7 phosphopeptides was attributed to the phosphorylations of Ser-22 and Ser-28 (see below).

The PKC-mediated phosphorylation of Nem1 and Spo7 was examined with respect to the time of the reaction, the amount of PKC used in the reaction, and the ATP concentration (Fig. 3). The stoichiometry for each reaction was consistent with the conclusion that Nem1 and Spo7 have at least one PKC phosphorylation site. That the stoichiometry of the reactions was less than a theoretical value of 1 (for Nem1) and 2 (for Spo7) indicated that some Nem1 and Spo7 molecules are endogenously phosphorylated by PKC. These data also indicated that PKC phosphorylates Spo7 to a greater extent when compared with Nem1. That the phosphorylation reactions were dependent on time (Fig. 3A) and the amount of protein kinase (Fig. 3B) indicated that PKC activity followed zero order kinetics using Nem1 and Spo7 as substrates. Additionally, the PKC activity towards Nem1 ($K_m = 10.5 \, \mu M$) and Spo7 ($K_m = 12.1 \, \mu M$) followed saturation kinetics with respect to the ATP concentration; the apparent Michaelis constants for ATP of both substrates were similar (Fig. 3C). We did not conduct a kinetic analysis of PKC activity with respect to Nem1 and Spo7 because of a limitation in the amount of pure Nem1-Spo7 complex.

**PKC phosphorylates Ser-201 in Nem1 and Ser-22/Ser-28 in Spo7 in vitro and in vivo**

We used a multifaceted approach to identify major PKC phosphorylation sites in the Pah1 phosphatase subunits. The NetPhosK (50) and NetPhosYeast (51) algorithms predict PKC phosphorylation sites in the cytosolic-facing regions of Nem1 or Spo7. Peptides containing the putative phosphorylation sites were synthesized and tested as substrates for the protein kinase (Fig. 4). The Nem1 peptide corresponding to residues 191-205 was majorly phosphorylated. Within this sequence are the putative PKC target sites of Ser-195, Ser-201, and Ser-204. The mutation of Ser-195, a site previously shown to be phosphorylated in response to rapamycin treatment (38), to a non-phosphorylatable alanine residue did not affect the phosphorylation by PKC. Thus, by this assay, Ser-195 is not a PKC target site. To test the hypothesis that Ser-201 and Ser-204 are the PKC sites of phosphorylation, the peptide corresponding to residues 197-207 was synthesized and used for the assay of PKC activity. This peptide was a good substrate of PKC, but when alanine was substituted for serine at position 201, the activity was reduced by 96% (Fig. 4A). This result supported the notion that Ser-201 is a major site of phosphorylation by PKC. If Ser-204 is a site of phosphorylation, it is a minor site. We also examined the PKC activity using the synthetic peptide of Nem1 residues 205-215. Among the serine residues in this peptide is Ser-210, previously identified as a major target site for PKA phosphorylation (40). The peptide was phosphorylated by PKC, and the alanine mutation of serine at the position of 210 reduced the activity by 85% (Fig. 4A). Yet, the PKC activity on the wild type peptide of residues 205-215 was only 7% of the activity on the peptide of residues 197-207. Thus, we concluded that Ser-210, as well as the other serine residues (e.g., Ser-208, Ser-212, and Ser-215), are not major sites of phosphorylation by PKC.

Peptides were synthesized that cover the soluble regions of Spo7 and we examined them for their phosphorylation by PKC (Fig. 4B). The Spo7 peptide of residues 21-50 was a PKC substrate. Shorter Spo7 peptides that contain Ser-22 (residues 17-27) and Ser-28 (residues 26-36) also served as substrates, but the kinase
activity on these peptides was 10- to 20-fold lower when compared with the longer peptide (residues 21-50). Nonetheless, the alanine mutations of the short peptides at residue 22 and 28 reduced the PKC activity by 85 and 64%, respectively. The Spo7 peptides of residues 125-150 and of residues 152-175 were relatively poor PKC substrates when compared with the peptide of residues 21-50. Accordingly, the identification of the residues being phosphorylated in these peptides was not pursued.

We performed a kinetic analysis of PKC activity on the Nem1 peptide (residues 197-207) and the Spo7 peptide (residues 21-50). In these experiments, the PKC activity on each peptide was measured with various peptide concentrations at the saturating concentration of ATP. The specificity constant, \( V_{\text{max}}/K_m \) (52), for the Spo7 peptide is 1.7-fold higher than that of the Nem1 peptide (Fig. 5), and thus, the Spo7 peptide is the better substrate.

To further confirm that Ser-201 in Nem1 and Ser-22/Ser-28 in Spo7 are target sites of PKC phosphorylation, the alanine mutations of these serine residues were constructed in the full length genes. The phosphorylation-deficient mutant forms of Nem1 (tagged with protein A for purification of the complex) and Spo7 were co-expressed and purified as a complex from S. cerevisiae cells. In the PKC phosphorylation of Nem1 \emph{in vitro}, the S201A mutation caused an 88% reduction in the extent of phosphorylation (Fig. 6A, \emph{left}). The phosphorylated S201A mutant protein was subjected to two-dimensional phosphopeptide mapping analysis. The major phosphopeptide labeled Ser-201 in the map of the wild type Nem1 (Fig. 2C, \emph{left}) was eliminated in the map of the S201A mutant protein, confirming that Ser-201 is the major site of phosphorylation by PKC \emph{in vitro}. To confirm that the S201A mutation affects the phosphorylation of Nem1 \emph{in vivo}, the wild type and mutant proteins expressed and purified from yeast were examined by immunoblotting with an antibody directed against the phosphoserine PKC substrate. The immunodetection of the wild type Nem1 with the antibody showed that the protein is endogenously phosphorylated by PKC (Fig. 6B, \emph{left}). The S201A mutation caused a 96% reduction in the amount of Nem1 detected by the antibody.

