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# Phosphorylation of Dgk1 Diacylglycerol Kinase by Casein Kinase II Regulates Phosphatidic Acid Production in *Saccharomyces cerevisiae*[*]

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**In the yeast *Saccharomyces cerevisiae*, Dgk1 diacylglycerol (DAG) kinase catalyzes the CTP-dependent phosphorylation of DAG to form phosphatidic acid (PA). The enzyme in conjunction with Pah1 PA phosphatase controls the levels of PA and DAG for the synthesis of triacylglycerol and membrane phospholipids, the growth of the nuclear/endoplasmic reticulum membrane, and the formation of lipid droplets. Little is known about how DAG kinase activity is regulated by posttranslational modification. In this work, we examined the phosphorylation of Dgk1 DAG kinase by casein kinase II (CKII). When phosphate groups were globally reduced using nonspecific alkaline phosphatase, Triton X-100-solubilized membranes from *DGK1*-overexpressing cells showed a 7.7-fold reduction in DAG kinase activity; the reduced enzyme activity could be increased 5.5-fold by treatment with CKII. Dgk1(1–77) expressed heterologously in *Escherichia coli* was phosphorylated by CKII on a serine residue, and its phosphorylation was dependent on time as well as on the concentrations of CKII, ATP, and Dgk1(1–77). We used site-specific mutagenesis, coupled with phosphorylation analysis and phosphopeptide mapping, to identify Ser-45 and Ser-46 of Dgk1 as the CKII target sites, with Ser-46 being the major phosphorylation site. *In vivo*, the S46A and S45A/S46A mutations of Dgk1 abolished the stationary phase-dependent stimulation of DAG kinase activity. In addition, the phosphorylation-deficient mutations decreased Dgk1 function in PA production and in eliciting *pah1*Δ phenotypes, such as the expansion of the nuclear/endoplasmic reticulum membrane, reduced lipid droplet formation, and temperature sensitivity. This work demonstrates that the CKII-mediated phosphorylation of Dgk1 regulates its function in the production of PA.**

The yeast[2] Dgk1 DAG[3] kinase, an integral membrane enzyme catalyzing the CTP-dependent phosphorylation of DAG to PA (1, 2), and Pah1 PA phosphatase, a peripheral membrane enzyme catalyzing the $Mg^{2+}$-dependent dephosphorylation of PA to DAG (3), have emerged as key regulators of the essential lipid intermediates PA and DAG (Fig. 1) (4, 5). PA is used for the synthesis of all membrane phospholipids via CDP-DAG (*CDP-DAG Pathway*) or DAG (*Kennedy Pathway*) and for the synthesis of TAG via DAG (4, 5) (Fig. 1). In addition, the mobilization of TAG to produce DAG and its subsequent phosphorylation to PA for the synthesis of membrane phospholipids (Fig. 1) play an important role in the growth resumption of yeast cells that exit stasis (*e.g.* stationary phase) (6). Moreover, PA and DAG serve as signaling molecules that control transcription, membrane proliferation, vesicular trafficking, and the activation of cell growth (7–16).

Disturbing the PA/DAG balance in yeast, as caused by the lack of Pah1 PA phosphatase activity, results in the abnormal regulation of phospholipid synthesis gene expression and phospholipid content, the aberrant growth of the nuclear/ER membrane, vacuole fragmentation, a defect in lipid droplet formation, an acute sensitivity to fatty acid-induced toxicity, and a reduction in chronological life span (1, 3, 17–27). Of the cellular defects imparted by the *pah1*Δ mutation, those related to the elevated PA content are dependent on Dgk1 DAG kinase activity and are mimicked by the overexpression of *DGK1* (1, 3, 6, 18, 21, 23, 25, 27). Consequently, the *pah1*Δ phenotypes that are conveyed by elevated PA content are suppressed by the *dgk1*Δ mutation (1, 6, 21, 25, 27).

The roles of DAG kinase and PA phosphatase in lipid metabolism and cell signaling are conserved throughout evolution.[4] For example, mammalian DAG kinase enzymes regulate cellular processes important to a variety of diseases, such as cancer, type II diabetes, autoimmunity, and nervous system disorders (28–33), whereas the PA phosphatase enzymes regulate cellular processes important to diseases that include lipodystrophy, insulin resistance, peripheral neuropathy, rhabdomyolysis, and inflammation (34–43).

Uncovering the regulation of yeast Dgk1 DAG kinase and Pah1 PA phosphatase is crucial to better understand their functional roles in the cell. In previous work, Pah1 has been shown to be phosphorylated by multiple protein kinases including

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[2] In this paper, the term "yeast" is used interchangeably with *Saccharomyces cerevisiae*.

[3] The abbreviations used are: DAG, diacylglycerol; ER, endoplasmic reticulum; PA, phosphatidic acid; TAG, triacylglycerol; CKII, casein kinase II; SC, synthetic complete.

[4] Unlike yeast that contains one DAG kinase (1) and one PA-specific PA phosphatase (3), mammalian cells possess multiple isoforms of DAG kinase (32, 87–90) and multiple forms of PA phosphatase (34, 120–122).

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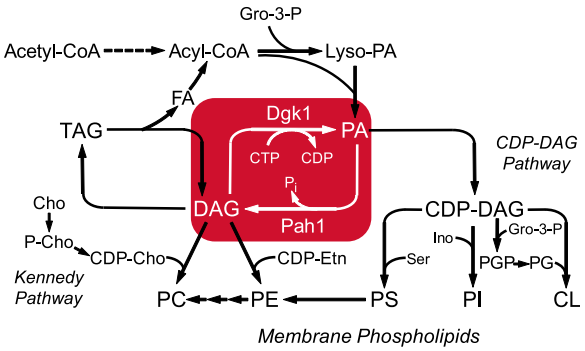


FIGURE 1. **Lipid synthesis in yeast.** The pathways in the figure include the steps of lipid synthesis discussed in this work. The reactions catalyzed by Dgk1 DAG kinase and Pah1 PA phosphatase, which produce PA and DAG, are highlighted by *red shading*. More detailed pathways for lipid synthesis and turnover in yeast may be found elsewhere (5). *Gro-3-P*, glycerol-3-phosphate; *FA*, fatty acid; *Ino*, inositol; *Ser*, serine; *PI*, phosphatidylinositol; *PS*, phosphatidylserine; *PE*, phosphatidylethanolamine; *PC*, phosphatidylcholine; *PG*, phosphatidylglycerol; *PGP*, phosphatidylglycerol phosphate; *CL*, cardiolipin; *Etn*, ethanolamine; *Cho*, choline.

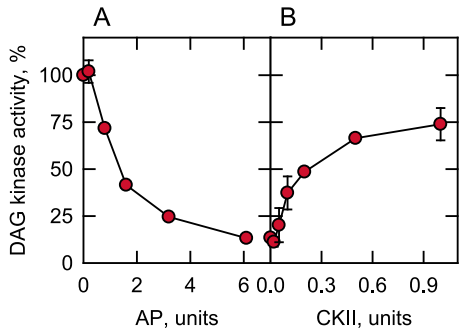


FIGURE 2. **DAG kinase activity is inhibited by dephosphorylation with alkaline phosphatase and stimulated by phosphorylation with CKII.** *A*, 10 $\mu$g of a Triton X-100-solubilized membrane fraction prepared from stationary phase cells overexpressing Protein A-tagged Dgk1 was incubated for 15 min at 30 °C with the indicated amounts of alkaline phosphatase immobilized on agarose. The enzyme reaction was terminated by removing alkaline phosphatase (*AP*) through filtration. The DAG kinase activity was normalized to that of the untreated Dgk1 control. *B*, the alkaline phosphatase-treated Dgk1 was incubated for 15 min at 30 °C with the indicated amounts of human CKII, followed by the measurement of DAG kinase activity. The enzyme kinase activity was normalized to that of the untreated Dgk1 control in *A*. The data shown are means ± S.D. (*error bars*) from triplicate determinations.

