Lack of Mitochondrial Anionic Phospholipids Causes an Inhibition of Translation of Protein Components of the Electron Transport Chain: A Yeast Genetic Model System for the Study of Anionic Phospholipid Function in Mitochondria*

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**Running Title:** Anionic Phospholipid Function in Mitochondria

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SUMMARY

Reduction of mitochondrial cardiolipin (CL) levels has been postulated to compromise directly the function of several essential enzymes and processes of the mitochondria. There is limited genetic evidence for the critical roles with which CL and its precursor phosphatidylglycerol (PG) have been associated. A null allele of the PGS1 gene from Saccharomyces cerevisiae, which encodes the enzyme responsible for the synthesis of the CL precursor PG phosphate, was created in a yeast strain in which PGS1 expression is exogenously regulated by doxycycline. The addition of increasing concentrations of doxycycline to the growth medium causes a proportional decrease to undetectable levels of PGS1 transcript, PG phosphate synthase activity, and PG plus CL. The doubling time of this strain with increasing doxycycline increases to senescence in non-fermentable carbon sources or at high temperatures, conditions that do not support growth of the pgs1Δ strain. Doxycycline addition also causes mitochondrial abnormalities as observed by fluorescence microscopy. Products of four mitochondrial-encoded genes (COX1, COX2, COX3, and COB) and one nuclear-encoded gene (COX4) associated with the mitochondrial inner membrane are not present when PGS1 expression is fully repressed. No translation of these proteins can be detected in cells lacking the PGS1 gene product, although transcription and splicing appear unaffected. Protein import of other nuclear-encoded proteins remains unaffected. The remaining proteins encoded by mitochondrial DNA are expressed and translated normally. Thus, the molecular basis for the lack of mitochondrial function in pgs1Δ cells is the failure to translate gene products essential to the electron transport chain.
INTRODUCTION

In eukaryotic cells, cardiolipin (CL\textsuperscript{1}) and its precursor phosphatidylglycerol (PG) are found almost exclusively in the inner mitochondrial membrane, where CL is the major anionic phospholipid (1). Due to their intracellular distribution, these anionic phospholipids have been postulated to be an essential component in many mitochondrial processes such as electron transport, ion permeability, membrane integrity, protein import, and solute transport (2,3).

Mitochondria house the primary oxygen utilization centers of cells and are therefore the major source of oxidative damage to tissue. Reduction of CL levels is correlated with a compromise in terminal oxidation and an increase in oxidative damage to tissue through the accumulation of highly reactive intermediates generated by the electron transport chain (ETC) (4,5). Oxidative damage to CL appears to be a major factor in aging (6,7). Because of the high level of unsaturation of its fatty acyl groups, CL is the primary and specific target of lipid peroxidation during ischemia and reperfusion (8,9). Alterations in mitochondrial functions reliant on CL also are associated with Alzheimer's and several other neurodegenerative diseases (7,10).

One of the most important mitochondrial processes with which anionic phospholipids are thought to participate is protein import (11). This function is inhibited by adriamycin, an antibiotic that specifically binds anionic phospholipids (12). The 25 amino acid presequence of cytochrome \textit{c} oxidase (Cox) subunit IV (Cox4p), widely utilized as a model peptide for mitochondrial targeting sequences, is both stabilized and properly oriented by CL (13). Another unique property of CL is its ability to adopt non-bilayer structures in the presence of divalent cations such as calcium (14,15) which is a known regulator of mitochondria function. These structures are believed necessary for protein import (16,17). Anionic phospholipids have also been shown to provide the membrane organization sites in \textit{Escherichia coli} for complexes
composed of both integral and peripheral membrane proteins that are responsible for initiation of DNA replication (18) and protein translocation across membranes (19).

A widely studied phospholipid/protein interaction is that of CL with Cox (20). Many studies have demonstrated the necessity of CL for the in vitro function of this enzyme complex (21). Adriamycin inhibits its function (22), and decreased CL levels are associated with a decline in the function of this enzyme in aged cells (23). In yeast, CL synthase activity is proportional to Cox activity at different stages of the cell cycle (24), and Cox activity has been shown to be diminished in mutants (crd1Δ) lacking CL synthase (25).

Only a few reports appear to demonstrate a requirement for CL in vivo at the molecular level for any of the above processes. The most direct evidence for the essential role of PG and CL in mammalian cells comes from Chinese hamster ovary cells with a mutation (26) in the gene encoding the PG phosphate synthase (PGPS). The enzyme catalyzes the committed step of CL biosynthesis, and the strain, therefore, has reduced levels of both PG and CL (27,28). These mutants have defects in electron transport and ATP production, show reduced oxygen utilization, possess gross alterations in mitochondrial morphology, are temperature sensitive at 37°C, and eventually lose viability even when grown with glucose. A yeast model system based on a null mutant of CL synthase has also been studied (25). However, the phenotypic changes brought about by this mutation are not dramatic (29-31) suggesting the major roles of CL are substituted by an increase in PG. More severe mitochondrial dysfunction occurs when PG levels are also reduced by growth of this mutant on glucose rather than a non-fermentable carbon source. However, any conclusions drawn from these experiments are compromised by the extreme effects changes in carbon source have on growth characteristics of cells in general and mitochondrial development and function in particular.
In the present study, all mitochondrial PG and CL were depleted by disruption of \textit{PGS1}, which encodes PGPS (32). Null mutants in \textit{PGS1} are temperature sensitive, cannot grow on non-fermentable carbon sources, and are petite lethal (32-34). A regulatable \textit{PGS1} gene was introduced on a plasmid into the \textit{pgs1Δ} strain in order to study the effects of attenuating anionic phospholipids without the need to alter growth conditions or compare different strains. The resulting strain was used to determine the molecular basis for dysfunction of mitochondria lacking these phospholipids.
EXPERIMENTAL PROCEDURES

Strains, Plasmids and Growth Conditions--The wild type yeast strain utilized in this study is YPH499 (ade2-101, his3Δ200, leu2Δ1, lys2-801, trp1Δ63, ura3-52, MATα) (35). Disruption of PGS1 was accomplished by homologous recombination with a polymerase chain reaction (PCR) product of TRP1 carried on plasmid pRS304 (35) by amplification with primers 5’-GCAGCCACACAAGAAAGTAGATATAATGTAGGACACCCAGCTTGTTGACAATTTG CTAATAGCATACTCAGGATAggtatcttcctagcatctgtg-3’ and 5’-GCTCTTCATCTCTTGTAATAATGAGAGCATTCGATCAAATCTAATGAATACGCC TTCTCGTATAGTTTGAAGGagtgcaccaacgacattactat-3’. (Capitol letters refer to regions of PGS1 homology [promoter for the first primer, terminator for the second], and lower case letters refer to regions of TRP1 homology.) The resulting PCR product contained the TRP1 gene flanked by DNA homologous to regions 5’ and 3’ to PGS1. PGS1 null strains were selected on tryptophan dropout minimal media glucose plates (36); pgs1Δ strains were identified by PCR analysis of isolated genomic DNA as described below. Many of the experiments were duplicated in strain YCD4 (his3-11, 15, leu2-3, 112, pgs1Δ::HIS3, ura3-251, 328, 372, MATα), another pgs1Δ strain, (32) together with its PGS1 wild type parent, DL1, completely unrelated to YPH499, as described in results. Because of problems associated with the recovery of pgs1Δ cells from stationary phase and the high mortality resulting from freezing, only mid-log phase cells were used for frozen stocks, and fresh culture plates were prepared every two to three weeks.