In the PKC phosphorylation of Spo7 \emph{in vitro}, the S22A, S28A, and S22A/S28A mutations had the effects of 30, 40, and 84% reductions in the extent of phosphorylation when compared with the wild type control (Fig. 6A, \emph{right}). Each of the phosphorylated mutant proteins was subjected to the phosphopeptide mapping analysis. The two phosphopeptides labeled as Ser-22 and the one phosphopeptide labeled as Ser-28 in the phosphopeptide map of the wild type Spo7 protein (Fig. 2C, \emph{right}) were not detected from the phosphopeptide maps of the S22A and S28A mutants, respectively. The anti-phosphoserine PKC substrate antibody was also used to assess the effects of the phosphorylation-deficient alanine mutations on the endogenous phosphorylation of Spo7 by PKC (Fig. 6B, \emph{right}). The antibody recognized wild type Spo7, confirming that the protein is phosphorylated by PKC \emph{in vivo}. Individually, the S22A and S28A mutations did not have a significant effect on the endogenous phosphorylation of Spo7 by PKC. Yet, the S22A/S28A double mutation caused an 80% reduction in the phosphorylation of Spo7 by PKC \emph{in vivo} (Fig. 6B, \emph{right}).

**The S201A mutation in Nem1 reduces Nem1-Spo7 phosphatase activity**

The effect of Nem1-Spo7 phosphorylation by PKC on the protein phosphatase activity was examined as a function of time. The phosphorylation of the Nem1-Spo7 complex had a small stimulatory effect on the activity (Fig. 7A). We considered that the effect of the phosphorylation on the phosphatase activity was dampened by the phosphorylation state of the complex. As indicated above, the isolated complex is subject to endogenous phosphorylation. As described previously (40), treatment of the purified complex with alkaline phosphatase reduced its phosphatase activity by 58%. Subsequent phosphorylation of the complex by PKC did not have a significant effect on Nem1-Spo7 phosphatase activity. The purified complex, with the S201A mutation in
Nem1 or the S22A/S28A mutations in Spo7, was examined for the Nem1-Spo7 phosphatase activity. The activity of the complex bearing the mutation in Nem1 was reduced by 53% when compared with the control complex (Fig. 7B). The mutations in Spo7 did not affect the Nem1-Spo7 phosphatase activity.

Effects of the PKC phosphorylation-deficient mutations of Nem1 and Spo7 on growth at elevated temperature and on lipid composition

Cells lacking Nem1 (e.g., nem1Δ mutant) or Spo7 (e.g., spo7Δ mutant) exhibit a temperature-sensitive phenotype (Fig. 8A) (34), which exemplifies the importance of the Nem1-Spo7 complex in regulating the phosphorylation state of Pah1 (4, 7). The inability of the nem1Δ and spo7Δ mutant to grow at 37 °C was complemented by the introduction of the NEM1 and SPO7 gene, respectively, on a single-copy plasmid into the respective mutant (Fig. 8A). The genes containing the PKC phosphorylation site mutant alleles of NEM1(S201A) and SPO7(S22A, S28A, and S22A/S28A) were introduced into the nem1Δ and spo7Δ mutant cells, respectively. The expression of the phosphorylation-deficient form of Nem1 or Spo7 afforded growth at the restrictive temperature (Fig. 8A). Thus, the PKC phosphorylation site mutations of Nem1 and Spo7 do not compromise the function of the Nem1-Spo7 complex at 37 °C.

We considered that the temperature-sensitivity assay was not sufficiently sensitive to reveal the partial loss-of-function effects of the phosphorylation-deficient mutations in Nem1 or Spo7 on the in vivo function of the phosphatase complex. Because Nem1-Spo7 is a major regulator of Pah1 function in the synthesis of TAG and phospholipids (4, 53), the effects of the PKC phosphorylation site mutations in Nem1 and Spo7 on the relative amounts of these lipids were examined (Fig. 8B and C). When compared with nem1Δ cells expressing the wild type NEM1 gene, those bearing empty plasmid exhibited decreases (70 and 80%, respectively) in the amounts of TAG in the exponential and stationary phases of growth. Also, the nem1Δ cells with empty vector exhibited 28 and 29% increases in the relative amounts of phospholipids in the exponential and stationary phases, respectively, when compared with nem1Δ cells expressing wild type NEM1. In the exponential phase, the S201A mutation in Nem1 caused a 28% decrease in TAG and a 7% increase in phospholipids when compared with the lipids of cells expressing the wild type NEM1 gene (Fig. 8B, left). The S201A mutation did not have a significant effect on the relative amounts of these lipids in the stationary phase (Fig. 8C, left).

Similar to that observed from nem1Δ mutant cells, the spo7Δ mutation caused 50 and 88% decreases in the relative amounts of TAG in the exponential and stationary phases, respectively, when compared with those expressing the wild type SPO7 gene (Fig. 8B and C, right). Additionally, the spo7Δ cells exhibited 23 and 29% increases in the levels of phospholipids in the exponential and stationary phases, respectively. In the exponential phase, the relative amounts of TAG in spo7Δ cells bearing the S28A and S22A/S28A alleles were 17 and 27% lower, respectively, when compared with those expressing the wild type SPO7. The relative amounts of phospholipids in the S28A and S22A/S28A cells were reduced by 20 and 12%, respectively. TAG and phospholipids were not affected by the S22A mutation in the exponential phase cells. Yet, in the stationary phase, the S22A mutation caused a 21% decrease in phospholipids, and the S22A/S28A double mutation caused a 18% increase in TAG (Fig. 8C, right).

Prephosphorylation of the Nem1-Spo7 complex by PKC inhibits the PKA phosphorylation of Nem1 and prephosphorylation of the phosphatase complex by PKA inhibits the PKC phosphorylation of Spo7

Owing that Pah1 phosphatase is also phosphorylated by PKA (40), we questioned what effect the phosphorylation by PKC would have on the phosphorylation by PKA and vice versa. To address this question, the Nem1-Spo7 complex was prephosphorylated by PKC with
unlabeled ATP and subsequently phosphorylated by PKA with \([\gamma^{32}\text{P}]\text{ATP}\) (Fig. 9A). PKC mediated a dose-dependent reduction (85%) of Nem1 phosphorylation by PKA. The PKA-mediated phosphorylation of Spo7 was not majorly affected by the prephosphorylation with PKC. In the next set of experiments, the Nem1-Spo7 complex was prephosphorylated by PKA with unlabeled ATP and then phosphorylated by PKC with \(32\text{P}\)-labeled ATP (Fig. 9B). PKA caused a dose-dependent reduction (86%) of the subsequent phosphorylation of Spo7 by PKC, but the prephosphorylation of the complex by PKA had little effect on the subsequent phosphorylation of Nem1 by PKC.