Pho85-Pho80 (44), Cdc28-cyclin B (45), protein kinase A (46), protein kinase C (47), and CKII (48). Collectively, the phosphorylations of Pah1 regulate its cellular location, PA phosphatase activity, and stability/degradation, which have a major impact on lipid metabolism and cell physiology (44–49). The reciprocal nature of the PA phosphatase and DAG kinase reactions raised a possibility that the latter enzyme is also regulated by its phosphorylation. In the N-terminal region, Dgk1 contains putative target sites for CKII,[5] an evolutionarily conserved serine/threonine protein kinase that is composed of two catalytic (*i.e.* Cka1 and Cka2) and two regulatory (*i.e.* Ckb1 and Ckb2) subunits (50–54) and is essential for the growth of yeast (54–57). The protein kinase is also known to phosphorylate yeast proteins (*e.g.* Pah1 and the transcriptional repressor Opi1) that are involved in physiological processes connecting with PA and/or DAG (48, 58). In this study, we showed that Dgk1 is a *bona fide* substrate for CKII and identified Ser-45 and Ser-46 as sites of phosphorylation. We also demonstrated that the CKII phosphorylation of Dgk1 stimulates DAG kinase activity and regulates its function for the production of PA and the phenotypes of the *pah1*Δ mutant in the nuclear/ER membrane growth and the formation of lipid droplets.

## Results

*Dgk1 Is a Bona Fide Substrate of CKII and Its Phosphorylation Stimulates DAG Kinase Activity*—Phosphoproteome analysis (59–62) has identified Dgk1 as a phosphoprotein whose phosphorylation occurs on Thr-3, Ser-26, Thr-36, Ser-44, Ser-45, and Ser-46. According to bioinformatics (63–65), the serines at residues 44–46 are contained within the CKII target motif. Initially, we questioned whether CKII affects DAG kinase activity of Triton X-100-solubilized membranes from the *DGK1*-overexpressing cells in the stationary phase.[6] When treated with CKII, the detergent-solubilized membrane did not show a change in DAG kinase activity. This result suggested that Dgk1 expressed in the cell had already been phosphorylated at CKII target sites. Accordingly, the Triton X-100-solubilized membrane was first treated with alkaline phosphatase to remove phosphates and then measured for DAG kinase activity. The alkaline phosphatase treatment caused a dose-dependent decrease (7.7-fold at the point of maximum inhibition) in DAG kinase activity (Fig. 2*A*). The CKII treatment of the alkaline phosphatase-treated membranes resulted in a dose-dependent (5.5-fold at the point of maximum stimulation) increase in the enzyme activity (Fig. 2*B*). These data suggested that CKII phosphorylates Dgk1, which stimulates DAG kinase activity. During the course of these experiments, we also questioned whether Nem1-Spo7, the protein phosphatase complex that dephosphorylates Pah1 (17, 66), has a similar inhibitory effect on Dgk1. Unlike alkaline phosphatase, the Nem1-Spo7 complex had no significant effect on Dgk1 for its DAG kinase activity.

To characterize the phosphorylation of Dgk1 in the absence of prephosphorylation, the enzyme was prepared through heterologous expression in *Escherichia coli* as a truncated form consisting of the N-terminal hydrophilic region (residues 1–77) (Fig. 3*A*). The truncated form of Dgk1 (Dgk1(1–77)), which contains the putative target sites of CKII, was utilized because the full-length protein was not tractable due to its poor expression. Purified Dgk1(1–77) (Fig. 3*B*) was incubated with CKII in the presence of [$\gamma$-$^{32}$P]ATP, and its phosphorylation was determined after electrophoretic separation by phosphorimaging. The *E. coli*-expressed Dgk1(1–77) was phosphorylated by yeast and human CKII (Fig. 3*C*). Phosphoamino acid analysis and phosphopeptide mapping showed that the CKII-treated Dgk1 (1–77) was phosphorylated on the serine residue (Fig. 4*A*) that is contained within one major phosphopeptide (Fig. 4*B*). Because the yeast and human forms of CKII phosphorylated

[5] CKII generally phosphorylates proteins with the motif (S/T)*XX*(E/D) but will also phosphorylate proteins with (S/T)*X*(E/D) or (S/T)(E/D) (50–52, 123).

[6] Using the Triton X-100-solubilized Dgk1 for this experiment facilitated the removal of the insoluble alkaline phosphatase-agarose from the phosphatase reaction for the subsequent measurement of DAG kinase activity. Alkaline phosphatase interfered with the DAG kinase assay because it dephosphorylates the substrate CTP. The removal the alkaline phosphatase-agarose from the reaction also facilitated the subsequent phosphorylation of the alkaline phosphatase-treated Dgk1 by CKII.

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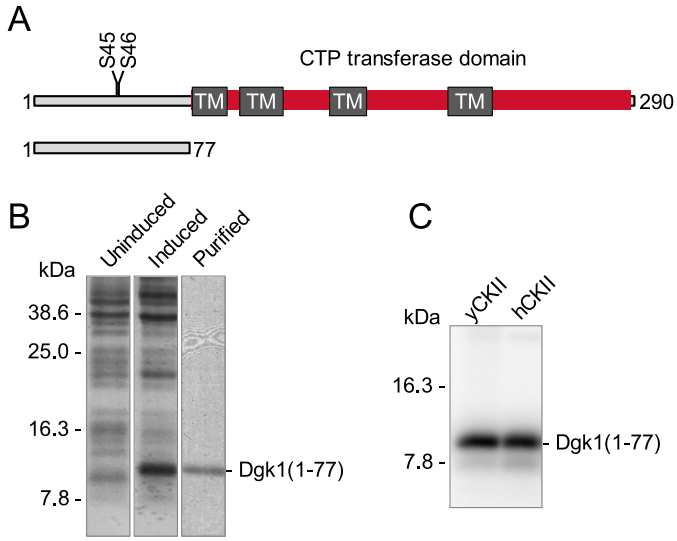


FIGURE 3. **Dgk1(1–77) is phosphorylated by yeast and human CKII.** *A*, the schematic diagram of Dgk1 shows a cytidylyltransferase domain, four transmembrane spanning domains, and two serine residues identified here as the sites of phosphorylation by CKII. The truncated form of Dgk1 (Dgk1(1–77)), which contains the phosphorylation sites, is shown *below* the full-length protein. *B*, the $His_6$-tagged Dgk1(1–77) was purified after heterologous expression in *E. coli* and subjected to SDS-PAGE (18% polyacrylamide gel, 1 $\mu$g loaded) analysis. *C*, the purified recombinant Dgk1(1–77) (0.42 $\mu$g) was incubated for 10 min with yeast CKII (*yCKII*; 0.36 $\mu$g) or human CKII (*hCKII*; 0.04 units) in the presence of [$\gamma$-$^{32}$P]ATP. Following the kinase reactions, phosphorylated Dgk1(1–77) was separated from ATP and CKII by SDS-PAGE (18% polyacrylamide gel) and subjected to phosphorimaging analysis. The positions of Dgk1(1–77) and molecular mass standards are indicated. The data shown in *B* and *C* are representative of three experiments.

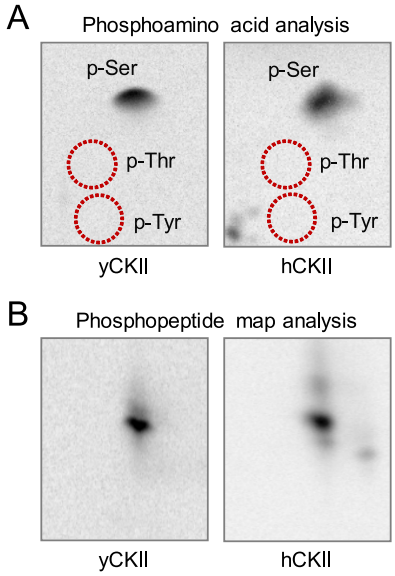


FIGURE 4. **Dgk1(1–77) is phosphorylated by CKII on the serine residue.** Purified Dgk1(1–77) was phosphorylated by yeast CKII (*yCKII*) or human CKII (*hCKII*) with [$\gamma$-$^{32}$P]ATP, separated in the SDS-polyacrylamide gel, and transferred to a PVDF membrane. The $^{32}$P-labeled Dgk1(1–77) on the PVDF membrane was hydrolyzed with 6 N HCl or digested with L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin. The phosphoamino acids produced by the acid hydrolysis were separated on cellulose TLC plates by two-dimensional electrophoresis (*A*), whereas 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 (*B*). Phosphoamino acids that were not detected from the $^{32}$P-labeled Dgk1(1–77) are indicated by *dotted circles*. *p-Ser*, phosphoserine; *p-Thr*, phosphothreonine; *p-Tyr*, phosphotyrosine. The data are representative of three independent experiments.

