REGULATION OF THE YEAST *DGK1*-ENCODED DIACYLGLYCEROL KINASE

By

YIXUAN QIU

A dissertation submitted to the
Graduate School-New Brunswick
Rutgers, The State University of New Jersey
In partial fulfillment of the requirements
For the degree of
Doctor of Philosophy
Graduate Program in Food Science
Written under the direction of
George M. Carman
And approved by

________________________________________
________________________________________
________________________________________
________________________________________
________________________________________

New Brunswick, New Jersey
October, 2016
ABSTRACT OF THE DISSERTATION

Regulation of the Yeast DGK1-encoded Diacylglycerol Kinase

By YIXUAN QIU

Dissertation Director:
Dr. George M. Carman

In the yeast *Saccharomyces cerevisiae*, the DGK1-encoded diacylglycerol (DAG) kinase catalyzes the CTP-dependent phosphorylation of DAG to form phosphatidate (PA). This enzyme, in conjunction with PAH1-encoded PA phosphatase, controls the levels of PA and DAG for the synthesis of triacylglycerol (TAG) and membrane phospholipids, nuclear/endoplasmic reticulum (ER) membrane growth, and lipid droplet formation. In this work, we showed that a functional level of DAG kinase is regulated by the Reb1 transcription factor. Purified recombinant Reb1 was shown to specifically bind its consensus recognition sequence (CGGGTAA, -166 to -160) in the DGK1 promoter. Analysis of cells expressing the P_{DGK1}-lacZ reporter gene showed that mutations (GT\rightarrow TG) in the Reb1-binding sequence caused an 8.6-fold reduction in \( \beta \)-galactosidase activity. The expression of DGK1(reb1), a DGK1 allele containing the Reb1-binding site mutation, was greatly lower than that of the wild type allele, as indicated by analyses of DGK1 mRNA, Dgk1, and DAG kinase activity. In the presence of cerulenin, an inhibitor
of de novo fatty acid synthesis, the dgk1Δ mutant expressing DGK1(reb1) exhibited a significant defect in growth as well as in the synthesis of phospholipids from TAG mobilization. Unlike DGK1, the DGK1(reb1) expressed in the dgk1Δ pah1Δ mutant did not result in the nuclear/ER membrane expansion, which occurs in cells lacking PA phosphatase activity. These results indicate that the Reb1-mediated regulation of DAG kinase plays a major role in its in vivo functions in lipid metabolism.

Treatment of membrane-solubilized and overexpressed Dgk1 with alkaline phosphatase caused a 7.7-fold decrease in DAG kinase activity, and the subsequent treatment with CKII caused a 5.5-fold increase in activity. A purified N-terminal fragment of Dgk1 (Dgk11-77) was phosphorylated by CKII on a serine residue in time- and dose-dependent manners, and the phosphorylation was dependent on the concentrations of Dgk11-77 and ATP. Ser-45 and Ser-46 were identified as the major CKII phosphorylation sites. Analysis of yeast expressing the phosphorylation-deficient mutants indicated that the stimulation of DAG kinase activity is attributed to the phosphorylation of Dgk1 by CKII. DAG kinase activity is required for deleterious phenotypes imparted by the pah1Δ mutation. The phosphorylation-deficient mutations inhibited the function that DAG kinase plays for defects in nuclear/ER membrane expansion and lipid droplet formation, as well as the temperature sensitivity caused by the pah1Δ mutation.
ACKNOWLEDGEMENTS

First and foremost, I would like to express my sincere gratitude to my mentor, Dr. George M. Carman, for his guidance, support and encouragement throughout these years. It has been honor and fortune for me to start my scientific research life in his extraordinary laboratory back in 2010. One of the greatest things is that he is always there with his office door widely open for his students. I feel grateful that whenever I need suggestions or inspiration, he would stop his work on hand right away and talk to me. His passion and rigorous attitude to research has been a role model to motivate me to walk firmly on the path of pursuing science. Under his guidance, I have developed the way of critical thinking and gained a wealth of knowledge, which are invaluable.

I also must express my appreciation to my committee members, Dr. Gil-Soo Han, Dr. Judith Storch, Dr. Michael Chikindas, and my oral exam committee member Dr. Loredana Quadro, for their constant support, the inspiring questions, and valuable comments and suggestions for my dissertation.

I am extremely grateful to my favorite Carman laboratory colleagues, who are not only my united research team coworkers, but more as my big family here in the United States. I would not be able to complete my research project and survive the graduate student life without them: Stylianos Fakas, Wen-Min Su, Gil-Soo Han, Yeonhee Park, Lu-Sheng Hsieh, Probuddha Dey, Azam Hassaninasab, Florencia Pascual, Hyeon-Son Choi and Minjung Chae. It has been an enjoyable time with them. Special thanks to Dr. Stylianos Fakas, who patiently taught me multiple laboratory techniques and how to plan and design experiments when I just joined the laboratory and was confused as a fresh
graduate student with the background of food science and engineering; his preliminary work and suggestions to this project are also appreciated. Special thanks also to Dr. Gil-Soo Han, whose critical review and troubleshooting ideas for my research has been always valuable and crucial to push my work forward; I also would like to acknowledge his contribution to my project: the preparation of total membranes from cells expressing overexpressed Dgk1 and the construction of plasmid pGH340. Special thanks also to Dr. Wen-Min Su, who has been willingly sharing her knowledge and experimental skills, and also being my best friend, whom I share laughter and tears with; her contribution to this work is also acknowledged: purifications of Pho85-Pho80 protein kinase complex and Nem1-Spo7 phosphatase complex. I would like to also acknowledge Dr. Antonio Daniel Barbosa and Dr. Symeon Siniossoglou for their contributions to my work: the experiment of fluorescence microscopy to observe the function of Reb1-mediated Dgk1 in nuclear/ER membrane structure and the construction of plasmid YCplac111-PtA-DGK1.

I would like to thank all my other friends in China, South Korea and here in United States, no matter the distance, they have been alongside me to encourage and support, and fill my life with joy.

Finally, I would like to thank my parents for their endless love and support, and my uncle and aunt for their encouragement and being my parents here in the States, and Victor for his love and understanding during my doctoral journey.
# TABLE OF CONTENTS

ABSTRACT OF THE DISSERTATION ................................................................. ii

ACKNOWLEDGEMENTS ................................................................................. iv

TABLE OF CONTENTS .................................................................................. vi

LIST OF TABLES ............................................................................................ ix

LIST OF ILLUSTRATIONS .............................................................................. x

LIST OF ABBREVIATIONS ............................................................................. xiii

INTRODUCTION ............................................................................................ 1

The Synthesis of Phospholipids ................................................................. 1

The Synthesis of TAG .................................................................................. 7

The Mobilization of TAG and the Involved Role of DGK1-encoded DAG Kinase ..................................................................................... 7

DGK1-encoded DAG Kinase and Its Functionally Conserved Homologs in Mammals ................................................................. 12

The Roles of Dgk1 DAG Kinase in the Maintenance of PA and DAG Balance ...................................................................................... 16

DAG Kinase Alleviates DAG Toxicity ...................................................... 18

Effectors of Dgk1 DAG Kinase Activity .................................................. 22

Truncation and Mutation Analyses of DAG Kinase Indicate Possible Regulatory Motifs ................................................................. 24

Genetic and Biochemical Mechanisms that Control Phospholipid Synthesis ................................................................. 25

HYPOTHESES ............................................................................................. 30
EXPERIMENTAL PROCEDURES
Materials
Strains and Growth Conditions
Plasmids and DNA Manipulations
RNA Isolation and Quantitative RT-PCR
Electrophoretic Mobility Shift Assays
Preparation of Cell Extract, Total Membrane Fraction, Solubilization of Dgk1, and Protein Determination
Purification of Recombinant Reb1, Dgk1^{1-77} and Yeast CKII
SDS-PAGE and Immunoblotting
Measurement of DAG Kinase and $\beta$-galactosidase Activity
Phosphorylation and Dephosphorylation Reactions
Analysis of Phosphoamino Acids and Phosphopeptides
Labeling and Analysis of Neutral Lipids and Phospholipids
Fluorescence Microscopy
Data Analysis
RESULTS
Reb1 Interacts with a Reb1-binding Site in the $DGK1$ Promoter
Reb1-binding Site Mutation Attenuates the Expression of $P_{DGK1}$-lacZ Reporter Gene Activity and the Abundance of $DGK1$ mRNA
Reb1-binding Site Mutation Abolishes the $DGK1$-mediated Nuclear/ER Membrane Expansion
Reb1-binding Site Mutation Compromises Growth Resumption from Stationary
Phase in the Presence of Cerulenin

Reb1-binding Site Mutation Attenuates the Expression of $P_{DGK1-lacZ}$ Reporter Gene Activity, Dgk1, and DAG Kinase Activity upon Nutrient Supplementation of Stationary Phase Cells

Reb1-binding Site Mutation Compromises the Mobilization of TAG for Phospholipid Synthesis upon Nutrient Supplementation of Stationary Phase Cells

Five-nucleotides Mutation in the Reb1-binding Site Causes the Same Compromised Growth Resumption from Stationary Phase in the Presence of Cerulenin

Dgk1 Is a Bona Fide Substrate of CKII and Its Phosphorylation Stimulates DAG Kinase Activity

CKII Phosphorylates Dgk1 on Ser-45 and Ser-46

The Stimulation of DAG Kinase Activity by CKII Regulates Its Function in Nuclear/ER Membrane Growth and Lipid Droplet Formation

PKA Phosphorylates Dgk1$^{1-77}$ on A Serine Residue

Ser-25 and Ser-26 in Dgk1$^{1-77}$ Are Phosphorylated by PKA

DAG Kinase Activity Is not Affected by PKA Phosphorylation

DISCUSSION

REFERENCES
LIST OF TABLES

I. Strains used in this study.................................................................34

II. Plasmids used in this study............................................................37

III. Oligonucleotides used in this study..............................................38
LIST OF ILLUSTRATIONS

1. Pathways for the synthesis of phospholipids and TAG in S. cerevisiae………………5
2. The role of yeast DAG kinase in lipid metabolism……………………………………10
3. The structure of Dgk1 DAG kinase…………………………………………………14
4. DAG kinase counterbalances the role that PA phosphatase plays…………………20
5. Interactions of Reb1 with its putative binding site in the DGK1 promoter………50
6. Effect of the Reb1-binding site mutation on P_{DGK1}-lacZ expression and DGK1 mRNA abundance ……………………………………………………………53
7. Effect of the Reb1-binding site mutation on the nuclear/ER membrane structure of cells lacking DGK1 and PAH1…………………………………………………55
8. Effect of the Reb1-binding site mutation on the resumption of cell growth from stasis in the absence of de novo fatty acid synthesis ……………………58
9. Effect of the Reb1-binding site mutation on P_{DGK1}-lacZ expression upon growth resumption from stasis in the absence of de novo fatty acid synthesis………61
10. Effect of the Reb1-binding site mutation on Dgk1 abundance upon growth resumption from stasis in the absence of de novo fatty acid synthesis ………63
11. Effect of the Reb1-binding site mutation on DAG kinase activity upon growth resumption from stasis in the absence of de novo fatty acid synthesis ………65
12. Effect of the Reb1-binding site mutation on the mobilization of TAG for phospholipid synthesis upon growth resumption from stasis in the absence of de novo fatty acid synthesis…………………………………………………………68
13. Effect of five-nucleotides mutation in Reb1-binding site on the interaction with Reb1
and the resumption of cell growth from stasis in the absence of de novo fatty acid synthesis .................................................................71

14. DAG kinase activity is inhibited by dephosphorylation with alkaline phosphatase and stimulated by rephosphorylation with CKII ..................................................74

15. DAG kinase activity is not affected by lambda protein phosphatase, protein phosphatase 1, and Nem1-Spo7 phosphatase complex .........................77

16. Expression and purification of Dgk1\textsuperscript{1-77} from \textit{E. coli} and its phosphorylation by yeast and human CKII ........................................................................79

17. CKII phosphorylates Dgk1\textsuperscript{1-77} on a serine residue ......................................82

18. Characterization of CKII activity using wild type or mutant Dgk1\textsuperscript{1-77} as substrates .................................................................................................84

19. Ser-45 and Ser-46 are CKII phosphorylation sites in Dgk1\textsuperscript{1-77} ......................86

20. Dgk1 is phosphorylated by CKII in the stationary phase causing the stimulation of its DAG kinase activity \textit{in vitro} .................................................................89

21. Phosphorylation does not affect DAG kinase activity when DAG and CTP are saturating .......................................................................................92

22. CKII phosphorylation site mutations in Dgk1 do not affect the resumption of cell growth from stasis in the absence of de novo fatty acid synthesis ..........94

23. Effect of CKII phosphorylation site mutations in Dgk1 on the complementation of the \textit{pah1Δ} temperature-sensitive phenotype .............................................97

24. Effect of CKII phosphorylation site mutations in Dgk1 on the nuclear/ER membrane structure in cells lacking \textit{DGK1} and \textit{PAHI} .................................................100

25. Effect of CKII phosphorylation site mutations in Dgk1 on the neutral lipid and the
major membrane phospholipid composition in cells lacking DGK1 and PAH1

26. Effect of CKII phosphorylation site mutations in Dgk1 on the number of lipid droplets in cells lacking DGK1 and PAH1

27. PKA phosphorylates Dgk11-77 on a serine residue

28. Ser-25 and Ser-26 are PKA phosphorylation sites in Dgk11-77

29. Effect of PKA phosphorylation on DAG kinase activity
LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAG</td>
<td>diacylglycerol</td>
</tr>
<tr>
<td>CTP</td>
<td>cytidine triphosphate</td>
</tr>
<tr>
<td>PA</td>
<td>phosphatidic acid</td>
</tr>
<tr>
<td>TAG</td>
<td>triacylglycerol</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>CKII</td>
<td>casein kinase II</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>PKA</td>
<td>cAMP-dependent protein kinase</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>CDP-DAG</td>
<td>cytidine diphosphate diacylglycerol</td>
</tr>
<tr>
<td>PI</td>
<td>phosphatidylinositol</td>
</tr>
<tr>
<td>CMP</td>
<td>cytidine monophosphate</td>
</tr>
<tr>
<td>PG</td>
<td>phosphatidylglycerol</td>
</tr>
<tr>
<td>CL</td>
<td>cardiolipin</td>
</tr>
<tr>
<td>PGP</td>
<td>phosphatidylglycerophosphate</td>
</tr>
<tr>
<td>PS</td>
<td>phosphatidylserine</td>
</tr>
<tr>
<td>PE</td>
<td>phosphatidylethanolamine</td>
</tr>
</tbody>
</table>
PC  phosphatidylcholine
Gro-3-P  glycerol-3-phosphate
DHAP  dihydroxyacetone phosphate
Glc-6-P  glucose-6-phosphate
Ins-3-P  Inositol-3-phosphate
UAS\textsubscript{INO}  inositol-responsive upstream activating sequence
UAS\textsubscript{ZRE}  zinc-responsive upstream activating sequence
PME  phosphatidylmonomethylethanolamine
PDE  phosphatidyldimethylethanolamine
Etn  ethanolamine
P-Etn  phosphoethanolamine
Cho  choline
P-Cho  phosphocholine
FA  fatty acid
CoA  coenzyme A
kDa  kilo Daltons
EDTA  ethylenediamine tetraacetic acid
EGTA  ethylene glycol bis-(aminoethyl ether) N,N',N'-tetraacetic acid
dCTP  deoxyctydine triphosphate
PKC  protein kinase C
cAMP  cyclic adenosine monophosphate
Reb1  RNA polymerase I enhancer binding protein
TLC  thin layer chromatography
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG</td>
<td>immunoglobulin G</td>
</tr>
<tr>
<td>HA</td>
<td>hemagglutinin</td>
</tr>
<tr>
<td>TAP</td>
<td>tandem affinity purification</td>
</tr>
<tr>
<td>LB</td>
<td>lysogeny broth (Luria-Bertani)</td>
</tr>
<tr>
<td>YEPD</td>
<td>yeast extract peptone dextrose</td>
</tr>
<tr>
<td>SC</td>
<td>synthetic complete</td>
</tr>
<tr>
<td>EMSA</td>
<td>electrophoretic mobility shift assay</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>dTTP</td>
<td>deoxythymidine triphosphate</td>
</tr>
<tr>
<td>PVDF</td>
<td>polyvinylidene difluoride</td>
</tr>
<tr>
<td>cpm</td>
<td>counts per minute</td>
</tr>
<tr>
<td>AP</td>
<td>alkaline phosphatase</td>
</tr>
<tr>
<td>LP</td>
<td>lambda protein phosphatase</td>
</tr>
<tr>
<td>PP1</td>
<td>protein phosphatase 1</td>
</tr>
<tr>
<td>DIC</td>
<td>differential interference contrast</td>
</tr>
<tr>
<td>ErgE</td>
<td>ergosterol ester</td>
</tr>
<tr>
<td>FFA</td>
<td>free fatty acid</td>
</tr>
<tr>
<td>Erg</td>
<td>ergosterol</td>
</tr>
<tr>
<td>PLs</td>
<td>phospholipids</td>
</tr>
</tbody>
</table>
INTRODUCTION

Lipids play a variety of cellular roles, as the principle forms of stored energy in most organisms, the major structural elements of cell membranes, signaling molecules, enzyme cofactors, surfactants, and hormones (1). Abnormal lipid metabolism results in various diseases. Excessive accumulation of TAG in adipose tissue and other organs can lead to obesity, which is associated with type II diabetes, coronary heart disease, hypertension, and certain types of cancers (2). Hence, a well-regulated lipid metabolism is critical for normal cellular physiology.

In our laboratory, we use the unicellular eukaryote yeast *Saccharomyces cerevisiae* to study the regulation of lipid synthesis. Yeast synthesizes membrane phospholipids and TAG via the pathways that are generally typical to those in multicellular higher eukaryotes (3-6). Compared with other eukaryotic organisms, its genetic manipulation is easily accomplished for gene knockout and overexpression. In addition, the availability of the genome database allows for the genomic and proteomic analyses of lipid metabolism. Furthermore, with its short generation time, yeast can be easily grown to a large quantity for the isolation of enzymes for biochemical studies (3, 5-8).

The Synthesis of Phospholipids

Phospholipids are the major building blocks of cellular membranes (9). Cell membranes are composed of lipids and protein molecules by non-covalent interactions
acting as selective barriers to maintain cell integrity, enabling communications between cells or organelles through the transduction of molecular signals, as well as providing a platform for crucial biological reactions taking place (1, 9, 10). Therefore, phospholipids are intimately linked to cellular growth (9). Phospholipids are amphipathic lipids composed of a glycerol backbone, two hydrophobic fatty acyl chains linked to sn-1 and sn-2 positions of the glycerol backbone, and a hydrophilic head group with a charged phosphate group linked to sn-3 position (1, 10, 11). The amphipathic property of phospholipids allows for the formation of phospholipid bilayers in cellular membranes (12). In addition, phospholipids function as lipid signaling molecules, molecular chaperones, and anchors for proteins to associate with cellular membranes (1, 3, 13).