**Discussion**

Siniossoglou et al. (34) originally identified Nem1 and Spo7 as proteins that form a phosphatase complex in the nuclear/ER membrane and participate in nuclear envelope morphogenesis. Subsequently, Santos-Rosa et al. (11) have shown that the complex is a protein phosphatase, which dephosphorylates Pah1 for its role in nuclear membrane growth as well as in the transcriptional control of phospholipid synthesis gene expression. The Pah1 function is a key regulation point of PA utilization and lipid metabolism, which in turn has an impact on many aspects of cell physiology (7). While much is known about the mode of action and the phosphorylation/dephosphorylation-mediated regulation of Pah1 (3-7), our understanding of the Nem1-Spo7 phosphatase regulation is limited. We know that the protein phosphatase has the pH optimum of 5.0 (29, 54). This is the approximate intracellular pH of yeast cells in the stationary phase (29, 54) when PA phosphatase activity and TAG levels are maximal and the bifurcation of PA toward lipid storage is favored over the synthesis of membrane phospholipids (12, 54, 55). Pah1 is phosphorylated on at least 40 sites (23, 37, 56-60, 60-65), but not all of the protein kinases that phosphorylate the sites have been identified (7). Of the known protein kinase-target site relationships (24-28), the specificity of the Nem1-Spo7 phosphatase-mediated dephosphorylations is in the order of the sites phosphorylated by Pho85 > PKA = casein kinase II > Cdc28 > PKC (28, 29).

While Nem1-Spo7 catalyzes the dephosphorylation of Pah1, the enzyme itself is subject to phosphorylation. Two of the protein kinases that are known to phosphorylate Pah1, namely PKA (26) and PKC (27), also phosphorylate Nem1 and Spo7 (39, 40). In a well-defined in vitro system, we demonstrated that Nem1 and Spo7 are phosphorylated by PKC. Both subunits were majorly phosphorylated on the serine residue with the threonine phosphorylation occurring to a minor extent. The enzymological studies performed with the purified phosphatase complex confirmed that Nem1 and Spo7 are bona fide PKC substrates. Moreover, we showed that the PKC-mediated phosphorylation of the subunits occurs in vivo; endogenously phosphorylated Nem1 and Spo7 were recognized by the anti-phosphoserine PKC substrate antibody. Our biochemical and mutagenic studies with synthetic Nem1 and Spo7 peptides as well as the full-length Nem1 and Spo7 proteins led to the identification of Ser-201 in Nem1 and Ser-22/Ser-28 in Spo7 as the major sites of phosphorylation by PKC in vitro. In vivo, the phosphorylation-deficient alanine mutations of Ser-201 in Nem1 and Ser-22 and Ser-28 in Spo7 reduced the PKC-mediated phosphorylation of the subunits by 96 or 80%, respectively.

The phosphorylation of the wild type complex had a small stimulatory effect on the Nem1-Spo7 phosphatase activity. We considered that this weak effect was influenced by the endogenous phosphorylations of the subunits. However, the phosphorylation of the alkaline phosphatase-treated complex by PKC did not have a significant effect on the phosphatase activity. Perhaps the phosphorylation of another site, as mediated by a different kinase, is required for the stimulation by PKC. Yet, the S201A mutation in Nem1 caused a 2-fold decrease in the Nem1-Spo7 phosphatase activity. This result supported the notion that the PKC-mediated phosphorylation of Nem1 stimulates the dephosphorylation of Pah1 by the complex. The phosphorylation of Spo7 by PKC does not appear to affect the Nem1-Spo7 phosphatase activity per se.
PKC phosphorylation of the Nem1-Spo7 phosphatase complex

The temperature-sensitive phenotype imparted by the nem1Δ or spo7Δ mutation (34), like that caused by the pah1Δ mutation (1, 11, 19), is attributed to the defect in the synthesis of TAG (4, 7). The lipid analysis of the cells expressing the phosphorylation-deficient mutant forms of Nem1 and Spo7 indicated that PKC does not majorly affect TAG synthesis through the phosphorylations of Nem1 or Spo7. Thus, it is not too surprising that the phosphorylation-deficient mutations of Nem1 and Spo7 did not elicit the temperature-sensitive phenotype characteristic of cells with the nem1Δ and spo7Δ mutations. That the cells expressing these phosphorylation-deficient mutant forms of Nem1 or Spo7 grew at the restrictive temperature and had near normal levels of TAG, indicated that the mutations did not affect their expression and the formation of the Nem1-Spo7 complex. This assertion is substantiated by the fact that the phosphorylation-deficient Nem1 and Spo7 were expressed and purified as a complex.

Although the lipid compositions of the cells expressing the phosphorylation-deficient Nem1 or Spo7 were not majorly different from that of the control cells, there were statistically significant effects imparted by the mutations. The most noticeable differences were in the exponential phase cells where the S201A mutation in Nem1 or the S22A/S28A mutations in Spo7 caused a near 30% decrease in TAG content. This is consistent with a reduction in the Pah1 phosphatase activity due to the S201A mutation in Nem1. Taken together, the data implies that PKC has a positive impact on TAG synthesis through the phosphorylation of the Pah1 phosphatase.

PKA also phosphorylates the Nem1-Spo7 complex (40), but the consequence differs from that imposed by the PKC-mediated phosphorylation. PKA, which phosphorylates Nem1 at Ser-140 and Ser-210 and Spo7 at Ser-28 (Fig. 1B), has a negative impact on the in vitro activity of Nem1-Spo7 and on TAG synthesis (40). Interestingly, in vitro, the prephosphorylation of the complex by PKC had an inhibitory effect on the phosphorylation of Nem1 by PKA, and the prephosphorylation by PKA had an inhibitory effect on the phosphorylation of Spo7 by PKC. Thus for Nem1, the phosphorylation of Ser-201 prevents phosphorylation at Ser-140 and/or Ser-210, but the opposite scenario of hierarchical phosphorylations does not apply. For Spo7, the inhibition of its PKC-mediated phosphorylation by PKA might be explained by the fact that Ser-28 is a phosphorylation site common to both kinases and/or the phosphorylation of Ser-28 by PKA prevents the phosphorylation at Ser-22 by PKC. How these in vitro observations translate into the hierarchical phosphorylation that presumably occurs in vivo is not yet clear, but the fact that PKA and PKC have opposite effects on Nem1-Spo7 function may provide an explanation why the phosphorylation site mutations of either PKC or PKA (40) do not have large effects on TAG content. This points to a fine-tuning mechanism that has been developed by nature to balance PA utilization and lipid metabolism.

