REGULATION OF PHOSPHOLIPID SYNTHESIS IN *SACCHAROMYCES CEREVISIAE* BY mRNA STABILITY

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ABSTRACT OF THE DISSERTATION

Regulation of Phospholipid Synthesis in *Saccharomyces cerevisiae* by mRNA Stability

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In the yeast *Saccharomyces cerevisiae*, the most abundant phospholipid phosphatidylcholine is synthesized by the complementary CDP-diacylglycerol and Kennedy pathways. Using a *cki1*Δ *eki1*Δ mutant defective in choline kinase and ethanolamine kinase, we examined the consequences of a block in the Kennedy pathway on the regulation of phosphatidylcholine synthesis by the CDP-diacylglycerol pathway. The *cki1*Δ *eki1*Δ mutant exhibited increases in the synthesis of phosphatidylserine, phosphatidylethanolamine and phosphatidylcholine via the CDP-diacylglycerol pathway. The increase in phospholipid synthesis correlated with increased activity levels of the CDP-diacylglycerol pathway enzymes phosphatidylserine synthase, phosphatidylserine decarboxylase, phosphatidylethanolamine methyltransferase, and phospholipid methyltransferase. However, other enzyme activities, including phosphatidylinositol synthase and phosphatidate phosphatase, were not affected in the *cki1*Δ *eki1*Δ mutant. For phosphatidylserine synthase, the enzyme catalyzing the committed step in the pathway, activity was regulated by increases in the levels of mRNA and protein. Decay analysis of *CHO1* mRNA indicated that a dramatic increase in transcript stability was a major component responsible for the elevated level of phosphatidylserine synthase.

We examined the decay pathway of *CHO1* mRNA by analyzing the rates of transcript degradation in mutants defective in a specific mRNA decay pathway. When compared with the decay ($t_{1/2}$ = 10-12 min) of the wild type control, the half-life of *CHO1* mRNA was increased ($t_{1/2}$ > 45 min) in the *ccr4*Δ, *dcp1*Δ, and *xrn1*Δ mutants defective in deadenylation, decapping, and 5'-to-3' exonucleolytic degradation, respectively. The stability of *CHO1* mRNA also increased in the *ski4-1* mutant defective in the 3'-to-5' exosome-mediated decay pathway. These results indicated that *CHO1* mRNA in *S. cerevisiae* is degraded through the 5'-to-3' and 3'-to-5' decay pathways. We also found that *CHO1* mRNA decay was defective in respiratory deficient mutants that were derived from wild type cells and from an *eki1* Δ mutant. The respiratory inhibitor KCN caused a dose dependent increase in *CHO1* mRNA stability. This increase in mRNA stability was recapitulated in a *cox4*Δ mutant defective in the cytochrome c oxidase enzyme. These results indicated that mitochondrial respiration was required for normal *CHO1* mRNA decay.

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LIST OF ABBREVIATIONS

AdoMet	S-adenosylmethionine
ADP	adenosine diphosphate
ARE	AU rich element
ATP	adenosine triphosphate
CDP	cytidine diphosphate
CDP-DAG	cytidine diphosphate diacylglycerol
CL	cardiolipin
CMP	cytidine monophosphate
CoA	coenzyme A
CTP	cytidine triphosphate
DAG	diacylglycerol
DGPP	diacylglycerol pyrophosphate
DSE	downstream sequence element
EDTA	ethylenediamine tetraacetic acid
ER	endoplasmic reticulum
FA	fatty acid
GFP	green fluorescent protein
bHLH	basic-helix-loop-helix
ICRE	inositol choline responsive element
KCN	potassium cyanide
NADH	nicotinamide adenine dinucleotide
NMD	nonsense mediated decay

opi$^-$	overproduction of inositol phenotype
P_i	inorganic phosphate
PA	phosphatidic acid
PC	phosphatidylcholine
PCR	polymerase chain reaction
PDME	phosphatidyldimethylethanolamine
PE	phosphatidylethanolamine
PG	phosphatidylglycerol
PI	phosphatidylinositol
PKA	protein kinase A
PKC	protein kinase C
PMME	phosphatidylmonomethylethanolamine
PL	phospholipid
PS	phosphatidylserine
PVDF	polyvinylidene difluoride
SC	synthetic complete
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
STE	stabilization element
TAG	triacylglycerol
TLC	thin layer chromatography
UAS	upstream activation sequence

UAS_{INO}	inositol-sensitive upstream activating sequence
UTP	uridine triphosphate

INTRODUCTION

Cell membranes are indispensable to the life of the cell (1). They define the boundaries of cells and their intracellular organelles (1). Membranes also serve as a site in which many important biological reactions occur (1). Membranes are composed of lipid bilayers and protein molecules that are held together by non-covalent interactions. Phospholipids are the most abundant membrane lipids (1). They are amphipathic molecules made up a hydrophilic head group and hydrophobic tails (1). They help modulate the function of membrane proteins and serve as a reservoir of lipid signaling molecules (2-4). Phospholipids and cellular growth are intimately related. The regulation of genes and enzymes in phospholipids synthesis is important for understanding the molecular basis for various diseases (5).

The unicellular microorganism, *Saccharomyces cerevisiae*, is used as a model system to study phospholipid metabolism. The phospholipids of yeast and regulation of their synthesis are typical of eukaryotic cells (6). *S. cerevisiae* has long been used for biochemical studies because they are easy to grow in large quantities (6). In addition, the ease of gene manipulation and complete genome sequences allow it to be an ideal organism for genetic research. These characteristics make *S. cerevisiae* an ideal model for studying phospholipid biosynthesis and its regulation (6, 7).

Phospholipids

The major phospholipids found in the membranes of *S. cerevisiae* are PC (phosphatidylcholine), PE (phosphatidylethanolamine), PI (phosphatidylinositol) and PS (phosphatidylserine) (6, 8). Phospholipids have a common structure containing a

glycerol backbone, in which the first and second carbons have long chain fatty acyl groups, and the third carbon has a phosphate group to which hydrophilic group is attached to (Fig. 1). The fatty acyl moieties provide a hydrophobic barrier, and the rest of the molecule has hydrophilic properties (9). These amphipathic properties form the phospholipid bilayer of the membrane. PC, PE, PS and PI contain choline, ethanolamine, serine, and inositol, respectively, as the hydrophilic group attached to the phosphate (Fig. 1) (9). The phospholipid composition of membranes can vary depending on growth conditions, but the average charge of the membrane phospholipids is relatively constant (8). Thus, *S. cerevisiae* has mechanisms that compensates for changes in the levels of phospholipids of one type by coordinating the changes in the level of phospholipids of another type (8). The mechanisms that control this coordinated process mediate the levels of genes and proteins of the biosynthetic enzymes as well as their enzyme activities (5-7). Phospholipid biosynthesis is a complex process including a number of branch points and connected pathways (Fig. 2) that are coordinated with other biological processes (5). Many structural genes encoding the enzymes required for the synthesis of membrane phospholipids in *S. cerevisiae* have been identified, and their gene products have been purified and studied (7, 10).

Phospholipid Biosynthetic Pathways

The phospholipid biosynthesis pathways in *S. cerevisiae* are generally similar to the pathways in higher eukaryotes. One exception is the synthesis of PS. In mammalian cells, PS is synthesized by an exchange reaction between PE and serine (11). PC is the

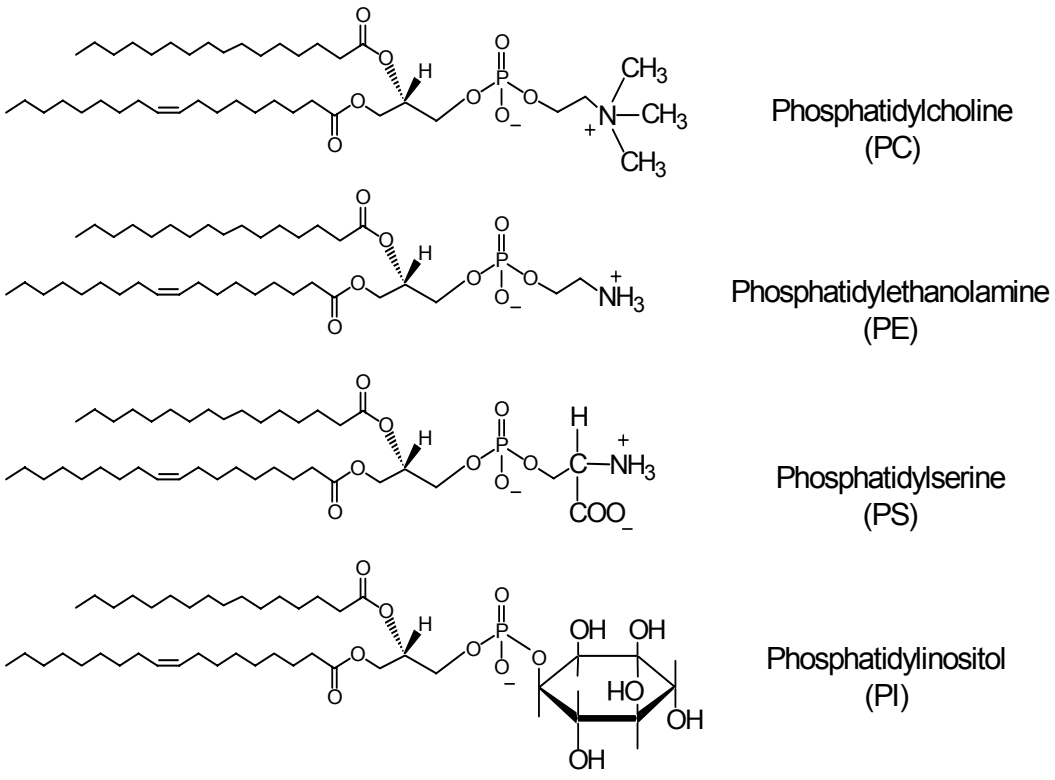
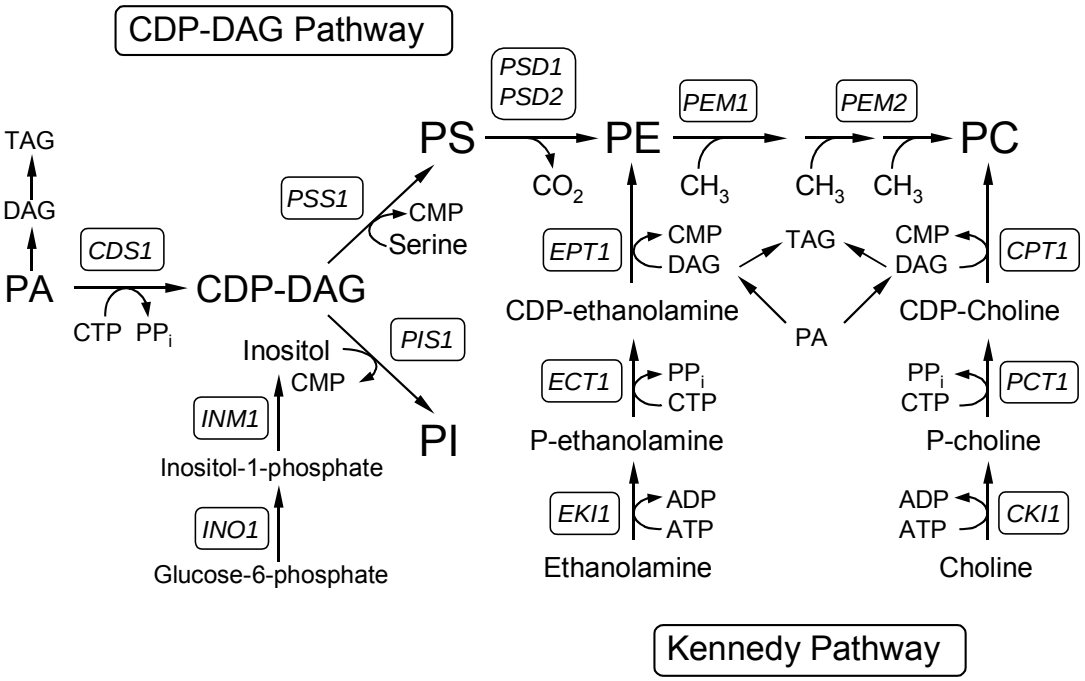


Figure 1. Major phospholipids in *S. cerevisiae*.

Figure 2. Phospholipid biosynthetic pathways in *S. cerevisiae*. The enzymes *CDS1*-encoded CDP-DAG synthase, *PSS1*-encoded PS synthase, *PSD1/PSD2*-encoded PS decarboxylase, *PEM1*-encoded PE methyltransferase and the *PEM2*-encoded PL methyltransferases catalyze reactions that lead to the formation of PC by the CDP-DAG pathway. *CKI1*-encoded choline kinase, *PCT1*-encoded choline-P cytidylyltransferase, and *CPT1*-encoded choline phosphotransferase catalyze reactions that lead to the formation of PC by the CDP-choline branch of the Kennedy pathway. *EKI1*-encoded ethanolamine kinase, *ECT1*-encoded ethanolamine-P cytidylyltransferase, and *EPT1*-encoded ethanolamine phosphotransferase catalyze reactions that lead to the formation of PE by the CDP-ethanolamine branch of the Kennedy pathway. *INO1*-encoded inositol 3-phosphate synthase and *INM1*-encoded inositol 3-phosphate phosphatase catalyze reactions that lead to the formation of inositol, which is utilized for the formation of PI. Abbreviations: PA, phosphatidate; CDP-DAG, cytidine 5'diphospho-diacylglycerol; PS, phosphatidylserine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PI, phosphatidylinositol; DAG, diacylglycerol; TAG, triacylglycerol.


CDP-DAG Pathway
PSD1
PSD2
PEM1
PEM2
PS
PE
PC
CO2
CH3
CH3
CH3
TAG
DAG
PA
CDS1
CTP
PPi
CDP-DAG
PSS1
CMP
Serine
EPT1
CMP
DAG
TAG
CMP
DAG
CPT1
CDP-ethanolamine
PA
CDP-Choline
PIS1
Inositol
CMP
PI
ECT1
PPi
CTP
PPi
CTP
PCT1
INM1
Inositol-1-phosphate
P-ethanolamine
P-choline
EKI1
ADP
ATP
ADP
ATP
CKI1
INO1
Glucose-6-phosphate
Ethanolamine
Choline
Kennedy Pathway

most abundant membrane phospholipids in *S. cerevisiae* and major structural component of the membrane (12). PC serves as a reservoir of bioactive lipids such as lyso-PC, PA, DAG, platelet activating factor, and arachidonic acid (12). In *S. cerevisiae*, PC is synthesized by way of the CDP-DAG pathway and the Kennedy pathway (Fig. 2) (6, 8). In the CDP-DAG pathway, CDP-DAG, a key branch point in phospholipid metabolism, is synthesized from PA by CDP-DAG synthase. PS is synthesized via displacement of CMP from CDP-DAG with serine by PS synthase (13-15). PS is decarboxylated to form PE by PS decarboxylase (16-19), and PC is formed through a series of methylation reaction of PE by PE methyltransferase, and phospholipid methyltransferase (20-23). When PC synthesis via the CDP-DAG pathway is blocked, PC is generated from the CDP-choline and CDP-ethanolamine branches of the Kennedy pathway (24). If supplemented with choline, choline is transported into the cell, (24-27) and phosphorylated to form PC by choline kinase (28). Then, phosphocholine is converted to CDP-choline by phosphocholine cytidylyltransferase (8, 28). CDP-choline is utilized by cholinephosphotransferase to produce PC (29, 30). Mutants defective in PS synthase, PS decarboxylase, PE methyltransferase and phospholipid methyltransferase can produce PC via CDP-choline pathway (31-34). When cells are supplemented with ethanolamine, it is converted to PE via the CDP-ethanolamine branch of the Kennedy pathway. This occurs by the reactions catalyzed by ethanolamine kinase, phosphoethanolamine cytidylyltransferase and ethanolamine phosphotransferase (31). The PE derived from this pathway undergoes the three-step methylation reactions to synthesize PC via the CDP-DAG pathway (35). Thus, mutants defective in PS synthase and PS decarboxylase are

able to produce PC via the CDP-ethanolamine branch of the Kennedy pathway (31, 34) when they are supplemented with ethanolamine.

CDP-DAG is also used to synthesize PI, another major phospholipid, by the PI synthase enzyme (36). This enzyme displaces CMP from CDP-DAG with inositol. PA, the precursor of the major phospholipids (9), is also used to synthesize DAG and TAG (10). PA phosphatase dephospholylates PA to make DAG and Pi (37). DAG synthesized from this reaction is used to produce PC and PE in the CDP-choline and CDP-ethanolamine branches of the Kennedy pathway, and to form TAG (37). The utilization of the CDP-DAG and Kennedy pathways for PC synthesis in wild type cells depends on whether or not choline is supplemented to the culture medium (24). When cells are grown in the presence of choline, PC is primarily synthesized by the Kennedy pathway. When grown in the absence of choline, yeast primarily synthesizes PC via the CDP-DAG pathway. However, even in the absence of choline, the Kennedy pathway still contributes to the synthesis of PC because choline is generated from the phospholipase D–mediated turnover of PC synthesized via CDP-DAG pathway (24, 38-40). The *cki1*Δ *eki1*Δ mutant lacking choline kinase and ethanolamine kinase is defective in the both the CDP-choline and CDP-ethanolamine branches of the Kennedy pathway (35, 41). In this mutant, PC can be only synthesized by the CDP-DAG pathway (35, 41).

Regulation of Phospholipid Synthesis

Phospholipid biosynthesis is regulated by many factors including water-soluble phospholipid precursors, nucleotides, lipids, growth phase, phosphorylation, and zinc availability (5, 7, 10, 42-47). The regulation of phospholipid synthesis by these factors is

mediated by biochemical mechanisms that regulate enzyme activity directly, and by genetic mechanisms that control gene expression and protein synthesis. (5, 10, 42, 43).

Biochemical regulation

The regulation of phospholipid synthesis is linked to nucleotide and lipid metabolism, and general nutrient control (10). Inositol regulates the activity of the phospholipid biosynthetic enzymes (42). PS synthase is a representative enzyme, which is regulated by inositol at a biochemical level. Inositol inhibits PS synthase activity by a noncompetitive mechanism (48). The inhibition of PS synthase contributes to an increase of PI synthesis at the expense of PS because these both enzymes utilize CDP-DAG as the common substrate (48).

Phosphorylation is another biochemical mechanism by which an enzyme activity is regulated. Mg^{2+}-dependent -PA phosphatase (45-kDa), choline kinase, CTP synthetase, and PS synthase are the phospholipid biosynthetic enzymes, which are regulated by phosphorylation (49-52). Protein kinase A phosphorylates these enzymes on a serine residue (49-52). PA phosphatase (45-kDa), choline kinase, and CTP synthetase enzymes are activated by phosphorylation whereas PS synthase is inactivated by phosphorylation. Protein kinase C phosphorylates CTP synthetase on both serine and threonine residues, and this activates the enzyme (53).