Phospholipid synthesis is a complex process involving numerous branch points and interconnected pathways with the precursor PA, the simplest membrane phospholipid, as the central point to be partitioned between CDP-DAG and DAG (3-5, 14, 15) (Fig. 1). PA is synthesized from the glycolysis product, glycerol-3-phosphate or dihydroxyacetone phosphate, after two acylation reactions catalyzed by SGT1/GPT2-encoded glycerol-3-phosphate acyltransferase and SLC1/ALE1-encoded lysosphospholipid acyltransferase (16-21). In yeast, the CDP-DAG pathway is the primary pathway for phospholipid synthesis. When enzymes in the CDP-DAG pathway are defective, the auxiliary Kennedy pathway becomes essential (4, 5, 14, 22) (Fig. 1).

In the CDP-DAG pathway, PA is converted to the energy-rich intermediate CDP-DAG by the CDS1-encoded CDP-DAG synthase (23, 24). The first branch of the CDP-DAG pathway is involved in the synthesis of PI via the replacement of CMP moiety in CDP-DAG with inositol, being catalyzed by the PIS1-encoded PI synthase (25-28). The
inositol used in this reaction can be obtained from the medium or can be derived from glucose-6-phosphate via two sequential reactions catalyzed by the INO1-encode inositol-3-phosphate synthase and the INM1-encoded inositol-3-phosphate phosphatase respectively (29-31). CDP-DAG is also used for the synthesis of PG and CL, phospholipids confined to inner mitochondrial membranes (5, 32). With the displacement of CMP from CDP-DAG with glycerol-3-phosphate, PGP is synthesized by the PGSI-encoded PGP synthase (33, 34). PGP is then dephosphorylated to form PG by the GEP4-encoded PGP phosphatase (34, 35). Next, PG is used to synthesize CL by the CRD1-encoded CL synthase (9). The CMP moiety of CDP-DAG can also be replaced with serine to form PS, a reaction catalyzed by the CHO1-encoded PS synthase (36-38). PS is next decarboxylated by the PSD1- and PSD2-encoded PS decarboxylase, resulting in PE (39-41). Finally, through three-sequential methylation reactions acting on the amine group of PE, PE is methylated to form PC catalyzed by the CHO2-encoded PE methyltransferase (42, 43) for the first step, and by the OPI3-encoded phospholipid methyltransferase for the next two steps (42, 44).

PC and PE can also be synthesized via the Kennedy pathway, when choline and ethanolamine are supplemented in the growth medium, respectively, or when these precursors are generated through the phospholipase D-mediated turnover of PC and PE, respectively. Choline and ethanolamine are phosphorylated by the CKII-encoded choline kinase (45) and EKII-encoded ethanolamine kinase (46), resulting in the phosphocholine and phosphoethanolamine, respectively. These intermediates are activated by CTP to form CDP-choline and CDP-ethanolamine by the PCTI-encoded phosphocholine cytidylyltransferase and ECTI-encoded phosphoethanolamine cytidylyltransferase,
respectively (9, 47, 48). Finally, CDP-choline and CDP-ethanolamine react with DAG derived from the dephosphorylation of PA by the PAH1-encoded PA phosphatase (9, 49-51) to generate PC and PE by the CPT1-encoded choline phosphotransferase and EPT1-encoded ethanolamine phosphotransferase (52-55), respectively.
FIGURE 1. Pathways for the synthesis of phospholipids and TAG in *S. cerevisiae*. The CDP-DAG pathway, Kennedy pathway, and TAG synthesis pathway are shown. The genes that are known to encode enzymes catalyzing individual steps in the pathways are indicated. UAS\textsubscript{INO}-containing genes are highlighted in *blue*. 
The Synthesis of TAG

TAG is an energy-dense molecule, composed of a glycerol backbone with three fatty acyl chains esterified, stored in lipid droplets, which consist of a lipophilic core enveloped by a phospholipid monolayer (56). In *S. cerevisiae*, the synthesis of TAG, which is derived from PA via DAG, occurs during vegetative growth, and it accumulates in lipid droplets at the stationary phase (49, 57-61). Cells defective in TAG synthesis are not able to survive with excess fatty acid supplementation, that is, the synthesis of TAG plays a role in protecting cells from fatty acid-induced toxicity through channeling the toxic excess free fatty acids into neutral lipids (59, 62, 63).

PA serves as the major precursor in *de novo* TAG synthesis (Fig. 1). PA is dephosphorylated by the *PAH1*-encoded PA phosphatase to form DAG (64, 65), which is then acylated by the *DGA1* - and *LRO1*-encoded acyltransferases to generate TAG (57, 66-68). In addition, this acylation can also be catalyzed by the *ARE1* - and *ARE2*-encoded acyltransferases, which are mainly involved in the synthesis of ergosterol esters (57).

The Mobilization of TAG and the Involved Role of *DGK1*-encoded DAG Kinase

The hydrolysis of TAG is equally important to cell physiology. In the stationary phase, when nutrient depletion occurs, FAs are produced as a result of TAG hydrolysis and subjected to peroxisomal β-oxidation, providing the required energy for cellular maintenance (69, 70). Moreover, rapid breakdown of TAG to generate free FAs for initiation of membrane phospholipid synthesis occurs when cells exit stationary phase and resume vegetative growth, because *de novo* FA synthesis is inadequate to fulfill the
cellular requirement for FAs (49, 57, 71-74). Due to the repression of peroxisomes under these conditions, released FAs from TAG degradation are directed toward making membrane phospholipids meant to be used for membrane synthesis, which is essential for the initiation of cellular growth and division (71). The mobilization of TAG due to transfer to fresh medium occurs during an initial lag phase; when cells enter vegetative growth, initiation of the de novo synthesis of FAs causes lipogenesis to outweigh lipolysis and TAG pools are replenished. Furthermore, TAG provides metabolites for sporulation (75), and cell cycle progression. That is, lipolysis requirement coincides with bud emergence (76).

TAG lipases are required for mobilization of TAG stored in lipid droplets. So far, three TAG lipases have been identified in yeast, encoded by TGL3, TGL4, and TGL5 genes, respectively, and all localized at lipid droplets with certain enzymatic specificities (71-73). Although they have no significant sequence homology to lipases identified from other sources, their deduced sequences contain the consensus sequence motif GXSXG typical for lipolytic enzymes (71-73). Studies showed that mutants (tgl3Δ and tgl4Δ) defective in TAG lipase activity exhibit a severe delay in their ability to resume growth from the stationary phase, indicating the requirement of TAG-derived FAs during the lag period (57, 71). The pathway, of that the incorporation of free FAs liberating from TAG hydrolysis to synthesize membrane phospholipids involves four sequential reactions, TAG → FA → fatty acyl-CoA → lysoPA → PA (Fig. 2). However, the most direct route for membrane phospholipid synthesis upon breakdown of TAG is via the conversion of DAG to PA by DGK1-encoded DAG kinase (77) (Fig. 2). In fact, dgk1Δ mutant cells also are retarded in entering vegetative growth from stationary phase (77). In addition,
when *de novo* FA synthesis is inhibited (Fig. 2), a condition of cellular FAs depletion, *dgk1Δ* mutant cells are unable to resume growth (77). Moreover, in wild type cells, the resumption of growth is accompanied by the induction of DAG kinase activity (77). Therefore, the *DGK1* gene, along with its encoded DAG kinase activity, is essential for growth resumption of static cells when fatty acid synthesis is inhibited (77).
FIGURE 2. The role of yeast DAG kinase in lipid metabolism. The reactions catalyzed by Dgk1 DAG kinase and Pah1 PA phosphatase, which control the relative amounts of PA and DAG, are highlighted by red shading. It also shows an abbreviated pathway for the essential role of Dgk1 DAG kinase in the mobilization of TAG for phospholipid synthesis when de novo fatty acid synthesis is blocked with cerulenin. The fatty acids derived from the hydrolysis of TAG are converted to fatty acyl-CoA molecules before their incorporation into PA (not shown in the figure) via glycerol-3-phosphate and lysoPA.
Cerulenin

Acetyl-CoA → Acyl-CoA → Lyso-PA

TAG

FA

Dagk1

CTP → CDP → P

PA

CDP-DAG Pathway

Pah1

CDP-Cho

CDP-Etn

PC ↔ PE ↔ PS ↔ PI ↔ CL

Membrane Phospholipids

Kennedy Pathway

Cho

P-Cho

Gro-3-P

Ino

Ser

PGP → PG
**DGK1-encoded DAG Kinase and Its Functionally Conserved Homologs in Mammals**

In *S. cerevisiae*, the *DGK1* (formerly known as *HSD1*) gene encoding DAG kinase is an ER-integral enzyme that phosphorylates DAG to yield PA (78). The yeast enzyme utilizes CTP as the phosphate donor (Fig. 3A) unlike the ATP-dependent DAG kinases of other organisms such as bacteria, plants, and mammals (79-83). *DGK1* was originally identified as a high copy number suppressor of the *sly1* temperature-sensitive mutation in ER-Golgi vesicular transport (84). Later, it was shown that the overexpression of *DGK1* suppresses the lethal effect caused by the overexpression of *PAH1-7A* or *NEM1-SPO7* (78). Overexpression of a hyperactive Pah1 mutant (*PAH1-7A*, constitutively dephosphorylated Pah1 generated by mutating its phosphorylation sites) causes the Opi1-mediated repression of phospholipid biosynthetic genes and inositol auxotrophy by possibly decreasing cellular PA through converting it to DAG (85). Similarly, overexpression of the Nem1-Spo7 phosphatase complex, which dephosphorylates Pah1, is also lethal (86). The lethality can be rescued by the overexpression of *DGK1*, presumably because Dgk1 catalyzes the phosphorylation of DAG and restores PA levels (78).

Dgk1 is a 32.8-kDa protein with an N-terminal hydrophilic region (residues 1-77), followed by four putative transmembrane domains containing a predicted cytidylyltransferase domain (residues 76-288) (Fig. 3B). There is no sequence similarity displayed between Dgk1 and DAG kinases found in higher eukaryotes (78). *S. cerevisiae* contains only a single DAG kinase (78), whereas multicellular eukaryotic organisms express more than one, and usually several, DAG kinases that are grouped into subfamilies by common structural elements. So far, ten isoforms (α, β, γ, δ, ε, ζ, η, θ, ι,
and κ) have been identified in mammals, and based on structural motifs, are divided into five subtypes (81, 83, 87-89). More structural diversities are generated by alternative splicing of some DAG kinase isotypes (88). All of the DAG kinases in mammals, as well as in other multicellular eukaryotic organisms, have two common structural features: at least 2 cysteine-rich C1 domains (act as protein-protein interaction sites) and a catalytic domain (composed of accessory and catalytic subunits, each of which has an ATP binding site) (88). Generally, other domains, in addition to C1 and catalytic domains, help regulate the level of DAG kinase activity and/or the localization of the enzyme (88).

In addition to the role in lipid metabolism, mammalian DAG kinases play an important role in cellular signaling by regulating the balance between DAG and PA, the two important lipid signaling molecules. Moreover, by connecting the molecular and cellular properties to physiological functions, DAG kinases are involved in the regulation of immune function, cell proliferation and cancer, brain function, cardiac function, glucose homeostasis and vision (88). Although the significance of the structural difference of DAG kinases in yeast and higher eukaryotes is not yet clear, it could have important physiological implications. Furthermore, though the very different structures, the roles of DAG kinases in lipid metabolism and cell signaling are conserved throughout evolution.
FIGURE 3. **The structure of Dgk1 DAG kinase.** The diagram shows the positions of the N-terminal hydrophilic region, the CTP transferase domain, and the transmembrane-spanning domains.
CTP transferase domain
The Roles of Dgk1 DAG Kinase in the Maintenance of PA and DAG Balance

Dgk1 has been demonstrated to be an important enzyme because its substrate DAG and product PA are key intermediates for the synthesis of membrane phospholipids and the lipid droplet constituent TAG, as described above (7, 49, 57) (Fig. 1).

In addition to their roles in lipid metabolism, PA and DAG are signaling molecules that affect various aspects of cell physiology that include activation of cell growth, membrane proliferation, the transcription of lipid synthesis genes, and vesicular trafficking (80, 90-98). For example, increased PA levels resulting from the lack of Pah1 cause the proliferation of the nuclear/ER membrane (86). High PA levels can have two distinct, signaling-independent/-dependent effects on lipid homeostasis and membrane biogenesis that are responsible for nuclear growth. First, PA plays a non-signaling role, implicated in many remodeling events like membrane budding and fission because of its conical structure. High PA levels cause changes in the physical properties of the double bilayer nuclear membrane and could contribute to its expansion (78). Second, PA acts as a signal that regulates the transcription of key phospholipid biosynthetic genes containing a UAS\textsubscript{INO} element (99). When the levels of PA are high, as in \textit{pah1}\textDelta cells, Opi1 is excluded from the nucleus and tethered to the nuclear/ER membrane through interactions with PA, resulting in derepression of the gene expression (100). In contrast, when the levels of PA are decreased, Opi1 is dissociated from the membrane and translocated into the nucleus where it represses the transcription of UAS\textsubscript{INO}-containing genes by binding to the Ino2 subunit of the Ino2-Ino4 activator complex (99, 100). Moreover, PA and DAG are known to facilitate membrane fission/fusion events in model systems (101-106), and they are also known to interact with and regulate enzymes (e.g., phosphatidylinositol 4-
phosphate kinase, protein kinase C, and protein kinase D) that play important roles in vesicular trafficking (107-111). App1 PA phosphatase, which is located at cortical actin patches (112), may regulate the local concentrations of PA and DAG. It is speculated that App1 PAP activity plays a role in vesicle formation through its recruitment from the cytosol to cortical actin patches via endocytic proteins (113). These proteins may tether App1 PA phosphatase to actin patches and/or serve to regulate the relative amounts of PA and DAG, which in turn contribute to the control of vesicle formation.

By working coordinately, Dgk1 and Pah1 are crucial regulators in controlling the cellular PA/DAG balance, which in turn regulates the synthesis of phospholipids, membrane growth, lipid droplet formation, cell sensitivity to hydrogen peroxide, growth on non-fermentable carbon sources, and the chronological life span (78, 114, 115, 115, 116) (Fig. 4). As mentioned above, cells bearing pah1 mutation have an increased PA content and exhibit an aberrant expansion of the nuclear/ER membrane (64, 65, 86). Moreover, the elevation of PA in pah1 mutant cells is associated with the derepression of phospholipid synthesis gene expression (65, 86). By contrast, the overexpression of PA phosphatase activity causes the loss of PA, the repression of phospholipid synthesis gene expression, and inositol auxotrophy (85). DAG kinase counterbalances the role that PA phosphatase plays in lipid metabolism and cell physiology (78, 114, 116). Though dgk1Δ mutant cells do not exhibit any remarkable phenotypes, DAG kinase activity increased in the pah1Δ mutant when compared with that of wild type cells (78). Furthermore, the overexpression of DAG kinase causes a series of pah1Δ-like phenotypes (i.e., the derepression of phospholipid synthesis genes, the abnormal nuclear/ER membrane expansion, and the temperature sensitivity at 37 °C) (65, 78, 86, 114). Moreover, the
overexpression of DAG kinase activity complements the inositol auxotrophy caused by the overexpression of PA phosphatase activity (78). Similarly, some of the phenotypes induced by the pah1Δ mutation require DAG kinase activity to produce PA, and consequently, its loss complements some pah1Δ phenotypes. The deletion of DGK1 gene in the pah1Δ mutant affects lipid composition displaying the normal level of PA, and the decrease in the level of phospholipids (78). However, the phospholipid levels are still higher than that of wild type cells because of the low level of TAG in the dgk1Δ pah1Δ mutant (78). The aberrant expansion of the nuclear/ER membrane and lipid droplet morphology of the pah1Δ mutant are complemented by the deletion of the DGK1 gene (78). In addition, the defects in chronological life span along with the hypersensitivity to the hydrogen peroxide and growth defect on non-fermentable carbon sources of the pah1Δ mutant are alleviated by the loss of Dgk1 DAG kinase (116).

**DAG Kinase Alleviates DAG Toxicity**

Fakas et al. (77) also indicated that DAG kinase alleviates the toxic effects of DAG. Cells exhibit inhibited growth resumption when dioctanoyl-DAG is supplemented into the growth medium (77). This indicates that the increased DAG level is toxic. In dgk1Δ mutant cells, the deletion of PAH1 or choline supplementation can partially restore the growth resumption (77). It is due to that the loss of PA phosphatase activity reduces DAG production (64, 65), and choline supplementation increases DAG consumption, converting DAG to PC (117). The role of DAG kinase in alleviating the toxic effects of elevated DAG levels was first reported in *E. coli* (118, 119). In addition, this function is supported by the fact that in the dga1Δ mutant cells, the deletion of DGK1 causes a loss-
of-growth phenotype (60). *DGA1*-encoded diacylglycerol acyltransferase consumes DAG for TAG synthesis, and the loss of this enzyme leads to an accumulation of DAG (67). The additional loss of DAG kinase exacerbates the accumulation of DAG. Moreover, the inhibited cell growth induced by overexpression of hyperactive hypophosphorylated PA phosphatase and the *NEM1*-encoded protein phosphatase that dephosphorylates and activates PA phosphatase can be partially restored by the overproduction of DAG kinase (85, 86). This is also due to the alleviation of the toxic levels of DAG.
FIGURE 4. DAG kinase counterbalances the role that PA phosphatase plays. The reactions catalyzed by Pah1 PA phosphatase and Dgk1 DAG kinase controls the balance of PA and DAG. The \textit{pah1}\Delta phenotypes suppressed by the \textit{dgk1}\Delta mutation are listed.
Phenotypes of the pah1Δ mutant

- Nuclear/ER membrane expansion
- Phospholipid synthesis gene expression
- Phospholipid content
- Lipid droplet formation
- Chronological life span
- Sensitivity to hydrogen peroxide
- Growth on non-fermentable carbon source

Suppressed by dgk1Δ

Not suppressed by dgk1Δ

Pah1

Mg2+

P

CDP

OH

Ca2+

CTP

DAG

Ugk1

PA

P

Fi

Suppressed by dgk1Δ

Not suppressed by dgk1Δ
**Effectors of Dgk1 DAG Kinase Activity**

Considering the roles of DAG kinase in the PA/DAG balance, lipid metabolism, and cell growth, to gain an understanding of how *DGK1* expression and DAG kinase activity are regulated is of vital importance. Although the regulation mechanism of DAG kinase in *S. cerevisiae* has barely been studied, Han *et al.* (114) investigated factors that affect the activity of DAG kinase biochemically by using enriched cell membrane fraction containing DAG kinase.