Dgk1(1–77) on the same site(s), the human enzyme, which is commercially available, could be used as an alternative for the yeast enzyme. Dgk1(1–77) was determined as a *bona fide* substrate of CKII by the dependence of its phosphorylation on the time of reaction, the amount of CKII, and the concentrations of ATP and Dgk1(1–77) (Fig. 5). At the point of maximum phosphorylation, CKII catalyzed the incorporation of 0.25 mol of phosphate/mol of Dgk1(1–77).

*CKII Phosphorylates Dgk1 on Ser-45 and Ser-46*—The three putative CKII phosphorylation sites (*i.e.* Ser-44, Ser-45, and Ser-46) of Dgk1 were individually mutated to the alanine residue. The phosphorylation-deficient forms of Dgk1(1–77) expressed heterologously in *E. coli* were affinity-purified, phosphorylated by CKII with [$\gamma$-$^{32}$P]ATP, and analyzed by SDS-PAGE and phosphorimaging. The S45A and S46A mutations reduced the phosphorylation of Dgk1(1–77) by 37 and 86%, respectively (yeast CKII; Fig. 6*A*), and by 39 and 93%, respectively (human CKII; Fig. 6*B*). However, the S44A mutation had no significant effect on the phosphorylation of Dgk1(1–77) by yeast or human CKII. These results indicate that Ser-46 in Dgk1 is the major site of phosphorylation by CKII. In contrast to the single mutations, the S44A/S45A/S46A triple mutation completely eliminated the CKII-mediated phosphorylation of Dgk1(1–77) (Fig. 6). (The S45A/S46A double mutation was not constructed in this analysis). In phosphopeptide mapping, the position of the major phosphopeptide was not affected by the S45A or S46A mutation (Fig. 6*C*). That Ser-46 is the major phosphorylation site of Dgk1(1–77) was further confirmed by the finding that the S46A mutation had a strong inhibitory effect on the phosphorylation with respect to time and the amounts of CKII, ATP, and Dgk1(1–77) (Fig. 5).

*The Stimulation of DAG Kinase Activity by CKII Regulates Its Function in the Production of PA, in Nuclear/ER Membrane Growth, and Lipid Droplet Formation*—We examined the physiological roles of phosphorylation-deficient (S46A and S45A/S46A) and -mimicking (S46D and S45D/S46D) alleles of *DGK1* by expressing them on a low copy plasmid in *dgk1*Δ or *dgk1*Δ *pah1*Δ cells. The *dgk1*Δ *pah1*Δ mutant was used to assess the function of Dgk1 that is required for the phenotypes imparted by the *pah1*Δ mutation. The *dgk1*Δ mutant expressing the *DGK1* allele was grown to the exponential and stationary phases and was examined for the level of Dgk1 by immunoblot analysis (Fig. 7*A*). ImageQuant analysis of triplicate immunoblot determinations showed that the Dgk1 level was 2.6-fold higher in the exponential phase than in the stationary phase. This indicated that the enzyme level is reduced during growth from the exponential to the stationary phase. However, no significant difference was shown between the levels of wild type Dgk1 and its phosphorylation site mutants.

Immunoblot analysis also showed that wild type Dgk1 from the stationary phase cells migrates as a doublet band on SDS-PAGE, whereas its alanine mutants migrate as a single band at the position corresponding to the faster migrating band of the doublet (Fig. 7*A*). This result suggested that the slower migrating band of Dgk1 represents the phosphorylated form of the protein. The phosphorylation-mediated electrophoretic mobility of Dgk1 was supported by the observation that alkaline

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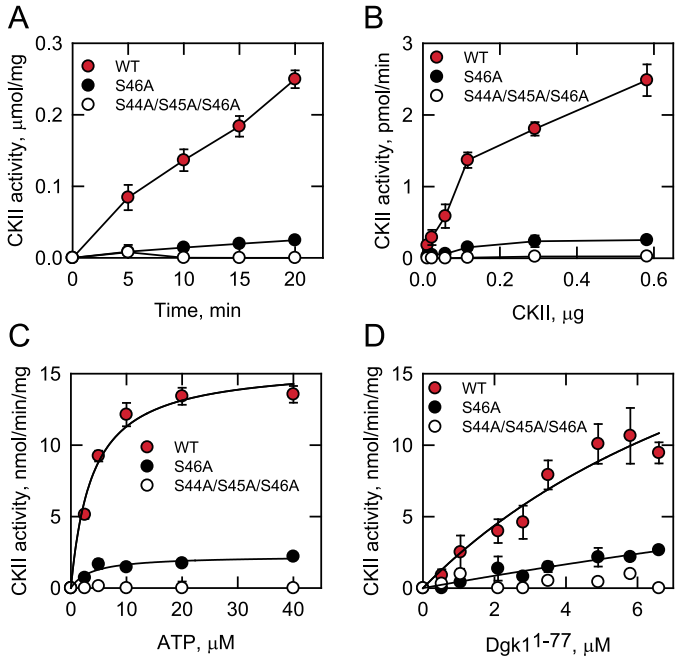


FIGURE 5. **Characterization of CKII activity on wild type or mutant Dgk1(1–77).** Purified wild type or mutant Dgk1(1–77) was incubated with human CKII and [$\gamma$-$^{32}$P]ATP. The enzyme reaction was terminated by spotting the reaction mixture onto a P81 phosphocellulose paper, which was then washed with 75 mM phosphoric acid and subjected to scintillation counting. The kinase reaction was conducted by varying the reaction time (*A*), the amount of human CKII (*B*), and the concentrations of ATP (*C*) and Dgk1(1–77) (*D*). The data shown in *A–D* are the averages of three experiments ± S.D. (*error bars*).

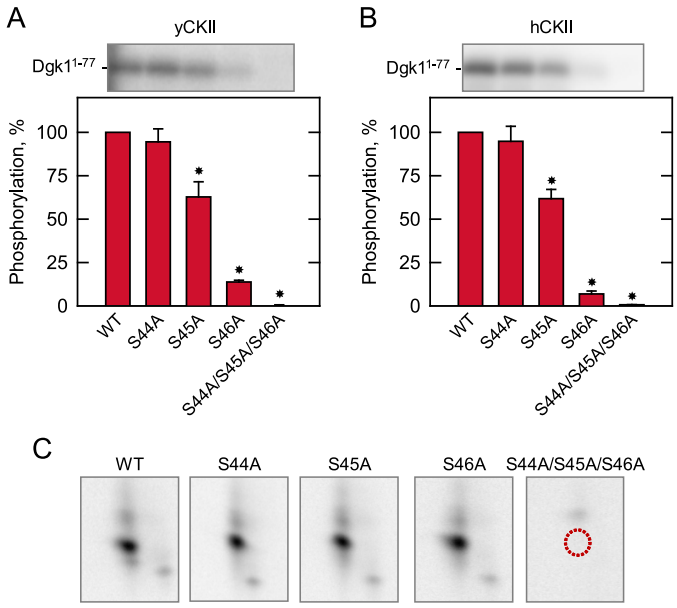


FIGURE 6. **Dgk1(1–77) is phosphorylated by CKII on Ser-45 and Ser-46.** *A* and *B*, purified recombinant Dgk1(1–77) (wild type or the indicated mutant, 0.21 $\mu$g) was incubated for 30 min with yeast CKII (*yCKII*; 0.36 $\mu$g) or human CKII (*hCKII*; 0.04 units) in the presence of [$\gamma$-$^{32}$P]ATP. The reaction mixtures were resolved by SDS-PAGE (18% polyacrylamide gel), transferred to a PVDF membrane, and subjected to phosphorimaging analysis. The radioactive intensities of wild type and mutant Dgk1(1–77) were quantified using ImageQuant software and were normalized to the intensity of the wild type Dgk1(1–77). *C*, the CKII-treated wild type and mutant Dgk1(1–77) in *B* were subjected to phosphopeptide mapping as described in the legend to Fig. 4. For wild type Dgk1 and its single mutants, almost equal amounts of radioactivity were used. The *dotted circle* indicates the position the phosphopeptide that was absent in the S44A/S45A/S46A triple mutant. The data shown are representative of three independent experiments. *, $p < 0.05$ *versus* the wild type.

phosphatase treatment collapsed its slower migrating band into the faster migrating band (Fig. 7*C*).