Lipids also regulate phospholipid biosynthetic enzymes. PA phosphatase (45-kDa and 104-kDa) is inhibited by sphingoid bases (54) and activated by CL, CDP-DAG, PI, and DGPP (55, 56). PI 4-kinase (45-kDa) is inhibited by CDP-DAG and PG (57). Nucleotides regulate the phospholid biosynthetic enzymes by controlling their activities. ATP and CTP inhibit 45 and 104-kDa Mg^{2+}-dependent PA phosphatase activity by

chelating the cofactor of the enzyme (58). The activity of choline kinase is regulated allosterically by its substrate ATP and its product ADP. ATP activates its activity by promoting the oligomerization of the enzyme, whereas ADP inhibits choline kinase activity by affecting the catalytic properties and the substrate affinity of the enzyme (59). CTP synthetase is inhibited by its product CTP. The inhibition is a result from an increase of the positive cooperativity of the enzyme for UTP (60). CTP synthetase with a Glu^{161}→Lys (E161K) mutation is less inhibited by CTP, and consequently, CTP levels accumulate in strains carrying an E161K mutation (61). In addition, the regulation of CTP synthetase by CTP controls the pathways by which PC is synthesized (61). Elevated levels of CTP result in an increase in the utilization of the CDP-choline pathway at the expense of the CDP-DAG pathway. The mechanism for this regulation is increased substrate availability for the P-choline cytidylyltransferase reaction and through inhibition of PS synthase (61, 62).

Genetic Regulation

Genetic regulation affects the activities of enzymes related to phospholipid synthesis by controlling gene expression and protein at the transcriptional level (5). The effect of inositol supplementation on the phospholipid synthesis is a representative example of genetic regulation. Inositol supplementation represses the transcription of *INO1* and other genes in the CDP-DAG and Kennedy pathways, and inositol starvation promotes the transcription of the genes. All genes responsive to inositol have a 10-base pair sequences 5'-CATGTGAA-3' called UAS_{INO} in their promoters (5, 7, 63). These genes include *CDS1*, *CHO1*, *PSD1, CHO2*, *OPI3*, *CKI1*, *PCT1*, *CPI1*, *EKI1*, *EPT1*, and *INO1* (5, 10, 41, 43, 64). However, the extent of transcriptional regulation in these genes

varies (10). For example, *INO1* is the most highly regulated gene in response to inositol whereas *OPI3* is the least regulated gene (10). The regulation of gene expression of UAS_{INO}–containing genes is coordinated with one another in the synthesis of phospholipids (5). The first 6 bases of the UAS_{INO} element, 5'CANNTG3' is the core sequence which serves as a binding site for the heterodimer Ino2p/Ino4p complex (6, 43, 65-67). The Ino2p/Ino4p complex contributes to maximum expression of phospholipid biosynthetic genes to which they bind. Ino2p contains an activation domain for transcriptional expression, and its binding to the UAS_{INO} element requires dimerization with Ino4p (5). Like mutations of the UAS_{INO} element, mutations of the *INO2* and *INO4* genes cause the down-regulation of phospholipid gene expression (10, 43, 63, 65, 66, 68, 69). Thus, Ino2p and Ino4p are required for maximal gene expression. Opi1p, the *OPI1* gene product, mediates repression of phospholipid biosynthetic gene expression in the response to inositol (5, 10). In other words, Opi1p is a negative regulator of UAS_{INO}-containing genes. Opi1p is localized to the endoplasmic reticulum (ER) through its interaction with PA and by Scs2p (70). Opi1p is released from the ER by PA utilization and translocated into the nucleus (70). However, Opi1p does not bind to DNA. Instead, Opi1p binds Ino2p to repress gene expression (70). Opi1p has a leucine zipper and glutamine rich region that is associated with transcriptional regulatory proteins (71). Strains harboring a *opi1*Δ mutation have a high level of expression of UAS_{INO}-containing genes. On the contrary, overexpression of Opi1p causes the repression of UAS_{INO}-containing genes (5, 43).

There is a general pattern of regulation by growth phase and nutrition availability of phospholipid biosynthetic genes that have UAS_{INO} *cis*-acting elements in their

promoters. The co-regulated genes of phospholipid biosynthesis are maximally expressed during exponential phase in the absence of inositol (7, 10). When inositol is added to the growth medium, these UAS_{INO} containing genes are repressed (10). The repression by inositol is increased with the addition of choline or ethanolamine to the growth medium. (5-7, 43, 66). The genes regulated by inositol and choline are repressed as cells enter stationary phase even in the absence of inositol and choline (72). In addition, because the deprivation in the growth medium is induced in the stationary phase, the depletion of nutrients also lead to a repression of UAS_{INO}-containing genes (73). The general pattern of regulation also occurs in mutants defective in the regulatory gene *INO2*, *INO4* and *OPI1* (10). Unlike most of the phospholipid biosynthetic genes containing a UAS_{INO} element, some structural genes involved with phospholipid synthesis do not contain this *cis*-acting sequences, leading to different regulation from UAS_{INO}–containing genes. For example, expression of genes including *INM1* (inositol-3-phosphate) (74), *DPP1* (DGPP phosphatase) (75), PA phosphatase (Mg^{2+}-dependent) (76, 77) is increased rather than decreased by inositol and in the stationary phase (10).

mRNA Stability Regulation in Lipid Metabolism

Transcriptional and biochemical regulation have been known as the main mechanisms for controlling phospholipids synthesis. However, a form of regulation not previously shown for phospholipids synthesis is post-transcriptional regulation by mRNA stability. In the lipid area, the regulation of transcript degradation in the cytoplasm has been described for *OLE1*, which encodes Δ-9 fatty acid desaturase, converting saturated fatty acyl-CoAs to *cis*-Δ-9 unsaturated fatty acids (78). Gonzalez and Martin (78)

observed that the half-life for the *OLE1* is 10-12 min in the absence of fatty acids and is less than 2 min in the presence of unsaturated fatty acids. The unsaturated fatty acid-induced destabilization of *OLE1* results from activation of exosomal 3'-to-5' degradation activities (79). The study on the *OLE1* mRNA stability revealed that ER-resident membrane protein, Mga2p, plays a key role in the regulation of *OLE1* mRNA stability in the absence and presence of fatty acids in the medium (79). In *mga*2Δ, the half-life of the *OLE1* mRNA is decreased by 2-fold and fatty acid-induced instability is lost (79). These findings indicated that Mga2p stabilizes the *OLE1* mRNA in wild-type cells grown in fatty acid free medium and is required to destabilize the transcript in cells exposed to unsaturated fatty acids (80). *OLE1* mRNA stability in the fatty acid free medium is controlled by a general 5'-to-3' decay pathway mechanism (81). Fatty acid synthase is another lipid-related gene regulated by mRNA stability. The increase of fatty acid synthase enzyme activity, protein, and mRNA abundance induced by glucose supplementation in HepG2 cells results from an increase in mRNA stability (82).

mRNA Stability

Eukaryotic gene expression is regulated by various steps following transcription in the nucleus (83). The regulation of posttranscriptional gene expression includes pre-mRNA processing, mRNA nucleo-cytoplasmic transport, subcellular localization, subsequent translation, and mRNA degradation (83, 84). The amount of any specific mRNA is determined by its rate of synthesis and its rate of degradation (mRNA stability) (83, 85). mRNA stability is an important control point in controlling gene expression (84-86). The half-lives of individual mRNAs, the time required for the disappearance of

50% of a given mRNA, vary as much as 10- to 100- fold (85-88). For example, unstable mRNAs have half-lives of 1-5 min, whereas the half-lives of stable mRNA are as long as 30-60 mins (89). The difference of decay rates of these mRNAs is likely to result from the modulation of the activity and gene expression of general trans-acting factors like Dcp1, Xrn1p, Ccr4p (83). The activities of these general factors can also be modulated by mRNA specific trans-acting factors. The mutation of these trans-acting factors specific for distinct decay pathway is usually used to determine the mRNA decay pathway (90).

The decay rate of these mRNA can be also regulated by ARE (AU-rich element) in 3'UTR. AREs can modulate poly (A) shortening rate and subsequent decay of the mRNA (83). These functions of AREs are mediated by specific binding protein like Pub1p. In mammalian cells, some proteins binding to ARE like AUF (ARE/ poly-(u) binding degradation factor) and TTP (tristetraprolin) stimulate mRNA degradation, while others like ELAV (embryonic lethal abnormal vision) it (83, 84). These interactions of ARE with trans-acting proteins are shown to modulate the rate of poly (A)-shortening by affecting the stability of the poly (A) and poly (A) binding protein complex.

Environmental metabolic signals can also affect mRNA stability (91). *OLE1* mRNA is destabilized when fatty acid is supplemented to growth medium (79, 81, 92). *SPO11* and *SPO13* transcripts are also degraded following a shift from acetate– to glycerol–containing medium. The stability of these meiotic mRNAs is regulated (93) by *UME2* and *UME5* gene products (94). The mammalian *FAS1*-encoded fatty acid synthase is also regulated by mRNA stability (82). The stability of *FAS* mRNA is increased when glucose is supplemented (82). So, the difference of mRNA stability

plays an important role in the expression of specific genes, providing the cell with adaptation to environmental change (95).

mRNA Decay Pathway

Several pathways for mRNA decay have been identified in eukaryotes (Fig. 3) (87, 96). In *S. cerevisiae*, general pathways of mRNA degradation are as follows:

The initial step of mRNA degradation is the shortening of the poly (A) tail at the 3' end of a transcript to an oligo (A) length of 10 to 15 nucleotides (91). This deadenylation requires mRNA deadenylase referred to as PAN (poly A-specific nuclease) (96, 97). PAN acts in concert with Pab1p, poly (A)-binding protein that stabilize the poly (A) tail by protecting it from other deadenylating nucleases (88, 98). Deadenylation by PAN removes the Pab1p binding site in poly (A) and leads to decapping (98-101). Following deadenylation, the cap structure is removed by a decapping enzyme consisting of two subunits, Dcp1p and Dcp2p (102-104), exposing the mRNA to degradation by 5' to 3' exonuclease Xrn1p. Dcp2p is required for the synthesis of Dcp1p (102, 105). The binding of the Dcp1p/Dcp2p complex to the transcript is increased by the cytoplasmic *LSM* complex, which facilitates the decapping step of mRNA degradation (106). Edc1p and Edc2p, enhancer of mRNA decapping proteins (107), also stimulate this decapping process. Then, decapped mRNAs are rapidly digested by the 5'-to-3' exonuclease Xrn1p. Xrn1p is a cytoplasmic member of a larger family of conserved exonucleases (108). Alternatively, after deadenylation, mRNAs can undergo another decay pathway in 3'-to-5' direction by 3'-to-5' exonuclease complex called the exosome (109, 110). The yeast exosome include at least 10 essential components: Rrp4p, Rrp40p, Ski6p, Rrp42p,

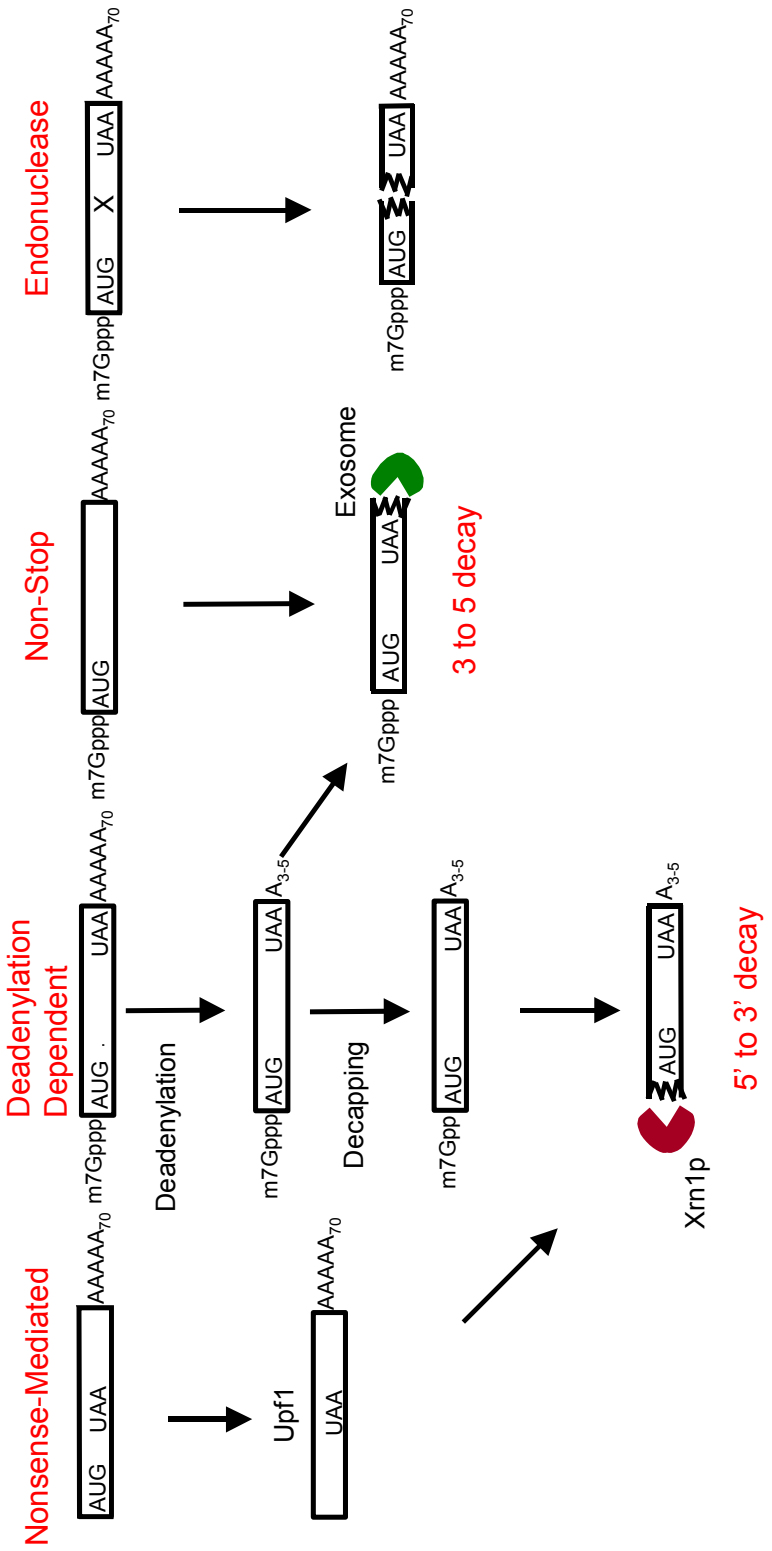


Figure 3. mRNA Decay Pathways.

Rrp43p, Rrp44p/Dis3p, Rrp45p, Rrp46p Mtr3p, Ski4p (110, 111). These 10 subunits are all essential for growth. In this pathway, the 5' cap structure is removed by the DcpS, scavenger-decapping enzyme (112). Unlike Dcp1p decapping enzyme that acts on the 5' cap structure with intact mRNA, DcpS scavenger enzyme functions on residual cap structure lacking on RNA moiety (112, 113).

Another degradation of mRNAs can be initiated by site-specific endonucleolytic cleavage (114, 115). Those mRNAs are cleaved into two parts by endonucleases and rapidly degraded by exonucleases (85). The degradation by endonucleolytic cleavage does not require poly (A) tail shortening. mRNA degradation through endonucleolytic cleavage has been generally found in transferring receptor of mammalian cells (116), cytokine (117), and growth factor (118, 119). Endonucleolytic cleavage site in many mammalian cells including TfR mRNA, IL2 is frequently located in 3' UTR, 5'UTR or ARE regions (85). The interaction of these cleavage sites with RNA-binding proteins can block access of endonuclease and stabilize the mRNA (85). In *S. cerevisiae*, several observations suggest that mRNA can be cleaved within its coding region (114, 115).

S. cerevisiae can also have specific mRNA decay pathway referred to as nonsense-mediated decay (NMD) (120-124). In this process, mRNAs harboring a premature translational termination codon enter into 5' to 3' decay pathway without the deadenylation step and then are decapped by Dcp1p, followed by a 5' to 3' degradation by Xrn1p (91, 121). The degradation of these nonsense-mediated mRNAs depends on the recognition of the premature termination codon by translational assembly (84, 121). This recognition of the premature nonsense codon requires surveillance complex such as Upf1, Upf2, Upf3 proteins and another *cis*-acting DSE (downstream sequence elements)

located at 3' of sequences (84, 125). Thus, the activation of NMD requires surveillance complex like Upf1p, premature termination codon, and 3' DSE (125, 126). Single or multiple mutations of these *UPF* genes inactivate the NMD pathway, stabilizing transcripts (84, 121, 123, 127). However, other transcripts containing specific genes, referred as stabilizer elements (STEs), do not undergo the NMD pathway. Pub1p, the poly (U)-binding protein, binds to the STE and interfere with activation of NMD pathway (125, 126).

mRNAs lacking translation termination codons are recognized and rapidly degraded by the exosome in a 3'→5' direction known as the non-stop pathway (128, 129). The recognition of these nonstop transcripts requires the exosome-associated protein Ski1p that binds to ribosome and brings the exosome to mRNA (129).

Phosphatidylserine Synthase

The *CHO1*-encoded (13-15) PS synthase (EC 2.7.8.8) is one of the most highly regulated enzymes of phospholipid synthesis in the yeast *Saccharomyces cerevisiae* (10, 42, 130, 131). This integral endoplasmic reticulum-associated enzyme (132) catalyzes the formation of PS by a Mn^{2+}-dependent sequential reaction by displacing CMP from CDP-DAG with serine (133). The reaction product PS is a major structural component of yeast membranes accounting for 4-18% of total phospholipids depending on growth conditions (6, 134, 135). PS also serves as the precursor for the synthesis of the most abundant membrane phospholipids PE (20-32%) and PC (35-55%) that are synthesized by the de novo CDP-DAG pathway (Fig. 2) (10, 134).

PS synthase is regulated by biochemical and genetic mechanisms, both of which have an impact on the synthesis of PC via the CDP-DAG and Kennedy pathways (10, 42, 130, 131, 136). The activity of PS synthase is modulated (i.e., inhibited or activated) by membrane phospholipids (e.g., PA, phosphatidylglycerol, cardiolipin,) (75, 137, 138), and is inhibited by inositol (48) and by the nucleotide CTP (62). The enzyme is also regulated by phosphorylated/dephosphorylation; phosphorylation inhibits activity, whereas dephosphorylation activates activity (52, 139, 139). In general, the inhibition of PS synthase activity favors PC synthesis via the Kennedy pathway (10, 42). The biochemical regulation of PS synthase activity also governs the partitioning of the substrate CDP-DAG between PS and PI; the inhibition of PS synthase activity favors PI synthesis (Fig. 2) (42).

The expression of the PS synthase (*CHO1*) gene is regulated by the supplementation of water-soluble phospholipid precursors (e.g., inositol) (140-143), zinc deprivation (46), and by growth phase (72, 144). *CHO1* is maximally expressed in exponential phase cells when grown in the absence of inositol (140-143) and grown in the presence of zinc (46). *CHO1* is repressed when inositol is supplemented to the growth medium (140-143) or when zinc is depleted from the growth medium (46). The zinc-mediated regulation of *CHO1* occurs in the absence of inositol supplementation (46). Repression of *CHO1* also occurs when cells enter the stationary phase of growth (72, 144). These forms of regulation are dependent on the UAS_{INO} *cis*-acting element in the promoter of the *CHO1* gene (136). The derepression of *CHO1* is mediated by a heterodimer complex of the positive transcription factors Ino2p and Ino4p that bind to a UAS_{INO} *cis*-acting element to drive transcription (5, 10, 43, 136). Repression of *CHO1* is mediated by the repressor

Opi1p, which interacts with Ino2p to attenuate transcription (5, 10, 43, 136). Opi1p repressor function is regulated by the cellular concentration of PA, which helps anchor the repressor to the nuclear/endoplasmic reticulum membrane apart from the Ino2p-Ino4p complex bound to the UAS_{INO} element (145). PA concentration and Opi1p repressor function is mediated in part by the *PAH1*-encoded Mg^{2+}-dependent PA phosphatase enzyme (70, 146-148) Data (149) are consistent with a model (147) whereby activation of Mg^{2+}-dependent PA phosphatase activity results in a decrease in PA concentration followed by the translocation of Opi1p into the nucleus for interaction with Ino2p to repress *CHO1* transcription. As discussed above for the biochemical regulation of PS synthase activity, the repression of *CHO1* favors PI synthesis and the Kennedy pathway for PC synthesis (10).