DAG kinase has a pH optimum at 7.0-7.5, requires Ca\(^{2+}\) or Mg\(^{2+}\) ions for activity, and is inhibited by N-ethylmaleimide, which reacts with sulphhydryl groups (114, 120). The addition of the chelating agent EDTA or EGTA, and Mn\(^{2+}\) or Zn\(^{2+}\) ions at 5 mM abolishes DAG kinase activity, but activity is not affected by Na\(^{+}\), K\(^{+}\), or Li\(^{+}\) ions at 100 mM (114). At 30 °C, its activity is observed as maximum, and not active at temperature above 40 °C (114). Reagents R59022 (121) and R59949 (122), inhibitors of activities of ATP-dependent DAG kinases in mammalian cells (100), are not particularly potent inhibitors of this CTP-dependent DAG kinase from *S. cerevisiae* (114). Like most lipid-dependent enzymes that use membrane-associated substrates (123), this DAG kinase enzyme follows surface dilution kinetics using a Triton X-100/DAG-mixed micellar substrate (114).

CTP-dependent DAG kinase utilizes dCTP as a phosphate donor as well (114). Based on its $K_m$ and $K_i$ values as both a substrate and competitive inhibitor, respectively, dCTP is as good a substrate as CTP (114). It is also known that dCTP is used as substrate for several phospholipid biosynthetic enzymes, including CDP-DAG synthase (124), phosphocholine cytidylyltransferase (125-128), and phosophoethanolamine
cytidylyltransferase(126-128). Although the deoxyribonucleotide phospholipid pathway intermediates have been identified in cells, the function for their existence has not been revealed (129). For Dgk1 DAG kinase, the utilization of dCTP may simply indicate that no specificity between CTP and dCTP as the substrate for this enzyme. This lack of specificity may provide an advantage for cells to utilize dCTP when CTP is limiting (114).

DAG kinase activity is stimulated by PC, PE, PI, PS, PG, and PA at various levels (114). The stimulation of DAG kinase activity by major membrane phospholipids may indicate the requirement of a phospholipid activator for this yeast-associated enzyme. The yeast DAG kinase is stable for long-term storage when the enzyme is associated with its native membrane environment. Without a maintained membrane-mimic environment, the solubilization and purification of Dgk1 leads to a great loss of its enzyme activity, which may due to the improperly folded enzyme structure, or the loss of a specific membrane component that is required for enzyme activity (114). The *E. coli* ATP-associated DAG kinase, which is membrane-associated, is also labile upon solubilization and purification (130), and requires phospholipid activators to elicit a maximum turnover number *in vitro* (131, 132). Maintaining activity during the purification of mammalian DAG kinases is not a problem because these enzymes are cytosolic that translocate to the membrane to catalyze their reactions (81-83).

Yeast DAG kinase is inhibited by DAG-pyrophosphate, CL, CDP-DAG, and lyso-PA (114). In particular, DAG kinase activity is sensitive to inhibition by the sphingoid bases, i.e., sphinganine, sphingosine, and phytosphingosine (114). CDP-DAG is converted from PA by the *CDS1*-encoded CTP-dependent CDP-DAG synthase. The
inhibition of DAG kinase activity by CDP-DAG might push the accumulated DAG to be used for the synthesis of PE and PC via Kennedy pathway, or favor the synthesis of TAG (114). Moreover, the accumulated DAG may regulate some other DAG-mediated functions, like signaling or membrane structure in *S. cerevisiae* (114). The inhibition of DAG kinase activity by sphingoid bases also requires further exploration. Sphingoid bases function as both precursors and turnover products in sphingolipid metabolism in *S. cerevisiae* (133). DAG-derived PE synthesis and DAG-involved sphingolipid synthesis through sphingoid base metabolism interact with each other (5). The inhibition of DAG kinase activity by sphingoid bases, leading to the DAG accumulation, might favor the utilization of DAG for other cellular functions as discussed above (114).

**Truncation and Mutation Analyses of DAG Kinase Indicate Possible Regulatory Motif**

Han *et al.* (114) also analyzed the effect of truncation and mutation in Dgk1 on DAG kinase activity by constructing several selected truncations and point mutations in low copy plasmid overexpressing Dgk1. The N-terminal truncated proteins, Δ66 and Δ70, display a sufficient level of DAG kinase activities to be functional *in vivo* (exhibited by the temperature sensitivity and nuclear/ER membrane expansion of cells with their overexpressed forms) (114). Interestingly, the Δ66 truncation form of Dgk1 exhibited a 45% increased enzyme activity compared with the wild type enzyme (114). This suggests that the N-terminal region may contain regulatory sequences. Deletion of the first 70 residues results in a 40% decrease in activity (114). The truncation Δ77, which includes first two residues contained within the CTP transferase domain, shows the
activity at the lower limit of detection (114). DAG kinase activity was also lost with the R76A, K77A (a mutation of a conserved residue found in other fungal homologs of Dgk1), D177A, and G184A mutations (114). The Arg-76, Asp-177, and Gly-184 are conserved residues that are found in the CTP transferase domains of Cds1 CDP-DAG synthase (24) and Sec59 dolichol kinase (134). A previous study on the human dolichol kinase indicates that Arg-76 and Gly-184 are important for CTP binding (135). The conserved amino acid residues within Dgk1 are also essential for CTP-dependent DAG kinase activity and its cellular functions.

**Genetic and Biochemical Mechanisms that Control Phospholipid Synthesis**

The synthesis of phospholipids is regulated by controlling the expression of enzymes and/or by modulating the catalytic activities (136). The regulation mechanisms of other enzymes involved in the phospholipid biosynthesis could provide the directions to explore the mechanism of how DAG kinase is regulated.

**Transcriptional Regulation**

The expression of phospholipid biosynthetic genes is controlled by multiple growth conditions, including growth phase, temperature, pH, and the availability of nutrients such as carbon, nitrogen, zinc, phosphate, and lipid precursors (3, 5, 6, 32, 86, 137-139). The mechanisms include a number of cis- and trans-acting elements (99, 137, 138). Of those, the inositol-responsive cis-acting element (UAS\textsubscript{INO}) and the corresponding trans-acting factors (Ino2, Ino4, Opi1), and zinc-responsive cis-acting
element (UAS\textsubscript{ZRE}) have been found to be the major mechanisms that transcriptionally regulate the phospholipid synthesis genes.

Genes encoding enzymes in the synthesis of PI (e.g., \textit{INO1}), in the CDP-DAG (e.g., \textit{CDS1, CHO1, PSD1, CHO2, and OPI3}) and Kennedy (e.g., \textit{EKII, EPT1, CKII, CPT1}) pathways, and the genes encoding the inositol (\textit{ITR1}) and choline/ethanolamine (\textit{HNMI}) permeases are coordinately regulated through a UAS\textsubscript{INO} element in the promoter (136). Under growth conditions (inositol depletion, zinc supplementation, and exponential phase) whereby the levels of PA are increased, the Opi1 repressor is tethered to the nuclear/ER membrane via interactions with the integral membrane protein Scs2 and PA, allowing the maximal expression of UAS\textsubscript{INO}-containing genes by the Ino2-Ino4 activator complex (99, 100, 136, 137, 140). Under growth conditions whereby the levels of PA are decreased, Opi1 is dissociated from the nuclear/ER membrane and enters into the nucleus, where it binds to Ino2 and attenuates the transcriptional activation by the Ino2-Ino4 complex (136). The PA level in the cell is reduced by the stimulation of PI synthesis in response to inositol supplementation and by the Zap1-mediated induction of \textit{PIS1} that occurs in response to zinc depletion (51, 136, 138, 141-143). The regulations that occur in response to zinc depletion and stationary phase take place in the absence of inositol supplementation (136). Like \textit{PIS1} and \textit{PAH1}, the \textit{CKII} and \textit{EKII} genes also contain a UAS\textsubscript{ZRE} in the promoter that interacts with Zap1 for gene activation (144, 145).

PA phosphatase and DAG kinase work coordinately in the regulation of PA levels, and thereby in the transcriptional regulation of UAS\textsubscript{INO}-containing genes. However, in contrast with phospholipid synthesis genes, the transcriptional regulation of DAG kinase has not been known yet. DAG kinase does not contain UAS\textsubscript{INO} and UAS\textsubscript{ZRE} elements in
the promoter. The mechanism of the transcriptional regulation of DAG kinase needs to be explored.

Regulation by Phosphorylation

Phosphorylation is a major covalent post-translational modification by which the activity of an enzyme or a transcription factor is regulated (146-151). The phosphorylation status (phosphorylated or unphosphorylated/dephosphorylated) of a protein can affect its catalytic activity, subcellular localization, stability, and interaction with DNA or other proteins (152, 153). Global analyses of protein phosphorylation indicates that several enzymes and transporters of glycerolipid metabolism are subject to phosphorylation (6). The identity of the protein kinases involved and the physiological consequences of their phosphorylations have been determined for a few proteins in the phospholipid synthesis (136).

In *S. cerevisiae*, phospholipid biosynthetic enzymes known to be *bona fide* substrates to protein kinases and regulated by phosphorylation include CTP synthase, PS synthase, choline kinase, PA phosphatase, Opi1 and TAG lipase (6). The major responsible protein kinases for regulating the function of these catalytic and regulatory proteins include PKA, PKC, CKII, and cyclin-dependent kinases Cdc28-cyclin B and Pho85-Pho80 (86, 154, 155, 155-165). PKA consists of three catalytic subunits (encoded by *TPK1*, *TPK2*, and *TPK3*) and one regulatory subunit (encoded by *BCY1*). Elevated cAMP levels, which are controlled by *CYRI*-encoded adenylate cyclase via the Ras-cAMP pathway, promote dissociation of the regulatory subunits from the catalytic subunit and thus allow the catalytic subunit to phosphorylate a variety of substrates (136,
PKA is the principle mediator of signals transmitted through the Ras-cAMP pathway (136, 156, 166). Its activity is required for proper regulation of growth, progression through cell cycle, and development in response to various nutrients (136, 156, 166). PKC (encoded by *PKC1*) is a monomeric enzyme required in cell cycle progression and cell wall formation (167, 168). CKII is composed of two catalytic and two regulatory subunits encoded by the *CKA1*, *CKA2*, *CKB1*, and *CKB2* genes, respectively (169-172). Its activity is essential for flocculation, cell cycle progression, cell polarity, and signal transduction (173-175). CKII was found to be associated with the cytoplasm, but when it phosphorylates proteins to control transcription and cell growth, it is primarily associated within the nucleus (162, 173, 176-178). Cdc28 is a master regulator of cell-cycle transitions whose activity is governed by interactions within various G1 and B-type cyclones (136, 179). Pho85, the partner of Pho80 cyclin in Pho85-Pho80 complex, is a multifunctional kinase involved in several signal transduction pathways that affect cell-cycle progression and the metabolism of nutrients (180-182).

In particular, enzyme activities of the CDP-DAG and Kennedy pathways are regulated by phosphorylation (152). CTP synthase is phosphorylated by both PKA and PKC, resulting in an increase in catalytic activity (157-159). PS synthase is phosphorylated by PKA, resulting in a decreased level of its activity, a reduction of PE and PC levels, and an increase of PI levels (183, 184). Opi1 is regulated by PKA and CKII, which stimulate its repressor function, and by PKC attenuating its function (160-162). Choline kinase is phosphorylated by PKA and PKC, resulting in an increase of its catalytic activity (163, 164). PA phosphatase is phosphorylated by Pho85-Pho80 (154), Cdc28-cyclin B (185), PKA (155), PKC (186), and CKII (187) to regulate its activity,
subcellular localization, susceptibility to the 20S proteasomal degradation, or the synthesis of TAG and the number of lipid droplets; PA phosphatase is dephosphorylated by the Nem1-Spo7 phosphatase complex to translocate it from cytosol to ER membrane along with the induced enzyme activity (85, 86, 154, 155, 165).

Phosphoproteomic analyses of *S. cerevisiae* (188-192) by mass spectrometry identified five phosphorylation sites (residue Thr-3, Thr-36, Ser-44, Ser-45, and Ser-46) located in the N-terminal hydrophilic region of Dgk1. Further studies are needed to investigate the phosphorylation of Dgk1 and determine the regulation of DAG kinase activity and its cellular functions by phosphorylation.
HYPOTHESES

Because Dgk1 DAG kinase and Pah1 PA phosphatase play important roles in the maintenance of the ER membrane PA/DAG for both lipid metabolism and cell physiology, the expression of these two enzymes and their activity must be controlled and the mechanisms of regulation need to be elucidated. However, before this study, the regulation mechanism of Dgk1 was poorly understood. Inspection of Dgk1 promoter revealed that it contains the consensus sequence for the binding of a transcription factor called Reb1 (RNA polymerase I enhancer binding protein). We hypothesized that Dgk1 might be regulated by the transcription factor Reb1. Phosphoproteomic analysis (188-192) indicated that Dgk1 is a phosphoprotein, and the N-terminal region contains putative sites of phosphorylation for CKII and PKA. Because it was known that phosphorylation regulates several phospholipid synthesis enzymes (6) including Pah1, which plays the reciprocal role of Dgk1, we hypothesized that DAG kinase is also regulated by phosphorylation.
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, calf alkaline phosphatase, protein phosphatase 1, and lambda protein phosphatase were purchased 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. Genosys Biotechnology, Inc., was the supplier of oligonucleotides used for PCRs and electrophoretic mobility shift assays. Ampicilin, carbenicillin, chloramphenicol, raffinose, PCR primers, cerulenin, nucleotides, IGEPAL CA-630, nucleoside 5’-diphosphate kinase, Triton X-100, protease inhibitors (phenylmethylsulfonyl fluoride, benzamidine, aprotinin, leupeptin, and pepstatin), β-mercaptoethanol, bovine serum albumin, phosphoamino acid standards, isopropyl-β-D-1-thiogalactoside, L-1-tosylamido-2-phenylethyl chloromethyl ketone-trypsin, and alkaline phosphatase-agarose were from Sigma-Aldrich. PerkinElmer 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 JT Baker. Protein assay reagents, electrophoresis reagents, DNA and protein size standards, Coomassie Blue-250, and iScript One-Step RT-PCR kit with SYBR Green were from Bio-Rad. Invitrogen was the source of the Ambion TURBO DNA-free kit and His$_6$-
tagged tobacco etch virus protease. ProbeQuant G-50 micro columns, polyvinylidene difluoride membrane, IgG-Sepharose, Sepharose 6B, SP-Sepharose, and the enhanced chemifluorescence western blot reagent were purchased from GE Healthcare. Roche Applied Science supplied the mouse anti-HA and anti-His\textsubscript{6} antibodies. PKA catalytic subunit and conventional PKC were from Promega. Alkaline phosphatase-conjugated goat anti-rabbit IgG antibodies, alkaline phosphatase-conjugated goat anti-mouse IgG antibodies, BODIPY 493/503, and the \textit{S. cerevisiae} strain expressing TAP-tagged Cka1 were from Thermo Scientific. P81 phosphocellulose paper was from Whatman.

**Strains and Growth Conditions**

Table I lists the \textit{E. coli} and \textit{S. cerevisiae} strains used in this study. \textit{E. coli} strains DH5\textalpha{} and BL21(DE3)pLysS were used for the propagation of plasmids, and for the expression of His\textsubscript{6}-tagged Reb1 and wild type and phosphorylation site mutant forms of His\textsubscript{6}-tagged Dgk1\textsuperscript{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\textsubscript{6}-tagged Reb1, His\textsubscript{6}-tagged Dgk1\textsuperscript{1-77} and its phosphorylation site mutants, \textit{E. coli} BL21(DE3)pLysS cells bearing pYQ3, or pYQ5 and its mutant forms were grown to \textit{A}_{600nm} = 0.5 at 30 °C in LB medium containing carbenicillin (100 µg/ml) and chloramphenicol (34 µg/ml) (193). The culture was then incubated for 3 hours with 0.5 mM isopropyl β-D-thiogalactoside to induce the expression of Reb1, and with 1 mM isopropyl β-D-thiogalactoside to induce the expression of Dgk1\textsuperscript{1-77} and its mutant forms. \textit{S. cerevisiae} cells expressing TAP-tagged Cka1 was grown at 30 °C in YEPD medium (1\%
yeast extract, 2% peptone, and 2% glucose) (194). For selection of *S. cerevisiae* cells bearing plasmids, cells were grown at 30 °C in standard SC medium containing 2% glucose with the appropriate amino acids omitted (194). *S. cerevisiae* mutants *dgk1Δ* and *dgk1Δ pah1Δ* were used to assess the physiological impact of phosphorylation-deficient and -mimicking Dgk1. *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$_{600nm}$=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. Liquid growth medium was supplemented with agar (2% for yeast or 1.5% for *E. coli*) to prepare solid growth medium.

