Transcription Factor Reb1p Regulates DGK1-encoded Diacylglycerol Kinase and Lipid Metabolism in Saccharomyces cerevisiae*

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Background: Diacylglycerol kinase produces phosphatidate, a major precursor for the synthesis of membrane phospholipids.

Results: The expression of diacylglycerol kinase is induced by the Reb1p transcription factor, and the resulting activity increase is essential for the enzyme function in phospholipid synthesis.

Conclusion: The Reb1p-mediated transcriptional activation regulates the expression of diacylglycerol kinase activity.

Significance: Diacylglycerol kinase is regulated at the level of transcription.

In the yeast Saccharomyces cerevisiae, the DGK1-encoded diacylglycerol kinase catalyzes the CTP-dependent phosphorylation of diacylglycerol to form phosphatidate. This enzyme, in conjunction with PAH1-encoded phosphatidate phosphatase, controls the levels of phosphatidate and diacylglycerol for phospholipid synthesis, membrane growth, and lipid droplet formation. In this work, we showed that a functional level of diacylglycerol kinase is regulated by the Reb1p transcription factor. In the electrophoretic mobility shift assay, purified recombinant Reb1p was shown to specifically bind its consensus recognition sequence (CGGGTTAA, −166 to −160) in the DGK1 promoter. Analysis of cells expressing the P<sub>DGK1</sub>-lacZ reporter gene showed that mutations (GT→TG) in the Reb1p-binding sequence caused an 8.6-fold reduction in β-galactosidase activity. The expression of DGK1(reb1), a DGK1 allele containing the Reb1p-binding site mutation, was greatly lower than that of the wild type allele, as indicated by analyses of DGK1 mRNA, Dgk1p, and diacylglycerol kinase activity. In the presence of cerulenin, an inhibitor of de novo fatty acid synthesis, the d<sub>g</sub>k<sub>1</sub>Δ mutant expressing DGK1(reb1) exhibited a significant defect in growth as well as in the synthesis of phospholipids from triacylglycerol mobilization. Unlike DGK1, the DGK1(reb1) expressed in the d<sub>g</sub>k<sub>1</sub>Δ pah1Δ mutant did not result in the nuclear/endoplasmic reticulum membrane expansion, which occurs in cells lacking phosphatidate phosphatase activity. Taken together, these results indicate that the Reb1p-mediated regulation of diacylglycerol kinase plays a major role in its in vivo functions in lipid metabolism.

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Materials—All chemicals were reagent grade. Difco was the source of growth medium components. Restriction endonucleases, modifying enzymes, and Phusion high fidelity DNA polymerase were from New England Biolabs. Qiagen was the supplier of the DNA purification kit and nickel-nitritoltriacetic acid-agarose resin. Clontech was the source of the yeast transformation kit. Genosys Biotechnology, Inc., was the supplier of oligonucleotides used for PCRs and electrophoretic mobility shift assays. Cerulenin, nucleotides, IGEPA CA-630, nucleoside 5’-diphosphate kinase, Triton X-100, and protease inhibitors (phenylmethylsulfonyl fluoride, benzamidine, aprotinin, leupeptin, and pepstatin) were from Sigma. PerkinElmer Life Sciences and National Diagnostics were the sources of radiochemicals and scintillation counting supplies, respectively. Lipids were obtained from Avanti Polar Lipids, and silica gel TLC plates were from EM Science. Protein assay reagents, electrophoresis reagents, DNA and protein size standards, 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. ProbeQuant G-50 micro columns, polyvinylidene difluoride membrane, and the enhanced chemifluorescence Western blot reagent were purchased from GE Healthcare. Roche Applied Science supplied the mouse anti-β and anti-His antibodies.