Hypothesis

The *cki1*Δ *eki1*Δ (41) and *cpt1 ept1* (38, 39) mutants are defective in both the CDP-choline and CDP-ethanolamine branches of the Kennedy pathway, and they can only synthesize PC via the CDP-DAG pathway. However, unlike mutants defective in the CDP-DAG pathway (17, 21-23, 31, 34, 150, 151) these Kennedy pathway mutants do not exhibit any auxotrophic requirements (39, 41). Moreover, even in the absence of the Kennedy pathway, the *cki1*Δ *eki1*Δ (41) and *cpt1 ept1* (39) mutants have an essentially normal complement of phospholipids including PC. I hypothesized that phospholipids synthesis via the CDP-DAG pathway is up-regulated in the *cki1*Δ *eki1*Δ to compensate for the block in the Kennedy pathway

EXPERIMENTAL PROCEDURES

Materials

All chemicals were reagent grade. Growth medium supplies were purchased from Difco Laboratories. Restriction endonucleases, modifying enzymes, recombinant Vent DNA polymerase, and NEBlot kit were purchased from New England Biolabs, Inc. RNA size markers were purchased from Promega. The plasmid DNA purification and DNA gel extraction kits were from Qiagen, Inc. ProbeQuant G-50 columns, polyvinylidene difluoride membranes, and enhanced chemifluorescence Western blotting detection kit were purchased from GE Healthcare. The Yeastmaker™ yeast transformation kit was obtained from Clontech. The DNA size ladder used for agarose gel electrophoresis, Zeta Probe blotting membranes, protein assay reagents, electrophoretic reagents, immunochemical reagents, isopropyl-β-D-thiogalactoside, protein molecular mass standards for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and acrylamide solutions were purchased from Bio-Rad. AdoMet, ampicillin, aprotinin, benzamidine, bovine serum albumin, leupeptin, *O*-nitrophenyl β-D-galactopyranoside, pepstatin, phenylmethylsulfonyl fluoride, Triton X-100, S-(5'-adenosyl)-L-methionine (SAM), *N*-ethylmaleimide, and hydrogen peroxide were purchased from Sigma. Radiochemicals and scintillation counting supplies were purchased from PerkinElmer Life Sciences and National Diagnostics, respectively. Phospholipids were purchased from Avanti Polar Lipids. Thin layer chromatography (TLC) plates were from EM science. Thiolutin was gifted from Pfizer Co. Potassium cyanide (KCN) was purchased from Fisher scientific Co.

Methods

Strains, Plasmids and Growth Conditions

The strains and plasmids used in this work are presented in Tables 1 and 2. Standard methods were followed for the growth of *S. cerevisiae* (152, 153). Cells were grown in complete synthetic medium without inositol (154), containing 2 % glucose or YPD medium (1% yeast extract, 2% peptone, 2% glucose) at 30 ^{o}C. For selection of cells bearing plasmids, appropriate nutrients were omitted from synthetic complete medium. Glucose-grown cells were also cultured in the presence of 1 mM hydrogen peroxide to induce oxidative stress (155, 156). Cells in liquid media were grown to the exponential phase (1-2 x 10^7 cells/ml), and cell numbers were determined spectrophotometrically at an absorbance of 600 nm. Viable cells were determined by scoring by the number of colonies on agar plates. Plasmids were maintained and amplified in *E. coli* strain DH5α, which was grown in LB medium (1% tryptone, 0.5% yeast extract, 1% NaCl, pH 7.4) at 37 °C. Ampicillin (100 μg/ml) was added to cultures of DH5α carrying plasmids. For growth on plates, yeast and bacterial media were supplemented with 2% and 1.5% agar, respectively. Respiratory sufficiency was scored by growth on YPG (1% YEAT extract, 2% peptone, 2% glycerol) and YPD (1% yeast extract, 2% peptone, 2% glucose) media plates (152, 153)

4'-6'-Diamidino-2-phenylindole (DAPI) – staining of Mitochondria

Mitochondrial DNA of *S. cerevisiae* cells was examined by 4'-6-diamidino-2-phenylindole (DAPI) staining (157) using a Nikon Eclipse E800 fluorescence microscope equipped with a Hamamatsu Orca digital camera. Images were captured in monochrome and processed using Improvision Openlab software.

TABLE 1
Strains used in this work

Strain	Relevant characteristics	Source or Ref.
E. coli		
DH5α	F$^-$ ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *deoR recA1 endA1 hsdR17*(r_k^- m_k^+) *phoA supE44* λ$^-$ *thi-1 gyrA96 relA1*	(153)
S. cerevisiae		
W303-1B	*MATα ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*	(158)
KS105	*cki1*Δ::*HIS3* derivative of W303-1B	(41)
KS101	*eki1*Δ::*TRP1* derivative of W303-1B [*rho*^{o}]	(41), This study
KS106	*cki1*Δ::*HIS3 eki1*Δ::*TRP1* derivative of W303-1B [*rho*^{o}]	(41), This study
HCY3	*ect1*Δ::*TRP1* derivative of W303-1B	This study
HCY4	*ept1*Δ::*TRP1* derivative of W303-1B	This study
HCY5	*eki1*Δ::*TRP1* derivative of strain W303-1B	This study
HCY6	*rho^o* derivative of HCY5	This study
HCY7	*cki1*Δ::*HIS3 eki1*Δ::*TRP1* derivative of W303-1B	This study
HCY8	*rho*$^-$ derivative of W303-1B	This study
YB1803	*MATa trp1 cho1*Δ::LEU2	(35)
W303 [*rho*^{o}]	*MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1* [*rho*^{o}]	M. Greenberg
MGY100	*MATa ade1 oxi2* [*rho*$^-$]	M. Greenberg
DL1	*MATα his3-11 15 leu2-3 12 ura3-251 328,372*	(159)
WD1	*cox4*Δ::*LEU2* derivative of DL1	(160)

TABLE 2
Plasmids used in this work

Plasmid	Relevant characteristics	Source or Ref.
pRS414	Single copy vector containing *TRP1*	(161)
pRS416	Single copy vector containing *URA3*	(161)
YEp352	Multicopy *E. coli*/yeast shuttle vector containing *URA3*	(162)
pAB709	Plasmid containing P_{CHO1}-*lacZ* reporter gene	(35)
pHC2	Plasmid containing mutated P_{CHO1}-*lacZ* reporter gene	(35)
pAS103	Plasmid containing a 1.2 kb fragment of the *CHO1* gene	(62)
pUC9-PGK1	Plasmid containing a 1.0 kb fragment of the *PGK1* gene	C. Martin
pKSK1	*EKI1* gene derived from PCR ligated into the *Srf*I site of pCRScript™ AMP SK(+)	(41)
pKSK2	*TRP1* disruption cassette from pJA52 ligated into the *Bgl*II/*BsaB*I sites of plasmid pKSK1	(41)
pHS9	*EKI1*gene from pKSK1 ligated into the *Pst*I/*Sac*I sites of YEp352	This study
pHS12	*EKI1* gene from pKSK1 ligated into the *BamH*I/*Sac*I site of pRS416	This study

DNA Isolation, Manipulations, and Site-directed Mutagenesis

Plasmid and genomic DNA preparation, restriction enzyme digestion, and DNA ligations were performed according to standard protocols (153). Transformations of yeast (163) and *E. coli* (153) were performed as described previously. Plasmid maintenance and amplifications were performed in *E. coli* strain DH5α. Conditions for the amplification of DNA by PCR were optimized as described previously (164). Mutagenesis was performed with the Stratagene QuikChange™ site-directed mutagenesis kit using plasmid pAB709 as the template and the mutagenic primers 5'-CCTCAGCCTTTGAGCTTTAAAAAAGACCCATCTAAAGATG-3' and 5'-CATCTTTAGATGGGTCTTTTTTAAAGCTCAAAGGCGTGAGG-3'. DNA sequencing confirmed the mutations in the UAS_{INO} sequence.

RNA Analysis

Total RNA (10 μg) was isolated from cells as described previously (165, 166). The RNA was resolved by 1.1 % agarose gel electrophoresis (167), and then transferred to Zeta Probe membranes by vacuum blotting. The *CHO1* (62) probe was labeled with [α-^{32}P] dTTP using the NEBlot random primer labeling kit, and unincorporated nucleotides were removed using ProbeQuant G-50 columns. Pre-hybridization, hybridization with the probes, and washes to remove non-specific binding were carried out according to manufacturer's instructions. Radioactive images were acquired by phosphorimaging. *CHO1* mRNA decay rate of specific strains was analyzed following the arrest of transcription as described by Gonzalez and Martin (92). Transcription of each gene was arrested by using the transcription inhibitor, thiolutin (15 μg/ml). Culture

samples (5ml) were taken every 5 min after 0 to 45 min of thiolutin treatment, Cells were centrifuged, and the pellet was frozen quickly on dry ice. Total RNA was extracted and analyzed as described above. The half-lives of *CHO1* mRNA in each strain was measured by plotting of the percentage of remaining mRNA versus time after the arrest of transcription. Target RNA amount was normalized by *PGK1* internal control.

Preparation of Subcellular Fractionations and Protein Determination

The cell extract and total membrane fraction were prepared as described previously (168). Cells were disrupted at 4°C by homogenization with glass beads in 50 mM Tris-maleate buffer (pH 7.0) containing 1 mM EDTA, 0.3 M sucrose, 10 mM 2-mercaptoethanol, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 5 μg/ml each of aprotinin, leupeptin, pepstatin. The cell extract was obtained by centrifugation of the homogenate at 1,500 x g for 10 min. The total membrane fraction was obtained from the cell extract by centrifugation at 100,000 x g for 1hr. The supernatant was used as the cytosolic fraction. Membranes were resuspended in buffer containing 50 mM Tris-maleate (pH7.0), 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 20 % glycerol (w/v), and 0.5 mM phenylmethylsulfonyl fluoride. Protein concentration was determined by the method of Bradford (169) using bovine serum albumin as the standard (170).

Preparation of Substrates

[3-^{3}H]PS was synthesized enzymatically from [3-^{3}H] serine and CDP-DAG using *S. cerevisiae* PS synthase as described previously (171).

Enzyme Assays

All assays were conducted in triplicate at 30 ^{o}C. CDP-DAG synthase activity was measured with 50 mM Tris-maleate buffer (pH 6.5), 20 mM $MgCl_2$, 15 mM Triton X-100, 0.5 mM PA, and 1.0 mM [5-^{3}H] CTP (172). PS synthase activity was measured with 50 mM Tris-HCl buffer (pH 8.0), 0.6 mM $MnCl_2$, 4 mM Triton X-100, 0.2 mM CDP-DAG, and 0.5 mM [3-^{3}H] serine (171). PS decarboxylase activity was measured with 50 mM Tris-HCl buffer (pH 7.2), 10 mM 2-mercaptoethanol, 5 mM EDTA, 2 mM Triton X-100, and 0.2 mM [3-^{3}H] PS (173, 174). PE methyltransferase activity was measured with 50 mM Tris-HCl buffer (pH 9.0), 0.2 mM PE, and 0.5 mM [*Me*-^{3}H] AdoMet (175). Phospholipid methyltransferase activity was measured with 50 mM Tris-HCl buffer (pH 7.5), 10 mM $MgCl_2$, 0.2 mM phosphatidylmonomethylethanolamine, and 0.5 mM [*Me*-^{3}H] AdoMet (175). PI synthase activity was measured with 50 mM Tris-HCl buffer (pH 8.0), 2 mM $MnCl_2$, 2.4 mM Triton X-100, 0.2 mM CDP-DAG, and 0.5 mM [2-^{3}H] inositol (176). Choline kinase activity was measured for 10 min at 30 °C with [*methyl*-^{3}H] choline (500 cpm/nmol), 67 mM glycine-NaOH buffer (pH 9.5), 5 mM choline, 0.5 mM ATP, 10 mM $MgSO_4$, 1.3 mM dithiothreitol, and enzyme protein in a total volume of 60 µl (177). The amount of labeled phosphocholine was identified by thin layer chromatography on silica gel plates using the solvent system methanol, 0.5% sodium chloride, ammonia hydroxide (50:50:1) (178). The position of the labeled phosphocholine on chromatograms was determined by fluorography and compared with a standard. β-galactosidase activity was measured with 100 mM sodium phosphate buffer (pH 7.0), 3 mM *O*-nitrophenyl β-D-galactopyranoside, 1 mM $MgCl_2$, and 100 mM 2-mercaptoethanol (179). All assays were linear with time and protein concentration. The

average standard deviation of the assays was ± 5%. A unit of phospholipid enzymatic activity was defined as the amount of enzyme that catalyzed the formation of 1 nmol of product per min. β-galactosidase activity was defined in μmol of product per min. Specific activity was defined as units/mg of protein.

Immunoblotting

The peptide sequence MVESDEDFAPQEFPH (residues 1-15 at the N-terminal end of the deduced protein sequence of *CHO1*) was synthesized and used to raise antibodies in New Zealand White rabbits by standard procedures (180) at Bio-Synthesis, Inc. SDS-PAGE (181) was routinely performed with 12% slab gels. Proteins were transferred from SDS-polyacrylamide gels to polyvinylidene difluoride membranes as described previously (182). The membrane was probed with a 1:500 dilution of anti-PS synthase antibodies (35). Goat anti-rabbit Immunoglobulin G alkaline phosphatase conjugate was used as a secondary antibody at a dilution of 1:5000. Immunoreactive proteins were detected using the enhanced chemifluorescence Western blotting detection kit, and fluorescent signals were acquired by Fluoroimaging. The relative density of the protein was analyzed using ImageQuant software. Immunoblot signals were in the linear range of detectability.

Construction of eki1Δ, ect1Δ, ept1Δ, and cki1Δ eki1Δ Mutants

The *eki1*Δ, *ect1*Δ, *ept1*Δ, and *cki1*Δ *eki1*Δ mutants were constructed using one-step gene replacement, as described previously (41). The 4-kb *EKI1* disruption cassette was released from the plasmid pKSK2 by digestion with *Sac*1/*Xho*1 (41). The *ECT1*

disruption cassette, which consisted of 1.2-kb *TRP1* DNA flanked with 25-bp nucleotides corresponding to the upstream and downstream sequence of the *ECT1* coding sequence, was prepared by PCR amplification using pRS414, which contains the *TRP1* gene, as template DNA with primers that consisted of the *TRP1* and *ECT1* sequences (forward primer, 5'-CAGAGACAAATGCTTTACAGGATCGGGACTTGAAATATAC TGACTGGCGTTTCGGTGATGAC-3'; reverse primer, 5'- AGTTTTCTTACATCCAT TTAATTTACGTTCGAAGAAGTTTTCAACATTTGTTTCCTGATGCGGTATTTTC TCCT-3'). Similarly, the *EPT1* disruption cassette was constructed by replacing the *ECT1* sequence with the *EPT1* sequences (forward primer, 5'-CGTAGTAGGAATTAGA AGTGTAGAATAAGAAAAACAAGCTAAGGTATAAACGTTTCGGTGATGAC-3'; reverse primer, 5'-CATTGTTGTGCCTTGTTTTCGAATAAAAAAAAGTAGATACAA AGTGCGATTTCCTGATGCGGTATTTTCTCCT-3' for *EPT1* disruption). These disruption cassettes were transformed into wild type (W303-1B) or *cki1*Δ. Yeast transformants were selected in synthetic complete medium without tryptophan. Disruption of *EKI1*, *ECT1*, and *EPT1* genes was confirmed by PCR. The loss of the function of *EKI1*, *ECT1*, and *EPT1* genes in cell was identified by labeling and analysis of [1,2-^{14}C]-ethanolamine as described previously (62).

Labeling and Analysis of CDP-ethanolamine Pathway Intermediates

The CDP-ethanolamine pathway intermediates were labeled with [1,2-^{14}C]-ethanolamine as described previously (62). The intermediates were isolated from whole cells after lipid extraction (183). The aqueous phase was neutralized and dried in vacuo, and the residue was dissolved in deionized water. Samples were subjected to

centrifugation at 12,000 x g for 3min to remove insoluble material. The CDP-ethanolamine pathway intermediates were then separated by thin layer chromatography with silica gel 60 plates using the solvent system 96% ethanol and 3% Ammonium hydroxide (1:2, v/v) (184). The positions of the labeled intermediates on chromatograms were determined by fluorography using EN^{3}HANCE and compared with standards.

Labeling and Analysis of Phospholipids

Labeling of phospholipids with $^{32}P_i$ or [^{14}C] serine was performed as described previously (31, 150, 185). Phospholipid synthesis was followed by labeling cells for 20 min, whereas the steady state composition of phospholipids was determined by labeling cells for six generations. Phospholipids were extracted from labeled cells by the method of Bligh and Dyer (183) and spotted on an oxalate-EDTA (1.2% potassium oxalate, 2 mM EDTA dissolved in methanol/water (2:3)) treated high performance TLC Silica Gel 60 plate. Plates were activated by heating at 110°C overnight, and cooled to room temperature in a desiccator immediately prior to the application of the sample (45). Phospholipids were analyzed by two-dimensional thin layer chromatography using chloroform/methanol/ammonium hydroxide/water (45:25:2:3, v/v) as the solvent for dimension one and chloroform/methanol/acetic acid/water (32:4:5:1 v/v) as the solvent for dimension two. The plate was allowed to dry completely in the hood. The identity of the labeled lipids on TLC plates was confirmed by comparison with standards after exposure to iodine vapor. Radiolabeled lipids were visualized by phosphorimaging analysis. The relative quantities of ^{32}P or ^{14}C-labeled phospholipids were analyzed using ImageQuant software.

Analyses of Data

Statistical significance was determined by performing the Student's *t*-test using SigmaPlot software. *P* values < 0.05 were taken as a significant difference.