Studies on the phosphorylation-mediated regulation of the yeast Nem1-Spo7 complex as well as of Pah1 are relevant to the regulation of PA utilization and lipid metabolism in higher eukaryotes including humans (66-71). The mouse and human counterparts to Pah1 are the lipins 1, 2, and 3 (72, 73), whereas the Nem1 and Spo7 counterparts are CTDNEP1 (C-terminal domain nuclear envelope phosphatase 1) (68) and NEP1-R1 (nuclear envelope phosphatase 1-regulatory subunit 1) (70), respectively. Lipin PA phosphatase enzymes are subject to phosphorylation and its phosphorylated forms are dephosphorylated by the CTDNEP1-NEP1-R1 protein phosphatase complex (70, 74-77). High throughput phosphoproteomics analyses (summarized on PhosphoSitePlus, https://www.phosphosite.org) indicate that both CTDNEP1 and NEP1-R1 are subject to phosphorylation. Analysis of the phosphorylation sites with the NetPhos3.1 algorithm (50) point to the possibility that they might be targets of PKC. This information, coupled with the findings reported herein, provide the incentive to pursue this avenue of investigation with higher eukaryotic systems.
Experimental procedures

Materials

Growth media were from Difco Laboratories. Avanti Polar Lipids was the source of all lipids. Enzyme reagents for DNA manipulations, carrier DNA for yeast transformation, the QuikChange site-directed mutagenesis kit were obtained from New England Biolabs, Clontech, and Stratagene, respectively. Bio-Rad supplied the DNA size ladders, molecular mass protein standards, and reagents for electrophoresis, immunoblotting, and protein determination. SYPRO Ruby protein gel stain was from Invitrogen. Qiagen was the supplier of ampicillin, kanamycin, chloramphenicol, bovine serum albumin, 2-mercaptoethanol, isopropyl-β-D-1-thiogalactoside, L-1-tosylamido-2-phenylethyl chloromethyl ketone, benzamidine, aprotinin, leupeptin, and pepstatin), phosphoamino acid standards, alkaline phosphatase-agarose, rabbit anti-protein A antibodies (product P3775, lot 025K4777) and TLC plates (cellulose and silica gel 60). GE Healthcare was the source of IgG-Sepharose, Sepharose, Q-Sepharose, polyvinylidene difluoride (PVDF) membrane, and the enhanced chemiluminescence Western blotting detection kit. DE53 anion exchange resin was purchased from Whatman. Promega was the source of bovine heart PKA catalytic subunit. Nem1 and Spo7 peptides used for PKC phosphorylation assays were synthesized by EZBioLabs. Rabbit anti-(phosphoserine) PKC substrate antibody (product 2261S, lot 23) was from Cell Signaling Technology. Thermo Scientific was the source of alkaline phosphatase-conjugated goat anti-rabbit IgG antibody (product 31340, lot NJ178812). P81 phosphocellulose paper was from Whatman. Radiochemicals were from Perkin-Elmer Life Sciences and scintillation-counting supplies were from National Diagnostics. All other chemicals were reagent grade.

Strains, plasmids, and growth conditions

Table 1 contains a list of the Escherichia coli and Saccharomyces cerevisiae strains, and the plasmids used in this study. The isolation of chromosomal and plasmid DNA, the digestion and ligation of DNA, and PCR were performed according to standard protocols (78). E. coli (78) and S. cerevisiae (79) transformations were performed as described previously. A 2.8-kb DNA fragment containing the NEM1 gene was amplified by PCR (80) from strain BY4741. The NEM1 gene was inserted (Xhol/NotI sites) into plasmid pRS415 to produce plasmid pPD221. The S201A mutation of NEM1 and the S22A, S28A, and S22A/S28A mutations of SP07 were made by PCR-mediated site-directed mutagenesis using the appropriate PCR primers and templates as described by Choi et al. (24) to produce the plasmids listed in Table 1. DNA sequencing of the PCR products was used to confirm the mutations of the NEM1 and SPO7 coding sequences. For the expression and purification of wild type and phosphorylation-deficient mutant proteins, the YCplac111-GAL1/10-NEM1-PtA and pRS313-GAL1/10-SP07 plasmids and their derivatives were transformed into the S. cerevisiae mutant nem1Δ::HIS3 spo7Δ::HIS (SS1010). To analyze the effects of the phosphorylation-deficient mutations on growth and lipid composition, the plasmids pPD221 (NEM1) or pGH443 (SPO7) and their derivatives were transformed into the yeast mutants nem1Δ::URA3 (WMY161) and spo7Δ::URA3 (GHY67), respectively.

All plasmids were propagated in the E. coli strain DH5α, whereas the E. coli strains BL21(DE3)pLysS and BL21(DE3) were used to express His6-tagged Pah1 and His6-tagged Pho85 and Pho80, respectively. The E. coli cells were grown at 37 °C in lysogeny broth medium (1% tryptone, 0.5% yeast extract, 1% NaCl, pH 7.0). For the selection of the transformant cells carrying plasmids, the growth medium was supplemented with antibiotics (e.g., ampicillin, 100 μg/ml; kanamycin, 30 μg/ml; chloramphenicol, 34 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 cells were routinely grown at 30 °C in synthetic complete medium (81) containing 2% glucose; appropriate amino acids were omitted from the growth medium to select for cells carrying specific plasmids. For the galactose-induced expressions of protein A-tagged Nem1 and Spo7, the yeast cells were first grown to the exponential phase in synthetic complete medium with 2% raffinose and then grown in the same medium for 8 h with 2% galactose. For the growth spot test, cells were grown on synthetic complete medium agar plates (81). Cell numbers in liquid cultures were determined spectrophotometrically at 600 nm. Solid medium plates were prepared by dissolving agar (1.5% for E. coli and 2% for S. cerevisiae) into the lipid growth medium.