Because fluorescence microscopy is difficult in an ade2 strain due to the buildup of a red pigment, ADE2 revertants were created in both the wild type parental and pgs1Δ strains. The gene was amplified from plasmid pRS402 (35) with primers
5'-TTTTCTTTAAAAGAATCAAAGACAGATAAAA-3'
and
5'-CACACCGCATAGATCTTATGTATGAAATTC-3', and this product used to transform the strains to white colonies on adenine dropout media. The ADE2 derivatives of YPH499 and its pgs1Δ derivative were named SDO224 (referred to as the “wild type” strain) and SDO225 (referred to as the “psg1Δ” strain), respectively. These were the strains used in this study. ρ0 derivatives of the wild type strain were created with ethidium bromide as previously described (37) as a control of the inability to create ρ0 derivatives of the psg1Δ strain.

The doxycycline regulated plasmid, pDO292 (CEN4, ARS1, URA3, CMVp/TetR DNA Binding Domain/VP16 Activation Domain, TetO/CYC1/pPGS1/CYC1t), was constructed as follows: Cloning of PGS1 was accomplished by PCR amplification from genomic DNA isolated (38) from strain DL1 (39) with primers

5'-ataggatccATGACGACTCGTTTGCTCCAACTCACTCGTCCTC-3'
and
5'-atagcggccgcCTAAAGTTTTTTACCCAAAATGGAGGTAGC-3'. (Capitol letters refer to regions of PGS1 homology with the start and stop codons underlined, respectively, and lower case letters refer to restriction sites and filler DNA.) In order to insert the gene 3’ of the tetracycline-regulated promoter, the 1.59 kb amplicon was cut with BamHI and NotI and ligated into plasmid pCM189 (40) cut with the same restriction enzymes. Because of problems associated with the stability of PGS1 plasmids in E. coli, ligations were transformed into and plasmids stored in yeast. Yeast spheroplast transformations of pgs1Δ strains were accomplished as described (41). Transformants were selected on yeast nitrogen base uracil dropout medium containing ethanol and glycerol (36). The construct was verified by sequencing PCR products obtained by amplification of DNA isolated from the strain. The psg1Δ strain carrying this plasmid is referred to throughout the text as the “psg1 regulatable” strain and was maintained at
10 µg/ml doxycycline (fully repressed) on YEP (1% yeast extract, 2% peptone) medium with 2% sucrose. Derepression was accomplished by growth for at least 24 h in lower doxycycline concentrations. Strain YCD4 (also a psg1Δ strain) was also transformed with the plasmid and tested in several experiments as described in results.

Quantitative Reverse Transcriptase (RT) PCR—Yeast strains were grown in YEP medium with 2% sucrose to the mid-exponential phase of growth. Total RNA was isolated using Trizol (Life Technologies) with 0.5 mm zirconia/silica beads and a Mini BeadBeater (BioSpec Products). RNA was quantified by spectroscopy. Primers (see Table 1 for a list used) were selected to amplify transcribed regions from 500 to 800 bp in length by Oligo Primer Analysis Software. RT-PCR was accomplished using the Access System (Promega). Primers used for COX1 and 21S RNA were designed to detect only properly spliced messages. Quantification was accomplished by varying either the number of cycles or RNA concentrations. Care was taken to ensure that the amplification had not plateaued and that the reactions produced bands of similar intensities upon replication (no variability in starting exponential PCR amplification). Each preparation of RNA was standardized using primers for either COX4, COB or ACT1. Standard reactions used 25 cycles, 50 pmol of each primer, and 100 ng/µl RNA in 50 µl. Amplicons were separated by agarose electrophoresis (Seakem, FMC), visualized with Sybr Green (Molecular Probes), and quantified using a Fluor-S Multi Imager (BioRad).

PGPS Enzymatic Assays and Phospholipid Quantification—Yeast strains were grown to the mid-exponential phase of growth in YEP medium with 2% sucrose. Crude mitochondrial preparations were isolated (42) using a Mini BeadBeater (BioSpec Products) and differential centrifugation. PGPS assays were performed essentially as previously described (32) by conversion of [14C]glycerol 3-phosphate (New England Nuclear) to [14C]PG phosphate.
dependent on CDP-diacylglycerol (Avanti, dioleoyl). The resulting mixture of \(^{14}\text{C}\)PG phosphate and \(^{14}\text{C}\)PG was isolated and quantified in a liquid scintillation counter (LKB). One unit is equivalent to the formation of one nmol PG phosphate plus PG per minute per mg protein.

Phospholipid quantification was performed essentially as previously described (32). Cells were labeled for 12 hr with 50 µCi \(^{32}\text{P}\)P\(_i\) (Amersham) in synthetic defined media (36) with 2% sucrose to the mid-exponential phase of growth. Cells were homogenized with a solution of 10:5:4 CH\(_3\)OH, CHCl\(_3\), 0.1N HCl in a Mini BeadBeater (BioSpec Products). Lipids were isolated by acid organic extraction (half volumes each of CHCl\(_3\) and 0.1N HCl, 0.5M NaCl), dried, and resuspended in an appropriate volume of CHCl\(_3\). Phospholipids were separated by thin layer chromatography using silica gel plates (Whatman) (43), and individual species were identified by co-migration of standards (Sigma). Phospholipid quantification was accomplished using both one- and two-dimensional thin layer chromatography. The spots were quantified by phosphoimaging using either an Instant Imager (Packard) or a Molecular Imager FX (BioRad) and normalized to the total amount of organic soluble \(^{32}\text{P}\).

**Fluorescent Dye Staining of Mitochondria**–The wild type and \(\text{psg1}\) regulatable strains were grown with no or 10 µg/ml doxycycline in YEP medium with 2% sucrose to the mid-exponential phase of growth. Cells were stained for 30 min with 50 nM 10-N-nonyl acridine orange (NAO) (44) or 20 µM 2-(4-dimethylaminostyryl)-1-methylpyridinium iodide (DASPMI) (45) and visualized with a 100X objective using a FITC filter. 50 µM carbonyl cyanide \(p\)-trifluoromethoxyphenylhydrazone (FCCP) was added to DASPMI stained cells grown without doxycycline to demonstrate complete dissipation of mitochondrial membrane potential.

**Mitochondrial Isolation**–The isolation of mitochondria was accomplished essentially by the method of Glick and Pon (46). Cells were grown for three days in increasing volumes of YEP
media with 2% sucrose to the mid-exponential phase of growth. Cells were harvested and spheroplasts prepared with zymolyase (ICN). Spheroplasted cells were lysed in hypotonic media by Dounce homogenization or with an Omnimixer (Omni). A crude mitochondrial preparation (pellet) was separated from the endoplasmic reticulum fraction (supernatant) by differential centrifugation. Highly purified mitochondria were obtained by sedimentation through a sucrose step gradient (47). A 70% sucrose cushion was employed when isolating mitochondria from the psg1 regulatable strain grown under repressing conditions. The mitochondrial associated microsomal membranes (MAM) fraction was isolated from the upper quarter of the gradient (48). Proteins from each fraction were concentrated using a TL100 ultracentrifuge (Beckman).