RESULTS

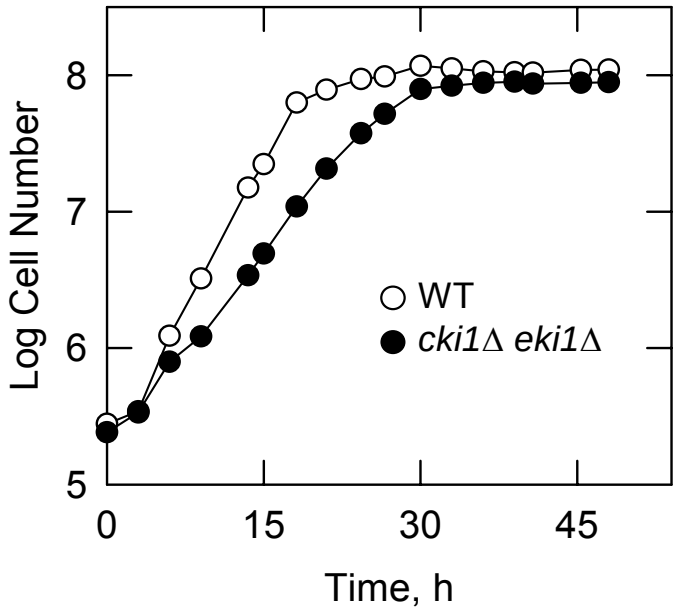
Effects of the cki1Δ eki1Δ Mutations on Cell Growth

The effect of the *cki1*Δ *eki1*Δ mutations on cell growth was examined. Unless otherwise indicated, cells were grown in medium without inositol to preclude the regulatory effects that this compound has on phospholipid synthesis (5-7, 10, 42, 43). The *cki1*Δ *eki1*Δ (KS106) mutant grew at a slower rate than the wild type control (Fig. 4). The doubling time for *cki1*Δ *eki1*Δ (KS106) mutant was 3 h, compared with 2 h for the wild type, in complete synthetic medium. Plate count analysis showed that the *cki1*Δ *eki1*Δ mutations did not affect cell viability. In addition, microscopic examination did not reveal any gross morphological abnormalities in the *cki1*Δ *eki1*Δ (KS106) mutant. However, I did notice that on plates, *cki1*Δ *eki1*Δ colonies were white whereas wild type colonies were pink. The cell density at the stationary phase of growth showed little difference between the wild type and the *cki1*Δ *eki1*Δ (KS106) mutant (Fig. 4).

Effects of the cki1Δ eki1Δ Mutations on the Synthesis and Steady-state Composition of Phospholipids

Wild type cells synthesize phospholipids by both the CDP-DAG and Kennedy pathways (38-40, 61, 62, 186) whereas the *cki1*Δ *eki1*Δ (KS106) mutant can only synthesize phospholipids via the CDP-DAG pathway (41). The effects of the *cki1*Δ *eki1*Δ mutations on the synthesis and steady state composition of

Figure 4. Effects of the *cki1*Δ *eki1*Δ mutations on cell growth. Wild type (WT, W3031-B) and *cki1*Δ *eki1*Δ (KS106) mutant cells were grown in complete synthetic medium containing 2 % glucose. Cell numbers were determined spectrophotometically at an absorbance of 600 nm. These values were consistent with the number of viable cells determined by plate counts. The data shown are representative of four independent growth studies.


Log Cell Number
8
7
6
5
0
15
30
45
Time, h
WT
cki1Δ eki1Δ

phospholipids were examined by labeling cells with $^{32}P_i$ and with [^{14}C] serine. In wild type cells, $^{32}P_i$ is incorporated into phospholipids synthesized by both the CDP-DAG and Kennedy pathways whereas the label from [^{14}C] serine is only incorporated into PS, PE, and PC synthesized by the CDP-DAG pathway (150). The *cki1*Δ *eki1*Δ mutations had a major effect on phospholipid synthesis. The ^{32}P-labeling of the mutant showed increases in PS (54%), PE (21%), and PA (27%), but a decrease in PC (80%) (Fig. 5A). The [^{14}C] serine-labeling of the mutant showed increases in the incorporation of label into PS (36%), PE (23%), and PC (28%) (Fig. 6A). The decrease in $^{32}P_i$ incorporation into PC in the *cki1*Δ *eki1*Δ (KS106) mutant reflected phospholipase D-mediated turnover of PC (40, 187, 187) and the inability to reutilize choline for PC synthesis via the Kennedy pathway. The *cki1*Δ *eki1*Δ mutations did not have a major effect on the steady state composition of phospholipids labeled with either $^{32}P_i$ (Fig. 5B) or with [^{14}C] serine (Fig. 6B). With the exception of a decrease in PS content (7.9 to 4.6%) for cells labeled with $^{32}P_i$, the phospholipid composition of the *cki1*Δ *eki1*Δ (KS106) mutant was not significantly different from that of the wild type control. Thus, even in the absence of the Kennedy pathway, the *cki1*Δ *eki1*Δ (KS106) mutant eventually contained an almost normal balance of phospholipids.

Effects of the cki1Δ eki1Δ Mutations on the Levels of CDP-DAG Pathway Enzyme Activities

The results of phospholipid labeling indicated that the *cki1*Δ *eki1*Δ (KS106) mutant compensated for the defect in the Kennedy pathway by increasing the synthesis of phospholipids by the CDP-DAG pathway. Accordingly, I questioned whether the *cki1*Δ

Figure 5. Effects of the *cki1*Δ *eki1*Δ mutations on the synthesis and steady-state composition of phospholipids synthesized by the CDP-DAG and Kennedy pathways. Wild type (WT, W3031-B) and *cki1*Δ *eki1*Δ (KS106) mutant cells were grown to the exponential (1×10^7 cells/ml) phase of growth. For pulse labeling of phospholipids (*panel A*), cells were incubated with $^{32}P_i$ (10 μCi/ml) for 30 min. The steady-state composition of phospholipids (*Panel B*) was determined by labeling cells for six generations with $^{32}P_i$ (5 μCi/ml). Phospholipids were extracted and analyzed as described under "Experimental Procedures." The percentages shown for phospholipids were normalized to the total $^{32}P_i$-labeled chloroform-soluble fraction, which included sphingolipids and other unidentified phospholipids. Each data point represents the average of two independent experiments ± S.D.

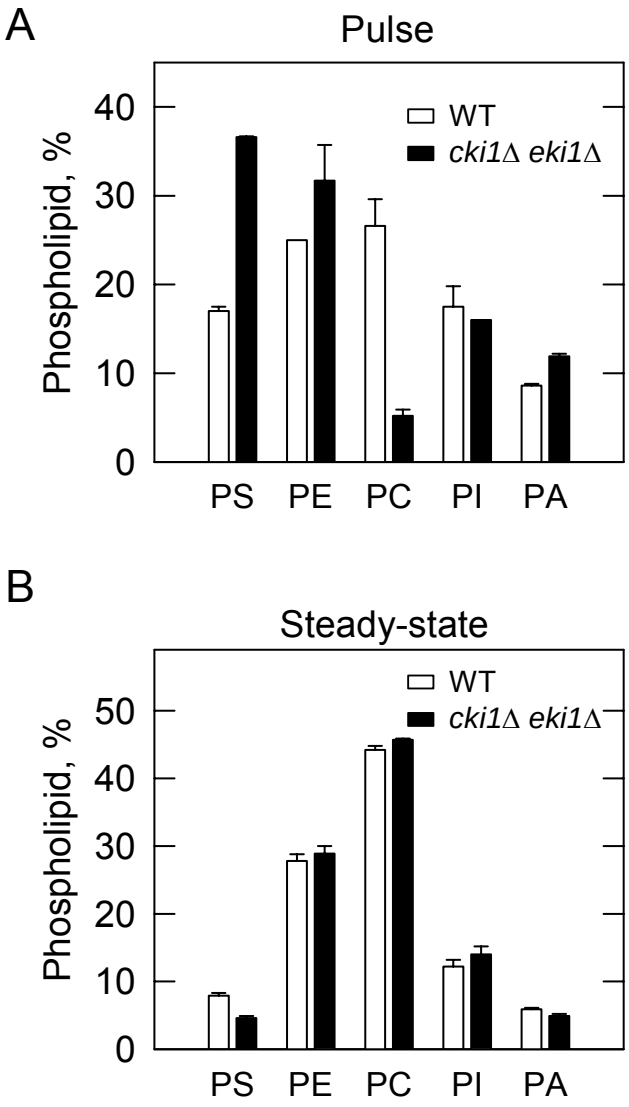
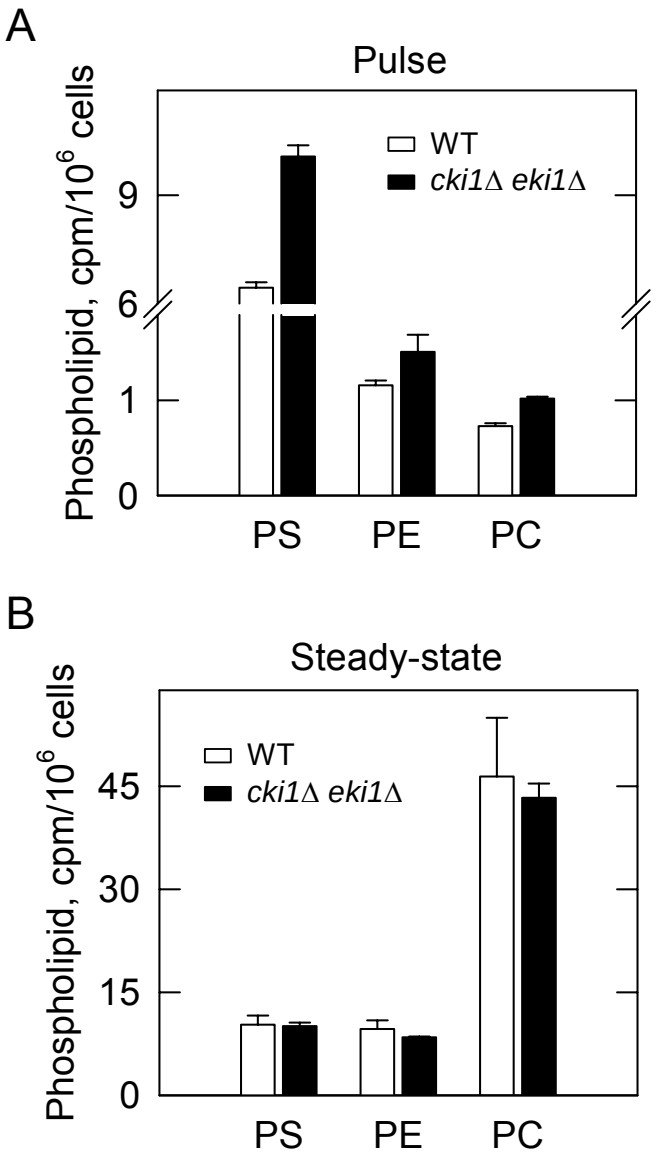

A
Pulse
Phospholipid, %
40
30
20
10
0
WT
cki1Δ eki1Δ
PS
PE
PC
PI
PA
B
Steady-state
Phospholipid, %
50
40
30
20
10
0
WT
cki1Δ eki1Δ
PS
PE
PC
PI
PA

Figure 6. Effects of the *cki1*Δ *eki1*Δ mutations on the synthesis and steady-state composition of phospholipids synthesized by the CDP-DAG pathway. Wild type (WT, W3031-B) and *cki1*Δ *eki1*Δ (KS106) mutant cells were grown to the exponential (1 x 10^7 cells/ml) phase of growth. For pulse labeling of phospholipids (*panel A*) cells were incubated with [^{14}C] serine (20 μCi/ml) for 30 min. The steady state composition of phospholipids (*panel B*) was determined by labeling cells for 6 generations with [^{14}C] serine (10 μCi/ml). Phospholipids were extracted and analyzed as described under "Experimental Procedures." Each data point represents the average of two independent experiments ± S.D.

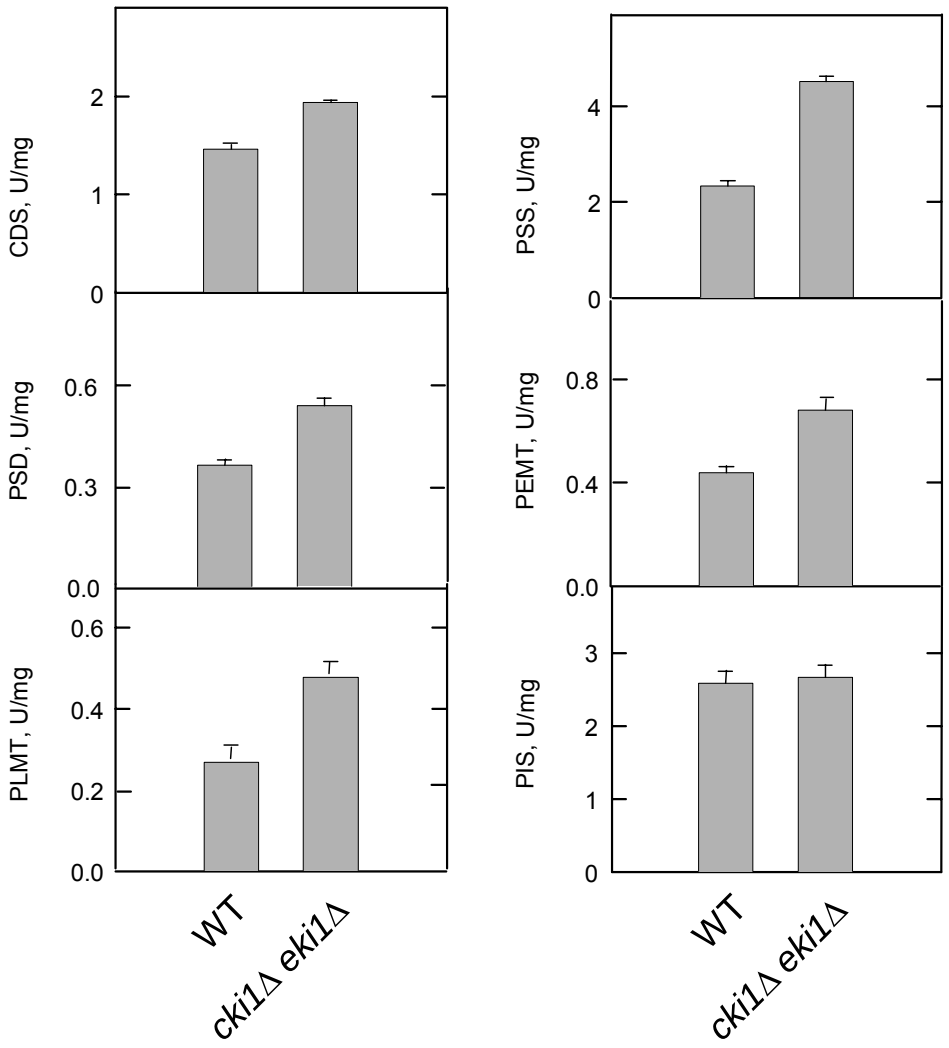

A
Pulse
Phospholipid, cpm/10^6 cells
WT
cki1Δ eki1Δ
9
6
1
0
PS
PE
PC
B
Steady-state
Phospholipid, cpm/10^6 cells
WT
cki1Δ eki1Δ
45
30
15
0
PS
PE
PC

*eki1*Δ mutations affected the activities of the CDP-DAG pathway enzymes PS synthase, PS decarboxylase, PE methyltransferase, and phospholipid methyltransferase. These enzymes, which are all associated with membranes (6, 188), were measured using the total membrane fraction isolated from cells grown to the exponential phase of growth. The *cki1*Δ *eki1*Δ (KS106) mutant showed elevated activity levels of PS synthase (50%), PS decarboxylase (33%), PE methyltransferase (36%), and phospholipid methyltransferase (44%) compared to the control (Fig. 7A-D). CDP-DAG synthase (189), which is responsible for the formation of CDP-DAG, and PI synthase (190, 191), which competes with PS synthase for the substrate CDP-DAG (Fig. 2), were also included in this analysis. The *cki1*Δ *eki1*Δ mutations caused a 27% increase in CDP-DAG synthase activity (Fig. 7E) whereas the level of PI synthase activity was not affected by the mutations (Fig. 7F). PA phosphatase is responsible for the formation of the DAG utilized for PE and PC synthesis via the Kennedy pathway (Fig. 2) (192). We questioned whether this enzyme would be regulated in cells blocked in the Kennedy pathway. The activities of the Mg^{2+}-dependent and Mg^{2+}-independent forms of PA phosphatase (192) were measured in both the membrane and cytosolic fractions of wild type and *cki1*Δ *eki1*Δ mutant cells. Neither of these activities was affected by the block in the Kennedy pathway.

Effects of the cki1Δ eki1Δ Mutations on the Expression of PS Synthase Protein and mRNA Levels

To gain insight into the mechanism by which the CDP-DAG pathway enzyme activities were elevated in the *cki1*Δ *eki1*Δ (KS106) mutant, we examined the expression

Figure 7. Effects of the *cki1*Δ *eki1*Δ mutations on the levels of CDP-DAG pathway enzyme activities. Wild type (WT, W3031-B) and *cki1*Δ *eki1*Δ (KS106) mutant cells were grown to the exponential (1 x 10^7 cells/ml) phase of growth. The total membrane fraction was isolated and used for the assay of CDP-DAG synthase (*CDS*), PS synthase (*PSS*), PS decarboxylase (*PSD*), PE methyltransferase (*PEMT*), phospholipid methyltransferase (*PLMT*), and PI synthase (*PIS*) activities as described under "Experimental Procedures." Each data point represents the average of triplicate enzyme determinations from a minimum of two independent experiments ± S.D. *U*, units.

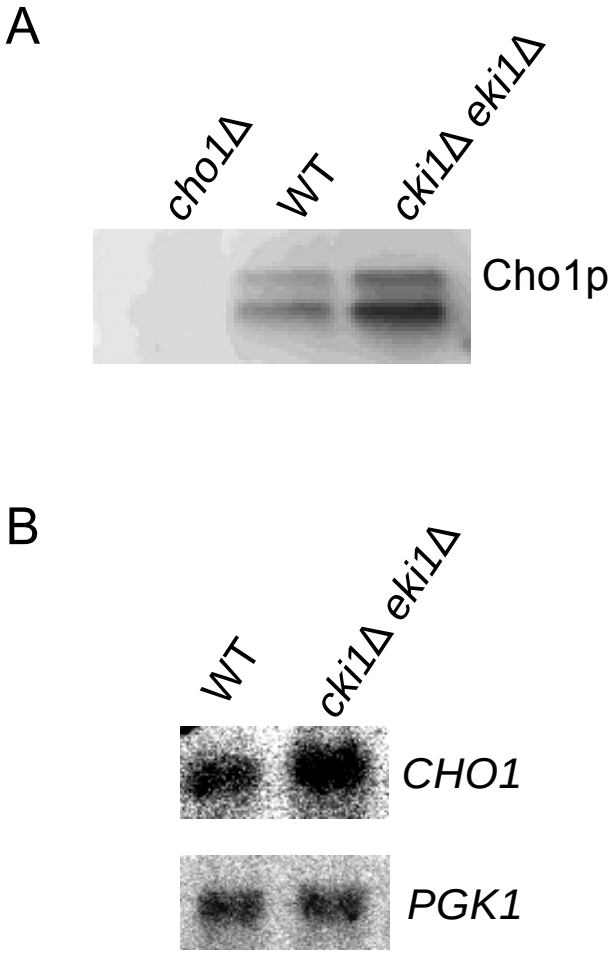

CDS, U/mg
PSD, U/mg
PLMT, U/mg
PSS, U/mg
PEMT, U/mg
PIS, U/mg
WT
cki1Δ eki1Δ

of the PS synthase enzyme. PS synthase was chosen as a representative enzyme because it catalyzes the committed step in the CDP-DAG pathway (Fig. 2), and its gene expression is coordinately regulated with the other structural genes in the pathway (5-7, 10, 42, 43). The levels of the PS synthase protein (Cho1p) were examined by immunoblot analysis using antibodies generated against a peptide sequence found at the N-terminal end of the protein. PS synthase is a 30-kDa protein that is susceptible to proteolytic degradation (15, 131, 193). These antibodies recognized PS synthase (and its proteolysis product) in the total membrane fraction (Fig. 8A). Immunoblot analysis of membranes derived from a *cho1*Δ mutant served as a negative control for the antibodies (Fig. 8A). The level of PS synthase protein was elevated (~ 2-fold) in the membranes of the *cki1*Δ *eki1*Δ (KS106) mutant when compared with the control (Fig. 8A). This indicated that the increase in PS synthase activity was a result of an increase in the level of enzyme protein. To determine whether the increase in enzyme content was due to an increase in gene expression, the level of *CHO1* mRNA was examined. Northern blot analysis of total RNA isolated from cells at the exponential phase of growth showed that the relative amount of *CHO1* mRNA in the *cki1*Δ *eki1*Δ (KS106) mutant was 2-fold greater than that present in the control wild type (Fig. 8B). These results indicated that a transcriptional mechanism was responsible for the regulation of PS synthase in the *cki1*Δ *eki1*Δ (KS106) mutant.