The yeast cells used for immunoblot analysis were measured for DAG kinase activity. To readily detect the effects of phosphorylation site mutations, the enzyme assay was conducted at the subsaturating concentrations of DAG and CTP (2). For wild type Dgk1, its DAG kinase activity was 52% higher in stationary phase than in the exponential phase (Fig. 7*B*). The growth phase-mediated regulation of DAG kinase activity was eliminated by the S46A and S45A/S46A mutations of the enzyme (Fig. 7*B*). For Dgk1 with the S46D or S45D/S46D mutation, its DAG kinase activity was ~22% higher in the stationary phase than in the exponential phase (Fig. 7*B*). Taken together, these results indicate that phosphorylation of Dgk1 on Ser-45 or Ser-46 by CKII stimulates DAG kinase activity.

Because the elevated PA content in the *pah1*Δ mutant is dependent on Dgk1 DAG kinase activity (1), we examined the effects of the phosphorylation site mutant alleles of *DGK1* on the PA levels in stationary phase *dgk1*Δ *pah1*Δ cells. The mass of PA in the cells was determined by the coupled enzyme assay that produces fluorescent resorufin (67). In *dgk1*Δ *pah1*Δ cells expressing the wild type allele of *DGK1* (*e.g. pah1*Δ mutant background), the level of PA was 3.5-fold greater than that found in the cells expressing *PAH1* (*e.g. dgk1*Δ mutant background) (Fig. 8). The S46A and S45A/S46A mutations in *DGK1* caused reductions in the levels of PA of 2.1- and 2.5-fold when compared with the level of PA found in cells expressing wild type *DGK1* (Fig. 8). The PA level was not significantly affected by the S45D and S45D/S46D mutations in *DGK1*. These data further confirmed that the phosphorylation of Dgk1 by CKII stimulates DAG kinase activity for the regulation of PA production *in vivo*.

The *pah1*Δ mutant exhibits a temperature-sensitive phenotype (3, 17, 68), which reflects the important role of Pah1 PA phosphatase activity in cell physiology. The dependence of this *pah1*Δ phenotype on Dgk1 function was utilized to examine the effects of the phosphorylation-deficient mutations on Dgk1 function (Fig. 9). In this assay, the expression of *PAH1* in the *dgk1*Δ *pah1*Δ mutant permitted its growth at 37 °C (the *dgk1*Δ mutant is not temperature-sensitive at 37 °C (1)), whereas the expression of *DGK1* in the double mutant inhibited its growth at the temperature (Fig. 9). That the expression of the S46A or S45A/S46A allele of *DGK1* permitted better growth at 37 °C indicated that the phosphorylation of Dgk1 on Ser-45 and Ser-46 causes an increase in DAG kinase activity *in vivo*. Like wild type *DGK1*, the expression of the S46D and S45D/S46D phosphorylation-mimicking mutations inhibited growth of *dgk1*Δ *pah1*Δ cells at 37 °C.

Cells lacking Pah1 PA phosphatase activity have irregularly shaped nuclei with the aberrant expansion of the nuclear/ER membrane (17, 18). This phenotype of the *pah1*Δ mutant is dependent on Dgk1 DAG kinase activity (1). To assess the effects of the phosphorylation-deficient mutations on the role of Dgk1 in the nuclear/ER membrane expansion of the *pah1*Δ mutant, we examined the nuclear morphology of the *dgk1*Δ

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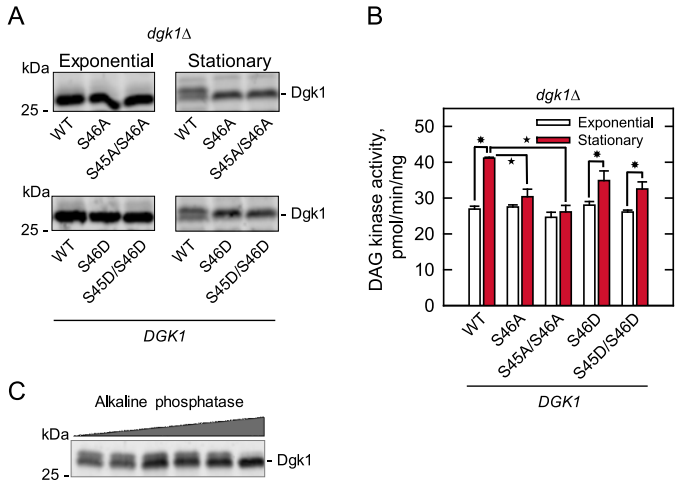


FIGURE 7. ***In vivo* phosphorylation of Dgk1 on the CKII target sites stimulates DAG kinase activity.** *A* and *B*, wild type and the indicated mutant alleles of *DGK1* on a low copy plasmid were transformed into *dgk1*Δ cells. The yeast transformants were grown to the exponential ($A_{600\ nm}$ = 0.6) and stationary ($A_{600\ nm}$ = 4.5) phases, followed by preparation of cell extracts. *A*, 35 $\mu$g of the total membrane fraction prepared from the cell extracts was resolved by SDS-PAGE (12% polyacrylamide gel) and subjected to immunoblot analysis with anti-Dgk1 antibody. *B*, the cell extracts were assayed for DAG kinase activity at subsaturating concentrations of DAG (6.25 mol %) and CTP (0.2 mM). *C*, 0.5 $\mu$g of the total membrane fraction from the *DGK1*-overexpressing cells in the stationary phase was incubated for 10 min with alkaline phosphatase by increasing its amount (0, 0.06, 0.125, 0.25, 0.5, and 1 $\mu$mol/min, indicated by the *gray triangle*). The alkaline phosphatase-treated samples were resolved by SDS-PAGE (12% polyacrylamide gel) and subjected to immunoblot analysis with anti-Dgk1 antibody. The immunoblots in *A* and *C* are representative of three experiments, whereas the data in *B* are means ± S.D. (*error bars*) from triplicate determinations. ✷, $p < 0.05$ *versus* the exponential phase; ⋆, $p < 0.05$ *versus* the wild type stationary phase.

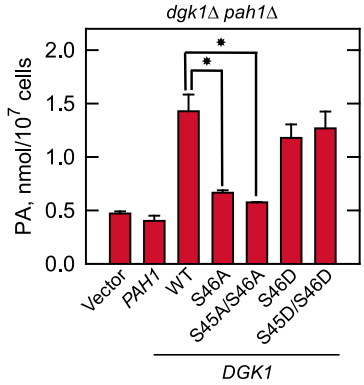


FIGURE 8. **PA level of *dgk1*Δ *pah1*Δ cells expressing Dgk1 with the CKII site mutation.** The *dgk1*Δ *pah1*Δ mutant was transformed with *DGK1*, its phosphorylation site mutant allele, or *PAH1* on a low copy plasmid. The yeast transformants were grown at 30 °C in SC-Ura-Leu medium to the stationary phase; lipids were extracted and digested with lipoprotein lipase, and the formation of PA-derived glycerol-3-phosphate was coupled to the formation of resorufin with glycerol-3-phosphate oxidase and peroxidase using Amplex Red. The data are averages ± S.D. (*error bars*) from quadruplicate determinations. ✷, $p < 0.05$ *versus* wild type *DGK1*.

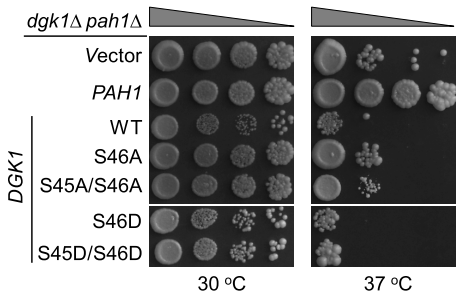


FIGURE 9. **Temperature sensitivity of *dgk1*Δ *pah1*Δ cells expressing Dgk1 with the CKII site mutation.** The *dgk1*Δ *pah1*Δ mutant was transformed with *DGK1*, its phosphorylation site mutant allele, or *PAH1* on a low copy plasmid. The yeast transformants were grown at 30 °C in SC-Ura-Leu medium to the stationary phase; the stationary cultures were serially diluted (5-fold) and spotted onto SC-Ura-Leu agar plates. Cell growth was scored after incubation for 5 days at 30 and 37 °C. The data are representative of three independent experiments.