The growth regime of Fakas *et al.* (77) was used to examine the effects of the Reb1-binding site mutation or the phosphorylation-deficient and -mimicking mutations of Dgk1 on the resumption of growth from the stationary phase. Cultures were grown for 48 hours in SC medium to reach stationary phase, harvested by centrifugation, and diluted with fresh SC medium. Cerulenin (10 µg/ml) was added to the cultures to inhibit fatty acid synthesis (195, 196). For growth curves, cultures (200 µl, A$_{650nm}$= 0.1) were incubated in 96-well plates, and the cell density was monitored at A$_{650nm}$ with a Thermomax plate reader. The modified Gompertz equation (197) was used to calculate growth parameters.
### TABLE I. Strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype or relevant characteristics</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E. coli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DH5α</td>
<td>F' φ80dlacZΔM15 Δ (lacZYA-argF)U169 deoR recA1 endA1 hsdR17 (rK− mK+) phoA supE44 thi-1 gyrA96 relA1</td>
<td>(198)</td>
</tr>
<tr>
<td>BL21(DE3)pLysS</td>
<td>F' ompT hsdS(b) (rB− mB−) gal dcm (DE3) pLysS</td>
<td>Novagen</td>
</tr>
<tr>
<td><strong>S. cerevisiae</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RS453</td>
<td>MATα ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-52</td>
<td>(199)</td>
</tr>
<tr>
<td>SS1144</td>
<td>dgk1Δ::HIS3 derivative of RS453</td>
<td>(78)</td>
</tr>
<tr>
<td>SS1147</td>
<td>dgk1Δ::HIS3 pah1Δ::TRP1 derivative of RS453</td>
<td>(78)</td>
</tr>
<tr>
<td>BY4741-CKA1-TAP</td>
<td>TAP-tagged Cka1 expressed in strain BY4741</td>
<td>Thermo Scientific</td>
</tr>
</tbody>
</table>
Plasmids and DNA Manipulations

All plasmids and oligonucleotides used in this study are listed in Table II and III, respectively. Plasmid pSF213, which was derived from plasmid pSF211, contains DGK1 with two transversion mutations in the Reb1-binding site. This plasmid was constructed by PCR-mediated site-directed mutagenesis using appropriate primers. Plasmid pSF211 was eliminated from the reaction by digestion with DpnI. Plasmids pYQ1 and pYQ2 contain the wild type and mutant DGK1 promoters, respectively, fused to the coding sequence of the lacZ gene of *E. coli*. They were constructed by replacing DPP1 promoter in pJO2 (200) with the wild type and mutant DGK1 promoter sequences at the EcoRI site. These DGK1 promoter sequences were obtained by PCR using plasmids pSF211 and pSF213, respectively, as the templates. For expression of Reb1 in *E. coli*, the REB1 coding sequence was amplified by PCR using strain RS453 genomic DNA as the template. The 2,448-bp PCR product was digested with NdeI and XhoI, and the product was ligated into pET-15b at its NdeI/XhoI sites. The resulting plasmid that bears the His6-tagged REB1 was named pYQ3. 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 GAL1/10 promoter in the multicopy plasmid pYES2. YEplac181-GAL1/10-DGK1 directs the GAL1/10-induced expression of Dgk1 (114). Plasmid pGH325 was constructed by the insertion of DGK1 coding sequence (78) into plasmid pET-15b. Plasmid pYQ5 was constructed by generating a nonsense mutation at the 232nd codon of DGK1 in pGH325, which directs the isopropyl β-D-1-thiogalactopyranoside-induced expression of His6-tagged Dgk11-77 in *E. coli*. Plasmid pSF211 directs low copy expression of Dgk1 in *S. cerevisiae* (77). 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 by Choi et al. (185). Plasmid pGH340 was constructed by inserting the PAHI gene (64) into plasmid pRS416. All plasmid constructions were confirmed by DNA sequencing, which was performed by GENEWIS, Inc. Standard methods were used to isolate plasmid and genomic DNA, and for the manipulation of DNA using restriction enzymes, DNA ligase, and modifying enzymes (198). PCRs were optimized as described by Innis and Gelfand (201). Plasmid transformations of E. coli (198) and S. cerevisiae (202) were performed as described previously.
<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Relevant characteristics</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pET-15b</td>
<td><em>E. coli</em> expression vector with the N-terminal His$_6$ tag fusion</td>
<td>Novagen</td>
</tr>
<tr>
<td>pYQ3</td>
<td>REB1 coding sequence inserted into pET-15b</td>
<td>This study</td>
</tr>
<tr>
<td>pGH325</td>
<td>DGK1 inserted into pET-15b</td>
<td>This study</td>
</tr>
<tr>
<td>pYQ5</td>
<td>DGK1 (1-231 truncation) derivative of pGH325</td>
<td>This study</td>
</tr>
<tr>
<td>pYQ5(S25A)</td>
<td>DGK1 (S25A) derivative of pYQ5</td>
<td>This study</td>
</tr>
<tr>
<td>pYQ5(S26A)</td>
<td>DGK1 (S26A) derivative of pYQ5</td>
<td>This study</td>
</tr>
<tr>
<td>pYQ5(S28A)</td>
<td>DGK1 (S28A) derivative of pYQ5</td>
<td>This study</td>
</tr>
<tr>
<td>pYQ5(S44A)</td>
<td>DGK1 (S44A) derivative of pYQ5</td>
<td>This study</td>
</tr>
<tr>
<td>pYQ5(S45A)</td>
<td>DGK1 (S45A) derivative of pYQ5</td>
<td>This study</td>
</tr>
<tr>
<td>pYQ5(S46A)</td>
<td>DGK1 (S46A) derivative of pYQ5</td>
<td>This study</td>
</tr>
<tr>
<td>pYQ5(S25A/S26A/S28A)</td>
<td>DGK1 (S25A/S26A/S28A) derivative of pYQ5</td>
<td>This study</td>
</tr>
<tr>
<td>pYQ5(S44A/S45A/S46A)</td>
<td>DGK1 (S44A/S45A/S46A) derivative of pYQ5</td>
<td>This study</td>
</tr>
<tr>
<td>pYQ5(S25A/S26A/S44A/S45A/S46A)</td>
<td>DGK1 (S25A/S26A/S44A/S45A/S46A) derivative of pYQ5</td>
<td>This study</td>
</tr>
<tr>
<td>pRS416</td>
<td>Low copy <em>E. coli</em>/yeast shuttle vector with URA3</td>
<td>(203)</td>
</tr>
<tr>
<td>pSF211</td>
<td>DGK1 inserted into pRS416</td>
<td>(77)</td>
</tr>
<tr>
<td>pSF213</td>
<td>Derivative of pSF211 with GT → TG mutations in the Reb1 binding site</td>
<td>This study</td>
</tr>
<tr>
<td>pSF214</td>
<td>Derivative of pSF211 with CGGGT → AAAAA mutations in the Reb1 binding site</td>
<td>This study</td>
</tr>
<tr>
<td>pJO2</td>
<td>P$_{DPP1}$-lacZ reporter gene with URA3</td>
<td>(200)</td>
</tr>
<tr>
<td>pYQ1</td>
<td>P$_{DGK1}$-lacZ reporter gene with URA3</td>
<td>This study</td>
</tr>
<tr>
<td>pYQ2</td>
<td>Derivative of pYQ1 with GT → TG mutations in the Reb1p binding site</td>
<td>This study</td>
</tr>
<tr>
<td>YCplac33-SEC63-GFP</td>
<td>SEC63-GFP fusion into the CEN/URA3 vector</td>
<td>(78)</td>
</tr>
<tr>
<td>YCplac33-PAH1</td>
<td>PAH1 into the CEN/URA3 vector</td>
<td>(86)</td>
</tr>
<tr>
<td>pSF211(S46A)</td>
<td>DGK1 (S46A) derivative of pSF211</td>
<td>This study</td>
</tr>
<tr>
<td>pSF211(S45A/S46A)</td>
<td>DGK1 (S45A/S46A) derivative of pSF211</td>
<td>This study</td>
</tr>
<tr>
<td>pSF211(S46D)</td>
<td>DGK1 (S46D) derivative of pSF211</td>
<td>This study</td>
</tr>
<tr>
<td>pSF211(S45D/S46D)</td>
<td>DGK1 (S45D/S46D) derivative of pSF211</td>
<td>This study</td>
</tr>
<tr>
<td>pYES2</td>
<td>Yeast 2µ/URA3 vector with GAL1/10 promoter fusion</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>YCplac111-PtA-DGK1</td>
<td>Protein A-tagged DGK1 inserted into CEN/LEU2 vector</td>
<td>S. Siniossoglou</td>
</tr>
<tr>
<td>pYQ4</td>
<td>Protein A-tagged DGK1 inserted into pYES2</td>
<td>This study</td>
</tr>
<tr>
<td>YEplac181-GAL1/10-DGK1</td>
<td>DGK1 under control of GAL1/10 promoter inserted into 2µ/LEU2 vector</td>
<td>(78)</td>
</tr>
<tr>
<td>pGH340</td>
<td>PAH1 inserted into pRS416</td>
<td>This study</td>
</tr>
<tr>
<td>YCplac111-SEC63-GFP</td>
<td>SEC63-GFP fusion into the CEN/LEU2 vector</td>
<td>(86)</td>
</tr>
</tbody>
</table>
### TABLE III. Oligonucleotides used in this study

<table>
<thead>
<tr>
<th>Oligonucleotides</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR</td>
<td></td>
</tr>
<tr>
<td>pSF213-F</td>
<td>5’-ATCCAGGGTGCTCACAGGAGGACAAAATTATTGGTT-3’</td>
</tr>
<tr>
<td>pSF213-R</td>
<td>5’-AACCAATAATTTGTTCACCGCTATGGAACCTGGAT-3’</td>
</tr>
<tr>
<td>pSF214-F</td>
<td>5’-ATCCAGGGTGCTCACAGGAGAAGAAATTATTGGTT-3’</td>
</tr>
<tr>
<td>pSF214-R</td>
<td>5’-AACCAATAATTTGTTCACAGGAGGACAAAATTATTGGTT-3’</td>
</tr>
<tr>
<td>$P_DGK1^+$-F</td>
<td>5’-GAGCTCGAGAATTCTCGTTTACCAACTGAA-3’</td>
</tr>
<tr>
<td>$P_DGK1^+$-R</td>
<td>5’-GAGCTCGAGAATTCTCGTTTACCAACTGAA-3’</td>
</tr>
<tr>
<td>REB1-F</td>
<td>5’-CAGCATTATGCTCTTACGGTATAACGATTTA-3’</td>
</tr>
<tr>
<td>REB1-R</td>
<td>5’-GCCGGGATCTCGAGAATTCTCTCTTTACATTGA-3’</td>
</tr>
<tr>
<td>pYQ5-F</td>
<td>5’-CuTACGGGGGAATTTTCTGTATTTTTA-3’</td>
</tr>
<tr>
<td>pYQ5-R</td>
<td>5’-TAACTCGAGGATCCCGCGCTTA-3’</td>
</tr>
<tr>
<td>S25A-F</td>
<td>5’-AAAGCAAGACTAGCTTCTCTAGAAGTCATCAAGTC-3’</td>
</tr>
<tr>
<td>S25A-R</td>
<td>5’-CAAAGCAAGACTAGCTTCTCTAGAAGTCATCAAGTC-3’</td>
</tr>
<tr>
<td>S26A-F</td>
<td>5’-CTATCATCTCTAGCTACAGATCTACGCGAAC-3’</td>
</tr>
<tr>
<td>S26A-R</td>
<td>5’-CTATCATCTCTAGCTACAGATCTACGCGAAC-3’</td>
</tr>
<tr>
<td>S25A/S26A-F</td>
<td>5’-AAAGCAAGACTAGCTCAGCTACAGATCTACGCGAAC-3’</td>
</tr>
<tr>
<td>S25A/S26A-R</td>
<td>5’-AAAGCAAGACTAGCTCAGCTACAGATCTACGCGAAC-3’</td>
</tr>
<tr>
<td>S25A/S26A/S28A-F</td>
<td>5’-AAAGCAAGACTAGCTCAGCTACAGATCTACGCGAAC-3’</td>
</tr>
<tr>
<td>S25A/S26A/S28A-R</td>
<td>5’-AAAGCAAGACTAGCTCAGCTACAGATCTACGCGAAC-3’</td>
</tr>
<tr>
<td>S44A-F</td>
<td>5’-AAAGAAGAAATTGTCTAGTCTAGGATGACCGATGCA-3’</td>
</tr>
<tr>
<td>S44A-R</td>
<td>5’-AAAGAAGAAATTGTCTAGTCTAGGATGACCGATGCA-3’</td>
</tr>
<tr>
<td>S45A-F</td>
<td>5’-GAAATTAGTCTAGTCTAGGATGACCGATGCAACCGTT-3’</td>
</tr>
<tr>
<td>S45A-R</td>
<td>5’-GAAATTAGTCTAGTCTAGGATGACCGATGCAACCGTT-3’</td>
</tr>
<tr>
<td>S45A/S46A-F</td>
<td>5’-GAAATTAGTCTAGTCTAGGATGACCGATGCAACCGTT-3’</td>
</tr>
<tr>
<td>S45A/S46A-R</td>
<td>5’-GAAATTAGTCTAGTCTAGGATGACCGATGCAACCGTT-3’</td>
</tr>
<tr>
<td>S44A/S45A/S46A-F</td>
<td>5’-GAAATTAGTCTAGTCTAGGATGACCGATGCAACCGTT-3’</td>
</tr>
<tr>
<td>S44A/S45A/S46A-R</td>
<td>5’-GAAATTAGTCTAGTCTAGGATGACCGATGCAACCGTT-3’</td>
</tr>
<tr>
<td>S46D-F</td>
<td>5’-GAAATTAGTCTAGTCTAGGATGACCGATGCAACCGTT-3’</td>
</tr>
<tr>
<td>S46D-R</td>
<td>5’-GAAATTAGTCTAGTCTAGGATGACCGATGCAACCGTT-3’</td>
</tr>
<tr>
<td>S45D/S46D-F</td>
<td>5’-GAAATTAGTCTAGTCTAGGATGACCGATGCAACCGTT-3’</td>
</tr>
<tr>
<td>S45D/S46D-R</td>
<td>5’-GAAATTAGTCTAGTCTAGGATGACCGATGCAACCGTT-3’</td>
</tr>
<tr>
<td>S44D/S45D/S46D-F</td>
<td>5’-GAAATTAGTCTAGTCTAGGATGACCGATGCAACCGTT-3’</td>
</tr>
<tr>
<td>S44D/S45D/S46D-R</td>
<td>5’-GAAATTAGTCTAGTCTAGGATGACCGATGCAACCGTT-3’</td>
</tr>
<tr>
<td>PtA-DGK1-F</td>
<td>5’-CCGAAGCTTTAATGCTCAGATCCTGTT-3’</td>
</tr>
<tr>
<td>PtA-DGK1-R</td>
<td>5’-CCGAAGCTTTAATGCTCAGATCCTGTT-3’</td>
</tr>
</tbody>
</table>
# Table III. Oligonucleotides used in this study

<table>
<thead>
<tr>
<th>Oligonucleotides</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>RT-PCR</strong></td>
<td></td>
</tr>
<tr>
<td><em>DGK1</em>-F</td>
<td>5’-CACCCAAAGTGGCAAGAAAT-3’</td>
</tr>
<tr>
<td><em>DGK1</em>-R</td>
<td>5’-AAGCAGCTACCACACCACCT-3’</td>
</tr>
<tr>
<td><strong>EMSA</strong></td>
<td></td>
</tr>
<tr>
<td>WT-F</td>
<td>5’-AGGGTCCATAGCGGGTAACAAATTATTGG-3’</td>
</tr>
<tr>
<td>WT-R</td>
<td>5’-CCAATAATTTGTTACCCGCTATGGACCCT-3’</td>
</tr>
<tr>
<td>Mutant1-F</td>
<td>5’-AGGGTCCATAGCGGAACAAATTATTGG-3’</td>
</tr>
<tr>
<td>Mutant1-R</td>
<td>5’-CCAATAATTTGTTACCCGCTATGGACCCT-3’</td>
</tr>
<tr>
<td>Mutant2-F</td>
<td>5’-AGGGTCCATAGAAAAAAACAAATTATTGG-3’</td>
</tr>
<tr>
<td>Mutant2-R</td>
<td>5’-CCAATAATTTGTTTTTTCTATGGACCCT-3’</td>
</tr>
</tbody>
</table>
RNA Isolation and Quantitative RT-PCR

Total RNA was isolated with hot phenol (204-206) and treated with the Ambion TURBO DNA-free kit to remove DNA contamination. DGK1 cDNA was synthesized and amplified on a Bio-Rad MyiQ single-color real time PCR detection system using the iScript one-step RT-PCR kit with SYBR Green and DGK1 primers. Quantification of each measurement was determined from a standard curve generated by PCR amplification run simultaneously with the RT-PCRs from plasmid pSF211 of known copy number. Each sample was run in triplicate, and PCR efficacy was 80-90%. Reactions without reverse transcriptase were included as a control for DNA contamination.

Electrophoretic Mobility Shift Assays

Double-stranded oligonucleotides for the wild type and mutant sequences for Reb1 binding were prepared (Table III), labeled with $[\alpha^{32}P]$ dTTP (400-800 Ci/mmol) and Klenow fragment (5 units), and then purified by gel filtration using ProbeQuant G-50 spin columns as described previously (145). The radiolabeled DNA probe (4 pmol, $8.0 \times 10^4$ cpm/pmol) and purified recombinant His$_6$-tagged Reb1 were mixed in a total reaction volume of 10 µl and were incubated for 15 min at room temperature. The reaction buffer contained 10 mM Tris-HCl (pH 8.0), 10 mM MgCl$_2$, 50 mM KCl, 1 mM dithiothreitol, 0.025 mg/ml poly(dI-dC)•poly(dI-dC), 0.2 mg/ml bovine serum albumin, 0.04% IGEPAL CA-630, and 10% glycerol. Following incubation, the reaction mixture was resolved for 45 min at 100 V on a 5% polyacrylamide gel (1.5-mm thickness) in 0.5 ×
Tris borate/EDTA buffer. Gels were dried onto a filter paper, and the radioactive signals were visualized by phosphorimaging analysis.

**Preparation of Cell Extract, 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 (207). 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 phenylmethylsulfonyl fluoride, 1 mM benzamidine, 5 µg/ml aprotinin, 5 µg/ml leupeptin, and 5 µg/ml pepstatin). The total membrane fraction was obtained by centrifugation of the cell extract at 100,000 × g for 70 min (207). 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 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 (208) using bovine serum albumin as the standard.

**Purification of Recombinant Reb1, Dgk1<sup>1-77</sup> and Yeast CKII**

All steps were performed at 4 °C. *E. coli*-expressed His<sub>6</sub>-tagged Reb1, wild type and mutant forms of yeast Dgk1<sup>1-77</sup> were disrupted by French press in breaking buffer containing 20 mM Tris-HCl (pH 7.5), 0.5 M NaCl, 5 mM imidazole, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor tablets. The Cell lysate was
centrifuged at 12,000 × g for 30 min, and the supernatant was subjected for purification to near homogeneity by affinity chromatography using nickel-nitrilotriacetic acid-agarose according to the procedure described by Han et al. (209). As described previously (210), the recombinant Reb1 with the predicted molecular mass of 92 kDa migrated on an 8% SDS-polyacrylamide gel as a 127-kDa protein (Fig. 5). The wild type and mutant Dgk11-77 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 Dgk11-77 proteins were eluted from the column with buffer containing 200 mM NaCl. The His6-tagged Pho85-Pho80 protein kinase complex expressed in *E. coli* was purified by nickel-nitrilotriacetic acid-agarose affinity chromatography as described by Jeffery et al. (211).

CKII (172, 173) 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. (85). 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-nitrilotriacetic acid-agarose chromatography (212, 213). The protein A-tagged Nem1-Spo7 complex was purified from *S. cerevisiae* by IgG-Sepharose affinity chromatography as described Siniossoglou et al. (214) and Su et al. (215).
**SDS-PAGE and Immunoblotting**

Proteins were separated by SDS-PAGE (216) using 8%, 12% or 18% slab gels. Proteins in polyacrylamide gels were visualized by staining with Coomassie Blue R-250. The samples for immunoblotting were normalized to total protein loading as determined by the Coomassie Blue-based assay of Bradford (208). Immunoblotting with PVDF membrane was performed as described previously (217-219). 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-Dgk1 antibodies (114) or with anti-Dpp1 antiserum (220) at a concentration of 1 µg/ml and a dilution of 1:1000, respectively, 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 and β-galactosidase Activities**

DAG kinase activity was measured by following the incorporation of the γ-phosphate of water-soluble [γ-\(^{32}\)P] CTP (70,000 cpm/nmol) into chloroform-soluble PA as described previously (114). The reaction mixture 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 [γ-\(^{32}\)P] CTP used in the reaction was synthesized enzymatically from CDP and [γ-\(^{32}\)P] ATP with nucleoside
5’-diphosphate kinase (221). β-galactosidase activity was measured by following the formation of O-nitrophenyl from O-nitrophenyl β-D-galactopyranoside at A_{410nm} (222). The reaction mixture contained 100 mM sodium phosphate (pH 7.0), 3 mM O-nitrophenyl β-D-galactopyranoside, 1mM MgCl₂, 100 mM 2-mercaptoethanol, and enzyme protein in a total volume of 0.1 ml. All enzyme assays were conducted in triplicate at 30 °C. The enzyme assays were linear with time and protein concentration. The units of DAG kinase and β-galactosidase activities were defined as the amount of enzymes that catalyzed the formation of 1 pmol of product/min and 1 nmol of product/min, respectively.