Effect of the UAS_{INO} Element on the Regulation of CHO1 Expression in the cki1Δ eki1Δ Mutant

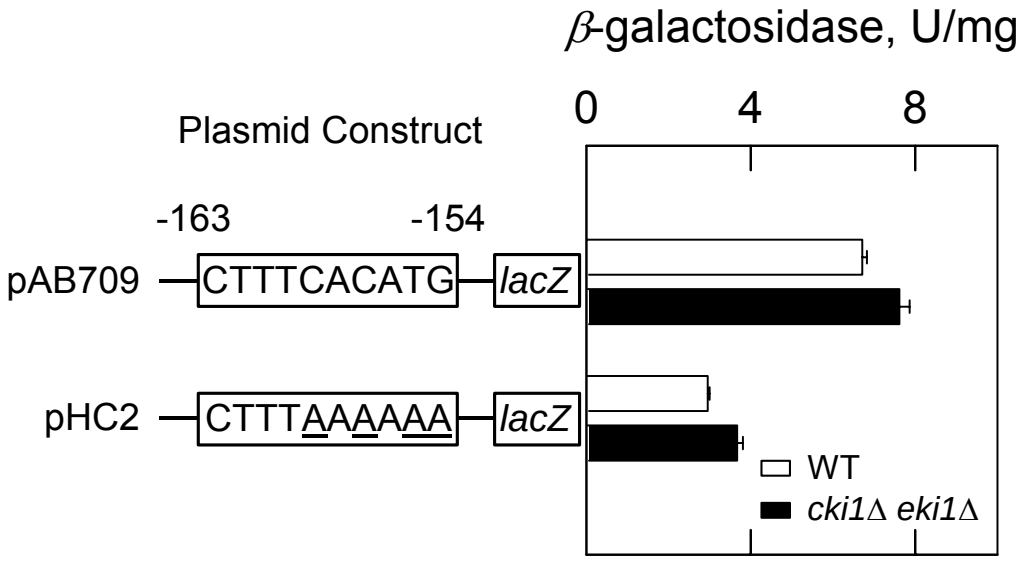
Maximum expression of *CHO1* in wild type cells grown in the absence of inositol is

Figure 8. Effects of the *cki1*Δ *eki1*Δ mutations on the levels of PS synthase protein and mRNA. Wild type (WT, W3031-B) and *cki1*Δ *eki1*Δ mutant (KS106) cells were grown to the exponential (1×10^7 cells/ml) phase of growth. *Panel A*, the total membrane fraction was isolated, and 12.5 μg of protein was subjected to immunoblot analysis using a 1:500 dilution of anti-PS synthase antibodies. A portion of the immunoblot is shown, and the position of the 30-kDa PS synthase protein (Cho1p) is indicated. The protein migrating below the 30-kDa protein is a proteolysis product. *Panel B*, total RNA was extracted, and the abundance of *CHO1* mRNA was determined with 25 μg of RNA by Northern blot analysis as described under "Experimental Procedures." Portions of Northern blots are shown, and the positions of *CHO1* mRNA and *PGK1* mRNA (loading control) are indicated. The data shown in *panels A* and *B* are representative of two independent experiments.


A
cho1Δ
WT
cki1Δ eki1Δ
Cho1p
B
WT
cki1Δ eki1Δ
CHO1
PGK1

dependent on the UAS_{INO} *cis*-acting element in its promoter (5, 7, 7, 143). The UAS_{INO} element contains a consensus-binding site (5'-CANNTG-3') for a heterodimer complex of the positive transcription factors Ino2p and Ino4p (5, 7, 66, 143). I questioned whether the UAS_{INO} element played a role in the regulation of PS synthase observed in the *cki1*Δ *eki1*Δ (KS106) mutant. To address this question, the UAS_{INO} element was mutated to a nonconsensus sequence in the P_{CHO1}-*lacZ* reporter gene where the expression of β-galactosidase activity is dependent on transcription driven by the *CHO1* promoter (143). Cell extracts were prepared from exponential wild type and *cki1*Δ *eki1*Δ (KS106) mutant cells bearing the wild type and mutant reporter genes, and then assayed for β-galactosidase activity. As might be expected (143), the mutations in the UAS_{INO} element caused 56% and 51% decreases in β-galactosidase activity in wild type and *cki1*Δ *eki1*Δ (KS106) mutant cells, respectively (Fig. 9). The β-galactosidase activity in the *cki1*Δ *eki1*Δ (KS106) mutant bearing the wild type reporter gene was 13% higher than that of wild type cells with the wild type reporter gene (Fig. 9). In addition, the β-galactosidase activity in the *cki1*Δ *eki1*Δ (KS106) mutant bearing the mutant reporter gene was 22% higher than that of wild type cells with the mutant reporter gene (Fig. 9). These results indicated that the mutations in the UAS_{INO} element did not affect the regulation of *CHO1* expression mediated by the *cki1*Δ *eki1*Δ mutations. Interestingly, the increase in *CHO1* expression in the *cki1*Δ *eki1*Δ (KS106) mutant as monitored by β-galactosidase activity was not as great as that observed by Northern blot analysis (Fig. 7B). This raised the suggestion that an increase in transcription was not a major reason for the increase in *CHO1* mRNA abundance in the *cki1*Δ *eki1*Δ (KS106) mutant.

Figure 9. Effects of the *cki1*Δ *eki1*Δ mutations on the expression of *β*-galactosidase activity in cells bearing the wild type and mutant P_{CHO1}-*lacZ* reporter genes. Wild type (WT, W3031-B) and *cki1*Δ *eki1*Δ mutant cells bearing the P_{CHO1}-*lacZ* reporter plasmid pAB709 or pHC2 were grown to the exponential phase of growth. Cell extracts were prepared and used for the assay of *β*-galactosidase activity. Each data point represents the average of triplicate enzyme determination from a minimum of two independent experiments $\pm$ S.D. Mutations in the UAS_{INO} element in plasmid pHC2 are *underlined*. *U*, units.


β-galactosidase, U/mg
0
4
8
Plasmid Construct
-163
-154
pAB709
CTTTCACATG
lacZ
pHC2
CTTTAAAAAA
lacZ
WT
cki1Δ eki1Δ

The expression of *CHO1* in wild type *S. cerevisiae* is repressed by the addition of inositol to the growth medium (141, 143). To examine whether the *cki1*Δ *eki1*Δ mutations affected this regulation, β-galactosidase activity was measured in wild type and mutant cells grown in the absence and presence of 50 μM inositol. Inositol supplementation caused a reduction (35-50%) in β-galactosidase activity in both wild type and mutant cells (data not shown), indicating that the *cki1*Δ *eki1*Δ mutations did not have a significant affect the regulation of *CHO1* expression by inositol.

Effects of the cki1Δ eki1Δ Mutations on CHO1 mRNA Stability

The abundance of mRNA in the cell reflects both its synthesis and decay. Because there was a small correlation between the levels of *CHO1* mRNA and reporter gene expression, I questioned whether mRNA stability was responsible for the increased level of *CHO1* transcript in the *cki1*Δ *eki1*Δ (KS106) mutant. To address this hypothesis, transcription was arrested in wild type and *cki1*Δ *eki1*Δ (KS106) mutant cells, followed by a kinetic analysis of *CHO1* mRNA decay. *PGK1* mRNA was included in this analysis as a loading control because it is a highly stable transcript (92, 194, 195). In wild type cells, *CHO1* mRNA decayed in a time-dependent manner with a half-life of 10 min (Fig. 10, Table 3). When compared with other mRNAs in yeast, which have half-lives ranging from 1 to 60 min (89), *CHO1* mRNA was a moderately stable transcript. In the *cki1*Δ *eki1*Δ (KS106) mutant, however, the *CHO1* mRNA was highly stable during the time course of the experiment with a half-life greater than 25 min (Fig. 10). These results indicated that an increase in the stability of *CHO1* mRNA had a major effect on the abundance of the *CHO1* transcript in the *cki1*Δ *eki1*Δ mutant.

Figure 10. Effects of the *cki1*Δ *eki1*Δ mutations on *CHO1* mRNA decay. *Panel A*, wild type (WT, W3031-B) and *cki1*Δ *eki1*Δ mutant (KS106) cells were grown to the exponential (1×10^7 cells/ml) phase of growth. Following the arrest of transcription, 4-ml samples were taken at the indicated time intervals, and total RNA was extracted. The levels of *CHO1* mRNA and *PGK1* mRNA were determined by Northern blot analysis as described in experimental procedure. Portions of Northern blots are shown, and the positions of *CHO1* mRNA and *PGK1* mRNA are indicated. *Panel B*, the relative amounts of *CHO1* and *PGK1* mRNAs from wild type and *cki1*Δ *eki1*Δ mutant cells were determined by ImageQuant analysis of the data in *panel A*. The figure shows a plot of the log of the relative amount of *CHO1* to *PGK1* mRNAs *versus* time. The *lines* drawn in *panel B* were the result of a least-squares analysis of the data. The data shown in the figure are representative of three independent experiments

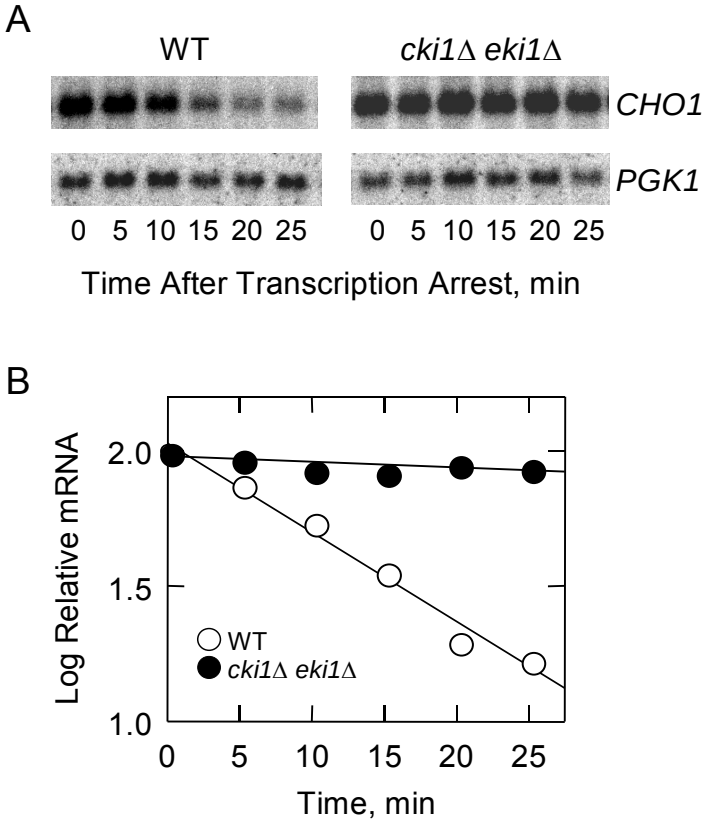

A
WT
cki1Δ eki1Δ
CHO1
PGK1
0 5 10 15 20 25
0 5 10 15 20 25
Time After Transcription Arrest, min
B
Log Relative mRNA
2.0
1.5
1.0
WT
cki1Δ eki1Δ
0 5 10 15 20 25
Time, min

TABLE 3
CHO1 mRNA half-lives determined in this work

Strain	Half-life (min)[1]
Wild type (W303-1B)	12.3 ± 1.3
*cki1*Δ (KS105)	14.5 ± 2.2
*eki1*Δ *rho*^{0} (KS101)	> 45
*eki1*Δ *cki1*Δ *rho*^{0} (KS106)	> 45
*ect1*Δ (HCY3)	12.9 ± 1.8
*ept1*Δ (HCY4)	14.6 ± 2.1
*eki1*Δ *rho*^{0}/*EKI1* (KS101 containing pHS9)	> 45
*eki1*Δ (HCY5)	18 ± 4.2
*eki1*Δ *rho*^{o} (HCY6)	> 45
rho$^-$ (MGY100)	> 45
rho^o (W303 [*rho*^{o}])	> 45
rho$^-$ (HCY8)	> 45
Wild type treated with 1 mM KCN	22.3 ± 1.3
Wild type treated with 2 mM KCN	32.0 ± 1.4
Wild type treated with 4 mM KCN	> 45
Wild type (DL1)	18.5 ± 4.5
*cox4*Δ (WD1)	> 45

[1]The half-life values are the average of triplicate determinations ± S.D.

Effects of the cki1Δ, eki1Δ, and cki1Δ eki1Δ Mutations on CHO1 mRNA Decay

Previous studies have shown that the *cki1*Δ *eki1*Δ mutations have a stabilizing effect on the abundance of *CHO1* mRNA (35). It is unclear however, whether the signal responsible for increased *CHO1* mRNA stability in the *cki1*Δ *eki1*Δ (KS106) mutant results from a defect in the CDP-choline branch, the CDP-ethanolamine branch, or from both branches of the Kennedy pathway. To address this question, *CHO1* mRNA decay was examined in the *cki1*Δ mutant (KS105) (41) defective in the first of the CDP-choline pathway and in the *eki1*Δ mutant (KS101) (41) defective in the first step of the CDP-ethanolamine pathway (Fig. 2). *CHO1* mRNA decay was also reexamined in the *cki1*Δ *eki1*Δ mutant. The rate (i.e., half-life) of *CHO1* mRNA decay in the *cki1*Δ mutant was similar to that observed in the wild type control (Fig. 11, Table 3). In contrast, *CHO1* mRNA was stabilized in the *eki1*Δ mutant (KS101) (Fig. 11, Table 3). Thus, the increase in *CHO1* mRNA stability in the *cki1*Δ *eki1*Δ mutant (KS106) mutant was due to the *eki1*Δ mutation (i.e., defect in the CDP-ethanolamine branch).

Effects of CDP-ethanolamine Pathway Mutations on CHO1 mRNA Stability

Mutations in the second (*ect1*Δ) and third (*ept1*Δ) steps of the CDP-ethanolamine pathway (i.e., phosphoethanolamine cytidylyltransferase and phosphocholine cytidylyltransferase, respectively) were constructed and examined for their effects on *CHO1* mRNA stability. Of the three mutants, *eki1*Δ (KS101) was the only mutant in which *CHO1* mRNA was stabilized (Fig. 11). The effects of the three CDP-ethanolamine pathway mutations on the abundance of *CHO1* mRNA and PS synthase protein (Cho1p) were also examined. The levels of *CHO1* mRNA and PS synthase

protein was elevated by about 2-fold in the *eki1*Δ mutant when compared with the wild type control (Fig. 12). On the other hand, the *CHO1* gene products were not affected in the *ect1*Δ and *ept1*Δ mutants (Fig. 12). These results correlated with the increase in *CHO1* mRNA stability observed in the *eki1*Δ mutant (Fig. 11).

Loss of the EKI1 Gene is Not Responsible for Increased CHO1 mRNA Stability in the eki1Δ Mutant (KS101)

To confirm that the deletion of the *EKI1* gene was responsible for the increased stability of *CHO1* mRNA, the *eki1*Δ mutant (KS101) was transformed with a single copy plasmid bearing the *EKI1* gene. Unexpectedly, the introduction of the *EKI1* gene into the *eki1*Δ mutant did not correct the increased *CHO1* mRNA stability phenotype of the mutant (Fig. 13). This result indicated that a second mutation in the strain KS101 was responsible for increasing *CHO1* mRNA stability. Accordingly, a new *eki1*Δ mutant (strain HCY5) was constructed by the same method (41) used to construct the original *eki1*Δ (KS101) mutant. The analysis of the new *eki1*Δ mutant confirmed that the loss of the *EKI1* gene was not responsible for the increased stability of *CHO1* mRNA (Fig. 14). Moreover, an *eki1*Δ (HCY7) mutant was construction by an alternative method (i.e., *HIS3* insertion), and it exhibited the normal rate of *CHO1* mRNA decay (data not shown).

The eki1Δ (KS101) and cki1Δ eki1Δ (KS106) Mutants are Respiratory Deficient

Unlike wild type (W303-1B) cells that formed pink colonies on agar plates, the *eki1*Δ (KS101) and *cki1*Δ *eki1*Δ (KS106) mutant colonies were white and relatively small (i.e., petite) in appearance.

Figure 11. Effects of the *cki1*Δ, *eki1*Δ, and *cki1*Δ *eki1*Δ mutations on *CHO1* mRNA decay. Wild type (WT, W303-1B), *cki1*Δ (KS105), *eki1*Δ (KS101), and *cki1*Δ *eki1*Δ (KS106) mutant cells were grown to the exponential phase ($1x10^7$ cells/ml) of growth. Following the arrest of transcription, 5-ml samples were taken every 5 min, total RNA was extracted, and the levels of *CHO1* mRNA and *PGK1* mRNA were determined by Northern blot analysis. The relative amounts of *CHO1* and *PGK1* mRNAs were determined by ImageQuant analysis. The figure shows a plot of the log of the relative amount of *CHO1* to *PGK1* mRNAs *versus* time. The lines drawn were the result of a least-squares analysis of the data. The data shown in the figure are representative of three independent experiments. Half-life values are presented in Table 3.

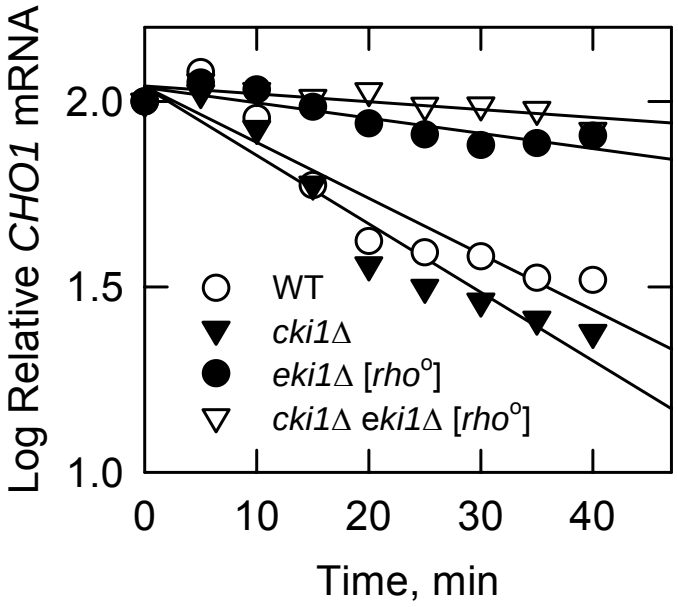

Log Relative CHO1 mRNA
2.0
1.5
1.0
0
10
20
30
40
Time, min
WT
cki1Δ
eki1Δ [rho°]
cki1Δ eki1Δ [rho°]

Figure 12. Effects of the *eki1*Δ, *ect1*Δ, and *ept1*Δ mutations on *CHO1* mRNA decay. Wild type (WT, W303-1B), *eki1*Δ (KS101), *ect1*Δ (HCY3), and *ept1*Δ (HCY4) mutant cells were grown to the exponential phase ($1x10^7$ cells/ml) of growth. Following the arrest of transcription, *CHO1* mRNA decay was quantified as described in the legend to Fig. 11. The figure shows a plot of the log of the relative amount of *CHO1* to *PGK1* mRNAs *versus* time. The lines drawn were the result of a least-squares analysis of the data. The data shown in the figure are representative of three independent experiments. Half-life values are presented in Table 3.