Strains and Growth Conditions—The strains used in this work are listed in Table 1. Yeast cells were grown in YEPD medium (1% yeast extract, 2% peptone, 2% glucose) or in SC medium containing 2% glucose at 30 °C as described previously (40, 41). For selection of yeast cells bearing plasmids, the appropriate amino acids were omitted from SC medium. Plasmid maintenance/amplifications (strain DH5α) and Reb1p expression (strain BL21(DE3)pLysS) were performed in Escherichia coli. 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 the cells carrying plasmid. For growth on solid media, agar plates were prepared with supplementation of either 2% (yeast) or 1.5% (E. coli) agar. For heterologous expression of His6-tagged Reb1p, E. coli BL21(DE3)pLysS cells bearing pYQ3 were grown to A600 nm = 0.5 at 30 °C in 1 liter of LB

EXPERIMENTAL PROCEDURES

TABLE 1

| Strain or plasmid | Relevant characteristics | Source or Ref. |
|-------------------|-------------------------|---------------|
| **Strain** | | |
| E. coli DH5α | F− 4808lacZΔM15Δ (lacZYA-argF)U169 deoR recA1 endA1 hisd17 trp1-1 ura3-52 thi-1 relA1 | 41 |
| BL21(DE3)pLysS & S. cerevisiae | F− ompT hsdS 5 (r− m− g− gal dcm) (DE3) pLysS | Novagen |
| RS453 | MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-52 | 100 |
| SS1144 | dkg1Δ::His3 derivative of RS453 | 1 |
| SS1147 | dkg1Δ::His3 pah1Δ::TRP1 derivative of RS453 | 1 |
| **Plasmid** | | |
| pRS416 | Low copy E. coli yeast shuttle vector with URA3 | 101 |
| pSF211 | DGK1 inserted into pRS416 | 34 |
| pSF213 | Derivative of pSF211 with GT→TG mutations in the Reb1p-binding site | This study |
| pIO2 | pYQ1 lacZ reporter gene with URA3 | 46 |
| pYQ1 | pYQ1 lacZ reporter gene with URA3 | This study |
| pYQ2 | Derivative of pYQ1 with GT→TG mutations in the Reb1p-binding site | This study |
| PET-15b | E. coli expression vector with the N-terminal His6 tag fusion | Novagen |
| pYQ3 | REB1 coding sequence inserted into pET-15b | This study |
| YCplac33-SEC63-GFP | SEC63-GFP fusion into the CEN/URA3 vector | 1 |
| YCplac33-PAH1 | PAH1 into the CEN/URA3 vector | 24 |
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medium containing ampicillin (100 µg/ml) and chloramphenicol (34 µg/ml). The culture was then incubated for 3 h with 0.5 mM isopropyl β-D-thiogalactoside to induce the expression of Reb1p.

The growth regime of Fakas et al. (34) was used to examine the effects of the Reb1p-binding site mutation on the resumption of growth from the stationary phase. Cultures were grown for 48 h in SC medium to reach stationary phase, harvested by centrifugation, and diluted with fresh SC medium. Cerulenan (10 µg/ml) was added to the cultures to inhibit fatty acid synthesis (42, 43). For growth curves, cultures (200 µl) were incubated in 96-well plates, and the cell density was monitored at A650 nm with a Thermomax plate reader. Generation times were calculated from the growth curves according to the modified Gompertz equation (44).

DNA Manipulations, Amplification of DNA by PCR, Construction of Plasmids, and DNA Sequencing—Standard methods were used to isolate plasmid and genomic DNA and for the manipulation of DNA using restriction enzymes, DNA ligase, and modifying enzymes (41). PCRs were optimized as described by Innis and Gelfand (45). The plasmids used in this work are listed in Table 1. Plasmid pSF213, which was derived from plasmid pSF211, contains DGK1 with two transversion mutations in the Reb1p-binding site. This plasmid was constructed by PCR-mediated site-directed mutagenesis (primers: forward, 5'-ATCCAGGGTCATAGGCTGAAAATATTGGTTGTTG-3'; reverse, 5'-AACAAATATTGGTTCAACCCTAGGACCC-TGAT-3'). 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. These were constructed by replacing the DPP1 promoter in plO2 (46) with the wild type and mutant DGK1 promoter sequences at the EcoRI site. These DGK1 promoter sequences were obtained by PCR (primers: forward, 5'-GAGCTCGAATTCTCGTTTACCAAACAGGAAATGGTTTG-3'; reverse, 5'-GAGCTGAAATTCTGTTTACCAAACAGGAAATGGTTTG-3') using plasmids pSF211 and pSF212, respectively, as the templates. For expression of Reb1p in E. coli, the REB1 coding sequence was amplified by PCR (primers: forward, 5'-CAGCCCATGCTTCTGCAGTCATACGATAA-3'; reverse, 5'-GGCGGAGTTCTGTTATTTTCTGTTTTGATTG-3') 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 Reb1p was named pYQ3. All plasmid constructions were verified by DNA sequencing, which was performed by GENEWIZ, Inc. Standard protocols were used to transform E. coli (41) and yeast (47) cells with plasmids.