**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 [γ-³²P]ATP into the protein. The reaction mixture contained 20 mM Tris-HCl (pH 7.5), 2 mM dithiothreitol, 10 mM MgCl₂, 0.1 mM EDTA, 0.01% Brij 35, 50 μM [γ-³²P]ATP (2,500 cpm/pmol), 21 μg/ml Dgk1^{1-77}, and the indicated amounts of CKII in a total volume of 20 μl. The phosphorylation reaction mixture for PKA contained 50 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 100 μM dithiothreitol, 50 μM [γ-³²P]ATP (3000 cpm/pmol), 21 μg/ml Dgk1^{1-77}, and the indicated amounts of PKA (223) in a total volume of 20 μl. The PKC reaction mixture contained 50 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 10 mM β-mercaptoethanol, 1.7 mM CaCl₂, 500 μM PS, 156 μM DAG, 50 μM [γ-³²P]ATP (3000 cpm/pmol), 21 μg/ml Dgk1^{1-77}, and the indicated amounts of PKC (223). The reaction mixture for Pho85-Pho80 contained 25 mM Tris-
HCl (pH 7.5), 10 mM MgCl₂, 100 μM dithiothreitol, 100 μM [γ-³²P]ATP (3000 cpm/pmol), 21 μg/ml Dgk1¹⁻⁷⁷, and 40 μg/ml purified recombinant Pho85-Pho80 (154). Reactions were terminated by the addition of 5x Laemmli sample buffer (216), subjected to SDS-PAGE to separate ³²P-labeled Dgk1¹⁻⁷⁷ and [γ-³²P]ATP, and transferred to PVDF membrane. Phosphorylated Dgk1¹⁻⁷⁷ was visualized by phosphorimaging, and the extent of phosphorylation was quantified by ImageQuant software. For the reactions to characterize CKII activity using Dgk1¹⁻⁷⁷ as substrate, the phosphorylation was terminated by spotting the reaction mixtures onto P81 phosphocellulose paper. The papers were 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. One unit of PKA activity was defined as 1 pmol/min. One unit of PKC activity was defined as 1 pmol/min.

Overexpressed Dgk1 in total membranes or in a Triton X-100-solubilized membrane extract were subjected to treatment with calf 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 amounts of the phosphatase in a final volume of 80 μl. Following the alkaline phosphatase treatment of membrane-associated Dgk1, samples were subjected to SDS-PAGE and immunoblotting with anti-Dgk1 antibodies. For the reaction containing Triton X-100-solubilized Dgk1 and alkaline phosphatase-agarose, the insoluble alkaline phosphatase-agarose was removed from the phosphatase reaction for the subsequent measurement of DAG kinase activity (alkaline phosphatase interferes with the DAG kinase assay because it dephosphorylates the substrate CTP).
The Samples treated with the alkaline phosphatase-agarose were subsequently used for the phosphorylation by CKII or PKA. Overexpressed Dgk1 in a Triton X-100-solubilized membrane extract was also subjected to treatment with lambda protein phosphatase, protein phosphatase 1, and Nem1-Spo7 phosphatase complex, at 30 °C, respectively. The reaction mixture for lambda protein phosphatase or protein phosphatase 1 contained 50 mM Tris-HCl (pH 7.5), 2 mM MnCl₂, 2 mM dithiothreitol, 0.01% Brij 35, 0.1 mM EGTA, 100 mM NaCl, and the indicated amounts of the phosphatase in a final volume of 80 μl. The reactions were directly subjected to the subsequent measurement of DAG kinase activity. The reaction mixture for Nem1-Spo7 contained 100 mM sodium acetate (pH 5.0), 10 mM MgCl₂, 0.25 mM Triton X-100, 1 mM dithiothreitol, and the indicated amounts of the phosphatase in a final volume of 80 μl. pH of this reaction was adjusted to be neutral before the following DAG kinase activity measurement. One unit of alkaline phosphatase activity was defined as 1 μmol/min. One unit of lambda protein phosphatase activity was defined as 1 μmol/min. One unit of protein phosphatase 1 activity was defined as 1 nmol/min.

**Analysis of Phosphoamino Acids and Phosphopeptides**

³²P-labeled Dgk1¹-⁷⁷ 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) (224). 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 (159, 224, 225). Radioactive phosphoamino acids and phosphopeptides were visualized by phosphorimaging analysis. Non-radioactive phosphoamino acid standards were visualized by ninhydrin staining.

**Labeling and Analysis of Neutral Lipids and Phospholipids**

For chase-labeling of lipids, cells were grown to stationary phase in the presence of [2-\(^{14}\)C] acetate (1 µCi/ml) to uniformly label lipids. The labeled stationary phase cells were washed with water and resuspended to A\(_{600\text{nm}}\) = 0.5 in fresh growth medium without label to follow the mobilization of TAG (77). The steady-state labeling of lipids with [2-\(^{14}\)C] acetate (1 µCi/ml) was performed as described previously (226). Total lipids were extracted (227) from the radiolabeled cells; neutral lipids were analyzed by one-dimensional TLC on silica gel 60 plates using the solvent system hexane/diethyl ether/glacial acetic acid (40:10:1, v/v) (228) and phospholipids were analyzed by one-dimensional TLC on Si250-PA plates using the solvent system chloroform/ethanol/water/triethylamine (30:35:7:35, v/v) (229). The identity of labeled lipids on TLC plates was confirmed by comparison with standards after exposure to iodine vapor. Radiolabeled lipids were visualized by phosphorimaging analysis, and the relative quantities of labeled lipids were analyzed using ImageQuant software.

**Fluorescence Microscopy**

For nuclear/ER membrane structure analysis in the transcriptional regulation study, cells grown at 30 °C in SC medium lacking leucine and uracil were collected at mid-exponential phase, resuspended in a reduced volume of the same medium, and
immediately imaged live at room temperature. Images were acquired with an epifluorescence microscope (Zeiss Axioplan) using a 100 X plan-apochromatic 1.4NA objective lens (Carl Zeiss Ltd), connected to a Hamamatsu Orca R2 CCD camera and controlled by the Simple PCI6 software (Hamamatsu). The brightness and contrast of the resulting images were adjusted using Adobe Photoshop. For the analysis of lipid droplets, cells were grown in the same medium and collected at exponential and stationary phase, stained for 30 min with 2 μM BODIPY 493/503, and washed with phosphate-buffered saline (pH 7.4). For nuclear/ER membrane structure and lipid droplet analyses in the phosphorylation study, they were visualized with a long pass green fluorescent protein filter. Microscopy was performed with a Nikon ECLIPSE Ni-U microscope using a 100× oil immersion objective. The brightness and contrast of the resulting images were adjusted using imaging software.

Data Analysis

SigmaPlot software was used for the statistical analysis of data using a student’s t test. The p values < 0.05 were taken as a significant difference.
RESULTS

Reb1 Interacts with a Reb1-binding Site in the DGK1 Promoter

The DGK1 promoter contains the core consensus sequence (CGGGTAA, -166 to -160) for binding of the transcription factor Reb1 (230-233). To determine whether the DGK1 promoter sequence interacts with Reb1, we performed an electrophoretic mobility shift assay with a double-stranded oligonucleotide probe containing the recognition sequence and pure His6-tagged Reb1 (Fig. 5A). The radiolabeled probe showed a decreased electrophoretic mobility in a dose-dependent manner with respect to Reb1 (Fig. 5C, left panel). Unlabeled probe competed with the labeled probe for Reb1 binding in a dose-dependent manner (Fig. 5C, middle panel), indicating the specificity of the protein-DNA interaction. However, when transverse mutations (GT→TG, Fig. 5B) that are known to abolish Reb1 binding to the Reb1-binding sequence (234) were introduced into the binding site, the electrophoretic mobility shift of the probes was greatly attenuated (Fig. 5C, right panel). Taken together, these data supported the conclusion that Reb1 directly interacts with the Reb1-binding sequence in the DGK1 promoter.

Reb1-binding Site Mutation Attenuates the Expression of P<sub>DGK1</sub>-lacZ Reporter Gene Activity and the Abundance of DGK1 mRNA

The expression of DGK1 was examined by use of a P<sub>DGK1</sub>-lacZ reporter gene where the DGK1 promoter was fused with the coding sequence of the lacZ gene of E. coli. The β-galactosidase activity was dependent on the transcription of lacZ driven by the
FIGURE 5. Interactions of Reb1 with its putative binding site in the *DGK1* promoter.  

*A.* purified preparation (2 µg) of the His<sub>6</sub>-tagged recombinant Reb1 (*rReb1*) was subjected to SDS-PAGE and stained with Coomassie Blue. The positions of the molecular mass standards (*lane 1*) and the purified *rReb1* (*lane 2*) are indicated.  

*B.* location (-166 to -160) and sequence of the putative Reb1-binding site in the *DGK1* promoter. Also shown is the sequence of the mutant (*Mt*) form of the Reb1-binding site.  

*C.* recombinant His<sub>6</sub>-tagged was mixed with 4 pmol of radiolabeled double-stranded oligonucleotide (8.0 × 10<sup>4</sup> cpm/pmol) with or without mutations in the Reb1-binding site. The left and right panels show assays with 0, 0.2, 0.5, and 1.0 µg of recombinant His<sub>6</sub>-Reb1. The middle panel shows an assay with 0.5 µg of recombinant His<sub>6</sub>-Reb1 in the presence of 0, 4, 8, and 16 pmol of unlabeled wild type oligonucleotide. Mixtures of His<sub>6</sub>-Reb1 with oligonucleotide probes were subjected to electrophoresis in a 5% polyacrylamide gel. The data shown are representative of two independent experiments. The positions of the Reb1- Reb1-binding site complex and free oligonucleotide probe are indicated in the figure.
DGK1 promoter. The β-galactosidase activity in wild type exponential phase cells expressing the reporter-gene was 86 ± 11 nmol/min/mg. The Reb1-binding site mutation in the P_{DGK1}-lacZ reporter gene reduced the β-galactosidase activity by 8.6-fold (Fig. 6A).

By using quantitative RT-PCR, we also examined whether Reb1 controls DGK1 transcription in exponential phase cells. The amount of DGK1 mRNA of DGK1(reb1)-expressing dgk1Δ cells was 7-fold lower when compared with that of cells expressing the wild type DGK1 allele (Fig. 6B).

Reb1-binding Site Mutation Abolishes the DGK1-mediated Nuclear/ER Membrane Expansion

Expression of the DGK1 gene is required for the aberrant expansion of the nuclear/ER membrane when the PAH1 gene is deleted (78). The basis for this phenotype is that the DGK1-encoded DAG kinase activity causes the accumulation of PA at the nuclear/ER membrane when the phospholipid is not hydrolyzed by PAH1-encoded PA phosphatase (78). To examine the dependence of DGK1 expression and function of the Reb1-binding site in vivo, we examined the effect of the Reb1-binding site mutation on the nuclear/ER membrane expansion. For this experiment, the DGK1 and DGK1(reb1) alleles were expressed in dgk1Δ pah1Δ cells. Expression of DGK1 in the double mutant caused membrane expansion, whereas the Reb1-binding site mutation did not (Fig. 7). As described previously (78), the dgk1Δ mutation alone (i.e. dgk1Δ pah1Δ/PAH1) and in combination with the pah1Δ mutation (i.e. dgk1Δ pah1Δ/vector) did not cause the aberrant nuclear/ER membrane expansion (Fig. 7). This result indicated that the Reb1-mediated regulation of DGK1 expression is crucial for its cellular function.
FIGURE 6. Effect of the Reb1-binding site mutation on P_{DGK1-}lacZ expression and DGK1 mRNA abundance.  

A, wild type cells bearing the wild type P_{DGK1-}lacZ or the mutant (Mt) P_{DGK1(reb1)}-lacZ reporter gene were grown in SC medium to the exponential phase; cell extracts were prepared and assayed for β-galactosidase activity. Each data point represents the average of triplicate enzyme determinations from five independent experiments ± S.D. (error bars).  

B, dgk1Δ cells expressing DGK1 or DGK1(reb1) from low copy plasmids were grown in SC medium to the exponential phase. Total RNA was isolated, and the absolute levels of DGK1 mRNA were quantified by real time-PCR. The level of DGK1 mRNA from the DGK1 expression is expressed relative to that from the DGK1(reb1) expression. Each data point represents the average of triplicate determinations from two independent experiments ± S.D. (error bars). ⋆, p< 0.05 versus the wild type DGK1.
FIGURE 7. Effect of the Reb1-binding site mutation on the nuclear/ER membrane structure of cells lacking *DGK1* and *PAH1*. *dgk1Δ pah1Δ* cells expressing *SEC63-GFP* (to label the nuclear/ER membrane) and *DGK1, DGK1(reb1)*, or *PAH1* from low copy plasmids were grown in SC medium to the exponential phase of growth. The fluorescence signal from the reporter protein was examined with a Zeiss Axioplan epifluorescence microscope equipped with a 100×plan apochromatic 1.4NA objective lens. The white bar indicates 5 µm.
$dgk1\Delta\ pah1\Delta$

<table>
<thead>
<tr>
<th>DGK1</th>
<th>DGK1(reb1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAH1</td>
<td>Vector</td>
</tr>
</tbody>
</table>
Reb1-binding Site Mutation Compromises Growth Resumption from Stationary Phase in the Presence of Cerulenin

Stationary phase cells resume vegetative growth upon replenishment with nutrients, and this process is dependent on the mobilization of TAG to synthesize phospholipids (71, 76, 77) (Fig. 2). Resumption of growth following stasis is dependent on DGK1 when fatty acid synthesis is blocked because the conversion of TAG-derived DAG to PA is needed for phospholipid synthesis (77) (Fig. 2). Accordingly, we questioned if Reb1-mediated DGK1 expression was important for growth resumption from stationary phase. dgk1Δ cells expressing the wild type DGK1 and DGK1(reb1) alleles were first grown to the stationary phase and then allowed to grow in fresh medium containing cerulenin, an inhibitor for fatty acid synthesis (195). As described previously (77), the expression of the wild type DGK1 gene complemented the loss-of-growth phenotype exhibited by dgk1Δ mutant cells (Fig. 8). However, the expression of the DGK1(reb1) mutant allele only partially complemented the growth defect (Fig. 8). The generation time (50.2 ± 1.4 h) of cells expressing DGK1(reb1) was 2.9 times longer than the generation time (17.4 ± 0.4 h) of cells expressing the wild type DGK1 gene. Thus, the Reb1-mediated expression of DGK1 is important for growth resumption from stasis.

Reb1-binding Site Mutation Attenuates the Expression of P_{DGK1-lacZ} Reporter Gene Activity, Dgk1, and DAG Kinase Activity upon Nutrient Supplementation of Stationary Phase Cells

To provide mechanistic information for the attenuation of growth in cells expressing the DGK1(reb1) allele, the DGK1 promoter activity was measured during
FIGURE 8. Effect of the Reb1-binding site mutation on the resumption of cell growth from stasis in the absence of de novo fatty acid synthesis. A, dgk1Δ cells expressing the DGK1 and DGK1(reb1) from low copy plasmids were grown to stationary phase in SC medium and then diluted in fresh medium containing 10 µg/ml cerulenin. Cell growth after the transfer to fresh medium was monitored with a plate reader. Each data point represents the average of three independent cultures. The generation times for dgk1Δ cells expressing DGK1, DGK1(reb1), and vector were 17.4 ± 0.4, 50.2 ± 1.4, 133.2 ± 0.7 h, respectively.
growth resumption from stasis. For these experiments, DGK1 promoter activity was monitored by the $\beta$-galactosidase activity from the P$_{DGK1}$-lacZ reporter gene expression. At stationary phase (at 0 h), the $\beta$-galactosidase activity of cells expressing P$_{DGK1(reb1)}$-lacZ was 9.6-fold lower than the activity of cells expressing the wild type reporter gene after nutrient supplementation, the level of expression was fairly constant during the course of the experiment. Likewise, the much reduced level of $\beta$-galactosidase activity from the mutant reporter gene expression was moderately constant after nutrient supplementation (Fig. 9)

Next, we questioned whether the Reb1-mediated control of DGK1 expression is translated into the levels of Dgk1. Western blot analysis showed that there was some variation in the level of the Dgk1 at different time points (Fig. 10). However, the major conclusion from this experiment was that the levels of Dgk1 in cells expressing the DGK1(reb1) were greatly reduced (~7-fold) when compared with cells expressing the wild type DGK1 gene (Fig. 10). To confirm that the levels of Dgk1 were from cells at different growth phases, we analyzed the levels of DPP1-encoded DAG pyrophosphate phosphatase (Dpp1) whose expression is known to be elevated in stationary phase and reduced in exponential phase (220). The growth phase-mediated regulation of Dpp1 expression was not altered in the dgk1Δ cells expressing DGK1 and DGK1(reb1) (Fig. 10).

The effect of Reb1-binding site mutation on the DGK1 expression was also examined by analysis of DAG kinase activity (Fig. 11). In stationary phase cells (at 0 h), the enzyme activity in dgk1Δ cells expressing the DGK1(reb1) allele was 4.3-fold lower
FIGURE 9. Effect of the Reb1-binding site mutation on $P_{DGK1}$-lacZ expression upon growth resumption from stasis in the absence of de novo fatty acid synthesis. Wild type cells bearing the wild type $P_{DGK1}$-lacZ or the mutant (Mt) $P_{DGK1(reb1)}$-lacZ reporter gene were grown to stationary phase in SC medium and then diluted in fresh medium containing 10 µg/ml cerulenin. At the indicated time intervals, cells were harvested; cell extracts were prepared and assayed for $\beta$-galactosidase activity. Each data point represents the average of triplicate enzyme determinations from five independent experiments ± S.D. (error bars).
FIGURE 10. Effect of the Reb1-binding site mutation on Dgk1 abundance upon growth resumption from stasis in the absence of de novo fatty acid synthesis. \(dgk1\Delta\) cells expressing \(DGK1\) and \(DGK1(\text{reb1})\) from low copy plasmids were grown to stationary phase in SC medium and then diluted in fresh medium containing 10 µg/ml cerulenin. At the indicated time intervals, cells were harvested; total membranes (20 µg) were prepared, and the amount of Dgk1 was determined by western blot analysis using anti-Dgk1 antibodies. The same blot was also probed with anti-Dpp1 antibodies to detect the \(DPP1\)-encoded DAG pyrophosphate phosphatase. Representative blots from three experiments are shown in the figure, and the positions of Dgk1 and Dpp1 are indicated.
$d_{gk1\Delta}$

<table>
<thead>
<tr>
<th>Time, h</th>
<th>$DGK1$</th>
<th>$DGK1(\text{reb1})$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

![Image showing Dgk1 and Dpp1 over time](image.png)
FIGURE 11. Effect of the Reb1-binding site mutation on DAG kinase activity upon growth resumption from stasis in the absence of de novo fatty acid synthesis. *dgk1Δ* cells expressing *DGK1* and *DGK1(reb1)* from low copy plasmids were grown to stationary phase in SC medium and then diluted in fresh medium containing 10 µg/ml cerulenin. At the indicated time intervals, cells were harvested; cell extracts were prepared, and DAG kinase activity was measured. Each data point represents the average of triplicate enzyme determinations from a minimum of six independent experiments ± S.D. (*error bars*).
than cells expressing the *DGK1*. The reduction in the levels of DAG kinase activity correlated with the reduction in the expression levels of the reporter gene and Dgk1. As described previously (77), a transient increase (~1.7-fold) was shown in the level of DAG kinase activity when *dgk1Δ* cells expressing *DGK1* resumed vegetative growth from stasis (Fig. 11). However, the reduced level of DAG kinase activity in cells expressing the *DGK1*(reb1) allele did not show change (Fig. 11).