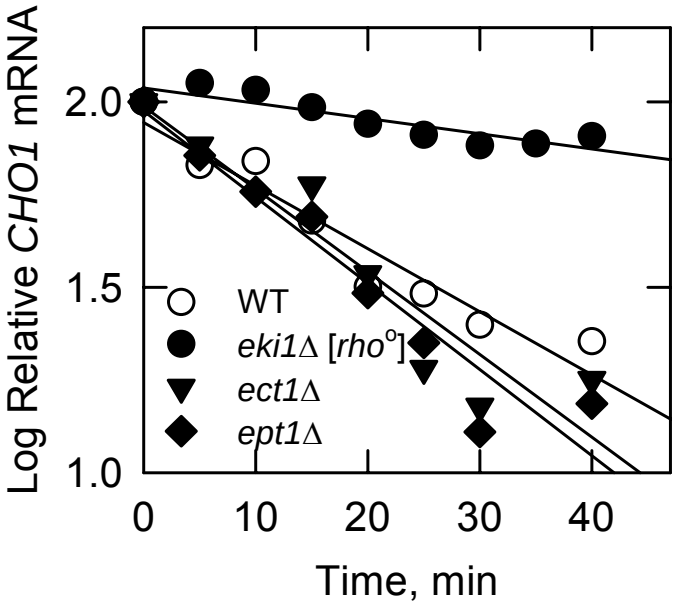

Log Relative CHO1 mRNA
2.0
1.5
1.0
0
10
20
30
40
Time, min
WT
eki1Δ [rho°]
ect1Δ
ept1Δ

Figure 13. Effects of Kennedy pathway mutations on the levels of *CHO1* mRNA and PS synthase protein. Wild type (WT, W303-1B), *cki1*Δ (KS105), *eki1*Δ (KS101), *cki1*Δ *eki1*Δ (KS106), *ect1*Δ (HCY3), and *ept1*Δ (HCY4) mutant cells were grown to the exponential phase ($1x10^7$ cells/ml) of growth. *Panel A*, total RNA was extracted and the abundance of *CHO1* mRNA was determined with 10 μg of RNA by Northern blot analysis. The relative amounts of *CHO1* and *PGK1* mRNAs from wild type and mutant cells were determined by ImageQuant analysis of the data. The relative amount of *CHO1* to *PGK1* mRNA in wild type cells was arbitrarily set at 1. *Panel B*, the total membrane fraction was isolated and 12.5 μg of protein was subjected to immunoblot analysis using a 1:500 dilution of anti-PS synthase antibodies. The relative amounts of the PS synthase protein from wild type and mutant cells were determined by ImageQuant analysis of the data. The amount of PS synthase protein found in wild type cells was arbitrarily set at 1. The data shown in panels *A* and *B* are the average of two experiments.

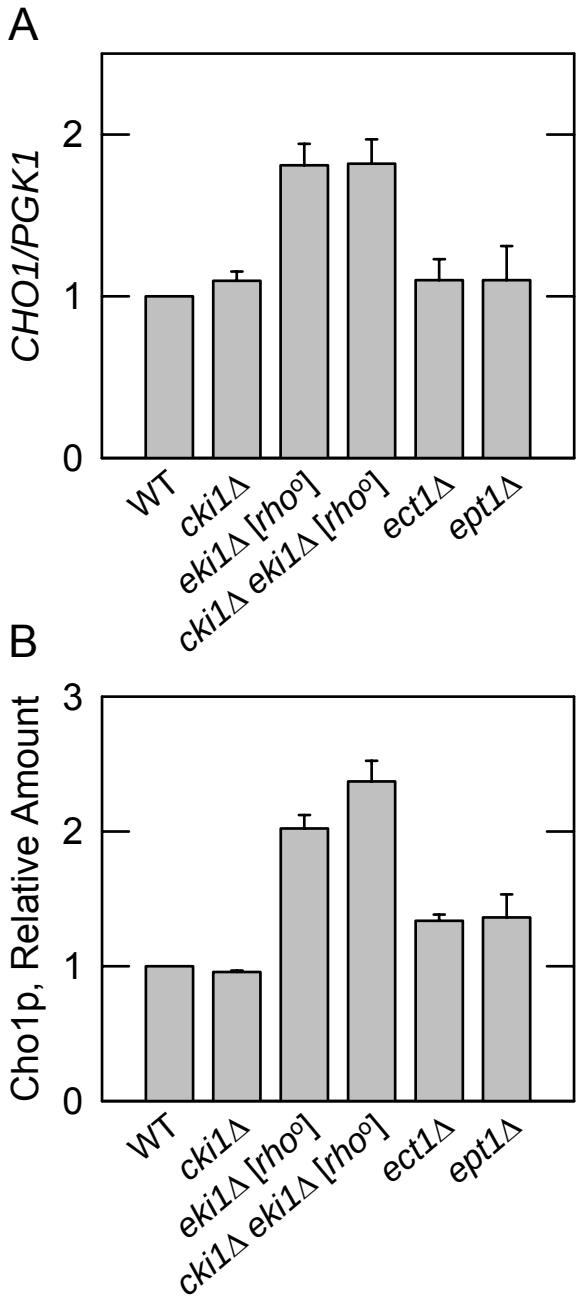
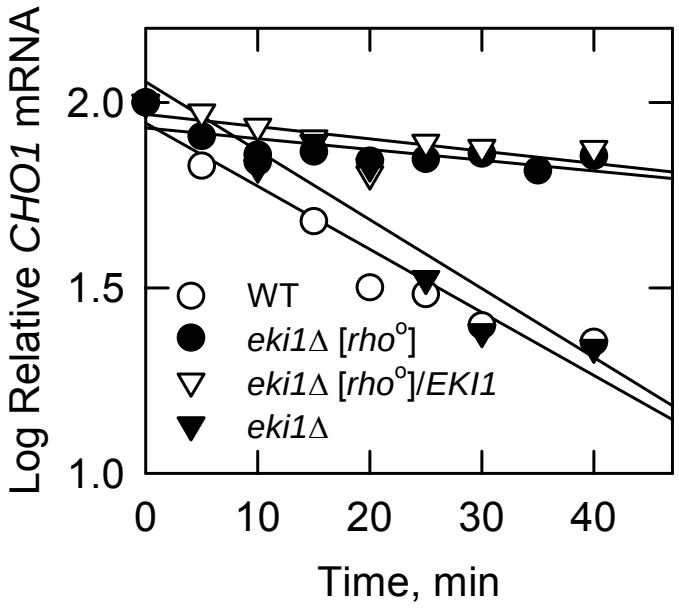

A
CHO1/PGK1
0
1
2
WT
cki1Δ
eki1Δ [rho°]
cki1Δ eki1Δ [rho°]
ect1Δ
ept1Δ
B
Cho1p, Relative Amount
0
1
2
3
WT
cki1Δ
eki1Δ [rho°]
cki1Δ eki1Δ [rho°]
ect1Δ
ept1Δ

Figure 14. Effect of the *EKI1* gene on *CHO1* mRNA decay. Wild type (WT, W303-1B), *eki1*Δ (KS101 containing plasmid YEp352), *eki1*Δ/*EKI1*(KS101 containing plasmid pHS9), and *eki1*Δ (HCY5) mutant cells were grown to the exponential phase ($1x10^7$ cells/ml) of growth. Following the arrest of transcription, *CHO1* mRNA decay was quantified as described in the legend to Fig. 11. The figure shows a plot of the log of the relative amount of *CHO1* to *PGK1* mRNAs *versus* time. The lines drawn were the result of a least-squares analysis of the data. The data shown in the figure are representative of three independent experiments. Half-life values are presented in Table 3.


Log Relative CHO1 mRNA
2.0
1.5
1.0
0
10
20
30
40
Time, min
WT
eki1Δ [rho°]
eki1Δ [rho°]/EKI1
eki1Δ

These characteristics were first thought to be due to the *eki1*Δ mutation. However, the colonies produced by the new *eki1*Δ (HCY5) and *cki1*Δ *eki1*Δ (HCY8) mutants were similar in appearance (i.e., normal size and pink) to their wild type parent W303-1B. Petite white colonies are characteristics of respiratory-deficient mutants (152). Respiratory deficient mutants are defective in mitochondrial function and cannot grow on non-fermentable carbon sources (152, 196). With this in mind, the respiratory sufficiency of the original *eki1*Δ (KS101) and *cki1*Δ *eki1*Δ (KS106) mutants were examined by growth on agar plates containing glucose (fermentable) or glycerol (non-fermentable) as the carbon source. Like known respiratory mutants (MGY100 and W303 [*rho*^{o}]), the original *eki1*Δ (KS101) and *cki1*Δ *eki1*Δ (KS106) mutants only grew on glucose agar plates, whereas the new *eki1*Δ (HCY5) and *cki1*Δ *eki1*Δ (HCY7) mutants grew on both glucose and glycerol agar plates (Table 4). These data indicated that the original mutants were respiratory deficient. The white colony phenotype can be attributed to respiratory deficiency because the development of the pink color in the *ade2* genetic background found in the *eki1*Δ (KS101) and *cki1*Δ *eki1*Δ (KS106) mutants and the parent W303-1B strain is dependent on oxidative phosphorylation (197). Another characteristic common to respiratory-deficient mutants is the inability to grow at elevated temperatures (198). Indeed, the original *eki1*Δ (KS101) and *cki1*Δ *eki1*Δ (KS106) mutants were temperature sensitive for growth at 37 ^{o}C, whereas the new mutants were not temperature sensitive for growth (Table 4). Interestingly, the new *eki1*Δ (HCY5) mutant had a tendency to give rise to spontaneous petite colonies that were white in color. Likewise, the wild type parent W303-1B also gave rise to petite white colonies, but at a lower frequency. One petite colony (strain HCY6) that was derived from the new *eki1*Δ

(HCY5) mutant and one petite colony (strain HCY8) that was derived from the parent strain W303-1B were examined for their growth on glucose and glycerol, and for their growth on glucose at 37 ^{o}C. Strains HCY6 and HCY8 exhibited the phenotypes of respiratory deficiency (Table 4). Respiratory deficient mutants are differentiated by the lack some part of the mitochondrial genome (*rho*$^{-}$) or lacking the entire mitochondrial genome (*rho*^{o}) (152, 196). Florescent microscopy of cells stained with 4',6-diamidino-2-phenylindole (152) indicated that KS101, KS106, and HCY6 were *rho*^{o}, whereas HCY8 was *rho*$^{-}$.

Respiratory Deficiency is Responsible for Increased CHO1 mRNA Stability

CHO1 mRNA stability was examined in two respiratory deficient mutants. These mutants included a known *rho*^{o} mutant (W303-1A [*rho*^{o}]), a known *rho*$^{-}$ mutant (MGY100). The two respiratory mutants exhibited an increase in the half-life of *CHO1* mRNA when compared with that of the respiratory sufficient control (Fig. 15). Thus, respiratory deficiency, whether due to a partial or total lack of the mitochondrial genome, gave rise to increased *CHO1* mRNA stability. These data also confirmed that the basis for the increased stability of *CHO1* mRNA in the old *eki1*Δ (KS101) was respiratory deficiency.

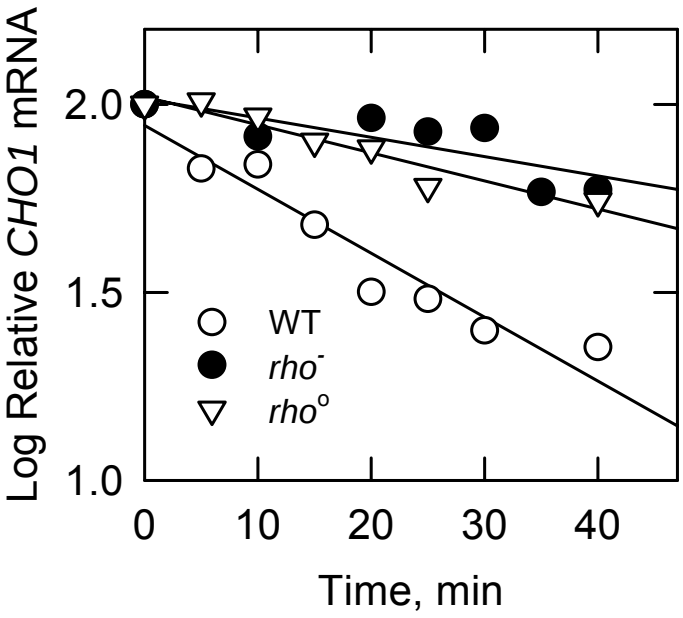
Respiration is a major function of the mitochondrion. Mitochondria contain a number of respiratory enzymes that participate in the tricarboxylic acid cycle and in the electron transport system (1). The electron transport system takes electrons from molecules such as NADH and $FADH_2$ and passes them along to oxygen, the final electron acceptor, to form water (1).

TABLE 4
Growth of *eki1*Δ and respiratory deficient mutants using glycerol and glucose as the carbon source

Relevant genotype	Growth[1]		
	Glycerol, 30 °C	Glucose, 30 °C	Glucose, 37 °C
Wild type	+	+	+
*eki1*Δ (KS101)	-	+	-
*eki1*Δ (HCY5)	+	+	+
rho$^-$ (MGY100)	-	+	-
rho^o (W303-1B [*rho*^{o}])	-	+	-

[1]The indicated cells were grown in YPD medium to stationary phase at 30 °C. Cells were diluted and spotted onto YPG and YPD media plates. The plates were incubated at the indicated temperatures, and colony growth was scored after 5 days. +, colony growth; -, no growth.

Figure 15. Effect of respiratory deficiency on *CHO1* mRNA decay. Wild type (WT, W303-1B), *rho*$^{-}$ (MGY100), and *rho*^{o} (W303 [*rho*^{o}]) mutant cells were grown to the exponential phase (1x10^{7} cells/ml) of growth. Following the arrest of transcription, *CHO1* mRNA decay was quantified as described in the legend to Fig. 11. The figure shows a plot of the log of the relative amount of *CHO1* to *PGK1* mRNAs *versus* time. The lines drawn were the result of a least-squares analysis of the data. The data shown in the figure are representative of three independent experiments. Half-life values are presented in Table 3.


Log Relative CHO1 mRNA
2.0
1.5
1.0
0
10
20
30
40
Time, min
WT
rho-
rho°

The enzymes of the electron transport system are located at the inner membrane of mitochondria, and include NADH dehydrogenase, succinate q reductase, cytochrome c dehydrogenase, and cytochrome c oxidase (1). KCN, a specific inhibitor of cytochrome c oxidase, is commonly used to halt the electron transport chain and mitochondrial respiration (1, 199). To determine the effects of inhibiting respiration on *CHO1* mRNA stability, respiratory-sufficient cells (W303-1B) were grown in the absence and presence of KCN. The addition of KCN to the growth medium resulted in a dose-dependent increase in the half-life of *CHO1* mRNA (Fig. 16, Table 3). To further confirm that a block in respiration was responsible for the increase in *CHO1* mRNA stability, the decay of the *CHO1* transcript was analyzed in the *cox4*Δ mutant. *COX4* is a nuclear gene that encodes an indispensable subunit of cytochrome c oxidase (160). The decay rate of *CHO1* mRNA was much longer in the *cox4*Δ mutant when compared with the wild type control (Fig. 17, Table3). Taken together, these data provided strong evidence that respiratory deficiency induces *CHO1* mRNA stability.

Effects of Respiratory Deficiency on the Synthesis and Steady-state Composition of Phospholipids

Respiratory deficient mutant (HCY8, *rho*$^-$) was isolated from agar plate of wild type (W3031-B) and examined for the synthesis and steady-state composition of phospholipids by labeling cells with $^{32}P_i$. Respiratory-deficient mutation had no significant effect on phospholipid synthesis (Fig. 18A). The $^{32}P_i$ labeling of the mutant showed small decrease in PS, PE, PC, PI, but an increase in PA. However, the difference

Figure 16. Effect of KCN on *CHO1* mRNA decay. Wild type (WT, W303-1B) cells were grown to the exponential phase ($1x10^7$ cells/ml) of growth in the absence and presence of the indicated concentrations of KCN. Following the arrest of transcription, *CHO1* mRNA decay was quantified as described in the legend to Fig. 11. The figure shows a plot of the log of the relative amount of *CHO1* to *PGK1* mRNAs *versus* time. The lines drawn were the result of a least-squares analysis of the data. The data shown in the figure are representative of three independent experiments. Half-life values are presented in Table 3.

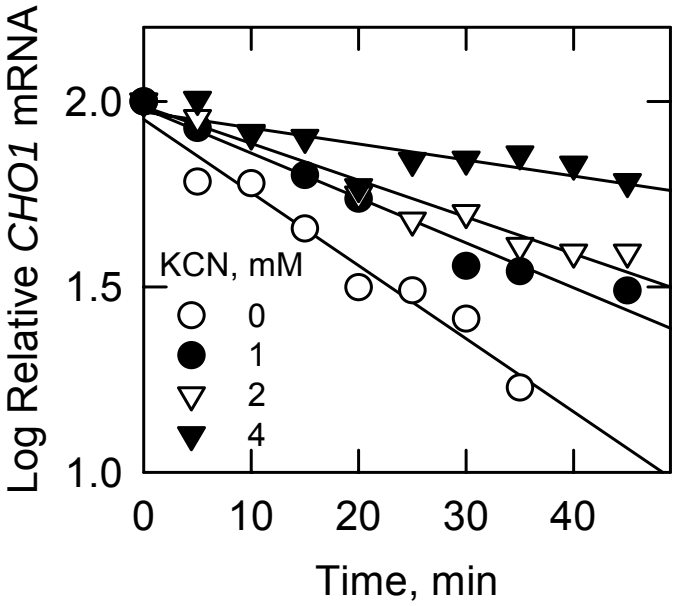

Log Relative CHO1 mRNA
2.0
1.5
1.0
KCN, mM
0
1
2
4
0
10
20
30
40
Time, min

Figure 17. Effect of the *cox4*Δ mutation on *CHO1* mRNA decay. Wild type (WT, W303-1B) and *cox4*Δ (WD1) mutant cells were grown to the exponential phase ($1x10^7$ cells/ml) of growth. Following the arrest of transcription, *CHO1* mRNA decay was quantified as described in the legend to Fig. 11. The figure shows a plot of the log of the relative amount of *CHO1* to *PGK1* mRNAs *versus* time. The lines drawn were the result of a least-squares analysis of the data. The data shown in the figure are representative of three independent experiments. Half-life values are presented in Table 3.