RNA Isolation and Quantitative RT-PCR—Total RNA was isolated with hot phenol (48-50) 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 (forward, 5'-CACCCCAAAGGGCAAGGAAAT-3'; reverse, 5'-AAGCAGCTACACACCAACT-3'). 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 the PCR efficiency 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 (5'-AGGGTCATAGGCGG-AAACAAATATTGGG-3'/5'-CCCAAATGATGCCCATTT- GTTAAAACC-5') and mutant (5'-AGGGTCATAGGCCGTGAAACAAATATTGGG-3'/5'-CCCAAATGATGCCCATTT- GTTAAAACC-5') sequences for Reb1p binding were prepared, labeled with [α-32P]dTTP (400–800 Ci/mmole) and Klenow fragment (5 units), and then purified by gel filtration using ProbeQuant G-50 spin columns as described previously (51). The radiolabeled DNA probe (4 pmol, 8.0 × 10^4 cpm/pmol) and purified recombinant His6-Reb1p 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 MgCl2, 50 mM KCl, 1 mM dithiothreitol, 0.025 mg/ml poly(dl-dc)poly(dl-dc), 0.2 mg/ml bovine serum albumin, 0.04% IGEPA 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.

Purification of His6-tagged Reb1p—His6-tagged Reb1p expressed in E. coli BL21(DE3)pLysS was purified to near homogeneity by affinity chromatography using nickel-nitrotriacetic acid-agarose (52). As described previously (53), the recombinant Reb1p with the predicted molecular mass of 92 kDa migrated on an 8% SDS-polyacrylamide gel as a 127-kDa protein (Fig. 2). Purified His6-Reb1p was stored at −80 °C.

Preparation of Cell Extracts and Total Membranes 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 (54). The cell disruption buffer contained 50 mM Tris-HCl (pH 7.5), 0.3 mM sucrose, 10 mM 2-mercaptoethanol, 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 (pellet) was prepared by centrifugation of the cell extract at 100,000 × g for 1 h (54). Protein concentration was estimated by the Coomassie Blue dye-binding method of Bradford (55) using bovine serum albumin as the standard.

SDS-PAGE and Western blot Analysis—SDS-PAGE (56) using 12% slab gels and Western blotting (57, 58) using polyvinylidene difluoride membrane were performed as described previously. Proteins in polyacrylamide gels were visualized by staining with Coomassie Blue R-250. The polyvinylidene difluoride membrane blots were probed with anti-Dgk1p antibodies (2) or with anti-Dpp1p antiserum (59) 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). The immune complexes were detected using the enhanced chemiluminescence Western blotting detection kit. Fluorimaging was used to acquire fluorescent signals from the immune complex reactions.
pyranoside, 1 mM 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.

Labeling and Analysis of Lipids—Cells were grown to stationary phase in the presence of [2-14C]acetate (1 μCi/ml) to uniformly label lipids. The labeled stationary phase cells were washed with water and resuspended to an A₅₀₀ of 0.5 in fresh growth medium without label to follow the mobilization of TAG (34). Total lipids were extracted (62) and analyzed by TLC using the solvent system hexane/diethyl ether/glacial acetic acid (40:10:1, v/v/v). The identity of labeled lipids on TLC plates was confirmed by comparison with standards after exposure to iodine vapor. Radiolabeled lipids were visualized by phosphor-imaging analysis, and the relative quantities of labeled lipids were analyzed using ImageQuant software.

Microscopy—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× plan-achromatic 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.

Data Analyses—Student’s t test (SigmaPlot software) was used to determine statistical significance, and p values of <0.05 were taken as a significant difference.