**Reb1-binding Site Mutation Compromises the Mobilization of TAG for Phospholipid Synthesis upon Nutrient Supplementation of Stationary Phase Cells**

To examine the role of the Reb1-mediated expression of *DGK1* in the resumption of growth from stationary phase when fatty acid synthesis is blocked, the mobilization of TAG to phospholipids was followed by a [2-\(^{14}\)C] acetate labeling chase experiment (77). The amount of lipids was determined up to 8 h following nutrient supplementation because maximum TAG hydrolysis has been shown in this time frame (77). As described previously (77), the mobilization of TAG was not shown in *dgk1Δ* mutant cells. This metabolic defect, however, was complemented by expression of the wild type *DGK1* allele (Fig. 12). In *dgk1Δ* cells expressing *DGK1*, the amount of TAG declined in a time-dependent manner to a maximum of 43% by 8 h (Fig. 12). Reciprocally, the amount of phospholipids increased in a time-dependent manner to a maximum of 100% by 8 h (Fig. 12). Over this time period, the level of fatty acids increased by 186%, whereas the level of DAG decreased by 70% (Fig. 12). However, the Reb1-binding site mutation attenuated the mobilization of TAG; the reduction in TAG content was only 22% and the increase in phospholipids was 78% by 8 h after the nutrient supplementation (Fig. 12).
FIGURE 12. Effect of the Reb1-binding site mutation on the mobilization of TAG for phospholipid synthesis upon growth resumption from stasis in the absence of de novo fatty acid synthesis. dgk1Δ cells expressing the DGK1 and DGK1(reb1) from low copy plasmids were grown to stationary phase in SC medium in the presence of [2-14C]acetate (1 µCi/ml) to uniformly label cellular lipids. The cells were then washed to remove the label and resuspended in fresh medium containing 10 µg/ml cerulenin. At the indicated time intervals, cells were harvested, and lipids were extracted and separated by one-dimensional TLC. The 14C-labeled lipids were visualized by phosphorimaging and quantified by ImageQuant analysis. The percentages shown for the individual lipids were normalized to the total 14C-labeled chloroform-soluble fraction. The values reported are the average of five separate experiments ± S.D. (error bars). *, p< 0.05 versus the 0 h. †, p< 0.05 versus the DGK1.
Five-nucleotides Mutation in the Reb1-binding Site Causes the Same Compromised Growth Resumption from Stationary Phase in the Presence of Cerulenin

Although it was shown that the introduction of the two-nucleotides transverse mutation (GT→TG) in the Reb1-binding site greatly attenuated the electrophoretic mobility shift of the probe by preventing the direct interaction with the recombinant Reb1, it still showed a limited level of the interacting protein-nucleotide complex (Fig. 5C, right panel, and Fig. 13A). To examine if this remaining interaction leads to the basal expression levels of β-galactosidase activity, DGK1 mRNA, Dgk1 and its activity as well as the partially complemented loss-of-growth phenotype and TAG mobilization for phospholipid synthesis, at first, five-nucleotides mutation (CGGGT→AAAAA) was introduced into Reb1-binding site in the double-stranded oligonucleotide probe and the electrophoretic mobility shift assay was performed again. The interactions of recombinant Reb1 with oligonucleotide probe without and with two- or five-nucleotides mutations in the Reb1-binding site were compared. Consistently, two-nucleotides mutation significantly reduced the interaction of Reb1 with the probe, compared to the interaction with the probe without mutation, as shown by the Reb1-Reb1-binding site complex (Fig. 5C, left and right panels, and Fig. 13A). The five-nucleotides mutation in Reb1p-binding site abolished the interaction (Fig. 13A).

Then, this five-nucleotides mutation in Reb1-binding site was constructed in the DGK1 promoter, and DGK1 coding sequence driven by this promoter was expressed in dgk1Δ cells. To determine if the abolished binding of Reb1 to DGK1 promoter resulted in repressing the expression of DAG kinase and its activity completely in vivo, resumption of cell growth from
FIGURE 13. Effect of five-nucleotides mutation in Reb1-binding site on the interaction with Reb1 and the resumption of cell growth from stasis in the absence of de novo fatty acid synthesis. A, recombinant His$_6$-Reb1 (0.5 µg) was mixed with 4 pmol of radiolabeled double-stranded oligonucleotide (8.0 ± 10$^4$ cpm/pmol) with (Mt1, two-nucleotides mutation; Mt2, five-nucleotides mutation) or without (WT, wild type) mutations in the Reb1-binding site. Mixtures of His$_6$-Reb1 with oligonucleotide probes were subjected to electrophoresis in a 5% polyacrylamide gel. The data shown are representative of three independent experiments. The positions of the Reb1-Reb1-binding site complex and free oligonucleotide probe are indicated in the figure. B, dgk1Δ cells expressing the DGK1 driven by wild type promoter (DGK1) and mutant promoter with two- (Mt1) /five- (Mt2) nucleotides mutations in the Reb1-binding site, respectively, from low copy plasmids were grown to stationary phase in SC medium and then diluted in fresh medium containing 10 µg/ml cerulenin. Cell growth after the transfer to fresh medium was monitored with a plate reader. Each data point represents the average of three independent cultures.
stasis in the presence of cerulenin was performed again. Being consistent with previous data (Fig. 8) (77), wild type DGK1 restored the growth resumption defect of dgk1Δ cells and DGK1 under the promoter with two-nucleotides mutation partially rescued this phenotype (Fig. 13B). However, cells expressing DGK1 driven by promoter containing five-nucleotides mutation in Reb1-binding site (Mt2) showed the same level of partial growth resumption restoration as cells expressing DGK1 controlled by promoter with two-nucleotides mutation (Mt1) (Fig. 13B). This indicated that the two-nucleotides mutation in the Reb1-binding site prevented the functional interaction of DGK1 promoter with Reb1, and thus inhibited the transcriptional regulation of DAG kinase by Reb1 thoroughly. Although Reb1 is the major transcription factor to regulate the expression of DGK1, because there was still DAG kinase activity left to support the partial growth resumption, there might be other transcription factor(s) unidentified.

Dgk1 Is a Bona Fide Substrate of CKII and Its Phosphorylation Stimulates DAG Kinase Activity

Phosphoproteomic analysis (188) has identified Ser-44, Ser-45, and Ser-46 as phosphorylation sites in Dgk1, and bioinformatics (235-237) has indicated that these sites are contained within the CKII phosphorylation target motif ((S/T)XX(E/D), (S/T)X(E/D) or (S/T)(E/D)) (169-171, 238). Initially, we questioned if the incubation of Triton X-100-solubilized membranes from stationary phase cells having overexpressed levels of Dgk1 with CKII would affect DAG kinase activity. The results of these experiments were negative. We considered that the Dgk1 expressed in stationary phase yeast cells had already been phosphorylated at CKII phosphorylation sites. Accordingly, the Triton X-
FIGURE 14. DAG kinase activity is inhibited by dephosphorylation with alkaline phosphatase and stimulated by rephosphorylation with CKII. A, 10 μg of a Triton X-100-solubilized membrane fraction containing overexpressed Protein A-tagged Dgk1 derived from stationary phase cells was treated with indicated amounts of alkaline phosphatase-agarose for 15 min at 30 °C. The reaction was stopped by removing alkaline phosphatase by filtration. The DAG kinase activity was normalized to that found in the untreated Dgk1 control. B, alkaline phosphatase-treated Dgk1 was phosphorylated by the indicated amounts of human CKII for 15 min at 30 °C, followed by the measurement of DAG kinase activity. The DAG kinase activity was normalized to that of the untreated Dgk1 control shown in panel A. The data shown are means ± S.D. (error bars) from triplicate determinations from two independent experiments.
76

-100-solubilized membrane preparation was first treated with alkaline phosphatase-agarose to remove phosphates and then incubated with CKII. The alkaline phosphatase treatment resulted in a dose-dependent decrease (7.7-fold at the point of maximum inhibition) in DAG kinase activity (Fig. 14A), and the subsequent treatment of the alkaline phosphatase-treated membranes with CKII resulted in a dose-dependent (5.5-fold at the point of maximum stimulation) increase in DAG kinase activity (Fig. 14B). These results suggested that Dgk1 is phosphorylated by CKII and its phosphorylation stimulates DAG kinase activity.

During the course of these experiments, we questioned if the Nem1-Spo7 protein phosphatase, which dephosphorylates Pah1 (86, 215), has a similar inhibitory effect on DAG kinase activity. Incubation of the detergent-solubilized membrane fraction with the Nem1-Spo7 complex had no effect on the DAG kinase activity (Fig. 15). We also examined if protein phosphatases lambda protein phosphatase and protein phosphatase 1 have inhibitory effect on DAG kinase activity. The results indicated that both lambda protein phosphatase and protein phosphatase 1 had no effect on the DAG kinase activity (Fig. 15).

To characterize the phosphorylation in a well-defined system where Dgk1 is not already phosphorylated in yeast, the N-terminal region (residue 1 through 77) of Dgk1 (i.e., Dgk1\textsuperscript{1-77}) (Fig. 16A) was expressed and purified to apparent homogeneity from \textit{E. coli} (Fig. 16B). The hydrophilic truncated form of Dgk1 was utilized because the expression and purification of the full-length protein was not tractable due to the low expression level. Purified Dgk1\textsuperscript{1-77} was incubated with CKII in the presence of [\textit{γ}-\textsuperscript{32}P]ATP, and its phosphorylation was examined by following the incorporation of the γ-
FIGURE 15. DAG kinase activity is not affected by lambda protein phosphatase, protein phosphatase 1, and Nem1-Spo7 phosphatase complex. 5 μg of a Triton X-100-solubilized membrane fraction containing overexpressed Protein A-tagged Dgk1 derived from stationary phase cells was treated without (control) or with 6 units of alkaline phosphatase-agarose, 3 units of lambda protein phosphatase, 12 units of protein phosphatase 1, and 1.5 μg of Nem1-Spo7 phosphatase complex, respectively, for 15 min at 30 °C. The reaction with alkaline phosphatase was stopped by removing alkaline phosphatase-agarose by filtration. DAG kinase activities were measured following phosphatase assays. The data shown are means ± S.D. (error bars) from triplicate determinations. *, p<0.05 versus the AP control.
FIGURE 16. Expression and purification of Dgk1^{1-77} from *E. coli* and its phosphorylation by yeast and human CKII. A, the diagram shows Dgk1 with its CTP transferase domain, its four transmembrane spanning domains, and the serine residues identified here to be phosphorylated by CKII. The diagram of the truncated form of Dgk1 (Dgk1^{1-77}) is shown below the full-length protein. B, SDS-PAGE (18% polyacrylamide gel) analysis of His_6-tagged Dgk1^{1-77} expressed and purified (1 μg loaded) from *E. coli*. C, 0.42 μg of purified recombinant Dgk1^{1-77} was phosphorylated by 0.36 μg of yeast CKII (yCKII) or 0.04 units of human CKII (hCKII) with [γ-^{32}P]ATP for 10 min. Following the reactions, Dgk1^{1-77} was separated from ATP and CKII by SDS-PAGE (18% polyacrylamide gel), and the gel was dried 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.
phosphate into the protein. Phosphorylated Dgk1\textsuperscript{1-77} was separated from ATP by SDS-PAGE and then detected by phosphorimaging. Dgk1\textsuperscript{1-77} was phosphorylated by yeast or human forms of CKII (Fig. 16C), and phosphoamino acid and phosphopeptide mapping analyses showed that Dgk1\textsuperscript{1-77} was phosphorylated on a serine residue (Fig. 17A) that is contained within one major phosphopeptide (Fig. 17B). Because the data indicated that the yeast and human forms of CKII phosphorylated Dgk1\textsuperscript{1-77} on the same site(s), we used the commercially prepared human CKII in some experiments to conserve on the yeast CKII that had to be purified. That Dgk1\textsuperscript{1-77} is a \textit{bona fide} substrate of CKII was demonstrated by the kinase activity being dependent on the time of reaction, the amount of CKII, and the concentrations of ATP and Dgk1\textsuperscript{1-77} (Fig. 18). At the point of maximum phosphorylation, CKII catalyzed the incorporation of 0.25 mol phosphate/mol Dgk1\textsuperscript{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) in Dgk1 were individually changed to an alanine residue. Mutant forms of Dgk1\textsuperscript{1-77} were expressed and purified from \textit{E. coli}; they were phosphorylated by CKII and analyzed by SDS-PAGE and phosphorimaging. The S44A mutation did not have a significant effect on the phosphorylation of Dgk1\textsuperscript{1-77} by either the yeast or human forms of CKII. However, the S45A and S46A mutations reduced the extent of phosphorylation of Dgk1\textsuperscript{1-77} by 37\% and 86\%, respectively, using yeast CKII (Fig. 19A) or by 39\% and 93\%, respectively, using human CKII (Fig. 19B). Thus, Ser-46 was the major site of phosphorylation. The CKII-mediated phosphorylation of Dgk1\textsuperscript{1-77} is eliminated by the S44A/S45A/S46A mutations (Fig. 19). Phosphopeptide mapping analysis confirmed that Ser-45 and Ser-46
FIGURE 17. CKII phosphorylates Dgk1<sup>1-77</sup> on a serine residue. <sup>32</sup>P-labeled Dgk1<sup>1-77</sup> phosphorylated by yeast CKII (yCKII) or human CKII (hCKII) in the SDS-polyacrylamide gel was transferred to the PVDF membrane and then incubated with HCl or 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). The positions of the standard phosphoamino acids phosphoserine (p-Ser), phosphothreonine (p-Thr), and phosphotyrosine (p-Tyr) (dotted lines) are indicated. The data are representative of three independent experiments.
A  Phosphoamino acid analysis

- yCKII
  - p-Ser
  - p-Thr
  - p-Tyr

- hCKII
  - p-Ser
  - p-Thr
  - p-Tyr

B  Phosphopeptide map analysis

- yCKII
- hCKII
FIGURE 18. Characterization of CKII activity using wild type or mutant Dgk1$^{1-77}$ as substrates. Phosphorylation of wild type or mutant Dgk1$^{1-77}$ by human CKII was measured by following the incorporation of the radiolabeled phosphate from [$\gamma$-$^{32}$P]ATP into purified recombinant wild type or mutant Dgk1$^{1-77}$ by varying the time ($A$), the amount of human CKII ($B$), and the concentrations of ATP ($C$) and wild type or mutant Dgk1$^{1-77}$ ($D$). After the kinase reactions, the samples were subjected to the P81 papers; the P81 papers were washed with 75 mM phosphoric acid to remove the unincorporated [$\gamma$-$^{32}$P]ATP and then dried and subjected to scintillation counting. The data shown in $A$-$D$ are the average of three experiments ± S.D. (error bars).
FIGURE 19. Ser-45 and Ser-46 are CKII phosphorylation sites in Dgk11-77. A and B, 0.21 μg of purified recombinant wild type or indicated mutant Dgk11-77 were phosphorylated with [γ-32P]ATP and 0.36 μg yeast CKII (yCKII) (A) or 0.04 units human CKII (hCKII) (B) for 30 min. The phosphorylated samples were resolved by SDS-PAGE (18% polyacrylamide gel), transferred to a PVDF membrane, and subjected to phosphorimaging analysis. The phosphorylation intensities of wild type and mutant Dgk11-77 were quantified using ImageQuant software and were normalized to the phosphorylation intensity of the wild type Dgk11-77. C, human CKII phosphorylated 32P-labeled wild type or mutant Dgk11-77 from the PVDF membrane was subjected to phosphopeptide mapping as described in the legend to Fig. 17. The samples used for the phosphopeptide mapping of the wild type and individual mutants contained near equal amounts of radioactivity. The positions of wild type and mutant Dgk11-77 (A and B) and the phosphopeptide that was absent in the S44A/S45A/S46A triple mutant Dgk11-77 (indicated by the dotted line circle) but present in the wild type Dgk11-77 are indicated. The data shown are representative of three independent experiments. *, p< 0.05 versus the wild type.
were contained within the same phosphopeptide and that this phosphopeptide was eliminated by the triple mutations (Fig. 19C). That Ser-46 is the major phosphorylation site in Dgk1\(^{1-77}\) was further confirmed by showing that the S46A mutation had a major inhibitory effect on the phosphorylation with respect to time and the amounts of CKII, ATP, and Dgk1\(^{1-77}\) (Fig. 18).

The Stimulation of DAG Kinase Activity by CKII Regulates Its Function in Nuclear/ER Membrane Growth and Lipid Droplet Formation

The physiological effects of phosphorylation-deficient and -mimicking forms of Dgk1 were examined. Serine-to-alanine and -aspartate mutations were constructed for Ser-46 alone and in combination with Ser-45, and the wild type and mutant alleles of \(DGK1\) were expressed in \(dgk1\Delta\) or \(dgk1\Delta\ pah1\Delta\) mutant cells from a low copy plasmid. The reason for expressing the phosphorylation site mutants in \(dgk1\Delta\ pah1\Delta\) was to assess the dependence of Dgk1 function on the phenotypes imparted by \(pah1\Delta\). The expression of Dgk1 was examined in the exponential and stationary phase of growth, and immunoblot analysis of cell extracts showed that none of the phosphorylation site mutations affected the abundance of Dgk1 (Fig. 20A). This experiment also showed that the abundance of Dgk1 was reduced in the stationary phase of growth. ImageQuant analysis of triplicate immunoblot determinations of the data indicated that the reduction of Dgk1 in the stationary phase was by 2.6-fold. The DAG kinase activity was measured from cells grown to the exponential and stationary phases. In these experiments, the DAG kinase activity was assayed with subsaturating concentrations of DAG and CTP.
FIGURE 20. Dgk1 is phosphorylated by CKII in the stationary phase causing the stimulation of its DAG kinase activity in vitro.  

A and B, wild type and the indicated phosphorylation site mutant alleles of \textit{DGK1} were expressed from a low copy plasmid in \textit{dgk1}\textsuperscript{Δ} cells. Cell extracts were prepared from mid exponential phase ($A_{600 \text{ nm}} = 0.6$) and stationary phase ($A_{600 \text{ nm}} = 4.5$) cells, and the total membrane fraction was isolated from the extract by differential centrifugation.  

\textit{A}, 35 μg of the membrane fraction was subjected to SDS-PAGE (12\% polyacrylamide gel) followed by immunoblot analysis using anti-Dgk1 antibodies.  

\textit{B}, cell extracts were used for the assay of DAG kinase activity using subsaturating concentrations of DAG (6.25 mol \%) and CTP (0.2 mM) (114).  