**Purification of enzymes and protein determinations**

Pkc1 PKC fused with a ZZ-tag (two repeats of the 60-amino acid IgG-binding domain of Staphylococcus aureus protein A) was expressed in yeast (confirmed by immunoblotting with anti-protein A antibody) and purified by chromatography with DE53 and IgG-Sepharose as described by Antonsson et al. (82) with minor modifications (43). The purified enzyme was dialyzed against 50 mM Tris-Cl (pH 7.5) buffer containing 15% glycerol. The WT and mutant forms of the Nem1-Spo7 complex (Nem1 tagged with protein A) were purified from the yeast cells by IgG-Sepharose affinity chromatography as described by Siniossoglou et al. (83) with minor modifications (29). His<sub>6</sub>-tagged Pah1 expressed in E. coli was purified by affinity chromatography with nickel-nitrilotriacetic acid agarose (1), followed by Q-Sepharose chromatography as described by Su et al. (29). The E. coli-expressed His<sub>6</sub>-tagged Pho85-Pho80 protein kinase complex was purified by nickel-nitrilotriacetic acid agarose affinity chromatography (84). SDS-PAGE (85) analysis indicated that the enzyme preparations were highly pure. The protein concentration was estimated by the method of Bradford (86) or by ImageQuant analysis of Coomassie Blue-stained or SYPRO Ruby-stained SDS-polyacrylamide gels. Bovine serum albumin was used as a standard to estimate protein concentrations in solution or in polyacrylamide gels.

**SDS-PAGE and immunoblotting**

SDS-PAGE (85) was routinely performed with a 12% slab gel and immunoblotting (87, 88) was performed with a PVDF membrane. Equal amounts of protein were loaded onto the SDS-polyacrylamide gels and Ponceau S staining was used to monitor the protein transfer from the polyacrylamide gel to the PVDF membrane. The affinity-purified IgG fractions of rabbit anti-Nem1 and anti-Spo7 antibodies (40) were used at a protein concentration of 1 μg/ml. The rabbit anti-protein A and anti-phosphoserine PKC substrate antibodies were used at dilutions of 1:3,000 and 1:1,000, respectively. Alkaline phosphatase-conjugated goat anti-rabbit IgG antibody was used at a dilution of 1:5,000. Immune complexes were detected using the enhanced chemifluorescence immunoblotting substrate. Fluorimaging was used to acquire fluorescence signals from immunoblots, and the intensities of the images were analyzed by ImageQuant software. A standard curve was used to determine that the immunoblot signals were in the linear range of detection.

**Phosphorylation reactions**

Phosphorylation reactions were performed in triplicate for 20 min at 30 °C in a total volume of 20 μl. The standard reaction mixture for PKC contained 50 mM Tris-Cl (pH 7.5), 10 mM MgCl<sub>2</sub>, 10 mM 2-mercaptoethanol, 1.7 mM CaCl<sub>2</sub>, 500 μM PS, 150 μM DAG, 50 μM [γ<sup>32</sup>P]ATP (3,000 cpm/pmol), and the indicated amounts of the Nem1-Spo7 complex and PKC (39). PS, DAG, and CaCl<sub>2</sub> were omitted from the PKC reaction mixture when the Nem1 and Spo7 peptides were used as substrates (39). The PKC reactions with the Nem1-Spo7 complex were terminated by mixing with Laemmli’s buffer (85), resolved by SDS-PAGE, and transferred to a PVDF membrane for phosphorimaging analysis. Alternatively, the SDS-polyacrylamide gel was dried and
subjected to the analysis, and the radioactive
signal was quantified with ImageQuant
software. In the PKC assays with Nem1 or Spo7
peptides, the enzyme reaction was terminated by
spotting the reaction mixture onto a P81
phosphocellulose paper. The paper was washed
three times with 75 mM phosphoric acid and
then subjected to scintillation counting. A unit
of PKC activity was defined as 1 nmol/min. The
phosphorylation by PKA was measured
similarly; the reaction mixture contained
25 mM Tris-HCl (pH 7.5), 10 mM MgCl$_2$, 2 mM
dithiothreitol, 100 µM [γ-$^{32}$P]ATP (3,000
cpm/pmole), and the indicated amounts of
the Nem1-Spo7 complex and PKA (40). A unit of
PKA activity was defined as 1 nmol/min.

**Phosphoamino acid analysis and phosphopeptide mapping**

PVDF membrane slices containing radioactively-labeled Nem1 or Spo7 were
subjected to hydrolysis with 6 N HCl at 110 °C
(for phosphoamino acid analysis) or proteolytic
digestion with L-1-tosylamido-2-phenylethyl
chloromethyl ketone-trypsin (for phosphopeptide mapping) as described
previously (42, 89, 90). The acid hydrolysates
were mixed with standard phosphoamino acids and
separated by two-dimensional electrophoresis on cellulose TLC plates (89).
Phosphoamino acid standards were visualized by
ninhydrin staining, whereas the $^{32}$P-labeled one
were observed by phosphorimaging analysis.
The tryptic digests were separated on the
cellulose plates first by electrophoresis and then
by TLC (89). The radioactive phosphopeptides
were visualized by phosphorimaging analysis.

**Nem1-Spo7 enzyme assay**

The phosphatase activity of Nem1-Spo7 was
measured for 10 min at 30 °C by following the
release of $^{32}$P$_i$ from $[^{32}$P]Pah1, which was
prepared by the Pho85-Pho80 phosphorylation of
*E. coli*-expressed Pah1 (29). The reaction
mixture contained 100 mM sodium acetate (pH
5.0), 10 mM MgCl$_2$, 0.25 mM Triton X-100, 1
mM DTT, 0.25 µM phosphorylated Pah1, and
the Nem1-Spo7 complex in a total volume of 50
µl. The amount of phosphate produced in the
reaction was calculated on the basis of the
specific activity of the [γ-$^{32}$P]ATP used to
prepare $[^{32}$P]Pah1 (29). A unit of Nem1-Spo7
phosphatase activity was defined as the amount
of enzyme that catalyzed the formation of 1
nmol phosphate/min.

**Radiolabeling and lipid analysis**

The steady-state labeling of lipids with [2-14C]acetate (91), the extraction of lipids from the
radiolabeled cells (92), and their separation one-
dimensional TLC (93) were performed as
described previously. The resolved lipids were
observed by phosphorimaging and quantified by
ImageQuant software. The identity of
radiolabeled lipids was confirmed by
comparison with the migration of authentic
standards visualized by staining with iodine
vapor.