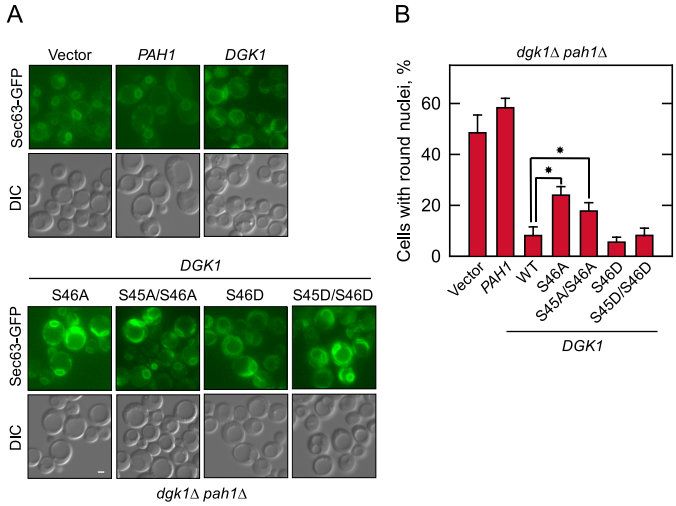


FIGURE 10. **Nuclear/ER membrane morphology of *dgk1*Δ *pah1*Δ cells expressing Dgk1 with the CKII site mutation.** The *dgk1*Δ *pah1*Δ mutant expressing *SEC63-GFP* (to label the nuclear/ER membrane) was transformed with *DGK1*, its phosphorylation site mutant allele, or *PAH1* on a low copy plasmid. The yeast transformants were grown in SC-Ura-Leu medium to the mid-exponential phase. *A*, the fluorescence signal of the Sec63-GFP localization marker was visualized by fluorescence microscopy. The images shown are representative of multiple fields of view from several experiments. *DIC*, differential interference contrast. *White bar*, 1 $\mu$m. *B*, the percentage of cells with round nuclear/ER membrane morphology was calculated from 300–400 cells. The data are averages ± S.D. (*error bars*) from three fields of view. ✷, $p < 0.05$ *versus* wild type *DGK1*.

*pah1*Δ mutant harboring the *DGK1* allele by coexpressing Sec63-GFP (1, 17), a localization marker for the nuclear/ER membrane (Fig. 10*A*). The *dgk1*Δ *pah1*Δ mutant expressing wild type *DGK1* exhibited a 7-fold lower number of cells with round-shaped nuclei than the double mutant expressing *PAH1* (Fig. 10*B*). When compared with wild type *DGK1*, the phosphorylation-deficient alleles (S46A and S45A/S46A) resulted in a 2–3-fold increase in the number of cells with round nuclei (Fig. 10*B*). In contrast, the phosphorylation-mimicking alleles (S45D and S45D/S46D) did not significantly change the number of cells with round nuclei. These results indicate that the phosphorylation-deficient Dgk1 is less functional for the nuclear membrane phenotype of the *pah1*Δ mutant, substantiating the conclusion that the CKII-mediated phosphorylation of Dgk1 increases its activity *in vivo*.

The number of lipid droplets, the organelle that stores TAG and steryl esters (69, 70), is reduced by the *pah1*Δ mutation, and this phenotype is suppressed by the *dgk1*Δ mutation (21, 23). Because the lipid droplet phenotype of the *pah1*Δ mutant is dependent on Dgk1 function (21, 23, 71), we examined the effects of the CKII phosphorylation-deficient mutations on the number of lipid droplets of stationary phase cells by staining with BODIPY 493/503 (Fig. 11*A*). A box plot analysis of the data

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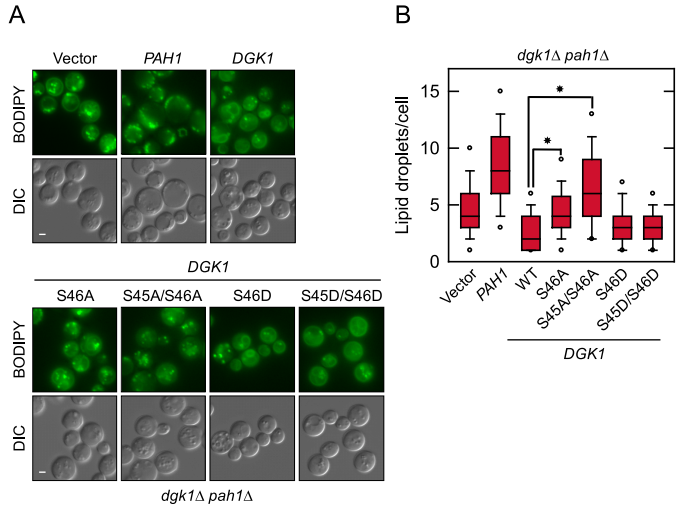


FIGURE 11. **Lipid droplet formation of *dgk1*Δ *pah1*Δ cells expressing Dgk1 with the CKII site mutation.** The *dgk1*Δ *pah1*Δ mutant was transformed with *DGK1*, its phosphorylation site mutant allele, or *PAH1* on a low copy plasmid. The yeast transformants were grown at 30 °C in SC-Ura-Leu medium to the stationary phase and stained with BODIPY 493/503. *A*, the stained lipid droplets were visualized by fluorescence microscopy. The images shown are representative of multiple fields of view from several experiments. *White bar*, 1 μm. *B*, the number of cellular lipid droplets was counted from three fields of view (200–300 cells/field) and presented by a box plot with the 5th and 95th percentile. ✱, $p < 0.05$ *versus* wild type *DGK1*.

(Fig. 11*B*) showed that for the mean values, the number of lipid droplets in the *dgk1*Δ *pah1*Δ cell expressing *DGK1* was 4-fold lower than that of the cell expressing *PAH1*. Compared with wild type *DGK1*, the S46A and S45A/S46A alleles caused a 2- and 3-fold increase, respectively, in the cellular number of lipid droplets. Similar data were obtained when the analysis was performed with mid-exponential phase cells. These results indicate that the lack of the CKII-mediated phosphorylation reduces the function of Dgk1 with respect to lipid droplet formation. The phosphorylation-mimicking mutations did not have a significant effect on Dgk1-mediated regulation of lipid droplet formation in the stationary (Fig. 11) and exponential phases of growth.

*The Dgk1 DAG Kinase-dependent Resumption of Growth from Stationary Phase Is Not Governed by the CKII-mediated Phosphorylation of the Enzyme*—Yeast cells resume vegetative growth from the stationary phase when they are supplemented with fresh nutrients; this physiological process is facilitated by the hydrolysis of the storage lipid TAG to produce DAG and fatty acid, which are both needed for the synthesis of new membrane phospholipids (6, 72, 73). The Dgk1-dependent conversion of DAG to PA plays an important role in phospholipid synthesis, especially when *de novo* fatty acid synthesis is blocked (6). We questioned whether the phosphorylation state of Dgk1 affects its function in the resumption of cell growth from stasis. As described previously (6), the *dgk1*Δ mutant could not resume growth from the stationary phase in the presence of cerulenin, a fatty acid synthesis inhibitor, and the growth defect was complemented by the expression of wild type *DGK1*. Similarly, the growth defect of the *dgk1*Δ mutant was also complemented by the expression of the phosphorylation-deficient (S46A and S45A/S46A) *DGK1* alleles. This result indicates that the phosphorylation of Dgk1 by CKII does not significantly affect the physiological role of the enzyme in growth resumption.

## Discussion

Dgk1 DAG kinase (1, 2), along with Pah1 PA phosphatase (3, 21, 71), plays an important role in controlling the balance of the lipid metabolism intermediates PA and DAG in *S. cerevisiae* (Fig. 1). The importance of maintaining the PA/DAG balance in yeast is typified by phenotypes resulting from mutations in the *PAH1* gene (3, 17, 18, 20, 21, 23, 71, 74–76), and many of the *pah1*Δ phenotypes (*e.g.* increased phospholipid synthesis gene expression, aberrant expansion of the nuclear/ER membrane, decreased lipid droplet formation, and reduced chronological life span) are dependent on Dgk1 DAG kinase activity (1, 21, 23, 27). The lack of *DGK1* does not impart a striking phenotype under standard laboratory culture conditions, but its overexpression causes many of the *pah1*Δ phenotypes that are attributed to an elevated PA content (1). Of the transcriptional (71, 77) and biochemical (49, 78–81) mechanisms that control Pah1 PA phosphatase, phosphorylation/dephosphorylation (19, 44–48, 66, 82) has the greatest impact on the functional role of the enzyme in lipid metabolism. In this work, we showed that Dgk1 DAG kinase, which counteracts the function of Pah1 PA phosphatase in controlling the levels of PA and DAG, is also phosphorylated and regulated by CKII.