RESULTS

 Reb1p Interacts with a Reb1p-binding Site in the DGK1 Promoter—The DGK1 promoter contains the core consensus sequence (CGGGTAA, −166 to −160) for binding of the transcription factor Reb1p (53, 63–65). To determine whether the DGK1 sequence interacts with Reb1p, we performed an electrophoretic mobility shift assay with a double-stranded oligonucleotide probe containing the recognition sequence and pure His₅-tagged Reb1p (Fig. 2A). The radiolabeled probe showed a decreased electrophoretic mobility in a dose-dependent manner with respect to Reb1p (Fig. 2C, left panel). Unlabeled probe competed with the labeled probe for Reb1p binding in a dose-dependent manner (Fig. 2C, middle panel), indicating the specificity of the protein-DNA interaction. However, when transverse mutations (GT → TG, Fig. 2B) that are known to abolish Reb1p binding to the Reb1p-binding sequence (66) were introduced into the binding site, the electrophoretic mobility shift of the probe was greatly attenuated (Fig. 2C, right panel). Taken together, these data supported the conclusion that Reb1p directly interacts with the Reb1p-binding sequence in the DGK1 promoter.

 Reb1p-binding Site Mutation Attenuates the Expression of PDGK1-lacZ Reporter Gene Activity and the Abundance of DGK1 mRNA—The expression of DGK1 was examined by use of a PDGK1-lacZ reporter gene where the DGK1 promoter was fused with the coding sequence of the lacZ gene of E. coli. The
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β-galactosidase activity was dependent on the transcription of lacZ driven by the DGK1 promoter. The β-galactosidase activity in wild type exponential phase cells expressing the reporter gene was 86 ± 11 nmol/min/mg. The Reb1p-binding site mutation in the P_{DGK1-lacZ} reporter gene reduced the β-galactosidase activity by 8.6-fold (Fig. 3A). By using quantitative RT-PCR, we also examined whether Reb1p 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. 3B).

Reb1p-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 (1). 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 (1). To examine the dependence of DGK1 expression and function of the Reb1p-binding site in vivo, we examined the effect of the Reb1p-binding site mutation on 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 Reb1p-binding site mutation did not (Fig. 4). As described previously (1), 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. 4). This result indicated that the Reb1p-mediated regulation of DGK1 expression is crucial for its cellular function.

Reb1p-binding Site Mutation Compromises Growth Resumption from Stationary Phase in the Presence of Cerulenin—Stationary phase (static) cells resume vegetative growth upon replenishment with nutrients, and this process is dependent on the mobilization of TAG to synthesize phospholipids (34, 36, 67). 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 (34). Accordingly, we questioned if Reb1p-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 (42). As described previously (34), the expression of the wild type DGK1 gene complemented the loss-of-growth phenotype exhibited by dgk1Δ mutant cells (Fig. 5). However, the expression of the DGK1(reb1) mutant allele only partially complemented the growth defect (Fig. 5). 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 Reb1p-mediated expression of DGK1 was important for growth resumption from stasis.

Reb1p-binding Site Mutation Attenuates Expression of P_{DGK1-lacZ} Reporter Gene Activity, Dgk1p, 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 growth resumption from sta-
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**FIGURE 5.** Effect of the Reb1p-binding site mutation on the resumption of cell growth from stationary phase 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. Cell growth after the transfer to fresh medium was monitored with a plate reader. Each data point represents the average of triplicate enzyme determinations from a minimum of two independent experiments ± S.D. (error bars).

**FIGURE 6.** Effect of the Reb1p-binding site mutation on P_{DGK1}lacZ expression upon growth resumption from stationary phase 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 β-galactosidase activity. Each data point represents the average of triplicate enzyme determinations from five independent experiments ± S.D. (error bars).

**FIGURE 7.** Effect of the Reb1p-binding site mutation on Dgk1p abundance upon growth resumption from stationary phase 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; total membranes (20 μg) were prepared, and the amount of Dgk1p was determined by Western blot analysis using anti-Dgk1p antibodies. The same blot was also probed with anti-Dpp1p antibodies to detect the DPP1-encoded DAG pyrophosphate phosphatase. Portions of representative blots from three experiments are shown in the figure, and the positions of Dgk1p and Dpp1p are indicated.

**FIGURE 8.** Effect of the Reb1p-binding site mutation on DAG kinase activity upon growth resumption from stationary phase 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 two independent experiments ± S.D. (error bars).

For these experiments, DGK1 promoter activity was monitored by the β-galactosidase activity from the P_{DGK1}lacZ reporter gene expression. At stationary phase (at 0 h), the β-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 (Fig. 6). Although there was a relatively small variation in the β-galactosidase 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 β-galactosidase activity from the mutant reporter gene expression was moderately constant after nutrient supplementation (Fig. 6).