**Data analysis**

Excel software was used for statistical
analyses where $p$ values < 0.05 were taken as a
significant difference. The Enzyme Kinetics
module of Sigma-Plot software, which uses the
Marquardt-Levenberg algorithm, was used to
determine kinetic parameters according to the
Michaelis-Menten equation.

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G.-S. H., and G. M. C. designed the study,
analyzed the results, and prepared the manuscript.
P.D., W.-M. S., and M. M performed the
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FOOTNOTES

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2 The abbreviations used are: DAG, diacylglycerol; ER, endoplasmic reticulum; PA, phosphatidic acid; PKA, protein kinase A; PKC, protein kinase C; PS, phosphatidylserine; PVDF, polyvinylidene difluoride; TAG, triacylglycerol,

3 In this paper, yeast is used interchangeably with *Saccharomyces cerevisiae*.

4 A. Hassaninasab, G.-S. Han, and G. M. Carman, unpublished data.
**Table 1**

Strains and plasmids used in this work

| Strain or plasmid | Genotype or relevant characteristics | Source or Ref |
|------------------|--------------------------------------|---------------|
| **Strain**       |                                      |               |
| *E. coli*        |                                      |               |
| DH5α             | F' φ80dlacZΔM15 Δ(lacZYA-argF)U169 deoR recA1 endA1 hsdR17(rK mK) phoA supE44 λ- thi-1 gyrA96 relA1 | (78)          |
| BL21(DE3)pLysS   | F' ompT hsdS8 (rK mK) gal dcm (DE3) pLysS | Novagen       |
| BL21(DE3)        | F' ompT hsdS8 (rK mK) gal dcm (DE3)    |               |
| **S. cerevisiae**|                                      |               |
| BY4741           | MATα his3Δ1 leu2Δ0 met15Δ0 ura3Δ0      | Invitrogen    |
| RS453            | MATα ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-52 | (20)          |
| **Derivatives**  |                                      |               |
| SS1010           | nem1Δ::His3 spo7Δ::His3               | (34)          |
| WMY161           | nem1Δ::URA3                          | (40)          |
| GHY67            | spo7Δ::URA3                          | (40)          |
| **Plasmid**      |                                      |               |
| pGH313           | PAH1 coding sequence inserted into pET-15b for *E. coli* expression | (1)           |
| EB1164           | PHO85-His6 derivative of pQE-60 for *E. coli* expression | (84)          |
| EB1076           | PHO80 derivative of pSBETA for *E. coli* expression | (84)          |
| YCplac111-GAL1/10-NE M1-PtA | NEM1-PtA under control of GAL1/10 promoter in CEN/LEU2 vector | (11)          |
| **Derivatives**  |                                      |               |
| pPD115           | NEM1(S201A)-PtA                      | This study    |
| pRS314-GAL1/10-S PO7 | SPO7 under control of GAL1/10 promoter in CEN/TRP1 vector | (40)          |
| **Derivatives**  |                                      |               |
| pPD121           | SPO7(S22A) derivative of pRS314-GAL1/10-SPO7 | This study    |
| pWM211           | SPO7(S28A) derivative of pRS314-GAL1/10-SPO7 | (40)          |
| pPD123           | SPO7(S22A/S28A) derivative of pRS314-GAL1/10-SPO7 | This study    |
| pRS415           | Low copy *E. coli*/yeast shuttle vector with LEU2 | (94)          |
| **Derivatives**  |                                      |               |
| pPD221           | NEM1 inserted into pRS415             | This study    |
| pPD221(S201A)    | NEM1(S201A)                          | This study    |
| pGH443           | SPO7 inserted into pRS415             | (40)          |
| pGH443(S22A)     | SPO7(S22A)                           | This study    |
| pGH443(S28A)     | SPO7(S28A)                           | (40)          |
| pGH443(S22A/S28A)| SPO7(S22A/S28A)                      | This study    |
PKC phosphorylation of the Nem1-Spo7 phosphatase complex

Figure Legends

FIGURE 1. Reactions catalyzed by the Nem1-Spo7 phosphatase complex and the Pah1 PA phosphatase and their roles in lipid synthesis; domains/regions and phosphorylation sites in Nem1 and Spo7. A, phosphorylated (indicated by small circles) Pah1 interacts with the Nem1-Spo7 complex at the ER membrane. Following its dephosphorylation, Pah1 that is associated with the ER membrane dephosphorylates PA to produce DAG. The DAG produced by the PA phosphatase reaction is used for the synthesis of TAG. PA is also utilized for the synthesis of membrane phospholipids via CDP-DAG. When the CDP-DAG-dependent pathway for phospholipid synthesis is blocked, phosphatidylincholine or phosphatidylethanolamine, respectively, may be synthesized from the DAG derived from the PA phosphatase reaction when cells are supplemented with choline or ethanolamine, respectively, via the CDP-choline or CDP-ethanolamine branches of the Kennedy pathway. Details of the lipid synthesis pathways maybe found elsewhere (95, 96). B, Nem1 and Spo7 are integral nuclear/ER membrane proteins possessing two transmembrane (TM) spanning domains (34). Nem1 binds to Spo7 through its conserved C-terminal domain, and this association is responsible for the formation of the complex (34). Nem1, which serves as the catalytic subunit, is a member of the haloacid dehalogenase superfamily (97, 98); its phosphatase activity is conferred by the DXD(T/V) catalytic motif within its haloacid dehalogenase (HAD)-like domain (11, 34). Spo7, which serves as the regulatory subunit (34), facilitates the formation of the Nem1-Spo7/Pah1 complex (31, 38).