Our studies to identify the sites of phosphorylation were facilitated by previous phosphoproteomic studies (59) and bioinformatics (63–65) that led to the hypothesis that the phosphorylation by CKII occurs at the N-terminal region of Dgk1 (Fig. 3). Through mutagenesis and phosphopeptide mapping analyses of Dgk1(1–77), we confirmed that the sites of phosphorylation by CKII occur at Ser-45 and Ser-46, with the latter residue being the major site of phosphorylation. Ser-44, which is predicted to be a CKII target site, was not identified as a phosphorylation site by the experiments performed here.

We observed that *dgk1*Δ cells expressing wild type *DGK1* or its phosphorylation-mimicking allele have higher DAG kinase activity in the stationary phase than in the exponential phase. The increase in DAG kinase activity was not ascribed to a greater abundance of Dgk1. In fact, the amount of Dgk1 was reduced by >2-fold in the stationary phase cells. The reduced level of Dgk1 in the stationary phase is consistent with the decrease in *DGK1* transcript abundance observed in global analyses of gene expression during the diauxic shift of growth (83, 84). The transcription factor Reb1 (85) is required for the maximum expression of *DGK1* (86). However, it is unknown whether the Reb1-mediated *DGK1* expression is regulated as yeast cells progress into the stationary phase. This question, along with the identification of other transcription factors that regulate the *DGK1* expression, will be the subject of future studies.

The finding that the growth phase-mediated regulation of DAG kinase activity was eliminated by the S46A and S45A/S46A mutations in *DGK1* indicated that the CKII phosphorylation of Dgk1 in the stationary phase is responsible for stimulating the activity. Consistent with this finding, the phosphorylation-mimicking mutations had a small, but statistically significant, stimulatory effect on DAG kinase activity in station-

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ary phase cells. These findings are consistent with the stimulatory effect that CKII had on the DAG kinase activity of the detergent-solubilized membrane fraction that had been treated with alkaline phosphatase. The DAG kinase activity in exponential phase cells expressing the wild type or phosphorylation site mutant forms of Dgk1 was essentially the same. This indicated that CKII does not have a major role in regulating DAG kinase activity in exponential phase cells. Dgk1 DAG kinase activity is required for cells to resume logarithmic growth from the stationary phase when fatty acid synthesis is blocked (6). The analysis of the phosphorylation-deficient and -mimicking mutants indicated that the CKII phosphorylation of Dgk1 is not involved in this process.

Three *pah1*Δ phenotypes were scored to assess the physiological effects of the CKII phosphorylation of Dgk1. In these assays, the S46A and S45A/S46A mutations in *DGK1* alleviated the *pah1*Δ phenotypes of temperature sensitivity, nuclear/ER membrane expansion, and reduced lipid droplet formation; the phosphorylation-deficient mutations permitted more growth at 37 °C and resulted in a greater population of cells with rounded nuclei and a population of cells with a greater number of lipid droplets, when compared with cells expressing the wild type *DGK1* gene. Moreover, the alleviation of these *pah1*Δ phenotypes correlated with the reduction in the cellular levels of PA in the cells expressing the phosphorylation-deficient mutations. These data further supported the conclusion that the phosphorylation of Dgk1 by CKII stimulates DAG kinase activity and PA production and that this regulation is relevant *in vivo*.

Yeast DAG kinase utilizes CTP as the phosphate donor and is localized to the ER as an integral membrane protein (1, 2). In contrast, mammalian DAG kinases utilize ATP as the phosphate donor, and their cellular localization is cytosolic in nature (32, 87–90). For catalytic function, the mammalian enzymes associate with the membrane in a peripheral manner that is governed by specific membrane interaction domains (32, 87–90). Despite these differences in the reaction mechanism and the membrane localization for catalytic activity, the yeast and mammalian DAG kinases have the same catalytic function and play important roles in regulating the cellular levels of PA. Moreover, both the yeast (as reported here) and mammalian enzymes are subjected to regulation by phosphorylation. For example, some isoforms (*e.g.* α, γ, θ, and ζ) of mammalian DAG kinase are phosphorylated by protein kinase C (91–94) or by the tyrosine kinase Src (95) to stimulate their activity and function in lipid signaling. Although CKII is evolutionarily conserved (54), it is unclear whether the protein kinase phosphorylates mammalian DAG kinase enzymes. Likewise, although protein kinase C is conserved in yeast (96), it is unknown whether this protein kinase phosphorylates Dgk1. Additional studies are required to address these questions.

CKII, which is essential to yeast growth, regulates diverse proteins involved in gene expression, growth control, signal transduction, and cell cycle progression (55, 97, 98). The findings reported here advance our understanding of the lipid metabolic processes that are regulated by this protein kinase. Interestingly, CKII phosphorylates the enzymes centered on the synthesis (*e.g.* Dgk1 DAG kinase) and degradation (*e.g.* Pah1 PA phosphatase (48)) of PA as well as the PA-regulated repressor Opi1 (58) of the Henry regulatory circuit of phospholipid synthesis (5, 99). CKII also phosphorylates acetyl-CoA carboxylase (100), the enzyme responsible for the first step in the synthesis of fatty acids (101, 102) constituting the hydrophobic moiety of PA and its derivative phospholipids and neutral lipids (5). With respect to Dgk1 and Pah1, which counteract each other in the production of PA and DAG, CKII exerts opposite effects on their catalytic functions; it inhibits PA phosphatase activity of the latter enzyme (48) but stimulates DAG kinase activity of the former enzyme. The CKII-mediated phosphorylations of Dgk1 and Pah1 should be coordinated to balance the levels of PA and DAG. However, the coordinate regulation of the enzyme phosphorylation by CKII is likely to be very complex. For example, the phosphorylation of Pah1 by CKII is inhibited by its prior phosphorylations by protein kinase A and by protein kinase C, and the physiological effects of the CKII-mediated phosphorylation are dependent on the Nem1-Spo7-mediated dephosphorylation of the sites phosphorylated by Pho85-Pho80 (48). With respect to the dephosphorylation of Dgk1, the protein phosphatase involved has yet to be identified. The *in vitro* studies performed here indicated that Nem1-Spo7 has no effect on DAG kinase activity. Phosphoproteomics (59) and bioinformatics (63–65) indicate that Dgk1 is also phosphorylated by other protein kinases (*e.g.* protein kinase A). Thus, additional studies are planned to unravel the complex regulation of Dgk1 by the multiple phosphorylations.

## Experimental Procedures

*Materials*—All chemicals were reagent grade or better. Difco was the source of growth medium constituents. Restriction endonucleases, modifying enzymes, Phusion high fidelity DNA polymerase, human CKII, T4 polynucleotide kinase, and calf alkaline phosphatase were obtained from New England Biolabs. Qiagen was the supplier of the DNA gel extraction kit, plasmid DNA purification kit, and nickel-nitrilotriacetic acid-agarose resin. Clontech was the source of carrier DNA for yeast transformation. Ampicillin, carbenicillin, chloramphenicol, raffinose, PCR primers, cerulenin, nucleotides, nucleoside 5′-diphosphate kinase, Triton X-100, protease inhibitors (phenylmethanesulfonyl fluoride, benzamidine, aprotinin, leupeptin, and pepstatin), 2-mercaptoethanol, bovine serum albumin, phosphoamino acid standards, isopropyl-β-D-1-thiogalactoside, L-1-tosylamido-2-phenylethyl chloromethyl ketone-trypsin, alkaline phosphatase-agarose, glycerol-3-phosphate oxidase (*Aerococcus viridans*), and peroxidase (horseradish) were from Sigma-Aldrich. Lipoprotein lipase (*Pseudomonas* sp.) was from Wako. PerkinElmer Life Science and National Diagnostics were the sources of radiochemicals and scintillation counting supplies, respectively. Lipids were obtained from Avanti Polar Lipids. Silica gel and cellulose TLC plates were from EMD Millipore, and Si250-PA TLC plates were from J. T. Baker. Protein assay reagents, electrophoresis reagents, DNA and protein size standards, and Coomassie Blue R-250 were from Bio-Rad. Invitrogen was the source of $His_6$-tagged tobacco etch virus protease. PVDF membrane, IgG-Sepharose, Sepharose 6B, SP-Sepharose, and the enhanced chemifluorescence Western blotting reagent were purchased from GE Healthcare. Alkaline phosphatase-conjugated goat anti-rabbit IgG antibodies (prod-