Proteolipid isolation of ATPase subunits was accomplished as described (49) by organic extraction of sonicated highly purified mitochondria. ATP6p, ATP8p, and ATP9p were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by silver staining.

In vitro Transcription/Translation and Mitochondrial Protein Import Assays—COX4 and COX6 were cloned into plasmid pCITE (CAP-independent translation enhancer, Novagen) by amplification of genomic DNA from strain DL1 using primer pairs

5'-ataccATGgTTTCACTACGTCATCTCTATAA-3' plus

5'-atagcatgcAGAAGGTTAAAAGTAAAAGAGAAAC-3' and

5'-ataccATGgTATCAAGGGCCATATTCAGAGAA-3' plus

5'-atagcatgcTTGTGGTTAGCTTTCTCTATTA-3', respectively. The amplicons and parent vector were cut with NcoI and SphI and ligated producing plasmids pDO290 (COX4) and pDO291 (COX6) in which the genes were expressed from the plasmid T7 promoter. Constructs were verified by sequencing. In vitro transcription/translation was accomplished using the STP3
kit (Novagen) with 500 ng of plasmid DNA in each 100 µl transcription/translation reaction. Ribosomes were removed by centrifugation and the biotinylated protein products were denatured with 5 M urea. The proteins were then prepared for import assays by ten-fold dilution.

Mitochondrial protein import assays were accomplished as described (50). Highly purified mitochondria were thawed on ice and potentiated with ATP. The assays were performed with 50 µg mitochondrial protein and 20 µl of the diluted biotinylation reaction. The reactions were performed for the times indicated and stopped with valinomycin (Sigma). Mitochondria were treated with proteinase K, extensively washed, and resuspended in protein loading reagent (BioRad), and subjected to SDS-PAGE in 5-20% gradient Ready gels (BioRad). Protein products were detected by Western blotting using streptavidin-linked horse radish peroxidase and chemiluminescence (Novagen) and quantified using either autoradiography and densitometry or a Molecular Imager FX (BioRad).

**Western Blotting Analysis**—Protein samples were subjected to SDS-PAGE in 5-20% gradient Ready gels and electrophoretically transferred to nitrocellulose sheets. Sheets were probed with the antibodies listed in Table 2 and detected using horseradish peroxidase secondary antibody and chemiluminescence (Amersham) as described above.

**Pulse/Chase Protein Labeling**—Yeast strains were grown in sulfate deficient growth medium (0.3% yeast extract, 1 g/l NH₄Cl, 1 g/l KH₂PO₄, 600 mg/l MgCl₂, 500 mg/l NaCl, 400 mg/l CaCl₂, 5 mg/l FeCl₂) with 2% sucrose to the mid-exponential phase of growth. Proteins were labeled with 50 µCi [³⁵S]methionine and [³⁵S]cysteine (Amersham) in spheroplasts for 10 min as described (51). The labeling was chased with cold amino acids, and the reactions stopped at the times indicated. Protein was precipitated with trichloroacetic acid and neutralized with NH₄OH prior to immunoprecipitation. Immunoprecipitations were optimized and performed as
described (52) using Pansorbin heat-killed *Staphylococcus aureus* (Calbiochem), separated by SDS-PAGE, and the gels dried. Detection and quantification were accomplished using a Molecular Imager FX (BioRad).
RESULTS

Construction of a Yeast System for the Exogenous Regulation of Mitochondrial Anionic Phospholipids–A complete PGS1 null mutant was created in a multiply auxotrophic laboratory strain. This was accomplished by substituting the PGS1 open reading frame with the TRP1 selectable marker by homologous recombination. Only a very short stretch of the gene's promoter and terminator were excised, and the entire open reading frame was deleted. Genomic PCR and Southern analysis confirmed the deletion (data not shown).

All of the phenotypes observed with the previously described psg1Δ strain, YCD4 (32), an incomplete disruption of the open reading frame, were verified in the new genetic background: the psg1Δ cells have no detectable PG or CL (see Fig. 3). This was true whether grown in glucose or sucrose. The strain is temperature sensitive at 37°C, and grows with the same doubling time as its wild type parent on YEP media containing glucose (Fig. 1A). The strain can grow on defined (minimal) media only if supplemented appropriately. The psg1Δ strain grows slowly on sucrose (Fig. 1B) and not at all on galactose, maltose, melibiose, ethanol, or glycerol. The strain enters stationary phase at a lower cell density than its wild type parent and has difficulty recovering from late stationary phase. The strain is also petite lethal. That is, it cannot be induced to lose its mtDNA as previously observed (53). Although growth of the psg1Δ strain on sucrose was slower than on glucose, the former carbon source was selected for subsequent experiments to avoid the complications of catabolite repression of mitochondrial biogenesis and function induced by glucose (54).

A low-copy “tet-off” plasmid system, pDO292, was employed to afford exogenous regulation of the PGS1 gene. (A high copy version of the plasmid was not fully repressible.) All
phenotypes were observed to return to wild type when strains containing this plasmid were
grown without the tetracycline derivative doxycycline. The following experiments were
performed in the two the \textit{psgl\Delta} strains SDO225 and YCD4 (32).

In order to test the effect of doxycycline on the regulation of the \textit{PGSI} plasmid in the
\textit{pgsl\Delta} strain, quantitative RT-PCR was employed with RNA isolated from the strain grown in
different concentrations of doxycycline. There is a well-regulated decrease to undetectable levels
of \textit{PGSI} mRNA as the concentration of doxycycline is increased (Fig. 2A, see also Fig. 9A and
B). The amount of \textit{PGSI} message without doxycycline is approximately 30\% higher than the
amount in the parental strain.

In order to test the effect of doxycycline on PGPS activity in the \textit{psgl} regulatable strain,
enzyme assays were employed with crude mitochondrial fractions from the strain grown in
different concentrations of doxycycline. There is a well-regulated decrease to undetectable levels
of PGPS specific activity as the concentration of doxycycline is increased (Fig. 2B). The specific
activity of PGPS in the parental strain is approximately 1.3 nmol/min/mg. Therefore, the enzyme
activity of this strain grown under fully derepressing conditions (without doxycycline) is
approximately 33\% above wild type levels.

In order to test the effect of doxycycline on the relative levels of PG and CL in the \textit{psgl}
regulatable strain, the strain was grown in different concentrations of doxycycline in the presence
of $[^{32}\text{P}]\text{P}_1$. Lipids were extracted and separated by thin layer chromatography. Phospholipids
were detected by autoradiography and quantified with a phosphoimager. All of the phospholipids
except PG and CL remained unchanged with different concentrations of doxycycline (Fig. 3).
There is a well-regulated decrease to undetectable levels of both PG and CL as the concentration
of doxycycline is increased. The relative percentage of PG in wild type cells is approximately
0.5%, and for CL, 1.5%, showing no discernible increase for these phospholipids in the fully derepressed strain.

Another method of CL detection is fluorescent staining with the CL-specific dye NAO (44). Fig. 4 shows mitochondrial staining of the psg1 regulatable cells with and without doxycycline. Without doxycycline, the mitochondrial network is clearly stained. When the strain is grown with doxycycline, no staining of CL is detected even when the image is overexposed.

