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. This work demonstrates that the CKII-mediated phosphorylation of Dgk1 regulates its function in the production of PA.

The yeast Saccharomyces cerevisiae, Dgk1 DAG 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 Mg2+-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 as 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 pah1Δ mutation, those related to the elevated PA content are dependent on Dgk1 DAG kinase activity and are mimicked by the overexpression of Dgk1Δ (1, 3, 6, 18, 21, 23, 25, 27). Consequently, the pah1Δ phenotypes that are conveyed by elevated PA content are suppressed by the dgk1Δ mutation (1, 6, 21, 25, 27).

The roles of DAG kinase and PA phosphatase in lipid metabolism and cell signaling are conserved throughout evolution. 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, rhadamylolysis, 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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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. Ckα1 and Ckα2) and two regulatory (i.e. Ckβ1 and Ckβ2) 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 pah1Δ 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 membrane fractions 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. 2A). 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. 2B). 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. 3A). The truncated form of Dgk1 (Dgk1(1–77)) 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. 3B) was incubated with CKII in the presence of [γ-32P]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. 3C). Phosphoamino acid analysis and phosphopeptide mapping showed that the CKII-treated Dgk1 (1–77) was phosphorylated on the serine residue (Fig. 4A) that is contained within one major phosphopeptide (Fig. 4B).

5 CKII generally phosphorylates proteins with the motif (S/T)XX(X/E) but will also phosphorylate proteins with (S/T)XXE(D) or (S/T)X(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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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 [γ-32P]ATP, and analyzed by SDS-PAGE and phosphorimaging analysis. The S44A mutation had no significant 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 (S44A and S45A/46A) and -mimicking (S46D and S45D/S46D) alleles of DGK1 by expressing them on a low copy plasmid in pah1Δ cells. The S46D pah1Δ mutant was used to assess the function of Dgk1 that is required for the phenotypes imparted by the pah1Δ 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. 7A). 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. 7A). 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 effect on the phosphorylation with respect to time and the amounts of CKII, ATP, and Dgk1(1–77) (Fig. 5).

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phosphorylation site mutants, 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 the stationary phase than in the exponential phase (Fig. 7B). The growth phase-mediated regulation of DAG kinase activity was eliminated by the S46A and S45A/S46A mutations of the enzyme (Fig. 7B). 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. 7B). Taken together, these results indicate that phosphorylation of Dgk1 on Ser-45 or Ser-46 by CKII stimulates DAG kinase activity for the regulation of PA production in vivo.

The pah1Δ 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 pah1Δ 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 dggk1Δ pah1Δ mutant permitted its growth at 37 °C (the dggk1Δ mutant is not temperature-sensitive at 37 °C (1)), whereas the expression of DGGK1 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 S45D and S45D/S46D phosphorylation-mimicking mutations inhibited growth of dggk1Δ pah1Δ 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 pah1Δ 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 pah1Δ mutant, we examined the nuclear morphology of the dggk1Δ

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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 [γ-32P]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 μg) was incubated for 30 min with yeast CKII (γCKII; 0.36 μg) or human CKII (hCKII; 0.04 units) in the presence of [γ-32P]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.
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pah1Δ mutant harboring the DGK1 allele by coexpressing Sec63-GFP (1, 17), a localization marker for the nuclear/ER membrane (Fig. 10A). The dgk1Δ pah1Δ mutant expressing wild type DGK1 exhibited a 7-fold lower number of cells with round-shaped nuclei than the double mutant expressing PAH1 (Fig. 10B). 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. 10B). 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 pah1Δ 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 pah1Δ mutation, and this phenotype is suppressed by the dgk1Δ mutation (21, 23). Because the lipid droplet phenotype of the pah1Δ 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. 11A). A box plot analysis of the data (Fig. 11B). 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 pah1Δ mutant, substantiating the conclusion that the CKII-mediated phosphorylation of Dgk1 increases its activity in vivo.

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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 stationary phase cells expressing wild-type DGK1 or its phosphorylation-mimicking allele. These results suggest that the phosphorylation of DGK1 by CKII is important for the regulation of PA metabolism in yeast, and that the phosphorylation of DGK1 by CKII plays a role in the resumption of cell growth after the yeast cells enter the stationary phase.

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 pah1Δ 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 pah1Δ 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 DGK1Δ 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 Rebl (85) is required for the maximum expression of DGK1 (86). However, it is unknown whether the Rebl-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.

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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, rifampin, PCR primers, cerulenin, nucleotides, nucleoside triphosphate kinase, Triton X-100, protease inhibitors (phenylmethanesulfonyl fluoride, benzamidine, aprotinin, leupeptin, and pepstatin), 2-mercaptoethanol, bovine serum albumin, phosphoamino acid standards, isopropyl-D-1-thiogalactopyranoside, and peroxidase (horseradish) were from Sigma-Aldrich. Lipoprotein lipase (from Pseudomonas sp.) was from Wako. PerkinElmer Life Science and National Diagnostics were the suppliers of radiochemicals and scintillation counting supplies, respectively. Lipids were obtained from Avanti Polar Lipids. Silica gel and cellulose TLC plates were from EMD Milipore, 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 His6-tagged tobacco etch virus protease. PVDF membrane, IgG-Sepharose, Sepharose 6B, SP-Sepharose, and the enhanced chemiluminescence 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− 6880d lacZΔM15A (lacZYA-argF) U169 deoR recA1 endA1 hisD17 (rE− mE+) phoA supE44 thi−1 gyrA96 relA1 | Ref. 105 |
| BL21(DE3)pLysS | F− ompT hsdS (rE− mE+) gal dcm (DE3) pLysS | Novagen |
| S. cerevisiae BY4741-CKA1-TAP | TAP-tagged Cka1 expressed in strain BY4741 | Thermo Scientific |
| RS453 | MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-52 | Ref. 1 |
| SS1144 | dgk1Δ::HIS3 derivative of RS453 | Ref. 1 |
| SS1147 | dgk1Δ::HIS3 pah1Δ::TRPI derivative of RS453 | Ref. 1 |
| **Plasmid** pET-15b | E. coli expression vector with the N-terminal His6 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 Surface-Amps, Ampex 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 His6-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 His6-tagged Dgk1(1–77) and its phosphorylation site mutants, E. coli BL21(DE3)pLysS cells bearing pYQ5 and its mutant forms were grown to A600 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 YEPl 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 A600 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-thiogalactopyranoside-induced expression of His6-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
(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-Beanbeater-16 (108). The cell disruption buffer contained 50 mM Tris-HCl (pH 7.5), 0.3 mM sucrose, 10 mM 2-mercaptoethanol, and protease inhibitors (0.5 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 5 µg/ml aprotinin, 5 µg/ml leupeptin, 5 µg/ml pepstatin). The total membrane fraction was obtained by centrifugation of the cell extract at 100,000 × 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 × 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. His6-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-nitritryptic acid-agarose chromatography (49, 110). E. coli-expressed His6-tagged wild type and mutant forms of yeast Dgk1(1–77) were purified by affinity chromatography with nickel-nitritryptic 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 µg/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 immuno-
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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 1-1-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 μM 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 fluorescence 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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