Next, we questioned whether the Reb1p-mediated control of DGK1 expression is translated into the levels of Dgk1p. Western blot analysis showed that there was some variation in the level of the Dgk1p at different time points (Fig. 7). However, the major conclusion from this experiment was that the levels of Dgk1p in cells expressing the DGK1(reb1) allele were greatly reduced (~7-fold) when compared with cells expressing the wild type DGK1 gene (Fig. 7). To confirm that the levels of Dgk1p were from cells at different growth phases, we analyzed the levels of DPP1-encoded DAG pyrophosphate phosphatase (Dpp1p) whose expression is known to be elevated in stationary phase and reduced in exponential phase (59). The growth phase-mediated regulation of Dpp1p expression was not altered in the dgk1Δ cells expressing DGK1 and DGK1(reb1) (Fig. 7).

The effect of the Reb1p-binding site mutation on the DGK1 expression was also examined by analysis of DAG kinase activity (Fig. 8). In stationary phase cells (at 0 h), the enzyme activity in dgk1Δ cells expressing the DGK1(reb1) allele was 4.3-fold lower than cell expressing DGK1. The reduction in the levels of DAG kinase activity correlated with the reduction in the expression levels of the reporter gene and Dgk1p. As described previously (34), 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. 8). However, the reduced level of DAG kinase activity in cells expressing the DGK1(reb1) allele did not show change (Fig. 8).

Reb1p-binding Site Mutation Compromises the Mobilization of TAG for Phospholipid Synthesis upon Nutrient Supplementation of Stationary Phase Cells—To examine the role of the Reb1p-mediated expression of DGK1 in the resumption of growth from stationary phase when fatty acid synthesis is...
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FIGURE 9. Effect of the Reb1p-binding site mutation on the mobilization of TAG for phospholipid synthesis upon growth resumption from stationary phase in the absence of de novo fatty acid synthesis. 

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 Reb1p. Through a detailed in vitro analysis, we showed that Reb1p specifically binds to its recognition sequence. Moreover, the Reb1p-binding site mutations greatly diminished the expression of DGK1 in vivo, which was translated into reduced expressions of DAG kinase protein and activity. The consequences of losing the Reb1p-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 Reb1p-binding site mutation supported some growth and the mobilization of TAG. Further proof that Reb1p medi-

DISCUSSION

The DGK1-encoded CTP-dependent DAG kinase has emerged as an important lipid metabolic enzyme in S. cerevisiae (1, 2, 30, 34). This ER-associated enzyme plays an important role in controlling the PA/DAG balance in the nuclear/ER membrane, which in turn regulates the synthesis of phospholipids, membrane growth, and lipid droplet formation (1, 2, 30). DAG kinase also alleviates the toxicity of DAG by virtue of its reaction to produce PA (34). The PAH1-encoded PA phosphatase, which catalyzes the conversion of PA to DAG (22), counteracts DAG kinase to control the PA/DAG balance (1, 22, 30). Interestingly, the dgk1Δ mutation does not impart any deleterious phenotypes under typical laboratory growth conditions (1). However, the DGK1 gene, along with its encoded DAG kinase activity, is essential for growth resumption of static cells when fatty acid synthesis is inhibited (34). In particular, DAG kinase participates in the mobilization of TAG to synthesize membrane phospholipids through PA (Fig. 1). Thus, the regulation of DGK1 expression and DAG kinase activity is likely to influence the balance of PA and DAG, lipid metabolism, and cellular growth.

The roles of DAG kinase in lipid metabolism and cell signaling are conserved throughout evolution. In mammalian cells, however, the enzyme utilizes ATP as the phosphate donor, and its localization is cytosolic in nature (4, 6, 68–70). The mammalian enzymes associate with membranes (governed by specific interaction domains) to convert DAG to PA (4, 6, 68–70). Unlike yeast containing only one DAG kinase (1), mammalian cells possess 10 isoforms (α, β, γ, δ, ε, ζ, η, θ, ι, and κ) that are differentiated by their primary structures, cellular locations, and functions (4, 6, 68–70). In controlling the balance of PA and DAG, whose concentrations impact on several signaling mechanisms, the mammalian enzymes influence numerous cellular processes important to diseases such as cancer, type II diabetes, autoimmunity, and nervous system disorders (e.g. epilepsy) (70–75). Clearly, 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 Reb1p. Through a detailed in vitro analysis, we showed that Reb1p specifically binds to its recognition sequence. Moreover, the Reb1p-binding site mutations greatly diminished the expression of DGK1 in vivo, which was translated into reduced expressions of DAG kinase protein and activity. The consequences of losing the Reb1p-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 Reb1p-binding site mutation supported some growth and the mobilization of TAG. Further proof that Reb1p medi-