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**TABLE 1**
**Stains and plasmids used in this study**

| Strain or plasmid | Relevant characteristics | Source/reference |
|---|---|---|
| **Strain** | | |
| *E. coli* | | |
| DH5α | F$^-$ ϕ80d*lacZ*ΔM15Δ (*lacZYA-argF*)U169 *deoR recA1 endA1 hsdR17* ($r_k^-m_k^+$) *phoA supE44*λ$^-$*thi-1 gyrA96 relA1* | Ref. 105 |
| BL21(DE3)pLysS | F$^-$ *ompT hsdS*$_B$ ($r_B^-m_B^-$) *gal dcm* (DE3) pLysS | Novagen |
| *S. cerevisiae* | | |
| BY4741-*CKA1*-TAP | TAP-tagged Cka1 expressed in strain BY4741 | Thermo Scientific |
| RS453 | *MAT***a** *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-52* | Ref. 124 |
| SS1144 | *dgk1*Δ*::HIS3* derivative of RS453 | Ref. 1 |
| SS1147 | *dgk1*Δ*::HIS3 pah1*Δ*::TRP1* derivative of RS453 | Ref. 1 |
| **Plasmid** | | |
| pET-15b | *E. coli* expression vector with the N-terminal His$_6$ tag fusion | Novagen |
| pGH325 | *DGK1* inserted into pET-15b | This study |
| pYQ5 | *DGK1* (1–231) derivative of pGH325 | This study |
| pYQ5(S44A) | *DGK1* (S44A) derivative of pYQ5 | This study |
| pYQ5(S45A) | *DGK1* (S45A) derivative of pYQ5 | This study |
| pYQ5(S46A) | *DGK1* (S46A) derivative of pYQ5 | This study |
| pYQ5(S44A/S45A/S46A) | *DGK1* (S44A/S45A/S46A) derivative of pYQ5 | This study |
| pRS416 | Low copy *E. coli*/yeast shuttle vector with *URA3* | Ref. 125 |
| pSF211 | *DGK1* inserted into pRS416 | Ref. 6 |
| pSF211(S46A) | *DGK1* (S46A) derivative of pSF211 | This study |
| pSF211(S45A/S46A) | *DGK1* (S45A/S46A) derivative of pSF211 | This study |
| pSF211(S46D) | *DGK1* (S46D) derivative of pSF211 | This study |
| pSF211(S45D/S46D) | *DGK1* (S45D/S46D) derivative of pSF211 | This study |
| pYES2 | Yeast 2μ/*URA3* vector with *GAL1* promoter fusion | Invitrogen |
| YCplac111-PtA-*DGK1* | Protein A-tagged *DGK1* inserted into *CEN/LEU2* vector | S. Siniossoglou |
| pYQ4 | Protein A-tagged *DGK1* inserted into pYES2 | This study |
| YEplac181-*GAL1/10-DGK1* | *DGK1* under control of *GAL1/10* promoter inserted into 2μ/*LEU2* vector | Ref. 1 |
| pGH340 | *PAH1* inserted into pRS416 | This study |
| YCplac111-*SEC63-GFP* | *SEC63-GFP* fusion inserted into the *CEN/LEU2* vector | Ref. 17 |

uct 31340, lot NJ178812), BODIPY 493/503, Triton X-100 Surfact-Amps, Amplex Red, and the *S. cerevisiae* strain expressing TAP-tagged Cka1 were from Thermo Scientific. P81 phosphocellulose paper was from Whatman.

*Strains and Growth Conditions*—Table 1 lists the *E. coli* and *S. cerevisiae* strains used in this study. *E. coli* strains DH5α and BL21(DE3)pLysS were used for the propagation of plasmids and for the expression of wild type and phosphorylation site mutant forms of His$_6$-tagged Dgk1(1–77), respectively. The bacterial cells were grown in LB medium (1% tryptone, 0.5% yeast extract, 1% NaCl, pH 7.4) at 37 °C, and ampicillin (100 μg/ml) was added to select for cells carrying plasmid. For heterologous expression of His$_6$-tagged Dgk1(1–77) and its phosphorylation site mutants, *E. coli* BL21(DE3)pLysS cells bearing pYQ5 and its mutant forms were grown to $A_{600\,nm}$ = 0.5 at 30 °C in LB medium containing carbenicillin (100 μg/ml) and chloramphenicol (34 μg/ml) (103). The culture was incubated for 3 h with 1 mM isopropyl β-D-thiogalactoside to induce the expression. *S. cerevisiae* cells expressing TAP-tagged Cka1 were grown at 30 °C in YEPD medium (1% yeast extract, 2% peptone, and 2% glucose) (104). For selection of *S. cerevisiae* cells bearing plasmids, cells were grown at 30 °C in standard synthetic complete (SC) medium containing 2% glucose with the appropriate amino acids omitted (104). *GAL1/10*-dependent overexpression of Dgk1 (untagged and Protein A-tagged) was performed by changing the carbon source of early log phase cells from 2% raffinose to 2% galactose. Cells were induced with 2% galactose for 12 h. For the measurement of growth on solid medium, the culture in liquid was adjusted to $A_{600\,nm}$ = 0.67, followed by 5-fold serial dilutions. The serially diluted cell suspensions were spotted onto solid medium, and cell growth was scored after incubation for 5 days. The growth regime of Fakas *et al.* (6) was used to examine the effect of the phosphorylation-deficient and -mimicking mutations of Dgk1 on the resumption of growth from stasis. Liquid growth medium was supplemented with agar (2% for yeast or 1.5% for *E. coli*) to prepare solid growth medium.

*Plasmids and DNA Manipulations*—All plasmids used in this study are listed in Table 1. Plasmid pYQ4 directs the *GAL1/10*-induced expression of Protein A-tagged Dgk1 in *S. cerevisiae*. pYQ4 was constructed by fusing Protein A-tagged *DGK1* amplified from YCplac111-PtA-*DGK1* to the *GAL1* promoter in the multicopy plasmid pYES2. YEplac181-*GAL1/10-DGK1* directs the *GAL1/10*-induced expression of Dgk1 (2). Plasmid pGH325 was constructed by insertion of the *DGK1* coding sequence (1) into plasmid pET-15b. Plasmid pYQ5 was constructed by generating a nonsense mutation at the 78th codon of *DGK1* in pGH325, which directs the isopropyl β-D-1-thiogalactopyranoside-induced expression of His$_6$-tagged Dgk1(1–77) in *E. coli*. Plasmid pSF211 directs low copy expression of Dgk1 in *S. cerevisiae* (6). The derivatives of pYQ5 and pSF211 that contain serine-to-alanine/aspartate mutations were constructed by PCR-mediated site-directed mutagenesis using appropriate primers. Plasmids containing multiple missense mutations were constructed by the general strategies described previously (45). Plasmid pGH340 was constructed by inserting the *PAH1* gene (3) into plasmid pRS416. All plasmid constructions were confirmed by DNA sequencing, which was performed by GENEWIZ, Inc. Standard methods were used for the isolation of plasmid and genomic DNA and for the manipulation of DNA using restriction enzymes, DNA ligase, and modifying enzymes (105). PCRs were optimized as described by Innis and Gelfand (106). Plasmid transformations of *E. coli*

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(105) and *S. cerevisiae* (107) were performed as described previously.

*Preparation of Cell Extracts, Total Membrane Fraction, Solubilization of Dgk1, and Protein Determination*—All steps were performed at 4 °C. Cell extracts were prepared by disruption of yeast cells with glass beads (0.5-mm diameter) using a Biospec Products Mini-Beadbeater-16 (108). The cell disruption buffer contained 50 mM Tris-HCl (pH 7.5), 0.3 M sucrose, 10 mM 2-mercaptoethanol, and protease inhibitors (0.5 mM phenylmethanesulfonyl fluoride, 1 mM benzamidine, 5 $\mu$g/ml aprotinin, 5 $\mu$g/ml leupeptin, 5 $\mu$g/ml pepstatin). The total membrane fraction was obtained by centrifugation of the cell extract at 100,000 $\times$ *g* for 70 min (108). Dgk1 was solubilized from the total membrane fraction (3.5 mg/ml protein) with 0.5% Triton X-100 in 20 mM Tris-HCl (pH 7.5), 300 mM KCl, and protease inhibitors. After a 2-h incubation, the solubilized enzyme (supernatant) was obtained by centrifugation at 100,000 $\times$ *g* for 70 min. Protein concentration was estimated by the Coomassie Blue dye-binding method of Bradford (109) using bovine serum albumin as the standard.