\textit{C}, 0.5 μg of the total membrane fraction containing overexpressed Dgk1 isolated from stationary phase cells was dephosphorylated by alkaline phosphatase with increasing amounts (0, 0.06, 0.125, 0.25, 0.5, and 1 unit, indicated by the grey triangle) for 10 min. Following treatment, the samples were subjected to SDS-PAGE (12\% polyacrylamide gel) and immunoblotting using anti-Dgk1 antibodies. The immunoblots shown in \textit{A} and \textit{C} are representative of three experiments, whereas the data shown in \textit{B} are means ± S.D. (error bars) from triplicate determinations.  

\textbullet, \textit{p}<0.05 versus the exponential phase;  

\textbullet\textbullet, \textit{p}<0.05 versus the wild type stationary phase.
In this manner, we could more readily observe the inhibitory effects of the mutations on DAG kinase activity. The inhibitory effects of the mutations were not observed when the activity was measured with saturating substrate concentrations (Fig. 21). For cells expressing the wild type Dgk1, the DAG kinase activity from stationary phase was 52% greater than that from exponential phase (Fig. 20B). This growth phase-mediated regulation in DAG kinase activity was eliminated by the S46A and S45A/S46A mutations in the enzyme (Fig. 20B). For cells expressing the phosphorylation-mimicking aspartate mutations, the DAG kinase activity in stationary phase was 22% higher than that in the exponential phase (Fig. 20B). Taken together, these results indicated that phosphorylation of Dgk1 at Ser-45 and Ser-46 by CKII stimulates DAG kinase activity in the stationary phase of growth in vitro.

The immunoblot analysis also indicated that in stationary phase cells, wild type Dgk1 existed as a doublet, whereas the alanine and aspartate mutant proteins existed as a single band (Fig. 20C). This result suggested that the slower migrating band in the doublet represents the phosphorylated form of the enzyme. This notion was supported by the observation that the slower migrating band collapsed into a faster migrating band upon treatment of the enzyme with alkaline phosphatase (Fig. 20C).

Yeast cells are able to resume vegetative growth from stationary phase if they are supplemented with fresh nutrients; this physiological process is dependent on the hydrolysis of TAG to form DAG and fatty acid that needed to synthesize new membrane phospholipids (71, 76, 77). The Dgk1-dependent conversion of DAG to PA plays an important role in this process, especially when de novo fatty acid synthesis is blocked (77) (Fig. 2). We questioned if the phosphorylation state of the enzyme affects the
FIGURE 21. Phosphorylation does not affect DAG kinase activity when DAG and CTP are saturating. Wild type and the indicated phosphorylation site mutant alleles of DGK1 were expressed from a low copy plasmid in dgk1Δ cells. Cell extracts were prepared from mid exponential phase (A_{600 nm} = 0.6) and stationary phase (A_{600 nm} = 4.5) cells. Cell extracts were used for the assay of DAG kinase activity using saturating concentrations of DAG (9.09 mol %) and CTP (1 mM) (114). The data shown are means ± S.D. (error bars) from triplicate determinations.
FIGURE 22. CKII phosphorylation site mutations in Dgk1 do not affect the resumption of cell growth from stasis in the absence of de novo fatty acid synthesis. $dgk1\Delta$ cells expressing the wild type and the indicated phosphorylation site mutant alleles of $DGK1$ from low copy plasmids were grown to stationary phase in SC-Ura medium and then diluted in fresh medium containing 10 μg/ml cerulenin. Cell growth after the transfer to fresh medium was monitored with a plate reader. Each data point represents the average ± S.D. (error bars) of three independent cultures.
resumption of growth from stasis as described by Fakas et al. (77). As described previously for cells grown in the presence of the fatty acid synthesis inhibitor cerulenin (77), the growth resumption from stationary phase was blocked by the \(dgk1\Delta\) mutation, and this \(dgk1\Delta\)-mediated growth defect was overcome by the expression of the \(DGK1\) gene. This experiment was repeated with \(dgk1\Delta\) cells that expressing the phosphorylation-deficient or -mimicking mutant alleles. The S46A, S45A/S46A, S46D or S45D/S46D mutations did not affect the ability of \(DGK1\) to correct the \(dgk1\Delta\) mutational defect in growth resumption (Fig. 22). Thus, the CKII phosphorylation of Dgk1 is not involved with the physiological role of the enzyme in growth resumption.

The \(pah1\Delta\) mutant exhibits a temperature sensitive phenotype (64, 86, 239) that reflects the important role of Pah1 PA phosphatase activity in controlling the balance of PA and DAG. This phenotype is dependent on Dgk1 DAG kinase activity (78). The dependence of Dgk1 function on this \(pah1\Delta\) phenotype was utilized to examine the effects of the phosphorylation-deficient mutants on Dgk1 function (Fig. 23). In this assay, the expression of \(PAH1\) in the \(dgk1\Delta pah1\Delta\) double mutant permitted growth at 37 °C (\(dgk1\Delta\) cells are not temperature sensitive at 37 °C (78)), whereas the expression of \(DGK1\) in the \(dgk1\Delta pah1\Delta\) mutant inhibited growth. That the expression of the S46A and S45A/S46A mutations in \(DGK1\) permitted better growth of the \(dgk1\Delta pah1\Delta\) mutant at 37 °C indicated a loss of Dgk1 function, and thus the phosphorylation at 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 \(dgk1\Delta pah1\Delta\) cells at 37 °C.
FIGURE 23. Effect of CKII phosphorylation site mutations in Dgk1 on the complementation of the *pah1Δ* temperature-sensitive phenotype. The indicated wild type and phosphorylation mutant alleles of *DGK1*, or wild type *PAH1* were expressed from a low copy plasmid in *dgk1Δ pah1Δ* mutant cells. The transformants were grown to stationary phase in SC-Ura-Leu medium at 30 °C; serial dilutions (5-fold) of the cells were spotted onto SC-Ura-Leu agar plates and incubated at 30 or 37 °C for 5 days. The data are representative of three independent experiments.
Cells lacking Pah1 PA phosphatase exhibit the aberrant expansion of the nuclear/ER membrane and have irregular shaped nuclei (65, 86). This phenotype is dependent on Dgk1 DAG kinase activity (78). To assess the effects of the phosphorylation-deficient mutations on the Dgk1-dependent expansion of the nuclear/ER membrane, the number of \( \text{dgk1}\Delta \text{pah1}\Delta \) cells with round nuclei was scored by fluorescence microscopy (Fig. 24A). As described previously (240), the \( \text{dgk1}\Delta \text{pah1}\Delta \) cells expressing wild type \( \text{DGK1} \) exhibited a 7-fold decrease in the number of cells with normally shaped nuclei when compared with the cells expressing \( \text{PAH1} \) (Fig. 24B). The expression of the S46A and S45A/S46A phosphorylation-deficient mutant alleles caused a 2- to 3-fold increase in the number of cells with round nuclei when compared with cells expressing the wild type \( \text{DGK1} \) (Fig. 24B). These results indicated that the alanine mutations decreased Dgk1 function and substantiated the conclusion that phosphorylation of Dgk1 by CKII increases its function \textit{in vivo}. The effects of the phosphorylation-mimicking mutations on the Dgk1-dependent \( \text{pah1}\Delta \) phenotype of nuclear/ER membrane expansion were similar to that of the wild type control.

Pah1 PA phosphatase activity plays a major role in the synthesis of TAG and membrane phospholipids (63, 64, 241). For example, the \( \text{pah1}\Delta \) mutation causes a dramatic decrease in TAG content and the increase in total phospholipid content in the stationary phase of growth (63, 64, 241). In contrast to most other phenotypes imparted by \( \text{pah1}\Delta \) (242), the gross changes in lipid composition are not dependent on Dgk1 DAG kinase activity (78). Nonetheless for experimental completeness, we examined the amounts of TAG and membrane phospholipids in \( \text{dgk1}\Delta \text{pah1}\Delta \) cells expressing the wild type and the phosphorylation site mutant alleles of \( \text{DGK1} \). Although some values were
FIGURE 24. Effect of CKII phosphorylation site mutations in Dgk1 on the nuclear/ER membrane structure in cells lacking *DGK1* and *PAH1*. *dgk1Δ pah1Δ* cells expressing *SEC63-GFP* (to label the nuclear/ER membrane), wild type and phosphorylation site mutant alleles of *DGK1* or *PAH1* from a low copy plasmid were grown in SC-Ura-Leu medium to the mid-exponential phase of growth. *A*, the fluorescence signal from the Sec63-GFP reporter protein was visualized by fluorescence microscopy. The data shown are representative of several fields of view from multiple experiments. *DIC*, differential interference contrast. *White bar*, 1 μm. *B*, the percentage of cells with round nuclear/ER membrane structure versus the number of cells in a field of view containing 300 to 400 cells. The data are averages ±S.D. (*error bars*) from three fields of view. *, *p*< 0.05 versus *DGK1*. 
FIGURE 25. Effect of CKII phosphorylation site mutations in Dgk1 on the neutral lipid and the major membrane phospholipid composition in cells lacking DGK1 and PAH1. dglΔ pahΔ cells expressing wild type and phosphorylation site mutant alleles of DGK1 or PAH1 from a low copy plasmid were grown in SC-Ura-Leu medium to exponential and stationary phases in the presence of [2-\(^{14}\)C]acetate (1 μCi/ml). Lipids were extracted and separated by one-dimensional Silica Gel 60 thin layer chromatography for neutral lipid composition analysis (A) and by one-dimensional Si250-PA thin layer chromatography for the major membrane phospholipid composition analysis (B). Images of \(^{14}\)C-labeled lipids were visualized by phosphorimaging and subjected to ImageQuant analysis. The percentages shown for the individual lipids were normalized to the total \(^{14}\)C-labeled chloroform-soluble fraction. Each data point represents the mean ± S.D. (error bars) of three experiments.
deviated from previous findings, generally, neither the wild type nor the mutants affected the neutral lipid composition or the composition of the major phospholipids (Fig. 25).

The number of lipid droplets, the organelle that stores TAG (243, 244), is reduced by the $pah1\Delta$ mutation, and this phenotype is suppressed by the $dgk1\Delta$ mutation (63, 115). Owing that the reduced lipid droplet phenotype of $pah1\Delta$ cell is dependent on Dgk1 function (63, 64, 115, 241), we examined the effects of the CKII phosphorylation-deficient mutations on lipid droplet numbers in both exponential and stationary phases by fluorescence microscopy (Fig. 26A and C). Because the number of lipid droplets per cell was variable among different cell populations, box plot analyses of the data were performed (Fig. 26B and D). In both growth phases, for the mean values, the number of lipid droplets per cell of $dgk1\Delta\ pah1\Delta$ cells expressing $DGK1$ was 4-fold lower than that of cells expressing the $PAH1$ gene. The S46A and S45A/S46A mutations caused an increase in the mean lipid droplets per cell of 2- to 3-fold. This indicated that the loss of phosphorylation by CKII reduces Dgk1 function with respect to lipid droplet formation. The phosphorylation-mimicking mutations did not have a significant effect on Dgk1-mediated regulation of lipid droplet formation.

**PKA Phosphorylates Dgk1^{1-77} on A Serine Residue**

Phosphoproteomic analysis (188-192) and bioinformatics (235-237) indicated putative target sites of several protein kinases other than CKII. Previous work has shown that Pah1 is phosphorylated by Pho85-Pho80, Cdc28-cyclin B, PKA, PKC, and CKII (154, 155, 185-187). Because of the reciprocal nature of the PA phosphatase and DAG kinase reactions, we questioned whether Dgk1 is also phosphorylated by the same protein
FIGURE 26. Effect of CKII phosphorylation site mutations in Dgk1 on the number of lipid droplets in cells lacking DGK1 and PAH1. \(\text{dgk1}\Delta \ pah1\Delta\) cells expressing wild type and phosphorylation site mutant alleles of \(\text{DGK1}\) or \(\text{PAH1}\) from a low copy plasmid were grown in SC-Ura-Leu medium to the exponential (A and B) and stationary (C and D) phases of growth. A and C, lipid droplets were visualized by fluorescence microscopy after staining cells with BODIPY 493/503. The data shown are representative of several fields of view during multiple experiments. \textit{White bar, 1 \(\mu\)m.}\ B and D, for each strain, the numbers of lipid droplets in each cell from three fields of view (200-300 cells/field) were counted. Box plot analyses of the data are presented. \(\star, p<0.05\) versus \(\text{DGK1}\).
kinases in addition to CKII. It was examined by incubating the purified Dgk1^{1-77} with [\gamma-^{32}P]ATP and protein kinases that phosphorylate Pah1 including PKA, PKC, and Pho85-Pho80 protein kinase complex. Mammalian orthologs of PKA and PKC were used because they are structurally and functionally similar to their yeast counterparts (245, 246). Pho85-Pho80 complex was from yeast (211). Similarly with previously described, after the kinase reactions, the phosphorylation of Dgk1^{1-77} was monitored by following the incorporation of the radioactive \(\gamma\)-phosphate from [\gamma-^{32}P]ATP into the protein (Fig. 27A). Phosphorimaging analysis of kinase reaction products resolved by SDS-PAGE indicated Dgk1^{1-77} serves as a substrate for PKA but not for PKC and Pho85-Pho80 complex.

PKA is a serine/threonine-specific protein kinase (247). To identify the target amino acid residue of PKA, \(^{32}P\)-labeled Dgk1^{1-77} phosphorylated by PKA with [\gamma-^{32}P]ATP was prepared and then was subjected to the phosphoamino acid analysis. The result indicated that PKA phosphorylates Dgk1^{1-77} only on a serine residue (Fig. 27B). Trypsin-digested \(^{32}P\)-labeled Dgk1^{1-77} was subjected to phosphopeptide mapping analysis. The result showed multiple signals, indicating that PKA phosphorylates Dgk1^{1-77} at several sites (Fig. 27C and 28B, WT).

**Ser-25 and Ser-26 in Dgk1^{1-77} Are Phosphorylated by PKA**

Bioinformatic analysis of Dgk1^{1-77} (237) suggested that Ser-25, Ser-26, and Ser-28 as PKA phosphorylation sites. To examine that, Dgk1^{1-77} with single (S25A, S26A or S28A) or triple (S25A/S26A/S28A) alanine mutations of these three sites were expressed in *E. coli*, purified, and phosphorylated with PKA and [\gamma-^{32}P]ATP (Fig. 28A).
FIGURE 27. PKA phosphorylates Dgk11-77 on a serine residue. A, 0.42 μg of purified recombinant Dgk11-77 was incubated with 0.5 units PKA, 1 μg Pho85-Pho80, or 2 units PKC, respectively, with [γ-32P]ATP for 10 min. Following the reactions, Dgk11-77 was separated from ATP and protein kinases by SDS-PAGE (18 % polyacrylamide gel), and the gel was dried and subjected to phosphorimaging analysis. The positions of Dgk11-77 and molecular mass standards are indicated. B, 32P-labeled Dgk11-77 phosphorylated by PKA in the SDS-polyacrylamide gel was transferred to the PVDF membrane and then incubated with HCl or 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 (B), 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 (C). The positions of the standard phosphoamino acids phosphoserine (p-Ser), phosphothreonine (p-Thr), and phosphotyrosine (p-Tyr) (dotted lines) are indicated. The data are representative of three independent experiments.
The result showed the significantly decreased phosphorylation intensities of Dgk1\(^{1-77}\) with S25A and S26A single mutations, and S25A/S26A/S28A triple mutations (Fig. 28A). However, Dgk1\(^{1-77}\) containing S28A mutation did not show noticeable difference in phosphorylation level compared to that of wild type Dgk1\(^{1-77}\) (Fig. 28A). Consistently, the result of phosphopeptide mapping analysis showed the loss of phosphopeptides in Dgk1\(^{1-77}\) with S25A, S26A, or S25A/S26A/S26A, but not in Dgk1\(^{1-77}\) with S28A (Fig. 28B). These indicated that only Ser-25 and Ser-26 are PKA phosphorylation sites in Dgk1\(^{1-77}\). However, the major phosphopeptide did not disappear along with any mutations of these three sites, indicating that there are other PKA target sites in Dgk1\(^{1-77}\). Although the consensus sequences of target sites are different between PKA and CKII phosphorylation, to exclude the possibility that PKA and CKII share the same target sites in Dgk1\(^{1-77}\), Dgk1\(^{1-77}\) with S25A/S26A/S44A/S45A/S46A (5A) quintuple mutations was expressed and purified to analyze phosphopeptides. It showed the same mapping as that of Dgk1\(^{1-77}\) with S25A/S26A/S28A triple mutations, which contains the loss of three phosphopeptides caused by S25A/S26A mutations (Fig. 28B), indicating Ser-44, Ser-45, and Ser-46 are not PKA target sites.

**DAG Kinase Activity Is not Affected by PKA Phosphorylation**

CKII study above indicated that the alkaline phosphatase-agarose treatment of detergent-solubilized membrane preparation from stationary phase cells resulted a dramatic decrease in DAG kinase activity (Fig. 14A and 15), and the subsequent treatment of the alkaline phosphatase-treated membranes with CKII resulted an increase (5.5-fold compared to the alkaline phosphatase-treated enzyme) in DAG kinase activity
FIGURE 28. Ser-25 and Ser-26 are PKA phosphorylation sites in Dgk1\(^{1-77}\). A, 0.21 μg of purified recombinant wild type or indicated mutant Dgk1\(^{1-77}\) were phosphorylated with \([γ-^{32}P]ATP\) and 0.5 units PKA for 30 min. The phosphorylated samples were resolved by SDS-PAGE (18 % polyacrylamide gel), transferred to a PVDF membrane, and subjected to phosphorimaging analysis. B, PKA phosphorylated \(^{32}P\)-labeled wild type or mutant Dgk1\(^{1-77}\) from the PVDF membrane was subjected to phosphopeptide mapping as described in the legend to Fig. 27C. The samples used for the peptide mapping of the wild type and individual mutants contained near equal amounts of radioactivity. The positions of wild type and mutant Dgk1\(^{1-77}\) (A and B) and the phosphopeptides that were absent in the S25A, S26A, S25A/S26A/S28A, and 5A mutant Dgk1\(^{1-77}\) (indicated by the dotted line circle) but present in the wild type Dgk1\(^{1-77}\) are indicated. 5A, S25A/S26A/S44A/S45A/S46A. The data shown are representative of three independent experiments.
FIGURE 29. Effect of PKA phosphorylation on DAG kinase activity. 1.25 μg of alkaline phosphatase-treated Dgk1 incubated without (control) and with 0.5 units of PKA or 2 units of CKII for 15 min at 30 °C, respectively, followed by the measurement of DAG kinase activity. The data shown are mean ± S.D. (error bars) from triplicate determinations. *, p< 0.05 versus the control.
(Fig. 14B and 29). We questioned whether PKA phosphorylation of Dgk1 also affects the DAG kinase activity. The Triton X-100-solubilized membrane preparation treated with alkaline phosphatase-agarose was incubated with PKA. The result showed that PKA treatment did not give a significant impact on DAG kinase activity (Fig. 29). Further study to investigate the effect of PKA phosphorylation on Dgk1 is required.
DISCUSSION

This work advanced the understanding of the regulation of Dgk1 DAG kinase in *S. cerevisiae*. The data supported the conclusion that *DGK1* expression was activated by the transcription factor Reb1 through its direct interaction with a Reb1-binding site in the promoter. That part of work also advanced the understanding of the role that Reb1 plays in the regulation of lipid metabolism. Here, we also showed that Dgk1 is a *bona fide* substrate for CKII and identified Ser-45 amd Ser-46 as major sites of phosphorylation. We also demonstrated that the CKII phosphorylation of Dgk1 stimulated DAG kinase activity, and a mutational analysis of Dgk1 indicated that its phosphorylation by CKII regulates its function in nuclear/ER membrane growth and lipid droplet formation.