FIGURE 2. Nem1 and Spo7 are majorly phosphorylated in vitro by PKC on the serine residue; Ser-201 in Nem1 and Ser-22 and Ser-28 in Spo7 are the major PKC phosphorylation sites. The protein A-tagged Nem1 was co-expressed with Spo7 in yeast cells and the Nem1-Spo7 complex was purified by IgG-Sepharose affinity chromatography. The complex (40 ng) was incubated at 30 °C for 20 min with 70 ng PKC, 40 μM [γ-32P]ATP, 10 mM MgCl₂, 500 μM PS, 150 μM DAG, and 1.7 mM CaCl₂. The reaction mixture was resolved by SDS-PAGE. A, the radioactive phosphorylations of Nem1 and Spo7 were visualized by phosphorimaging of the SDS-polyacrylamide gel (left). The proteins were transferred to PVDF membrane, which was split into the upper and lower portions and used for immunoblot analysis using anti-Nem1 and anti-Spo7 antibodies, respectively (right). The positions of Nem1, Spo7, and molecular mass standards are indicated. B, PVDF membrane containing 32P-labeled Nem1 or Spo7 was incubated with 6 N HCl for 90 min at 110 °C. The acid hydrolysates were separated by two-dimensional electrophoresis on cellulose TLC plates followed by phosphorimaging analysis. The positions of the standard phosphoamino acids phosphoserine (p-Ser), phosphothreonine (p-Thr), and phosphotyrosine (p-Tyr) (dotted lines) are indicated. C, PVDF membrane containing 32P-labeled Nem1 or Spo7 was digested with L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin. The phosphopeptides produced by the proteolytic digestion were separated on cellulose TLC plates by electrophoresis (from left to right) in the first dimension and by chromatography (from bottom to top) in the second dimension. The identity of the phosphorylation sites in the radioactive phosphopeptides of Nem1 or Spo7 were determined from phosphopeptide maps of the phosphorylation-deficient alanine mutations of Ser-201 of Nem1 and Ser-22 and Ser-28 of Spo7. The data shown in panels A-C are representative of three independent experiments.
FIGURE 3. Phosphorylations of Nem1 and Spo7 by PKC are dependent on time, the amount of PKC, and ATP concentration. Purified Nem1-Spo7 complex (40 ng) was phosphorylated by PKC using $[\gamma^{32}P]$ATP as described in the legend to Figure 2. The PKC reaction was conducted by varying the reaction time (A), amount of PKC (B), and the concentration of ATP (C). A and B, 40 $\mu$M ATP; A and C, 20 ng PKC; B and C, 15 min. The amount of radioactive phosphate incorporated into Nem1 or Spo7 was determined from a standard curve using $[\gamma^{32}P]$ATP. The amounts of Nem1 and Spo7 were determined by comparing their band intensities from a SYPRO Ruby-stained polyacrylamide gel with a standard curve of bovine serum albumin. The data shown are averages of three experiments ± S.D. (error bars).

FIGURE 4. PKC phosphorylates Nem1 or Spo7 synthetic peptides that contain sites of phosphorylation. PKC activity was measured with 100 $\mu$M of the indicated Nem1 (A) or Spo7 (B) peptides at 100 $\mu$M $[\gamma^{32}P]$ATP. The enzyme reaction, which was performed without PS, DAG, or CaCl$_2$, was terminated by spotting the mixture onto a P81 phosphocellulose paper, which was then washed with 75 mM phosphoric acid and subjected to scintillation counting. The numbers at the beginning and end of the Nem1 (A) and Spo7 (B) peptides represent the positions in the full-length sequences of the respective proteins. The underlined residues within the Nem1 and Spo7 peptides designate the serine-to-alanine substitutions in the sequence. The data are the averages of three experiments ± S.D. (error bars). a, $p < 0.05$ versus WT peptide, residues 197-207; b, $p < 0.05$ versus WT peptide, residues 205-215; c, $p < 0.05$ versus WT peptide, residues 17-27; d, $p < 0.05$ versus WT peptide, residues 26-36.

FIGURE 5. Kinetics of PKC activity on Nem1 and Spo7 synthetic peptides. PKC activity was measured as a function of the concentrations of the Nem1 peptide (residues 197-207) (A) or the Spo7 peptide (residues 21-50) (B) at 1 mM $[\gamma^{32}P]$ATP. The enzyme reaction, which was performed without PS, DAG, or CaCl$_2$, was terminated by spotting the mixture onto P81 phosphocellulose paper, which was then washed with 75 mM phosphoric acid and subjected to scintillation counting. The apparent $V_{\text{max}}$ and $K_m$ values were determined by analysis of the data with the Enzyme Kinetics module of Sigma-Plot software according to the Michaelis-Menten equation. The data are averages of three experiments ± S.D. (error bars).

FIGURE 6. Alanine mutations of Ser-201 in Nem1 and Ser-22/Ser-28 in Spo7, respectively, reduce their phosphorylations by PKC in vitro and in vivo. The WT and indicated mutant forms of Nem1 or Spo7 were co-expressed in yeast cells and purified by IgG Sepharose affinity chromatography based on the protein A tag on Nem1. A, the purified Nem1-Spo7 complex (40 ng) was phosphorylated by PKC (40 ng) with $[\gamma^{32}P]$ATP (40 $\mu$M) as described in the legend to Figure 2. Following the reaction, samples were subjected to SDS-PAGE. The phosphorylations of Nem1 and Spo7 were visualized by phosphorimaging. The proteins on the gel were visualized with SYPRO ruby stain (not shown). B, the purified Nem1-Spo7 complex (20 ng) was subjected to SDS-PAGE, followed by transfer to a PVDF membrane and probing with anti-phosphoserine PKC substrate antibody. A duplicate PVDF membrane was split into the upper and lower portions and used for immunoblot analysis with anti-Nem1 and anti-Spo7 antibodies, respectively. The phosphorimages, SYPRO ruby-stained polyacrylamide gels, and immunoblots were quantified by ImageQuant analysis. The positions of Nem1’, Spo7’, and molecular mass standards are indicated. The PKC-mediated phosphorylation levels of the mutant proteins were compared with the respective WT proteins.
that were set at 100%. The phosphorimages (A) and immunoblots (B) are representative of three experiments, whereas the quantification data shown below the images are averages of three experiments ± S.D. (error bars). *, p< 0.05 versus WT.

FIGURE. 7. Effects of PKC-mediated phosphorylation and PKC phosphorylation site mutations on Nem1-Spo7 phosphatase activity. The wild type and PKC phosphorylation site mutant forms of the Nem1-Spo7 complex were expressed and purified from yeast. A, 10 ng of the wild type Nem1-Spo7 complex was phosphorylated by 40 ng PKC with unlabeled ATP for 15 min. The PKC phosphorylated and unphosphorylated (control) forms of the complex were assayed for Nem1-Spo7 phosphatase activity for the indicated time intervals. B, 10 ng of the wild type and the indicated mutant forms of the Nem1-Spo7 complex were assayed for its phosphatase activity for 10 min. Nem1-Spo7 phosphatase activity was measured by following the release of $^{32}$P, from $[^32P]$Pah1 that was phosphorylated by the Pho85-Pho80 protein kinase. The data shown are means ± S.D. (error bars) from triplicate assays. *, p< 0.05 versus the wild type Nem1-Spo7 complex.