\textit{psg1Δ} yeast cells cannot grow on non-fermentable carbon sources and are temperature sensitive at 37°C (32). In order to observe the effect of doxycycline on these phenotypes, the optical density of a culture using non-fermentable carbon sources was measured in different doxycycline concentrations. Increased doxycycline caused an increased doubling time of the strain to the point of senescence (Fig. 5). This lack of growth corresponds to the concentration of doxycycline that showed no expression of the gene, no detectable enzymatic activity, and no detectable PG or CL. The same results were observed with growth at 37°C in rich medium with 2% sucrose with increasing concentrations of doxycycline.

In order to determine the effect of doxycycline on overall mitochondrial membrane potential, the fluorescent dye DASPMI, a vital stain sensitive to mitochondrial membrane potential (45), was used to stain cells in a growing culture. The mitochondrial potential, which is observed for the psg1 regulatable cells in the absence of doxycycline, is indistinguishable from that of the wild type parental cells (Fig. 6). The mitochondrial network is clearly stained in these cells. However, no energized mitochondria are detectable when the cells are grown with doxycycline even when the image is overexposed. This lack of staining is indistinguishable from that of the strain grown without doxycycline when the mitochondrial membrane potential is collapsed with the uncoupler FCCP.
Analysis of Protein Levels in Anionic Phospholipid Depleted Mitochondria—In order to test protein import into mitochondria lacking the major anionic phospholipids, highly purified mitochondria are required. Specifically, the amount of contaminating microsomal fraction must be minimized. Crude mitochondrial preparations were purified by sedimentation through a sucrose gradient that separates purified mitochondria from MAM (47). Immunoblotting was utilized to determine the purity of the respective fractions. The amount of signal from antibodies to porin (the outer mitochondrial membrane voltage dependent anion channel) increased on a per mg of protein basis as the mitochondrial fraction was purified (Fig. 7A). However, a significant amount of this protein was observed in the MAM fraction. In order to determine the amount of microsomal protein contaminating the purified mitochondrial fraction, antibody to dolichyl-phosphate β-D-mannosyltransferase (DPMT, an endoplasmic reticulum lumenal enzyme) was employed (Fig. 7B). Significant amounts of this protein were observed in the MAM fraction. However, no detectable amounts were observed in the purified mitochondrial fraction.

Because of the possibility that the mitochondrial isolation protocol was significantly decreasing the mitochondrial yield, immunoblotting was performed to determine the amount of mitochondrial inner membrane protein associated with the MAM fraction. Insignificant amounts of Cox2p can be seen in this fraction (Fig. 7C, lane 2). However, significant amounts of outer membrane proteins are again observed (Fig. 7D, lane 2). As discussed below, Cox2p is only detected in cell fractions from cells containing PG and CL (Fig. 7C, lanes 1 and 2 versus lanes 3 and 4) while porin is present independent of anionic phospholipid content (Fig. 7D).

As a first step in determining if the loss of anionic phospholipids had any effect on protein import, purified mitochondria were used to study the relative amounts of various mitochondrial proteins. Proteins (integral membrane, peripheral, and soluble) from all four
compartments (outer membrane, inter membrane space, inner membrane, and matrix), both nuclear and mitochondrial-encoded, were quantified by immunoblot analysis. The majority of proteins tested in doxycycline grown cells were not altered in amount from cells grown without doxycycline (see Table 2 for summary of proteins tested); the results with porin (Fig. 8F) are representative of this group of proteins. The only proteins found to be in lower abundance, indeed practically absent, in the doxycycline grown \textit{psg1} regulatable strain were cytochrome \textit{b} and the largest four subunits of Cox (Fig. 8A-E). Cox subunits 5, 6, and 7 were present at reduced levels from wild type but clearly well above the levels of the larger subunits of Cox. Densitometry analysis of immunoblots with different preparations of isolated mitochondria were used to obtain relative measures of these proteins in cells grown with and without doxycycline. Cox1p is reduced six-fold in doxycycline grown \textit{psg1} regulatable cells, while Cox3p is reduced fifteen-fold. Cox2p, Cox4p, and Cobp are all reduced more than twenty five-fold. These results were also confirmed by comparison of protein amounts in the \textit{psg1}\textit{Δ} strain and the wild type parental strain (not shown) to rule out any effect of doxycycline. The amount of each protein in the doxycycline grown \textit{psg1} regulatable strain was indistinguishable from that of the \textit{psg1}\textit{Δ} strain, and the amount of each protein in non-doxycycline grown cells was indistinguishable from that of wild type cells.

In order to determine if the amount of these ETC proteins is in proportion to the amount of anionic phospholipid present in mitochondria, immunoblots were performed on mitochondria isolated from the \textit{psg1} regulatable strain grown with different doxycycline concentrations. When the strain is grown under derepressing conditions, the amount of Cox4p is indistinguishable from that found in the \textit{PGS1} parental strain (Fig. 8G). However, the amount of Cox4p diminishes with increasing repression of \textit{PGS1} expression until no protein is detectable at doxycycline
concentrations consistent with complete repression of the *PGS1* gene expression. A proportional decrease is also observed for the mitochondrial-encoded protein Cox2p (Fig. 8H).

A common mechanism for regulation of protein levels in cells occurs at the level of transcription. In order to determine if lack of these proteins in the doxycycline grown *psg1* regulatable strain is due to reduced transcription of their respective genes, quantitative RT PCR was employed to determine the amount of message expressed from these genes (see Table 1 for primers used). In no case were message levels affected by doxycycline concentration except the *PGS1* message which is clearly observed to decrease with increasing concentrations of doxycycline (Fig. 9A and B). No change is observed for either nuclear-encoded *COX4* (Fig. 9A) or mitochondrial-encoded *COB* (Fig. 9B) message. RT PCR experiments with *COX2*, *ATP6*, and 21S rRNA show that mitochondrial gene transcription is unaffected and that proper splicing occurs (Fig. 9C).

Of the five proteins found absent in the doxycycline grown *psg1* regulatable strain, four are encoded by mitochondrial DNA. In order to determine if the other four mitochondrial-encoded proteins (55) are down regulated, it was first necessary to determine their presence in the *pgs1Δ* strain. No antibodies are available to the three ATP synthase subunits (*F₀* encoded by mtDNA. All three proteins are myristolated and, therefore, are the only mitochondrial proteins soluble in chloroform (56). Therefore, an organic extraction was prepared of highly purified mitochondria from both the wild type and the *psg1Δ* strains. Proteins from these preparations were identified by silver staining of SDS-PAGE gels. All three subunits are present in the *psg1Δ* strain, and densitometry demonstrated that the proteins are found in the same amount as in wild type cells (Fig. 10). This analysis also reveals that the eighth mitochondrial-encoded protein,
Var1p, is also present, as the subunits of Fₜ could not be produced without this ribosomal protein (57).

Mechanism of Mitochondrial ETC Protein Down Regulation—In order to directly test protein import into anionic phospholipid depleted mitochondria, in vitro mitochondrial protein import assays were performed with highly purified mitochondria isolated from both the psg1 regulatable strain and the wild type strain. Experiments with in vitro transcribed and translated Cox4p (Fig. 11) and Cox6p (data not shown) showed that protein import into mitochondria is unaffected by the absence of the major anionic phospholipids. Cox4p shows proper cleavage of signal sequences and is imported and retained in a time dependent manner in mitochondria isolated from pgs1Δ cells (Fig. 11B). This is true although Cox4p is not detectable in cells grown with high concentrations of doxycycline. When the ionophore valinomycin is added to the reaction to dissipate the membrane potential, no protein import is observed. Therefore, membrane potential, although low as indicated by DASPMI staining, can be induced to a level sufficient to support normal protein import even of proteins not present in pgs1Δ cells.