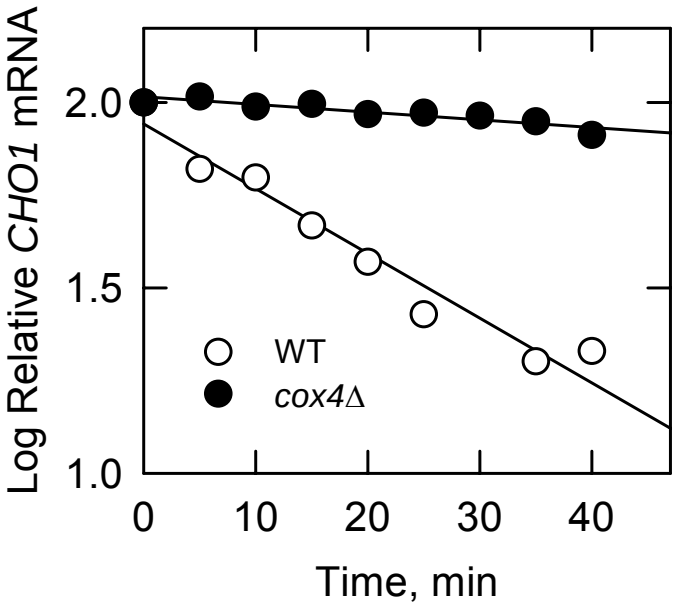
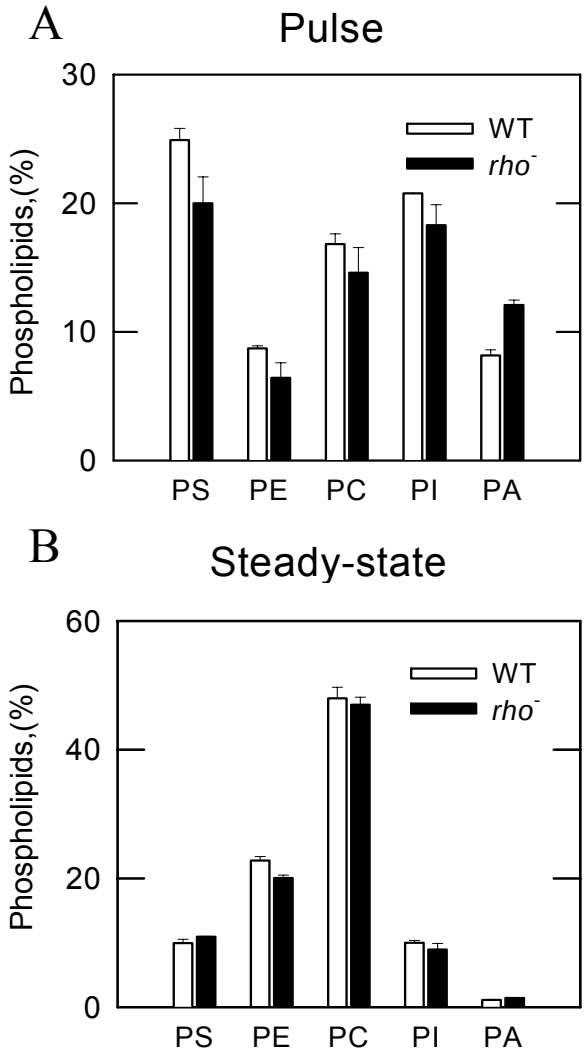

Log Relative CHO1 mRNA
2.0
1.5
1.0
0
10
20
30
40
Time, min
WT
cox4Δ

Fig. 18. Effects of the respiratory deficiency on the synthesis and steady-state composition of phospholipids synthesized by the CDP-DAG and Kennedy pathway. Wild type (WT) and respiratory deficient (HCY8) cells were grown to the exponential (1x 10^7 cells/ml) phase of growth. For pulse labeling of phospholipids (*Panel A*), cells were incubated with $^{32}P_i$ (10μCi/ml) for 20 min. The steady-state composition of phospholipids (*Panel B*) was determined by labeling cells for six generations with $^{32}P_i$ (5μCi/ml). Phospholipids were extracted and analyzed as described under " Experimental Procedure" The percentage shown for phospholipids were normalized to the total $^{32}P_i$-labeled chloroform-soluble fraction, which included sphingolipids and other unidentified phospholipids. Each data points represnts the average of two independent experiments ± S.D.

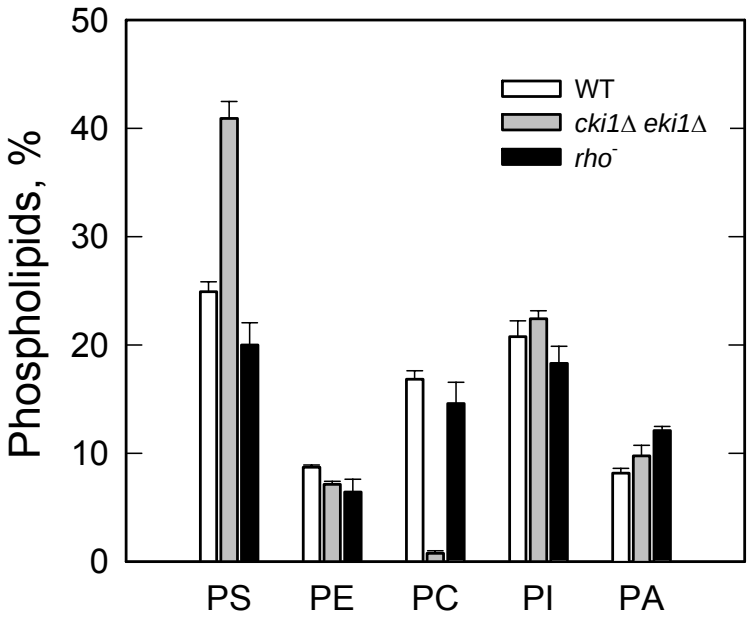

A
Pulse
30
20
10
0
Phospholipids,(%)
WT
rho-
PS
PE
PC
PI
PA
B
Steady-state
60
40
20
0
Phospholipids,(%)
WT
rho-
PS
PE
PC
PI
PA

of major phospholipid (PC, PS, PE, PI) synthesis between wild type and respiratory-deficient mutant was not significant by standard deviation.

This result was not correlated to the consequence (Fig. 5) from *cki1*Δ *eki1*Δ which showed significant increase in the rate of PS and PE synthesis. This result indicated that up-regulated phospholipid synthesis in the CDP-DAG pathway was due to not the respiratory-deficient mtation, but the block in the Kennedy pathway by *cki1*Δ *eki1*Δ. To confirm the difference of effects of respiratory-deficient mutation and *cki1*Δ *eki1*Δ mutation for phospholipid synthesis, new *cki1*Δ *eki1*Δ without respiratory-deficiency (HCY7) was constructed and analyzed for phospholipids synthesis (Fig. 19). The rate of PS synthesis in the *cki1*Δ *eki1*Δ was greatly increased compared to wild-type and the *rho*$^-$ mutant. This result correlated to the effect (Fig. 5) of *cki1*Δ *eki1*Δ (KS106) on the phospholipids synthesis. The elevated level of PE in *cki1*Δ *eki1*Δ (KS106) with respiratory-deficiency was not observed in *cki1*Δ *eki1*Δ (HCY7). The increase of only PS synthesis is likely to due to the shorter 20-min pulse labeling. The great decrease in PC synthesis in the *cki1*Δ *eki1*Δ (KS106) was also observed in the *cki1*Δ *eki1*Δ (HCY7), suggesting the phospholipase D-mediated choline reutilization by Kennedy pathway (40, 187). These results indicated that the block in the Kennedy pathway caused the elevation of phospholipids synthesis by CDP-DAG pathaway. On the other hand, the steady-state phospholipid composition had no significant difference between wild type and respiratory-deficient mutant (Fig. 18B). This result indicated that the respiratory-deficient mutant maintains almost normal level of phospholipids.

Thus, respiratory deficientcy, that still has Kennedy pathway, has not shown significant effect on the phospholipid synthesis and composition. The result of

Figure. 19. Effects of the *cki1*Δ *eki1*Δ mutations on the synthesis of phospholipids synthesized by the CDP-DAG pathway. Wild type (WT), *cki1*Δ *eki1*Δ, and respiratorydeficient mutant (*rho*$^-$, HCY8) cells were grown to the exponential (1 x 10^7 cells/ml) phase of growth. For pulse labeling of phospholipids cells were incubated with $^{32}P_i$ (20 μCi/ml) for 20 min. Phospholipids were extracted and analyzed as described under "Experimental Procedures." Each data point represents the average of two independent experiments ± S.D.

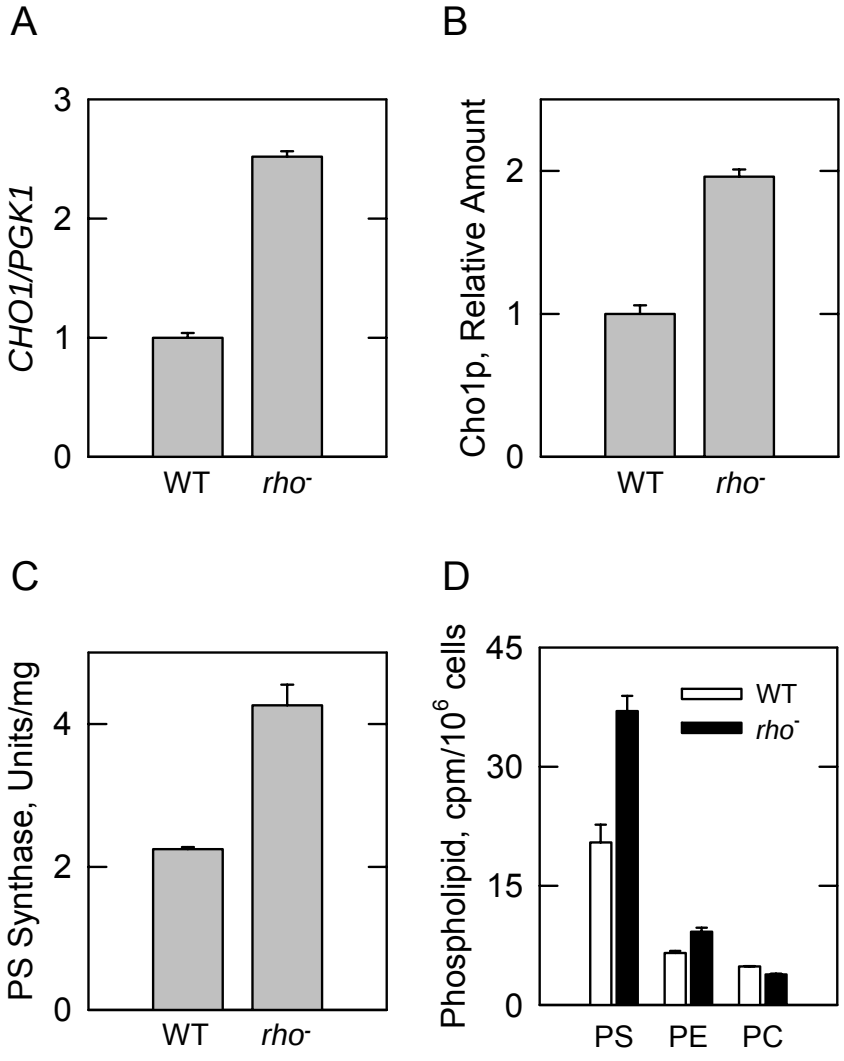

50
40
30
20
10
0
Phospholipids, %
WT
cki1Δ eki1Δ
rho-
PS
PE
PC
PI
PA

phospholipid synthesis of *cki1*Δ *eki1*Δ without respiratory-deficiency suggests that Kennedy pathway is still important for PC synthesis even in the absence of choline and ethanolamine in the media. .

Effects of Respiratory Deficiency on the Abundance of CHO1 mRNA, PS Synthase Protein, PS Synthase Activity, and on the Synthesis of PS In Vivo

The work presented above showed that the *cki1*Δ *eki1*Δ (KS106) mutant exhibited increased levels of the *CHO1* gene products (mRNA and protein) and PS synthase activity (Fig. 7 and 12). As indicated above, the *cki1*Δ *eki1*Δ (KS106) mutant was also respiratory deficient. Thus, it was unclear whether the regulation of PS synthase was mediated by the *cki1*Δ *eki1*Δ mutations or the *rho*$^-$ mutation. Accordingly, Northern blot and Western blot analyses for the *CHO1* gene products were performed with a respiratory deficient mutant and the results are presented in Figs. 20 and B, respectively. The levels of both *CHO1* mRNA and PS synthase protein (Cho1p) were about 2-fold higher in the respiratory deficient mutant when compared with the levels in the wild type control. The increased levels of *CHO1* mRNA and PS synthase protein in the respiratory mutant correlated with an increased level of PS synthase activity (Fig. 20C). To examine the effect of respiratory deficiency on the synthesis of PS *in vivo*, exponential phase cells were labeled with [^{14}C] serine for 30 min followed by the extraction and analysis of phospholipids. [^{14}C] serine is directly incorporated into PS via the PS synthase enzyme, followed by the incorporation of the label into PE and PC via the reactions catalyzed by the PS decarboxylase and phospholipid methyltransferase enzymes (i.e., CDP-DAG pathway) (10, 150). The respiratory mutant showed a 1.9-fold increase in the

Figure 20. Effects of respiratory deficiency on the levels of *CHO1* mRNA, PS synthase protein, PS synthase activity, and the synthesis of PS *in vivo*. Wild type (WT, W303-1B) and *rho*$^-$ (HCY8) mutant cells were grown to the exponential phase ($1x10^7$ cells/ml) of growth. *Panel A*, total RNA was extracted and the abundance of *CHO1* mRNA was determined with 10 μg of RNA by Northern blot analysis. The relative amounts of *CHO1* and *PGK1* mRNAs from wild type and mutant cells were determined by ImageQuant analysis of the data. The relative amount of *CHO1* to *PGK1* mRNA in wild type cells was arbitrarily set at 1. *Panel B*, the total membrane fraction was isolated and 12.5 μg of protein was subjected to immunoblot analysis using a 1:500 dilution of anti-PS synthase antibodies. The relative amounts of the PS synthase protein from wild type and mutant cells were determined by ImageQuant analysis of the data. The amount of PS synthase protein found in wild type cells was arbitrarily set at 1. *Panel C*, the total membrane fraction was isolated and used for the assay of PS synthase activity. *Panel D*, cells were incubated with [^{14}C]serine for 30 min. Phospholipids were extracted and analyzed by two-dimensional TLC. The data shown in panels *A-D* are the average of three experiments ± S.D.


A
B
C
D
CHO1/PGK1
Cho1p, Relative Amount
PS Synthase, Units/mg
Phospholipid, cpm/10^6 cells
WT
rho-
PS
PE
PC
0
1
2
3
4
15
30
45

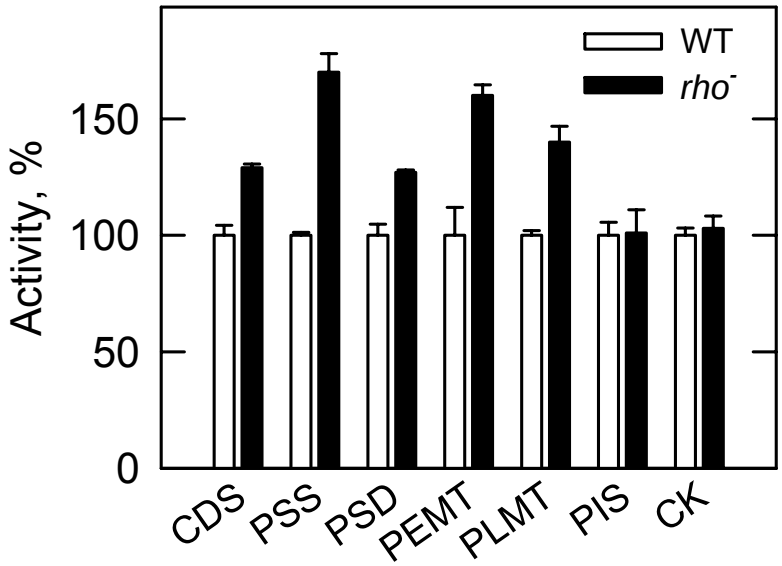
incorporation of [^{14}C] serine into PS after the 30 min labeling period (Fig. 20D). The effects of respiratory deficiency on the incorporation of the label into PE and PC were less dramatic (Fig. 20D). Respiratory deficiency did not have a significant affect on the steady state composition of phospholipids labeled [^{14}C] serine (data not shown).

Effects of Respiratory Deficiency on the Levels of CDP-DAG Pathway Enzyme Activities

In addition to PS synthase, the activities of other CDP-DAG pathway enzymes are elevated in the original *cki1*Δ *eki1*Δ mutant (KS106) (35). To address whether this regulation was due to the Kennedy pathway mutations or due to respiratory deficiency, the enzyme activity analyses were performed with the *rho*$^{-}$ (HCY8) mutant. The levels of CDP-DAG synthase (29%), PS decarboxylase (26%), PE methyltransferase (60%), and phospholipid methyltransferase (34%) activities were elevated in respiratory deficient cells when compared with the control (Fig. 21). On the other hand, the activities of PI synthase and choline kinase were not affected by respiratory deficiency (Fig. 21). The analyses of these activities in the new *cki1*Δ *eki1*Δ mutant (HCY7) showed that the Kennedy pathway mutations did not affect the activity levels of the CDP-DAG pathway enzymes (data not shown).

Effects of Oxidative Stress and Respiratory Growth on CHO1 mRNA Deacay/Stability

Figure 21. Effects of the *rho*$^-$ mutation on the levels of phospholipid synthesis enzyme activities. Wild type (WT, W303-1B) and *rho*$^-$ (HCY8) mutant cells were grown to the exponential phase ($1x10^7$ cells/ml) of growth. The total membrane fraction was isolated and used for the assay of CDP-DAG synthase (*CDS*), PS synthase (*PSS*), PS decarboxylase (*PSD*), PE methyltransferase (*PEMT*), phospholipid methyltransferase (*PLMT*), and PI synthase (*PIS*). The cell extract was used for the assay of choline kinase (*CK*) activity. The specific activities (nmol/min/mg) of these enzymes from wild type cells were 0.92 $\pm$ 0.04, 2.2 $\pm$ 0.03, 0.41 $\pm$ 0.02, 0.4 $\pm$ 0.05, 0.64 $\pm$ 0.01, 2.5 $\pm$ 0.14, and 4.5 $\pm$ 0.14. Each data point represents the average of triplicate enzyme determinations from two independent experiments ± S.D.