FIGURE. 8. Effects of the PKC phosphorylation site mutations in Nem1 and Spo7, respectively, on the complementation of the nem1Δ or spo7Δ temperature-sensitive and the aberrant lipid composition phenotypes. The indicated WT and phosphorylation-deficient forms of Nem1 and Spo7 were expressed in the nem1Δ and spo7Δ mutants, respectively, and grown in liquid synthetic complete-Leu medium. A, saturated cultures were diluted to the density of 0.67 at $A_{600}$, followed by 10-fold serial dilutions. The diluted cultures were spotted (2.5 µl) onto agar plates and incubated for 5 days at 30 and 37 °C. B, C, the cells were grown at 30 °C to the exponential (B) and stationary (C) phases of growth in synthetic complete-Leu medium containing [2-14C]acetate (1 µCi/ml). The lipids were extracted and separated by one-dimensional TLC, and the phosphorimages were subjected to ImageQuant analysis. The percentages shown for TAG and phospholipids (PLs) were normalized to the total 14C-labeled chloroform-soluble fraction. Each data point represents the average of three experiments ± S.D. (error bars). a, p< 0.05 versus TAG of WT, b, p< 0.05 versus phospholipid of WT.

FIGURE. 9. Prephosphorylation of the Nem1-Spo7 complex by PKC reduces the subsequent phosphorylation of Nem1 by PKA, and prephosphorylation of the complex by PKA reduces the subsequent phosphorylation of Spo7 by PKC. A, the purified Nem1-Spo7 complex was prephosphorylated by the indicated amounts of PKC for 60 min with unlabeled ATP (40 µM). The prephosphorylated complex was then phosphorylated by 8 units of PKA with 40 µM [$\gamma$-$^{32}$P]ATP for 20 min. B, the purified Nem1-Spo7 complex was prephosphorylated by the indicated amounts of PKA for 60 min with unlabeled ATP (40 µM). The prephosphorylated complex was then phosphorylated by 500 units of PKC with 40 µM [$\gamma$-$^{32}$P]ATP for 20 min. The $^{32}$P-labeled Nem1 and Spo7 were separated from each other and the labeled ATP by SDS-PAGE and subjected to phosphorimaging and ImageQuant analyses. The amount of the phosphorylated Nem1 (A) or Spo7 (B) that was not subjected to prephosphorylation was set at 100%. The amount PKC used in the experiment was greater than that of PKA because the PKA preparation is more active. Also, the units of the PKA and PKC activities are based on different peptide substrates. The data reported are the result of three independent experiments ± S.D. (error bars).
Fig. 2

A  
**Phosphorylation**

| kDa | Phosphorimage | kDa | Immunoblot |
|-----|---------------|-----|------------|
| 100-75 | - Nem1 | 100-75 | - Nem1 |
| 25- | - Spo7 | 25- | - Spo7 |

PKC

| Nem1-Spo7 |
|-----------|
| +         |

| Nem1-Spo7 |
|-----------|
| +         |

B  
**Phosphoamino acid analysis**

| Nem1 |
|------|
| p-Ser |
| p-Thr |
| p-Tyr |

| Spo7 |
|------|
| p-Ser |
| p-Thr |
| p-Tyr |

C  
**Phosphopeptide mapping**

| Nem1 |
|------|
| p-Ser-201 |

| Spo7 |
|------|
| p-Ser-28 |
| p-Ser-22 |
**Fig. 4**

**A**

PKC activity, nmol/min/mg

- Nem1 peptide
  - 135 KRNRG SNASEN 145
  - 135 KRNRG A NASEN 145
  - 191 NVK GS LLRAQSVKSR 205
  - 191 NVK GALLRAQSVKSR 205
  - 197 LAQSVKSRPR 207
  - 197 LAQAQAVKSRPR 207
  - 205 RPRSYSKSELS 215
  - 205 RPR SYAKSELS 215
  - 371 NGKGS SSSSL 379

**B**

PKC activity, nmol/min/mg

- Spo7 peptide
  - 21 VSGPRRRSTS KTSSAK NIRNSSNSISPASMI 50
  - 17 SAVS GPRRR 27
  - 17 SAVG GPRRR 27
  - 26 RRTS KTSSAK 36
  - 26 RR ATS KTSSAK 36
  - 35 AKNIRNSSNI 44
  - 125 SGQYRRTIV PRRFFTSTNKGIRQFN 150
  - 152 KLKVQSTWDEKY TDSVRFSRTI 175
  - 197 KFWKS VTIQSQPRI 210
  - 216 KVLN PRAFSAEI RE 230
  - 247 RRQAHELRPKSE 259
Fig. 5

A

PKC activity, nmol/min/mg

Nem1 peptide, μM

$V_{max} = 481$ nmol/min/mg

$K_m = 101$ μM

$V_{max}/K_m = 4.8$

B

PKC activity, nmol/min/mg

Spo7 peptide, μM

$V_{max} = 1538$ nmol/min/mg

$K_m = 188$ μM

$V_{max}/K_m = 8.2$
A. Phosphorylation \textit{in vitro}

B. Phosphorylation \textit{in vivo}
Fig. 7

A

Nem1-Spo7 phosphatase activity, nmol/mg

Time, min

PKC
Control

B

Nem1-Spo7 phosphatase activity, nmol/min/mg

Nem1-Spo7
Nem1(S201A)-Spo7
Nem1-Spo7(S22A/S28A)

*
**A**  

![Images showing mutants under different conditions.](image)

**B**  

**Exponential**

![Bar charts showing lipid content in different conditions.](image)

**C**  

**Stationary**

![Bar charts showing lipid content in different conditions.](image)
Fig. 9

A

Phosphorylation by PKA, %

Prephosphorylation by PKC, nmol/min

Nem1
Spo7

B

Phosphorylation by PKC, %

Prephosphorylation by PKA, nmol/min

Nem1
Spo7
Protein kinase C mediates the phosphorylation of the Nem1-Spo7 protein phosphatase complex in yeast
Prabuddha Dey, Wen-Min Su, Mona Mirheydari, Gil-Soo Han and George M. Carman
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