In order to determine whether the proteins found to be missing in pgs1Δ cells are translated and subsequently degraded, pulse/chase labeling of spheroplasts was performed. No immunoprecipitable translation products of any of the proteins found to be absent in the doxycycline grown psg1 regulatable cells could be detected (Fig. 12A and B). This includes mitochondrial-encoded proteins, such as Cox2p, as well as the single nuclear-encoded protein found absent, Cox4p. The translation and proper processing of these proteins upon import into mitochondria was found normal in wild type cells. Other nuclear-encoded mitochondrial proteins, such as aconitase, were translated, imported and processed as expected in psg1Δ cells (Fig. 12C).
Mitochondrial-encoded proteins are individually regulated at the level of translation by specific nuclear-encoded mitochondrial translation activation factors (NEMTAFs) (58). In order to determine if the lack of translation observed was caused by repression of these factors, quantitative RT-PCR was performed with primers specific for messages of these factors. In every case, message levels were found somewhat lower in the \textit{psg1}Δ strain than in the wild type strain (Fig. 13A). When the actual differences in message levels were quantified, only five of the eight NEMTAFs were shown to be significantly repressed in the \textit{psg1}Δ strain: \textit{MSS51} and \textit{PET309}, which activate the translation of \textit{COX1} message; \textit{PET111} for \textit{COX2}; \textit{PET54} for \textit{COX3}; and \textit{CBS2} for \textit{COB} (Fig. 13B). No statistical difference could be discerned in message levels for \textit{PET122} and \textit{PET494}, which also activate the translation of \textit{COX3} message, and for \textit{CBS1} for \textit{COB}. However, Western blot analysis of mitochondrial proteins using antibody specific for PET111p showed no difference in the level of this NEMTAF between wild type and \textit{psg1}Δ cells (Table 2). Therefore, it is unlikely that the levels of the NEMTAFs are limiting in the absence of PG and CL.
DISCUSSION

The construction of a "biological reagent" to probe the role of anionic phospholipids in mitochondrial function has allowed us to determine the molecular basis for the dependence of *pgs1Δ* cells on a fermentable carbon source for growth. By introducing into a *pgs1Δ* strain a plasmid carrying a copy of *PGS1* under "tet-off" regulation, a dose-response relationship between *PGS1* message, PGPS activity, level of PG plus CL, and mitochondrial properties dependent on PG plus CL was demonstrated.

Not surprising cells repressed for expression of *PGS1* lack components of the ETC such as mitochondrial-encoded Cobp, Cox1p, Cox2p, and Cox3p. Prior to identifying the *PEL1* (renamed *PGS1*) gene product as PGPS, *pel1* mutants were shown to be respiratory deficient and lacking the spectra associated with Cox and cytochrome b (34,59). The surprising finding is that the molecular basis for lack of these proteins is failure to translate their respective messages that are present in normal amounts. In addition, nuclear-encoded Cox4p is missing for the same reason. However, other nuclear-encoded proteins found in all sub-compartments of the mitochondria as well as the remaining mitochondrial encoded proteins are present in normal amounts.

The lack of the mitochondrial-encoded components of the ETC cannot be attributed to the lack of Cox4p. In *cox4Δ* strains, all other subunits of Cox are easily detected although at reduced levels due to failure of assembly resulting in some degradation (60) as we observed for Cox5, Cox6 and Cox7. Cytochromes c, c1 and b are all also present in normal amounts in *cox4Δ* strains. Lack of cytochrome c (not determined in our studies) results in low levels of the mitochondrial-encoded subunits of Cox that can be readily detected in pulse/chase experiments.
Lack of translation in *pgs1Δ* cells rather than rapid degradation of these ETC components is further supported by the lack of their detection in pulse/chase experiments reported here.

Some mitochondrial-encoded proteins are inserted into the membrane co-translationally and utilize nuclear-encoded proteins that must themselves be imported into the mitochondria (62). Cox4p may also be co-translationally imported (63). Therefore, lack of the machinery for co-translational insertion of proteins or a dependence on anionic phospholipids to couple translation with insertion might result in the lack of their detection at both steady state and in pulse/chase experiments. Since Cox4p is still posttranslationally imported *in vitro* into PG/CL depleted mitochondria and no precursor is detected *in vivo*, the anionic phospholipid dependent step is at the level of translation. Because general protein insertion of most nuclear- and mitochondrial-encoded proteins seems unaffected without PG and CL, perhaps the lack of translation possibly coupled with insertion is specific for mitochondrial-encoded proteins of the ETC and for Cox4p. However, lack of the Oxa1p, which is required for membrane insertion of Cox2p and Cox3p, results in accumulation of these proteins in the matrix and not in lack of translation (64,65). The bacterial Sec-dependent membrane translocation system shows a strong dependence on anionic phospholipids for function (19). Although there are no homologues of the Sec system in the mitochondria, mitochondria may possess similar uncharacterized systems that are dependent on anionic phospholipids for organization of functional membrane associated complexes.

Although lack of DASPMI staining indicates a low membrane potential in the absence of anionic phospholipid, there must be sufficient membrane potential to support the minimal requirements of protein import (50) that were inhibited *in vitro* by addition of an ionophore. Our results indicate there is little or no dependence of protein import on anionic phospholipids. A
previous study showed there was a detectable reduction in protein import when a crd1Δ strain (lacks CL synthase) was grown on glucose resulting in depletion of both PG and CL in this particular background (25). However, mitochondrial assembly and function are themselves significantly reduced in glucose media (66) compromising any conclusions implicating the anionic phospholipids. Nevertheless, the effect of altering PG levels in a crd1 null strain by carbon source cannot be reproduced in our laboratory. In all carbon sources tested in three genetic backgrounds, PG levels remain elevated at least five-fold in crd1Δ strains2.

Since lack of anionic phospholipids results in failure to translate components of the ETC, our biological reagent cannot be used to test whether components of the ETC require these lipids for function (2,3). However, in vitro reconstitution experiments show a necessity for CL for ATP synthase activity (67) and particularly the ADP/ATP translocase (68,69). Activation of mitochondria from psglΔ cells for protein import by ATP indicates that both of these mitochondrial components are present and at least partially functional in psglΔ cells. Studies in crd1Δ cells indicated that the ADP/ATP translocase is compromised in vivo due to the lack of CL and that the presence of PG does not substitute (25). In vitro reconstitution studies showed a requirement by the translocase for CL. Therefore, the translocase may only be marginally active in the absence of PG and CL which may be the basis for both the petite lethal and temperature sensitive phenotypes of pgs1Δ cells. Cells lacking the ADP/ATP translocase are also petite lethal (70). It has been postulated that in the absence of both the ETC and the F0 portion of the ATP synthase, the only means of generating a membrane potential across the mitochondrial membrane is by continuous exchange of cytoplasmic ATP4 for mitochondrial ADP3 mediated by the translocase and driven by F1-ATPase activity in the mitochondrial matrix (for review, see Ref. (71)). In pgs1Δ cells, F0F1-ATPase activity coupled to proton export may be sufficient to
compensate for a poorly functional translocase (still necessary for import of ATP) in maintaining mitochondrial membrane potential. However, loss of $F_0$ by eliminating mtDNA in a $pgs1\Delta$ strain may make a marginally functional translocase coupled to remaining $F_1$ ATPase activity insufficient to maintain membrane potential. Above 37°C the function of either the translocase or the ATPase in a $pgs1\Delta$ strain may be further compromised. For example, the nuclear-encoded $FMC1$ gene product is required only at elevated growth temperatures for proper assembly of the $F_1$ component of the ATP synthase (72). It is not known whether FMC1p is expressed or functional in $pgs1\Delta$ cells above 37°C.