The *DGK1*-encoded CTP-dependent DAG kinase has emerged as an important lipid metabolic enzyme in *S. cerevisiae* (77, 78, 114, 115). This ER-associated enzyme, along with Pah1 PA phosphatase (63, 64, 241), plays an important role in controlling the balance of the lipid intermediates PA and DAG in the nuclear/ER membrane, which in turn regulates the synthesis of phospholipids, membrane growth, and lipid droplet formation (78, 114, 115). The importance of maintaining the PA/DAG balance in yeast is typified by several phenotypes caused by mutations in the *PAH1* gene (63-65, 86, 115, 241, 248-251), and many of the *pah1Δ* phenotypes (e.g., increased phospholipid synthesis gene expression, aberrant expansion of the nuclear/ER membrane, decrease in lipid droplet formation, and the loss of chronological life span) are dependent in part on Dgk1 DAG kinase activity (63, 78, 115, 116). 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 (89, 252-256), whereas the PA phosphotase enzymes regulate cellular processes important to diseases that include lipodystrophy, insulin resistance, peripheral neuropathy, rhabdomyolysis, and inflammation (257-266). Interestingly, the \textit{dgk1}\textDelta~mutation, itself, does not impart any deleterious phenotypes under typical laboratory growth conditions (78). However, the overexpression of \textit{DGK1} causes many of the same phenotypes attributed to the elevated levels of PA induced by the \textit{pah1}\textDelta~mutation (78). DAG kinase also plays an important role in the metabolic process whereby yeast cells in stasis resume vegetative growth upon nutrient supplementation (77). Especially when \textit{de novo} fatty acid synthesis is inhibited, DAG kinase activity is essential for TAG mobilization to synthesize membrane phospholipids to resume the growth of cells (77). In addition, DAG kinase facilitates cellular health by alleviating the toxic effects caused by DAG (60, 77).

Although the DAG kinase-mediated conversion of DAG to PA is conserved between yeast and mammalian systems, the yeast and mammalian enzymes differ significantly with respect to their reaction mechanisms and how they interact with the membrane to convert DAG to PA. Yeast DAG kinase utilizes CTP as the phosphate donor in the reaction and is localized as an integral ER membrane protein (78, 114). In mammalian cells, however, the DAG kinase utilizes ATP as the phosphate donor and its cellular localization is cytosolic in nature (81, 83, 87-89). Mammalian DAG kinases associate with membranes in a peripheral nature that is governed by special membrane interaction domains (81, 83, 87-89). Despite these differences, the yeast and mammalian
enzymes play important roles in regulating the cellular levels of PA/DAG, understanding the regulation of DAG kinase expression and activity will facilitate its control in abnormal cellular processes.

We sought to gain an understanding of the transcriptional regulation of yeast DGK1. Inspection of the promoter revealed that it contains the consensus sequence for interaction with the transcription factor Reb1. Through a detailed \textit{in vitro} analysis, we showed that Reb1 specifically binds to its recognition sequence. Moreover, the Reb-binding site mutations greatly diminished the expression of DGK1 \textit{in vivo}, which was translated into reduced expression of Dgk1 and its activity. The consequences of losing the Reb1-mediated activation of DGK1 expression included the misregulation of the nuclear/ER membrane growth, and when fatty acid synthesis was inhibited, a significant defect in growth as well as in the synthesis of phospholipids from TAG mobilization. However, the residual DAG kinase activity remaining in cells expressing the Reb1-binding site mutation supported some growth and the mobilization of TAG. Further proof that Reb1 mediated this regulation could not be obtained from the analysis of the \textit{reb1}Δ mutant because the \textit{REB1} gene is essential for cell growth (230). Because the abolition (five-nucleotides mutation) of the interaction between Reb1 and DGK1 promoter did not aggravate the growth resumption of cells, it is reasonable to speculate that other transcription factor(s) may regulate DAG kinase expression.

Yeast cells resuming vegetative growth from stasis exhibit an increase in DAG kinase activity; this regulation occurs whether or not fatty acid synthesis is inhibited (77). The mechanism for this regulation was not attributed to the Reb1-mediated activation of DGK1 expression since the levels of P\textsubscript{DGK1}-\textit{lacZ} reporter gene activity and Dgk1 did not
show changes that correlated with the transient increase in DAG kinase activity. Thus, the change in DAG kinase activity appears to be regulated by a biochemical mechanism. It was previously hypothesized that it might be regulated by phosphorylation/dephosphorylation. The study of CKII phosphorylation of Dgk1 in this work showed that CKII phosphorylation on Ser-45 and Ser-46 of Dgk1 did not affect the growth resumption of cells. Additional work will be needed to address the mechanism.

The essential nature of Reb1 emanates from the fact that it is required for activation of genes (e.g., ACS1, ACT1, ENO1, FAS1, FAS2, GCY1, ILV1, PGK1, RAP1, and REB1) involved in various aspects of cell physiology that include lipid metabolism (267-275). In particular, Reb1 interacts with the promoters of FAS1 and FAS2 to activate their transcription (270). Fas1 (β-subunit) and Fas2 (α-subunit) comprise the fatty acid synthase complex (organized as α6/β6) that catalyzes a multistep process leading to the formation of fatty acids that are incorporated into lipids (276-279). Interestingly, the promoters of ACC1 and ACB1, whose protein products function before and after the fatty acid synthase reactions, also contain the Reb1-binding site (270). Acc1 acetyl-CoA carboxylase catalyzes the conversion of acetyl-CoA to malonyl-CoA that is used by fatty acid synthase to produce fatty acyl-CoA molecules (280, 281), where Acb1 acyl-CoA binding protein delivers fatty acyl-CoA molecules into lipid biosynthetic pathways (282-284). Also, the TGL3 promoter possesses the consensus Reb1-binding sequence (74). Furthermore, the promoters of DGA1 and LROI, which encode acyltransferase enzymes responsible for the synthesis of TAG, and CKII and EKII, which encode kinase enzymes responsible for the synthesis of PC and PE, respectively, via the Kennedy Pathway (6), contain putative sequences for Reb1 interactions. It is unknown whether Reb1 plays a
role in the transcriptional activation of ACC1, ACB1, TGL3, DGA1, LRO1, CKII, and EKI1. However, given their roles in lipid metabolic processes for the synthesis of TAG and its mobilization for phospholipid synthesis and growth resumption from stasis, it is reasonable to hypothesize that these genes might be regulated in a coordinate manner with DGK1 by the transcription factor Reb1. Reb1 is subjected to positive and negative autoregulation (275), but whether it is regulated under these growth conditions is unknown.

Of the transcriptional (51, 241) and biochemical (213, 285-288) mechanisms that control Pah1 PA phosphatase function, phosphorylation/dephosphorylation (85, 154, 155, 185-187, 215, 289) has the greatest impact on enzyme regulation and lipid metabolism. Because of the complementary functions of PA phosphatase and DAG kinase in controlling PA/DAG, we considered that Dgk1 might also be regulated by phosphorylation/dephosphorylation.

Phosphoproteomic analysis (188-192) indicated phosphorylation sites (Thr-3, Thr-36, Ser-44, Ser-45 and Ser-46) in the N-terminal region of Dgk1. Bioinformatic analysis (235-237) predicted Ser-44, Ser-45 and Ser-46 as CKII target sites. So in this study, we examined the hypothesis that Dgk1 is phosphorylated by CKII. Studies with Triton X-100-solubilized membrane fraction of stationary phase cells that overexpressed full-length Dgk1 (114) showed that treatment with alkaline phosphatase resulted in the loss of DAG kinase activity, whereas the subsequent incubation with CKII resulted in the stimulation of activity. That CKII did not affect DAG kinase activity without the prior treatment with alkaline phosphatase indicated that Dgk1 is endogenously phosphorylated in yeast in stationary phase. Owing that our studies were performed with membrane
samples derived from stationary phase cells, we cannot rule out that the enzyme might also be phosphorylated in exponential phase cells. The protein phosphatase that dephosphorylates Dgk1 \textit{in vivo} is unknown, but we did question whether the Nem1-Spo7 complex, the protein phosphatase that dephosphorylates Pah1 (86, 215), would have a similar effect on DAG kinase activity as that of alkaline phosphatase. Nem1-Spo7 had no effect on DAG kinase activity, indicating that this protein phosphatase does not dephosphorylate Dgk1.

In more direct studies that followed the transfer of radiolabeled phosphate from \([\gamma^{-32}P]ATP\) into protein, we showed that Dgk1 is a \textit{bona fide} substrate of CKII. Our work to identify the sites of phosphorylation was facilitated by previous phosphoproteomic studies (188) and bioinformatics (235-237) that led to hypothesis that the phosphorylation by CKII occurs at the N-terminal region of Dgk1. Through mutagenesis and phosphopeptide mapping analyses of Dgk1\textsubscript{1-77}, we confirmed that the sites of phosphorylation by CKII occur at Ser-45 and Ser-46 with Ser-46 being the major site of phosphorylation. Ser-44, which was predicted to be a CKII phosphorylation site, was not identified as a site by the studies performed here.

Phosphorylation-deficient and –mimicking mutant forms of Dgk1 were expressed in \textit{dgk1}\textsubscript{Δ} or \textit{dgk1}\textsubscript{Δ} \textit{pah1}\textsubscript{Δ} cells to examine the physiological relevance of the CKII phosphorylation of the enzyme. In the \textit{in vitro} analysis, we observed that DAG kinase activity in stationary phase \textit{dgk1}\textsubscript{Δ} cells that expressing wild type \textit{DGK1} was elevated when compared with that of exponential phase cells. 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 reduction in Dgk1 abundance
in the stationary phase was consistent with the decrease in \textit{DGK1} transcript levels observed in global analyses of gene expression during the diauxic shift of growth (290). It is known that the essential transcription factor Reb1 (230) is required for maximum \textit{DGK1} expression and DAG kinase function with respect to its role in phospholipid synthesis, nuclear/ER membrane growth, and the resumption of growth from the stationary phase (240). It is unknown, however, whether the Reb1-mediated regulation of \textit{DGK1} affects Dgk1 abundance and DAG kinase activity as cell progress in the stationary phase. This question, along with the identification of other transcription factors that regulate the expression of \textit{DGK1} will be subjected to future studies. Pah1 PA phosphatase also shows a stimulation of activity as cells enter stationary phase (241), it suggests that a correlation with the elevated activity of its reciprocal role, Dgk1, to maintain the balance of PA/DAG in cells. This correlation requires further exploration.

Based on the \textit{in vitro} studies, that the growth phase-mediated regulation of DAG kinase activity was eliminated by the S46A and S45A/S46A mutations in \textit{DGK1} indicated that 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. These findings are consistent with the stimulatory effect CKII had on the DAG kinase activity in 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 \textit{in vitro}. While these studies supported the conclusion that CKII phosphorylation of Dgk1 stimulates DAG kinase activity in stationary phase, the \textit{in
vivo studies examining nuclear/ER membrane structure and lipid droplet formation in the mid- to late- exponential phase indicated that phosphorylation by CKII also occurs in non-stationary phase cells. In fact, CKII is a constitutive protein kinase that phosphorylates proteins throughout growth (291).

Wild type Dgk1 existed as a single band in exponential phase cell. In comparison, in stationary phase cells, wild type Dgk1 existed as a doublet, along with the elevated activity. Alkaline phosphatase treated membranes containing overexpressed Dgk1 from stationary phase cells exhibited a dose-dependent band collapse from the slower migrating band to the faster migrating band, indicating the slower migrating band in the doublet represents the phosphorylated form of the enzyme. This suggests that DAG kinase in stationary phase is phosphorylated, but also not necessarily means it is not phosphorylated in exponential phase since phosphorylation does not always induce band shift of protein in SDS-PAGE analysis. Because S46A and S45A/S46A in Dgk1 abolished the slower migrating band in stationary phase cells, it suggests that the slower migrating band might be specifically the form of CKII phosphorylated DAG kinase.

Dgk1 DAG kinase activity is required for cells to resume growth from the stationary phase when fatty acid synthesis is blocked (77). The analysis of the phosphorylation-deficient and -mimicking mutants indicated that the CKII phosphorylation of Dgk1 is not involved in this process. This is not too surprising because for growth resumption, the conditions where CKII did not appear to regulate DAG kinase activity.

Of the numerous pah1Δ phenotypes that depend on Dgk1 DAG kinase activity, we examined three phenotypes to confirm the physiological relevance of the CKII
phosphorylation of the enzyme. In these assays, the S46A and S45A/S46A mutations in *DGK1* alleviated the *pah1Δ* phenotypes of temperature sensitivity, nuclear/ER membrane expansion, and reduced lipid droplet formation; the phosphorylation-deficient mutations permitted more grown at 37 °C, 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. These *in vivo* data supported the conclusion that the phosphorylation of Dgk1 by CKII stimulates DAG kinase activity.

It was not clear that how *dgk1Δ*, or the S45A and S46A mutations in Dgk1 lead to the partial restoration of lipid droplet formation in *pah1Δ*. Consistent with previous finding (78), lipid analysis showed neutral storage lipid TAG was not brought back much by *dgk1* deletion in *pah1Δ*. Adeyo *et al.* also showed that the disruption of *PAH1* gene did not change the total neutral lipid level, although there was a dramatic decrease in lipid droplet number (115). These indicate that the lipid droplet biogenesis defect may not be a result of alterations in neutral lipid. Adeyo *et al.* also provided evidence that DAG is important for lipid droplet formation by showing the elevated lipid droplet formation in *dgk1Δ pah1Δ and dgk1Δ dga1Δ lro1* cells when compared with that in *pah1Δ* and *dga1Δ lro1* cells, respectively, even if TAG synthesis was completely blocked (115). However, *dgk1Δ* mutation has increased DAG as well as decreased PA (115). It didn’t rule out the possibility that the elevated PA levels might be responsible for the decreased lipid droplet number. Fei *et al.* showed that PA might facilitate the coalescence of contacting lipid droplets, resulting in the formation of ‘supersized’ lipid droplets (292). It provided evidence that increased PA levels might be the cause of the decreased lipid droplet number in *pah1Δ*, and the repressed PA might help restore lipid droplet formation in
Data in this work didn’t explain the mechanism of restored lipid droplet formation in *dgk1Δ pah1Δ* and *dgk1Δ pah1Δ* expressing Dgk1 carrying S45A and S46A, due to the limitation of methodology sensitivity. Higher sensitive measurement of PA and DAG needs to be developed to address this mechanism.

It has been long known that CKII is essential to yeast cell growth because it phosphorylates and regulates proteins involved in gene expression, growth control, signal transduction, and cell cycle progression (173, 174, 293). This CKII phosphorylation study advanced our understanding of the lipid metabolic processes that are regulated by this protein kinase. Interestingly, CKII phosphorylates enzymes centered on the synthesis (i.e., Dgk1 DAG kinase) and degradation (i.e., Pah1 PA phosphatase (187)) of PA, as well as a transcription factor (i.e., Opi1 (162)) whose repressor function in the Henry regulatory circuit that involves Ino2/Ino4/Opi1 is governed by the levels of PA (100). CKII phosphorylation of Opi1 stimulates its function (162). In addition, CKII phosphorylates acetyl-CoA carboxylase (294), the enzyme responsible for the first step in the synthesis of fatty acids (280, 281), the hydrophobic moiety of PA and its derivative lipids (6). With respect to Dgk1 and Pah1, CKII has an inhibitory effect on PA phosphatase activity (187), whereas as shown here, CKII has a stimulatory effect on DAG kinase activity. The phosphorylations of these enzymes, that catalyze opposing reactions, must work together to coordinate the balance of PA and DAG. Yet, the coordinate regulation of these enzymes by CKII is likely to be very complex. For example, the phosphorylation of Pah1 by CKII is inhibited by its phosphorylations by PKA and by PKC, and the physiological effects of phosphorylation by CKII are
dependent on the Nem1-Spo7-mediated dephosphorylation of the sites phosphorylated by Ph85-Pho80 (187).

Because bioinformatic (237) analysis also predicted PKA target sites in the N-terminal region of DAG kinase, we also examined if Dgk1 is phosphorylated by PKA. The preliminary study of PKA phosphorylation identified Ser-25 and Ser-26 as the target sites. The mutation of S25A/S26A did not abolish all the phosphopeptides, indicating further study is required to identify the major PKA sites in Dgk1. It is determined that PKA only phosphorylates serine residues in Dgk1\textsuperscript{1-77}. The future work to identify the other PKA target sites was narrowed down to three candidate sites, Ser-12, Ser-32, and Ser-61. Interestingly, PKA also phosphorylates Pah1 PA phosphatase and Opi1(155, 161). PKA-mediated phosphorylation of Pah1 inhibits PA phosphatase activity(155). The phosphorylation of Opi1 at Ser-31 and Ser-251 stimulates its repressor activity (161). Treatment of alkaline phosphatase-treated Dgk1 with PKA did not show a change in enzyme activity. The cellular function of PKA phosphorylation of DAG kinase needs further exploration.

Mammalian DAG kinases are also subjected to regulation by phosphorylation. Some DAG kinase isoforms are phosphorylated by PKC (295-298) or by the tyrosine kinase Src (299) to stimulate their activity and functions in lipid signaling. Although PKC is conserved in yeast (167), yeast DAG kinase is not phosphorylated by PKC as shown in this study. CKII and PKA are also evolutionary conserved (291, 300), it is unclear whether they phosphorylate mammalian DAG kinase enzymes.

As mentioned above, there are other phosphorylation sites (Thr-3 and Thr-36) located at the N-terminal hydrophilic region of Dgk1, identified by phosphoproteomic
analysis (188-192). Furthermore, additional protein kinases and phosphorylation sites are predicted by bioinformatic analysis (237), additional studies are planned to unravel the complex regulation by multiple phosphorylations anticipated for Dgk1. Previous work by Han et al. (114) showed that a Dgk1 mutant lacking the first 66 amino acid residues exhibited a higher level of DAG kinase activity compared with the wild type protein. This suggests that the N-terminal region may contain regulatory sequences, and the regulatory mechanism is not due to CKII phosphorylation on Ser-45 and Ser-46 of Dgk1 based on the current study. Further study is also needed to investigate this mechanism. The mechanisms for the regulation of Dgk1 DAG kinase function uncovered in this work have opened the paths that warrant further investigation.
REFERENCES


*FEBS J.* **275**, 5552-5563


272. Remacle, J. E. and Holmberg, S. (1992) A REB1-binding site is required for GCN4-independent ILV1 basal level transcription and can be functionally replaced by an ABF1-binding site. Mol. Cell Biol. 12, 5516-5526