block the mobilization of TAG to phospholipids was followed by a [2-14C]acetate labeling chase experiment (34). The amounts of lipids was determined up to 8 h following nutrient supplementation because maximum TAG hydrolysis has been shown in this time frame (34). As described previously (34), 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. 9). In dgk1Δ cells expressing DGK1, the amount of TAG declined in a time-dependent manner to a maximum of 43% by 8 h (Fig. 9). Reciprocally, the amount of phospholipids increased in a time-dependent manner to a maximum of 100% by 8 h (Fig. 9). Overall, the level of fatty acids increased by 186%, whereas the level of DAG decreased by 70% (Fig. 9). However, the Reb1p-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. 9).

FIGURE 9. Effect of the Reb1p-binding site mutation on the mobilization of TAG for phospholipid synthesis upon growth resumption from stationary phase in SC medium in the presence of [2-14C]acetate (1 Ci/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). FA, fatty acids; PL, phospholipids.
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A phosphorylation-regulated amphipathic helix controls the membrane translocation and function of the yeast phosphatidate phosphatase enzyme.

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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 (34). The mechanism for this regulation was not attributed to the Reb1p-mediated activation of DGK1 expression because the levels of P<sub>DGK1-lacZ</sub> reporter gene activity and Dgk1p 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. Several phosphorylation sites have been identified in the N-terminal region of Dgk1p by phosphoproteome analyses of *S. cerevisiae* (77–81). Thus, DAG kinase activity during growth resumption from stasis might be regulated by phosphorylation/dephosphorylation. Additional work will be needed to address this hypothesis.

The essential nature of Reb1p emanates from the fact that it is required for activation of genes (e.g. *ACS1*, *ACT1*, *ENO1*, *FAS1*, *FAS2*, *GCC1*, *ILV1*, *PGK1*, *RAP1*, and *REB1*) involved in various aspects of cell physiology that include lipid metabolism (82–90). In particular, Reb1p interacts with the promoters of *FAS1* and *FAS2* to activate their transcription (85). Fas1p (β-subunit) and Fas2p (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 (91–94). Interestingly, the promoters of *ACCI* and *ACBI*, whose protein products function before and after the fatty-acid synthase reactions, also contain the Reb1p-binding site (85). Accp1 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 (95, 96), whereas the Acb1p acyl-CoA-binding protein delivers fatty acyl-CoA molecules into lipid biosynthetic pathways (97–99). Also, the TGL3 promoter possesses the consensus Reb1p-binding sequence (85). Tgl3p is a major TAG lipase required for the mobilization of TAG (10, 11, 37, 39). Furthermore, the promoters of *DGAl* and *LRO1*, which encode acyltransferase enzymes responsible for the synthesis of TAG, and *CKII* and *EIKI*, which encode kinase enzymes responsible for the synthesis of phosphatidylcholine and phosphatidylethanolamine, respectively, via the Kennedy pathway (9), contain putative sequences for Reb1p interactions. It is unknown whether Reb1p plays a role in the transcriptional activation of *ACCI*, *ACBI*, *TGL3*, *DGAl*, *LRO1*, *CKII*, and *EIKI*. 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 speculate that these genes might be regulated in a coordinate manner with *DGK1* by the transcription factor Reb1p. Reb1p is subject to positive and negative autoregulation (90), but whether it is regulated under these growth conditions is unknown.

In summary, this work advanced the understanding of the regulation of DAG kinase in *S. cerevisiae*. Our data supported the conclusion that *DGK1* expression was activated by the transcription factor Reb1p through its direct interaction with a Reb1p-binding site in the promoter. This study also advanced the understanding of the role that Reb1p plays in the regulation of lipid metabolism.
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