In summary, we have constructed a biological reagent that has proven useful in defining the molecular basis for the dysfunction of mitochondria lacking PG and CL. This reagent should be useful in determining the apparent requirement of these lipids in the translation of a subset of proteins integral to ETC function. Such studies may lead to uncovering additional factors involved in the complex process of translocation of proteins across membranes and their insertion into the membrane bilayer.
FOOTNOTES

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1The abbreviations used are: Aac2p, ADP/ATP translocase; CCPO, cytochrome c peroxidase; Cox, cytochrome c oxidase; CL, cardiolipin; Cyt, cytochrome; DASPMI, 2-(4-dimethylaminostyryl)-1-methylpyridinium iodide; DPMT, dolichyl-phosphate β-D-mannosyltransferase; ETC, electron transport chain; FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone; MAM, mitochondrial associated microsomal membrane; Mge, protein required for import of proteins into the mitochondria; mtDNA, mitochondrial DNA; NAO, 10-N-nonyl acridine orange; NEMTAF, nuclear-encoded mitochondrial translation
activation factor; PG, phosphatidylglycerol; PGPS, phosphatidylglycerol phosphate synthase; RT, reverse transcriptase; PCR, polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; Tim, mitochondrial protein translocator of the inner membrane; Tom, mitochondrial protein translocator of the outer membrane; YEP, yeast extract, peptone.

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| Gene  | Primer Sequence                          |
|-------|-----------------------------------------|
| ATP6  | TACATCACCATTAGATCAATTGGAGATTAGACTA      |
|       | AATTAAATAGTAAACCAGCTAAAAATAACCATTAA    |
| CBS1  | GTGAAGTCCTTTGAGTTAATCTGACGGATATATAA    |
|       | TTTGGTTATCCTTTTAATTCGAGTAAATTTCAG     |
| CBS2  | AGTTTTATGGCATTTTGTCTCCGCCCATTCTAAAG   |
|       | TATCTTGTGCTCCGGGGGATTTTCAAAGGTTTCT    |
| COB   | TGGCTAAAGGTTTATTATGGTTCATATAGTACACCAAAAGAGTAC |
|       | CAGGTACAATAGTGCTGGTACAGGGTGAACTGTCTAT |
|       | AATTGCAATAATCATTAGTGCACTAGGAGAATATGCTCTAAGATC |
| COX1  | TCAACTTTTAGTAGAATCAGGTCTGGTACAGGGTGAACTGTCTAT |
|       | AATTGCAATAATCATTAGTGCACTAGGAGAATATGCTCTAAGATC |
| COX2  | CATGTTATTCTTCAAGGATTCAGCAACACCAATCAAGGAAGGTATTT |
|       | AAAAAATTTAGGTAATGATACTGCTCCGCATCTAAATTGGCGATTTT |
| COX3  | TCATTTCGATTATTATCATTAGCATTATCAGTACATTAAACACCAGATGCAGATG |
| MSS51 | CCCTTCCCGGGAGTTTGATTTTTCCCACAGCAAGAAG |
|       | AACCCTAGGATCTGGAGATGCTAGCGCTGGATG     |
| PET54 | AGCAGTTGCAGGAAAAGAAAACCCTCTGTCCTACA |
|       | TGAAGCTAGACCTTTATGTTGTAAGAAACCAAAATA |
| PET111| TAGGTTGATTTGAATTTTGTCATTCTCTGTATTGC |
|       | TTTAGAGTCACAAGGATAGGCATCTTGTAAATTTTCTT |
| PET122| GCGTCGACGACGACGACGACCAGCTGGATGTTGAG |
|       | GCCAAAGGGACACCCAGCAGTAGTGCGGTCAGTGA |
| PET309| ATACCGACATACGCTTGTTTTACAATCTTTACTAT |
|       | CTATGATGCTCAATGCCTGGCTTTTCGCAACTTTGCTT |
| PET494| CTTAGTGCGAGGAGCAGAAGCGAAAAGTGTAGACACC |
|       | CGTACATATTATTGCGGAGAAGGAAGGAATGGTC |
| PGS1  | ATATGGGTGGTATAACGAGGTCATTCTTTTCGGGAGCACAACCTTTTC |
|       | TGCAGTGAACGACGACGACCAGCAGAACGAGATTAGGATG |
| 21s rRNA | GATCGAAAGATTTGATCCAGTTATTAGGATG |
|       | AAACCTTTCGACCGAAGACTAAAGGCAACAATGTA |
Table 2. Mitochondrial Proteins Screened for Dependence on Anionic Lipids

| Protein | Location | Presence in pgs1Δ | Antibody Source |
|---------|----------|-------------------|-----------------|
| Aac2p   | MIM      | +                 | Schatz          |
| Aconitase | Matrix   | +                 | Koehler         |
| ATP6p   | MIM      | +                 |                 |
| ATP8p   | MIM      | +                 |                 |
| ATP9p   | MIM      | +                 |                 |
| CCPO    | IMS      | +                 | Schatz          |
| Cox1p   | MIM      | -                 | Molec Probes    |
| Cox2p   | MIM      | -                 | Molec Probes    |
| Cox3p   | MIM      | -                 | Molec Probes    |
| Cox4p   | MIM      | -                 | Schatz          |
| Cox5p   | MIM      | +                 | Koehler         |
| Cox6p   | MIM      | +                 | Koehler         |
| Cox7p   | MIM      | +                 | Koehler         |
| Cyt b   | MIM      | -                 | Schatz          |
| Cyt b2  | IMS      | +                 | Schatz          |
| F0J     | MIM      | +                 | Schatz          |
| Mge1p   | Matrix   | +                 | Schatz          |
| Pet111p | MIM      | +                 | Fox             |
| Porin   | MOM      | +                 | Molec Probes    |
| Tim22p  | MIM      | +                 | Koehler         |
| Tim23p  | MIM      | +                 | Koehler         |
| Tim54p  | MIM      | +                 | Koehler         |
| Tom22p  | MOM      | +                 | Schatz          |

1 IMS, intermembrane space; MIM, mitochondrial inner membrane; MOM, mitochondrial outer membrane

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FIGURE LEGENDS

Fig. 1. **Glucose and sucrose growth curves.** SDO224 (wild type, WT) and SDO225 (pgs1Δ) were grown for eight generations in rich media cultures using carbon sources of either 2% glucose (A) or sucrose (B). Late logarithmic cultures were used to inoculate media of the same composition at an OD_{540} of 0.02 (A) or 0.005 (B). Optical density readings were taken at the indicated times. The experiment was performed in duplicate at least three times, and the points shown have less than 5% standard deviation. The results for SDO225 (pgs1Δ) are indistinguishable from those obtained with strain YCD4 (pgs1Δ) and represent averages of data obtained with both strains. Doubling times were calculated by finding the least squares best line fit to the points in the exponential phase of growth. The doubling time in glucose of SDO224 pgs1Δ (wild type) is 1.52 +/- 0.11 h, while SDO225 (pgs1Δ) is 1.51 +/- 0.11 h; in sucrose, SDO224 (wild type) is 2.45 +/- 0.08 h, while SDO225 (pgs1Δ) is 3.10 +/- 0.14 h.