*Purification of Yeast CKII, Dgk1(1–77), and Nem1-Spo7 Phosphatase Complex*—CKII (53, 55) was purified from *S. cerevisiae* cells expressing the TAP-tagged Cka1 by IgG-Sepharose affinity chromatography using the procedures described by O'Hara *et al.* (19). The purification of Protein A-tagged Cka1 was confirmed by immunoblot analysis using anti-Protein A antibodies. $His_6$-tagged tobacco etch virus protease was used to remove the Protein A tag from the purified fusion protein, and the protease was removed by nickel-nitrilotriacetic acid-agarose chromatography (49, 110). *E. coli*-expressed $His_6$-tagged wild type and mutant forms of yeast Dgk1(1–77) were purified by affinity chromatography with nickel-nitrilotriacetic acid-agarose according to the procedures described by Han *et al.* (3). The wild type and mutant Dgk1(1–77) proteins were further purified by ion exchange chromatography with SP-Sepharose. The affinity-purified proteins were diluted with 10 volumes of 10 mM Tris-HCl (pH 6.8) to reduce the concentrations of NaCl and imidazole. They were then applied to a 0.5-ml SP-Sepharose column equilibrated with 10 mM Tris-HCl (pH 6.8) buffer. The column was washed with 25 ml of the same buffer containing 100 mM NaCl to remove contaminating proteins. The wild type or mutant Dgk1(1–77) proteins were eluted from the column with the buffer containing 200 mM NaCl. The Protein A-tagged Nem1-Spo7 protein phosphatase complex was isolated from yeast by IgG-Sepharose affinity chromatography (19, 66).

*SDS-PAGE and Immunoblotting*—Proteins were separated by SDS-PAGE (111) using 12 or 18% slab gels. The samples for immunoblotting were normalized to total protein loading, as determined by the Coomassie Blue-based assay of Bradford (109). Immunoblotting with PVDF membrane was performed as described previously (112–114). Ponceau S staining was used to monitor the protein transfer from the polyacrylamide gels to the PVDF membrane. The PVDF membrane blots were probed with anti-Dgk1p antibodies (2) at a concentration of 1 $\mu$g/ml, followed by goat anti-rabbit IgG antibodies conjugated with alkaline phosphatase (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 ensure that the immunoblot signals were in the linear range of detection.

*Measurement of DAG Kinase Activity*—DAG kinase activity was measured by following the incorporation of the $\gamma$-phosphate of water-soluble [$\gamma$-$^{32}$P]CTP (70,000 cpm/nmol) into chloroform-soluble PA as described by Han *et al.* (2). The standard assay contained 50 mM Tris-HCl (pH 7.5), 0.1 mM dioleoyl-DAG, 1 mM Triton X-100, 1 mM CTP, 1 mM $CaCl_2$, 10 mM 2-mercaptoethanol, and enzyme protein in a total volume of 0.1 ml. The [$\gamma$-$^{32}$P]CTP used in the reaction was synthesized enzymatically from CDP and [$\gamma$-$^{32}$P]ATP with nucleoside 5′-diphosphate kinase (115). The enzyme assay was conducted in triplicate at 30 °C and was linear with time and protein concentration. A unit of DAG kinase activity was defined as the amount of enzyme that catalyzed the formation of 1 pmol of product/min.

*Phosphorylation and Dephosphorylation Reactions*—The phosphorylation of wild type and mutant forms of Dgk1(1–77) by yeast or human CKII was routinely measured in triplicate at 30 °C by following the incorporation of radiolabeled phosphate from [$\gamma$-$^{32}$P]ATP into the protein. The reaction mixture contained 20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 50 $\mu$M [$\gamma$-$^{32}$P]ATP (2,500 cpm/pmol), 21 $\mu$g/ml Dgk1(1–77), and the indicated amounts of CKII in a total volume of 20 $\mu$l. The kinase reactions were terminated by the addition of 5$\times$ Laemmli sample buffer (111), subjected to SDS-PAGE to separate $^{32}$P-labeled Dgk1(1–77) from [$\gamma$-$^{32}$P]ATP, and transferred to a PVDF membrane. Radioactively phosphorylated Dgk1(1–77) was visualized by phosphorimaging, and the extent of its phosphorylation was quantified by ImageQuant software. For the reactions to characterize CKII activity using Dgk1(1–77) as substrate, the phosphorylation 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. The phosphorylation reactions were linear with time and protein concentration. One unit of CKII activity was defined as 1 nmol/min.

Overexpressed Dgk1 in total membranes or in a Triton X-100-solubilized membrane extract was subjected to treatment with alkaline phosphatase or alkaline phosphatase-agarose, respectively, at 30 °C. The reaction mixtures contained 20 mM Tris acetate (pH 7.9), 50 mM potassium acetate, 10 mM magnesium acetate, 0.1 mg/ml bovine serum albumin, and the indicated amount of the phosphatase in a final volume of 80 $\mu$l. Following the alkaline phosphatase treatment of membrane-associated Dgk1, samples were subjected to SDS-PAGE and immunoblotting with anti-Dgk1 antibodies. Samples treated with the alkaline phosphatase-agarose were subsequently used for the phosphorylation by CKII. The reaction mixture for treatment with Nem1-Spo7 contained 100 mM sodium acetate (pH 5.0), 10 mM $MgCl_2$, 0.25 mM Triton X-100, and 1 mM dithiothreitol in a final volume of 80 $\mu$l. Following the incubation, the reaction was neutralized before using a sample to measure DAG kinase activity.

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*Analysis of Phosphoamino Acids and Phosphopeptides*—$^{32}$P-labeled Dgk1(1–77) was resolved by SDS-PAGE, transferred to the PVDF membrane, and hydrolyzed with 6 N HCl at 110 °C (for phosphoamino acid analysis) or proteolytically digested with L-1-tosylamido-2-phenylethyl chloromethyl ketone-trypsin (for phosphopeptide mapping analysis) (116–118). The acid hydrolysates were mixed with standard phosphoamino acids and were separated by two-dimensional electrophoresis on cellulose TLC plates, whereas the tryptic digests were separated on the cellulose plates first by electrophoresis and then by TLC (116–118). Radioactive phosphoamino acids and peptides were visualized by phosphorimaging analysis. Non-radioactive phosphoamino acid standards were visualized by ninhydrin staining.

*Analysis of PA*—PA was analyzed by the coupled enzyme assay of Morita *et al.* (67). For this assay, cellular lipids were extracted (119), solubilized with Triton X-100 (Surfact-Amps), and digested with lipoprotein lipase. The formation of PA-derived glycerol-3-phosphate was coupled to the formation of resorufin with glycerol-3-phosphate oxidase to produce hydrogen peroxide, which is reduced by peroxidase using Amplex Red.[7]

*Fluorescence Microscopy*—For nuclear/ER membrane morphology analysis, cells were grown at 30 °C in SC medium lacking leucine and uracil, collected at the mid-exponential phase, and resuspended in a reduced volume of the same medium. The average number of cells with normal nuclear/ER membrane structure (*i.e.* round- to oval-shaped circle) were scored from 300 to 400 cells. For the analysis of lipid droplets, cells were grown in the same medium and collected at the stationary phase, stained for 30 min with 2 $\mu$M BODIPY 493/503, and washed with phosphate-buffered saline (pH 7.4). The number of lipid droplets per cell was scored from three fields of view (200–300 cells/field). In both analyses, fluorescence images were observed under a microscope (Nikon Eclipse Ni-U, Japan) with a long pass green fluorescent protein filter, captured by the DS-Qi2 camera with the imaging software NIS-Elements BR.

*Data Analyses*—SigmaPlot software was used for the statistical analysis of data. The $p$ values $<0.05$ were taken as a significant difference.

*Author Contributions*—Y. Q. and A. H. performed the experiments and prepared the manuscript. G.-S. H. and G. M. C. directed the research and contributed to the preparation of the manuscript. All authors analyzed the results and approved the final version of the manuscript.

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[7] The inherent fluorescent background in the assay was minimized by using highly purified Triton X-100 (Surfact-Amps with reduced levels of $H_2O_2$) to solubilize phospholipids in lipid extracts and by using black 96-well plates for the fluorescent measurements.

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# Phosphorylation of Dgk1 Diacylglycerol Kinase by Casein Kinase II Regulates Phosphatidic Acid Production in *Saccharomyces cerevisiae*

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