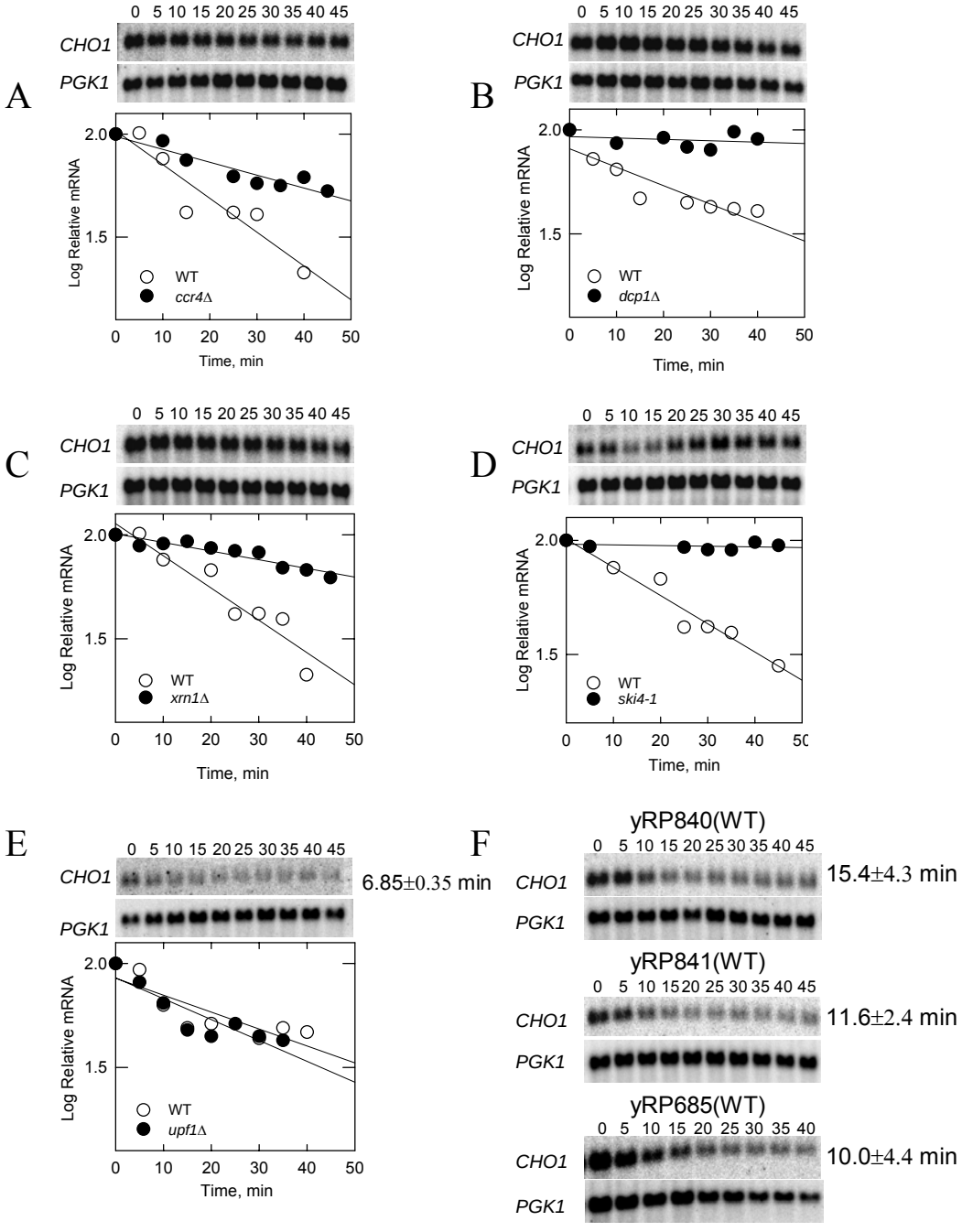

WT
rho-
Activity, %
150
100
50
0
CDS
PSS
PSD
PEMT
PLMT
PIS
CK

We questioned whether oxidative stress affected the rate of *CHO1* mRNA decay. For this experiment, glucose-grown wild type cells were incubated with hydrogen peroxide, a treatment commonly used to induce oxidative stress (155, 156). This treatment did not affect the rate of *CHO1* mRNA decay. We also questioned whether respiratory growth affected the decay of *CHO1* trranscript. The rate of *CHO1* mRNA decay of wild type cells grown with glycerol was not significantly different from cells grown with glucose.

CHO1 mRNA Decay Pathways

To investigate the mechanism of *CHO1* mRNA decay, the decay rates were examined in specific *S. cerevisiae* mutants that are defective in trans-acting factors that are involved in specific pathways (90). An increase of *CHO1* mRNA stability in a particular mutant indicates the involvement of that decay pathway. Many mRNAs in *S. cerevisiae*, are degraded by a general deadenylation-dependent pathway involving the deadenylation of the poly(A) tail by Ccr4p, the removal of the 5' cap by the Dcp1p-Dcp2p complex, and the 5'-3' exonuclease cleavage by Xrn1p (91). To examine whether *CHO1*mRNA is degraded by this general pathway, a decay analysis was performed in mutants defective in each of the three steps. The rate of *CHO1* mRNA decay was reduced in the *ccr4*Δ mutant defective in deadenylation, in the *dcp1*Δ mutant defective in decapping, and in the *xrn1*Δ mutant defective in 5'-3' exonuclease cleavage (Fig 22, Table3). That *CHO1* mRNA was stabilized in these mutants indicated that the *CHO1* transcript is degraded by the general decay pathway (91).

Figure 22. **Decay of *CHO1* mRNA in mRNA decay pathway mutants.** Decay pathway mutant cells were grown to the exponential (1 x 10^7 cells/ml) phase of growth. Following the arrest of transcription, 5-ml samples were taken at the indicated time intervals, and total RNA was extracted. The levels of *CHO1* mRNA and *PGK1* mRNA were determined by Northern blot analysis in the *ccr4*Δ (*panel A*), *dcp1*Δ (*panel B*), *xrn1*Δ (*panel C*), *ski4-1* (*panel D*), *upf1*Δ (*panel E*) and each wild-type cells (*panel F*). yRP840, yRP841, and yRP685 were used as wild-type control to the each decay mutants (*panel F*). Portions of Northern blots are shown, the half-lives of *CHO1* mRNA are indicated. The relative amount of *CHO1* and *PGK1* mRNAs from decay pathway mutant cells were determined by ImageQuant analysis. The figure shows a plot of the log of the relative amount of *CHO1* to *PGK1* mRNAs versus time. The data shown in the figure are representative of three independent experiments.


A
0 5 10 15 20 25 30 35 40 45
CHO1
PGK1
Log Relative mRNA
2.0
1.5
WT
ccr4Δ
0 10 20 30 40 50
Time, min
B
0 5 10 15 20 25 30 35 40 45
CHO1
PGK1
Log Relative mRNA
2.0
1.5
WT
dcp1Δ
0 10 20 30 40 50
Time, min
C
0 5 10 15 20 25 30 35 40 45
CHO1
PGK1
Log Relative mRNA
2.0
1.5
WT
xrn1Δ
0 10 20 30 40 50
Time, min
D
0 5 10 15 20 25 30 35 40 45
CHO1
PGK1
Log Relative mRNA
2.0
1.5
WT
ski4-1
0 10 20 30 40 50
Time, min
E
0 5 10 15 20 25 30 35 40 45
CHO1
PGK1
6.85±0.35 min
Log Relative mRNA
2.0
1.5
WT
upf1Δ
0 10 20 30 40 50
Time, min
F
yRP840(WT)
0 5 10 15 20 25 30 35 40 45
CHO1
PGK1
15.4±4.3 min
yRP841(WT)
0 5 10 15 20 25 30 35 40 45
CHO1
PGK1
11.6±2.4 min
yRP685(WT)
0 5 10 15 20 25 30 35 40
CHO1
PGK1
10.0±4.4 min

DISCUSSION

In this work, I hypothesized that the defect of the Kennedy pathway is compensated by the up-regulation of the CDP-DAG pathway. We examined the consequences of a block in the Kennedy pathway on the regulation of phospholipid synthesis by the CDP-DAG pathway. The *cki1*Δ *eki1*Δ mutant compensated for the block in the Kennedy pathway by increasing the activity levels of the CDP-DAG pathway enzymes PS synthase, PS decarboxylase, PE methyltransferase, and phospholipid methyltransferase. The increase in these activities was reflected by an increase in the rate of phospholipid synthesis by the CDP-DAG pathway. CDP-DAG synthase, which supplies CDP-DAG for the pathway, was also elevated in *cki1*Δ *eki1*Δ mutant cells. However, other enzyme activities (i.e., PI synthase and PA phosphatase), which are not CDP-DAG pathway enzymes, were unaffected by the *cki1*Δ *eki1*Δ mutations. Although the steady-state level of PC (and overall phospholipid composition) of *cki1*Δ *eki1*Δ mutant cells was not much different from that of wild type cells, the mutation caused a slower rate of growth.

We then focused our attention to the *CHO1*-encoded PS synthase. The *CHO1*-encoded PS synthase is one of the most highly regulated phospholipid biosynthetic enzymes in *S. cerevisiae* (10, 42). Regulation of this enzyme by biochemical and transcriptional mechanisms have an impact on the partitioning of CDP-DAG between PS and PI, and the utilization of the pathways by which cells synthesize the major membrane phospholipid PC (10, 42, 43, 136). Most studies of *CHO1* regulation are focused on mechanisms that control its transcription (10, 136) (5, 43). Transcription of *CHO1*, like that of several other genes involved in the synthesis of phospholipids, is mediated by a

regulatory circuit comprised of a UAS_{INO} *cis*-acting element, the positive transcriptions factors Ino2p and Ino4p, and the repressor Opi1p (5, 10, 43, 136). This regulatory circuit controls *CHO1* transcription in response to inositol supplementation (140-143), zinc deprivation (46), and by growth phase (72, 144).

We showed that the *cki1*Δ *eki1*Δ mutations caused an increase in *CHO1* mRNA abundance, and the corresponding increase in the levels of PS synthase protein and activity played a role in the activation of the CDP-DAG pathway to compensate for the block in the Kennedy pathway. However, this regulation was not mediated by the UAS_{INO} element in the *CHO1* promoter that is required for maximum gene expression (143). Moreover, the *cki1*Δ *eki1*Δ mutations did not affect the inositol-mediated repression of the *CHO1* gene. A dramatic increase in *CHO1* mRNA stability, as opposed to an increase in *CHO1* transcription, contributed to the elevated levels of *CHO1* transcript in the *cki1*Δ *eki1*Δ mutant. The rate of mRNA decay plays an important role in the control of gene expression (199). For example, the half-life of an mRNA governs the number of times a transcript can be translated, which in turn governs the amount of protein that can be produced at a given rate of transcription (199). *CHO1* mRNA was a moderately stable transcript ($t_{1/2}$=10-12 min) when compared with other mRNAs in *S. cerevisiae* that have half-lives from 1-60 min (200). However, *CHO1* mRNA was greatly stabilized with a half-life >45 min in the *cki1*Δ *eki1*Δ (KS106) mutant (35).

We sent out to identify the Kennedy pathway component(s) that controlled *CHO1* mRNA decay. Through *CHO1* mRNA decay analyses of Kennedy pathway mutants, we narrowed down the regulation point for increased *CHO1* mRNA stability to the ethanolamine kinase reaction. Upon further analysis however, we found that the loss of

ethanolamine kinase (i.e., *eki1*Δ mutation) was not responsible for the increased stability of the *CHO1* transcript. Instead, transcript stability was the result of *rho* mutation (i.e., respiratory deficiency) that spontaneously arose in the *eki1*Δ mutant background. In fact, the *rho* mutation was also present in the *cki1*Δ *eki1*Δ mutant that was originally examined for identification of *CHO1* regulation by mRNA stability (35). It was not too surprising that the *eki1*Δ mutation gave rise to respiratory deficient cells. A number of workers have noted the tendency of phospholipid synthesis mutants (e.g., *cho1*, *psd1*Δ, *opi3*, *crd1*Δ) to give rise to respiratory deficient petite colonies (31, 201, 202). The *eki1*Δ, *ect1*Δ, and *ept1*Δ were indeed observed to give rise to respiratory-deficient mutant (petite) easily compared to wild-type (data not shown). Although it is not yet clear how the *eki1*Δ mutation caused respiratory deficiency, the reason for this general phenomenon may be attributed to the important roles that phospholipids (e.g., cardiolipin and PE) play in both the composition and function of mitochondrial membranes (201, 203-212). Thus, *eki1*Δ might provide the condition for the occurrence of respiratory-deficient mutant by blocking the small contribution in synthesis of mitochondrial PE. In addition, if PE can be also reutilized by PE specific phospholipase D (213), ethanolamine accumulated in *eki1*Δ may provide the condition which mitochondrial function is inhibited (214).

mRNA stability can be regulated in response to the various developmental, environmental, metabolic factors which play as stress conditions (83, 84, 86, 215-217). For example, studies with *E.coli* (218) and mammalian cells (87, 219, 220) have shown that the stability of mRNA increases under stress conditions. Defects in phospholipid synthesis, especially that of phosphatidylglycerol, cardiolipin, and PE, have a negative effect on mitochondrial function (201, 203-212, 221). Respiratory-dysfunction of

mitochondria can be a severe stressful condition, which might contribute to the stability of the *CHO1* mRNA. Mitochondrial-dysfunction actually induces the alteration of gene expression (210-212, 222). Respiratory-deficiency causes up-regulation of many genes (222). In this work, we showed that respiratory deficiency has a significant effect on the genetic regulation of the key phospholipid synthesis enzyme PS synthase. The increase in *CHO1* transcript stability in response to respiratory deficiency translated into elevated levels of *CHO1* mRNA, PS synthase protein and activity, and an increase in the synthesis of PS *in vivo*. Translocation of transcription factors (Rtg1p and Rtg3p) from the cytoplasm to the nucleus acts as a signal for the regulation of respiratory-deficiency responsive genes known as a retrograde regulation (211, 212, 222). *CHO1* mRNA might be another gene regulated by retrograde regulation mechanism.

During this work, we also hypothesized that *S. cerevisiae* cells compensated for the stress of respiration deficiency by conserving the *CHO1* transcript for translation to the PS synthase enzyme for enhanced phospholipid synthesis via the CDP-DAG pathway.

The activities of other CDP-DAG pathway enzymes (e.g., CDP-DAG synthase, PS decarboxylase, and the phospholipid methyltransferase enzymes) were also elevated in respiratory deficient cells. For the *OPI3*-encoded phospholipid methyltransferase, the increase in activity may also be attributed to an increase in mRNA stability. These results were similar to that found in the respiratory deficient *cki1*Δ *eki1*Δ (KS106) mutant (35). A reanalysis of these enzyme activities in the respiratory sufficient *cki1*Δ *eki1*Δ (HCY7) mutant confirmed that the regulation was solely due to the *rho*^{o} mutation. The regulation of phospholipid synthesis enzymes in response to respiratory deficiency was not a universal response. The PI synthase and choline kinase activities were not affected

by the *rho*$^{-}$ mutation. This raised the suggestion that there must be a specific mechanism(s) that controls the rate of deadenylation, decapping, and/or exonuclease cleavage of *CHO1* mRNA in response to respiratory deficiency. Studies to address this mechanism(s) awaits the determination of the elements within the 5'-untranslated region, 3'-untranslated region, and/or coding sequence of *CHO1* mRNA and the trans-acting RNA-binding protein(s) that interact with these elements to stabilize or destabilize the *CHO1* transcript (81, 160, 219, 222, 223).

On the other hand, even if $^{32}P_i$ labeled PS synthesis in *cki1*Δ *eki1*Δ (HCY7) was greatly increased compared to wild type. The elevation was not due to the up-regulation of gene expression. Instead, the biochemical mechanism such as post-translational modification might be resulted from the regulation of PS synthesis in the 20-min pulse in *cki1*Δ *eki1*Δ (HCY7). The phosphorylation of PS synthase is known to inhibit its enzymatic activity (49). For example, the PS synthase in *cki1*Δ *eki1*Δ might be less phosphorylated.

Decay analysis of *CHO1* mRNA using respiration-deficient mutants (*rho*$^{-}$ and *rho*^{o}), the *cox4*Δ mutant defective in the cytochrome c oxidase, and wild type cells treated with KCN (a cytochrome c oxidase inhibitor) confirmed that respiratory deficiency was responsible for the increase in the half-life of the *CHO1* transcript. Mitochondria have several components such as electron transport system necessary for respiration. Cytochrome c oxidase is the last complex in the electron transport chain to deliver electron to the oxygen (1). There are different mitochondrial inhibitors to the other electron transport chains and ATP synthase complex (1). For example, antimycin A is the specific inhibitor of cytochrome c oxidoreductase, one of electron transport system (1,

222). Carbonyl cyanide m-chlorophenylhydrazone (CCCP) uncouples electron transport system and ATP synthase complex by removing proton gradient between mitochondria membranes (222). The effects of mitochondrial dysfunction by these inhibitors on gene expression are different (222). Up-regulation of genes by antimycin A is similar to the effect in respiratory-deficient (*rho*^{o}) cell, however, the effect of CCCP and oligomycin is very little (222) which suggest that up-regulation of transcript in *rho*^{o} is affected by the perturbation of the electron transport chain rather than the loss of mitochondrial ATP synthesis (222). Whether or not the effect of respiratory deficiency on *CHO1* mRNA stability is also dominated by the block of electron transport system will require the test for CCCP or oligomycin on the *CHO1* mRNA stability.

Mitochondria function (respiration) provides eukaryotic cells with energy for life process including gene expression (9). All genes undergo the synthesis and decay. This process is regulated at various cellular levels (9). Genetic regulation in nucleus may require more energy than cytoplasmic regulation. However, respiratory deficient cells are not able to generate enough energy as respiratory deficient cells. Thus, the control of transcript on the cytoplasmic level may be an easier way to regulate gene expression without consumption of much energy unlike a respiratory competent, which has sufficient energy.

S. cerevisiae provides a well-suited system for analyzing how cells respond to mitochondrial dysfunction. To study the cellular responses to mitochondrial dysfunction, many researches use respiratory-deficient strain called petite (196). In general, such study is performed by comparing transcriptional level of the target gene in respiratory-sufficient strain to that in respiratory- deficient petite strain (*rho*^{o}). However, the study

on the regulation of post-transcriptional expression between respiratory-sufficient strain and respiratory-deficient petite is yet to be reported.

CHO1 mRNA was stabilized in mutants defective in deadenylation (*ccr4*Δ), mRNA decapping (*dcp1*), and the 5'-3' exonuclease (*xrn1*). These results indicated that the *CHO1* transcript is primarily degraded through the general 5'-3' mRNA decay pathway (177). The specific mechanisms and regulators that control the stabilization of *CHO1* mRNA in response to respiratory deficiency are unknown. Given that *CHO1* mRNA decays by the primary 5'-3' decay when cells are respiratory sufficient, it is reasonable to predict that the rate of deadenylation and/or decapping may be reduced when respiration is blocked. Extensive studies will be required to identify *cis*-acting elements in the transcript as well as components of the signal transduction system that lead from the respiratory defect to the mRNA stabilization response.

OLE1, which encodes the Δ-9 fatty acid desaturase, is another yeast gene of lipid metabolism whose expression is independently regulated at the levels of transcription and by mRNA stability (81, 223, 224). The *OLE1* transcript is destabilized when cells are supplemented with unsaturated fatty acids (81, 223, 224). This fatty acid-regulated decay of *OLE1* mRNA occurs through both the 5'-3' general pathway and via exosomal 3'-5' degradation activities (223). Interestingly, like *CHO1* mRNA, *OLE1* transcript levels are more abundant under oxygen-deprived conditions (223). It is not clear, however, whether this regulation is due to an increased rate of transcription, a decreased rate of mRNA decay or contributions from both regulatory systems.

In summary, we clarified that a block in the Kennedy pathway for phospholipid synthesis was not responsible for the regulation of PS synthase by mRNA stability.

Instead, we discovered that the stress of respiratory deficiency triggers *S. cerevisiae* cells to stabilize the *CHO1* transcript for increased levels of *CHO1* mRNA, PS synthase protein, and synthesis of PS. The rate of mRNA decay plays an important role in the control of gene expression (81). The half-life of an mRNA governs the number of times a transcript can be translated, which in turn governs the amount of protein that can be produced at a given rate of transcription. This work underscores the importance of mitochondrial function to the regulation of phospholipid synthesis in *S. cerevisiae*.

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