Fig. 2. **Effect of doxycycline on PGS1 transcript level and PGPS enzymatic activity.**

A. SDO225/pDO292 (pgs1 regulatable strain) was grown for eight generations in rich media with 2% sucrose with the indicated concentrations of doxycycline to the mid-exponential phase of growth. Total RNA was isolated and subjected to quantitative RT PCR with PGS1 specific primers (see Table 1, Fig. 9A and B). The results are expressed as percentage of fully derepressed levels. The experiments were performed in duplicate on at least two occasions with two distinct RNA preparations. The results are indistinguishable from those obtained with strain YCD4/pDO292 (pgs1 regulatable strain) and represent averages and standard deviations of data obtained with both strains. B. SDO225/pDO292 (pgs1 regulatable strain) was grown for eight generations in rich media with 2% sucrose with the indicated concentrations of doxycycline to the mid-exponential phase of growth. Crude mitochondrial preparations were isolated and PGPS
assays performed. The assays were performed in duplicate on two occasions with at least three distinct membrane preparations. The results are indistinguishable from those obtained with strain YCD4/pDO292 (pgs1 regulatable strain) and represent averages and standard deviations of data obtained with both strains.

Fig. 3. **Phospholipid analysis.** SDO225/pDO292 (pgs1 regulatable strain) was grown for 24 h in rich media with 2% sucrose and the indicated concentrations of doxycycline ([Doxy], µg/ml). The cells were subsequently grown for eight generations in synthetic complete sucrose medium with \( ^{32}\text{P} \) to the mid-exponential phase of growth. Lipids were extracted and separated by thin layer chromatography. Panel A shows one-dimensional analysis, although the data in panel B are a representation of both single and two-dimensional analyses. The phospholipid species were identified with standards and quantified by both phosphoimager analysis and scintillation counting of the isolated spots. PG and PS, which are not visible in the autoradiogram reproduction, were nevertheless identified by phosphoimaging and can be visualized by overexposure of the TLC plate to film. The results in panel B are expressed as a percentage of total phospholipid counts. The experiment was performed using four distinct phospholipid preparations. The results are indistinguishable from those obtained with strain YCD4/pDO292 (pgs1 regulatable strain) and represent averages of data obtained with both strains. The data has a standard deviation of less than 8%. Abbreviations: CL, cardiolipin; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; SL, sphingolipids.

Fig. 4. **Nonyl acridine orange staining.** SDO225/pDO292 (pgs1 regulatable strain) was grown in rich sucrose media with no (-) or 10 µg/ml (+) doxycycline to the mid-exponential phase of growth. Cells were stained for 30 min with 50 nM 10-N-nonyl acridine orange and
visualized with a 100X oil immersion phase contrast objective using a FITC filter. Exposure times were 1/60th second for phase contrast, one second for no doxycycline, and an eight second overexposure for doxycycline fluorescent images.

Fig. 5. Effect of doxycycline on growth rate in non-fermentable carbon sources. A late logarithmic culture of SDO225/pDO292 (pgs1 regulatable strain) grown in rich media containing ethanol and glycerol was used to inoculate cultures at an OD of 0.025 with the same composition but with the indicated concentrations of doxycycline ([Doxy], µg/ml). The optical density of the culture was determined at the times indicated. The experiment was performed in duplicate on four occasions. The results are indistinguishable from those obtained with strain YCD4/pDO292 (pgs1 regulatable strain) and represent averages and standard deviations of data obtained with both strains.

Fig. 6. DASPMI staining. SDO224 (wild type) and SDO225/pDO292 (pgs1 regulatable strain) were grown in rich sucrose media with no or 10 µg/ml doxycycline (Doxy). Cells were stained with 20 µM DASPMI and visualized with a 100X objective using a FITC filter. 50 µM FCCP was also added to the no doxycycline strain as an uncoupling agent (+ Unc). Exposure times were 1/60th second for phase contrast, one second for parental and no doxycycline fluorescent, and an eight second overexposure for doxycycline and uncoupler fluorescent images.

Fig. 7. Mitochondrial isolation. In panels A and B, samples of identical protein amounts (25 µg) from various steps in the isolation of purified mitochondria from SDO224 (wild type) were analyzed by immunoblotting. Panel A is an immunoblot with antibodies to porin, a mitochondrial outer membrane protein. Lane 1, cell lysate; 2, crude mitochondrial fraction; 3, purified mitochondria; 4, MAM. Panel B is an immunoblot with antibodies to dolichyl-phosphate
ß-D-mannosyltransferase, an endoplasmic reticulum protein. Lane 1, cell lysate; 2, microsomal fraction; 3, crude mitochondria; 4, purified mitochondria; 5, MAM. In panels C and D, purified mitochondria containing 12 µg of protein from SDO225/pDO292 (pgs1 regulatable strain) grown without (lane 1) or with 10 µg/ml (lane 3) doxycycline were separated by SDS-PAGE. Lanes 2 and 4 contain the MAM fraction from SDO225/pDO292 (pgs1 regulatable strain) grown without or with doxycycline, respectively. Panel C is an anti-Cox2p (inner mitochondrial membrane) immunoblot, and panel D is an anti-porin immunoblot as a loading control for the results in panel C.

Fig. 8. Immunoblot analyses of anionic phospholipid depleted mitochondria. Purified mitochondria were analyzed by immunoblotting with the indicated antibodies. In panels A-F, purified mitochondria containing 12 µg protein from either no (lanes 1) or 10 µg/ml (lanes 2) doxycycline grown SDO225/pDO292 (pgs1 regulatable strain) were separated by SDS-PAGE. Immunoblots used the antibodies indicated. Protein quantification was accomplished by densitometry in duplicate at least three times using three different highly purified mitochondria preparations. Panel F is an anti-porin immunoblot as a loading control. Panel G is a Cox4p immunoblot. Lane 1 is mitochondria isolated from the parental wild type strain, while lanes 2-5 are from SDO225/pDO292 (pgs1 regulatable strain) grown with different concentrations of doxycycline (µg/ml): Lane 2, 0; 3, 0.1; 4, 1.0; 5, 10. Panel H is a Cox2p immunoblot of SDO225/pDO292 (pgs1 regulatable strain) grown with different concentrations of doxycycline (µg/ml): Lane 1, 0; 2, 0.1; 3, 1.0; 4, 10. In blots with multiple bands, the arrows indicate the protein of interest.

Fig. 9. Quantitative RT PCR. SDO225/pDO292 (pgs1 regulatable strain) was grown for eight generations in rich sucrose media with different concentrations of doxycycline to the mid-
exponential phase of growth. Total RNA was isolated and subjected to quantitative RT PCR with primers specific for the messages indicated (see Table 1). Lanes 1-4 of panels A and B are \textit{PGS1}. Note that a low molecular weight non-specific amplicon is amplified when \textit{PGS1} message is in low abundance. Lanes 5-8 of panel A are \textit{COX4}; lanes 5-8 of panel B are \textit{COB} (cytochrome \textit{b}). The cells were grown in the following concentrations of doxycycline (µg/ml): Lanes 1 and 5, 0; 2 and 6, 0.1; 3 and 7, 1.0; 4 and 8, 10. In panel C, RT-PCR was performed alternatively with RNA obtained from SDO224 (wild type, odd lanes) and SDO225 (\textit{pgs1A}, even lanes). Lanes 1 and 2, \textit{PGS1}; lanes 3 and 4, \textit{COX2}; lanes 5 and 6, \textit{COB}; lanes 7 and 8, \textit{ATP6}; lanes 9 and 10, 21S rRNA. The markers shown in panels A and C are a 100 bp ladder, while the markers in panel B are a 1 kb ladder (New England Biolabs). The experiments were performed in duplicate on at least two occasions with two distinct RNA preparations. The reason for the apparent difference in sizes of some of the amplicons from the different strains is unknown. Sequencing was performed to ensure that the amplicons were the same.

Fig. 10. \textbf{Isolation of F}_{0} \textbf{subunits of ATPase.} Highly purified mitochondria with 2 mg of protein from SDO224 (wild type, lane 1) and SDO225 (\textit{pgs1A}, lane 2) were homogenized by sonication and lipids isolated by organic extraction. Proteolipids were separated by SDS-PAGE and visualized by silver staining. Proteolipid isolation and quantification were accomplished twice in duplicate.

Fig. 11. \textbf{In vitro protein import assays.} \textit{COX4} was subcloned behind a T7 CAP-independent translation enhancer and transcribed \textit{in vitro}. The message was translated with biotinylated amino acids \textit{in vitro}, and protein denatured with urea. The resulting biotinylated Cox4p (Panel A, lane 1, no mitochondria) was added to ATP-activated highly purified mitochondria from SDO224 (wild type, A) or SDO225 (\textit{pgs1A}, B). A 60 min assay was
performed in the presence of 1 µg/ml valinomycin as a negative control in lane 2 of panel A and lane 1 of panel B. Aliquots of the reactions were stopped with valinomycin at 20 min. intervals up to an hour. The first time point (lane 3 of panel A, and lane 2 of panel B) was approximately five min. The mitochondria were treated with proteinase K, extensively washed, and isolated by centrifugation. Protein was separated by SDS-PAGE, blotted to membrane, and detected with streptavidin conjugated chemiluminescence reagents. The in vitro transcription/translation and mitochondrial protein import assays were performed in duplicate on three occasions with the same results.

Fig. 12. Pulse/chase labeling. Spheroplasted cells from SDO224 (wild type, lanes 1-4) and SDO225 (psg1Δ, lanes 5-8) were labeled for 10 min with [35S]-methionine and [35S]-cysteine. The cells were then chased with a high concentration of cold amino acids. Aliquots were stopped with cold TCA at 20 min intervals up to one hr. The first time point was taken at approximately 5 min. The above aliquots were divided into multiple aliquots for immunoprecipitation to isolate the respective labeled proteins that were separated by SDS-PAGE and detected by autoradiography. Panel A, Cox2p; B, Cox4p; C, Aconitase. The upper arrow in each panel denotes the precursor, while the lower denotes the mature form of the protein. Immunoprecipitations were performed in duplicate at least twice from at least three different pulse/chase labelings with the same results.

Fig. 13. NEMTAF message levels. A. Total RNA was isolated from SDO224 (wild type, odd lanes) or SDO225 (psg1Δ, even lanes). RT-PCR was performed with primers (see Table 1) specific for the messages indicated. Lanes 1 and 2, MSS51; 3 and 4, PET309; 5 and 6, PET111; 7 and 8, PET54; 9 and 10, PET122; 11 and 12, PET494; 13 and 14, CBS1; 15 and 16, CBS2. The experiments were performed in duplicate on at least two occasions with two distinct RNA
preparations. The markers shown are a 100 bp ladder (New England Biolabs). The reason for the apparent difference in sizes of some of the amplicons from the different strains is unknown. Sequencing was performed to ensure that the amplicons were the same. B. The image in panel A as well as duplicate experiments were quantified by densitometry and the changes in mRNA concentration calculated. The message levels in SDO225 (psg1Δ) are plotted as a percentage of SDO224 (wild type) message for each gene.
Figure 1

(A) OD_{540} vs. Time (h) for WT and pgs1Δ strains.

(B) OD_{540} vs. Time (h) for WT and pgs1Δ strains.
Figure 2

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**A**

Transcript Level

| Doxycycline µg/ml | 100 | 50 | 0 |
|-------------------|-----|----|---|
| 0.01              |     |    |   |
| 0.1               |     |    |   |
| 1                 |     |    |   |
| 10                |     |    |   |

**B**

nmol PGP/min/mg

| Doxycycline µg/ml | 2 | 1 | 0 |
|-------------------|---|---|---|
| 0.01              |   |   |   |
| 0.1               |   |   |   |
| 1                 |   |   |   |
| 10                |   |   |   |
Ostrander et al. Figure 3

A

CL > PA > PG > PE > PS > PI > PC > SL >

[Doxy] 0 0.1 1 10

B

% Total

|     | SLs | PC | PI | PS | PE |
|-----|-----|----|----|----|----|
| 0   |     |    |    |    |    |
| 0.1 |     |    |    |    |    |
| 1.0 |     |    |    |    |    |
| 10  |     |    |    |    |    |

% Total

|     | PG  | PA  | CL  |
|-----|-----|-----|-----|
| 0   |     |     |     |
| 0.1 |     |     |     |
| 1.0 |     |     |     |
| 10  |     |     |     |
FITC Fluorescence

|                | Parent | No Doxy | 10µg/ml Doxy | No Doxy + Unc |
|----------------|--------|---------|--------------|---------------|
| **FITC**       | ![Image](image1.png) | ![Image](image2.png) | ![Image](image3.png) | ![Image](image4.png) |
| **Phase Contrast** | ![Image](image5.png) | ![Image](image6.png) | ![Image](image7.png) | ![Image](image8.png) |
Figure 7

A

Porin

B

DPMT

C

Cox2p

D

Porin

52 >
30 >
7.5 >

1 2 3 4

1 2 3 4 5
Figure 8

A

120 >
84 >
52 >
30 >
22 >
7.5 >

Cox1p

B

1 2

Cox2p

C

1 2

Cox3p

D

120 >
84 >
52 >
30 >
22 >
7.5 >

Cox4p

E

1 2

Cobp

F

1 2

Porin

G

84 >
52 >
30 >
22 >
7.5 >

H

Cox2p
Figure 9

A

PGS1  COX4

B

PGS1  COB

C

1  2  3  4  5  6  7  8
1  2  3  4  5  6  7  8  9 10
Figure 10

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- Atp6p
- Atp8p
- Atp9p

Lane 1: 52, 36, 7.5
Lane 2: 52, 36, 7.5
Figure 12

A

B

C

1 2 3 4 5 6 7 8

52 >
30 >
22 >

84 >
52 >
Lack of mitochondrial anionic phospholipids causes an inhibition of translation of protein components of the electron transport chain: a yeast genetic model system for the study of anionic phospholipid function in mitochondria

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