Aup1p, a Yeast Mitochondrial Protein Phosphatase Homolog, Is Required for Efficient Stationary Phase Mitophagy and Cell Survival*

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Autophagy is a catabolic membrane-trafficking process that occurs in all eukaryotic cells and leads to the hydrolytic degradation of cytosolic material in the vacuolar or lysosomal lumen. Mitophagy, a selective form of autophagy targeting mitochondria, is poorly understood at present. Several recent reports suggest that mitophagy is a selective process that targets damaged mitochondria, whereas other studies imply a role for mitophagy in cell death processes. In a screen for protein phosphatase homologs that functionally interact with the autophagy-dedicated protein kinase Atg1p in yeast, we have identified Aup1p, encoded by Saccharomyces cerevisiae reading frame YCR079w. Aup1p is highly similar to a family of protein phosphatase homologs in animal cells that are predicted to localize to mitochondria based on sequence analysis. Interestingly, we found that Aup1p localizes to the mitochondrial intermembrane space and is required for efficient mitophagy in stationary phase cells. Viability studies demonstrate that Aup1p is required for efficient survival of cells in prolonged stationary phase cultures, implying a pro-survival role for mitophagy under our working conditions. Our data suggest that Aup1p may be part of a signal transduction mechanism that marks mitochondria for sequestration into autophagosomes.

Mitochondria perform numerous essential physiological functions in all eukaryotic cells. Apart from their role in oxidative phosphorylation and fatty acid oxidation, they are also essential for biosynthesis of central building blocks such as amino acids and nucleotides. At the same time, mitochondria are a threat to cellular well-being. Mitochondria are a major source of reactive oxygen species in cells. In addition, disruption of mitochondrial compartmentalization results in leakage of cytochrome c and other cytotoxic factors, and mitochondria with defective chemiosmotic coupling can cause an energy drain on the cell. Accumulation of mitochondrial genetic variation and mitochondrial damage are widely considered to underlie many age-related metabolic diseases and late-onset genetic disorders (1, 2). It is commonly postulated that in normal cells defective mitochondria are broken down in the lysosomal compartment through autophagy, and inability to clear defective mitochondria is thought to underlie numerous pathological conditions (3, 4).

Autophagy is a set of catabolic membrane trafficking mechanisms that allow import of cytosolic material into the vacuole/lysosome. The best understood form of autophagy is macroautophagy, in which intracellular membranes of undetermined origin engulf cytosolic material to form a double or multi-bilayer membrane-bound intermediate called the autophagosome (reviewed in Refs. 3 and 5–8). This intermediate then goes on to fuse with the vacuole/lysosome, releasing a single-bilayer bound vesicle called an autophagic body into the lumen of the lytic compartment where it is broken down, releasing the cytosol-derived material for further degradation to biosynthetic building blocks. Classical macroautophagy is induced under starvation conditions and is largely thought to be a non-specific process, although some central cellular structures are clearly off limits under normal conditions, and there have been reports of selective degradation of some proteins (9). This type of starvation-induced autophagy is essential for surviving nutrient deprivation in all eukaryotes, from yeast to mammals. Microautophagy is a related process whereby the vacuolar/lysosomal membrane invaginates and pinches into the lumen, forming an autophagic body without first forming a free autophagosome. There are a number of selective autophagic pathways, which appear to target specific cellular components. The Cvt pathway, for example, is an autophagic pathway in yeast that is highly similar to macroautophagy yet functions in the biosynthetic routes of two known proteins, aminopeptidase I and α-mannosidase, without delivering any additional known cargo to the vacuolar lumen (10–12). Aminopeptidase I, or Ape1p, is synthesized on cytosolic ribosomes as a 61-kDa precursor and is then specifically recruited to Cvt vesicles that are analogous to autophagosomes. Upon fusion of Cvt vesicles with the limiting membrane of the vacuole the protein is released into the vacuole lumen. Within the vacuole, prApe1 is proteolytically processed to yield the mature 50-kDa form in a Pep4p-

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§ The abbreviations used are: Cvt, cytoplasm to vacuole targeting; HA, influenza virus hemagglutinin; GFP, green fluorescent protein; RFP, red fluorescent protein; SLM, synthetic lactate medium; PP2C, protein phosphatase 2C; PP1K, protein phosphatase 1K; PAS, preautophagosomal structure.
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and Prb1p-dependent fashion. Whereas most of the protein factors required for Cvt trafficking and for macroautophagy are identical, there exist Cvt pathway-specific factors that are dispensable for macroautophagy, and autophagy-specific factors that are not required in the Cvt pathway (3).

An additional example of a selective autophagic pathway is pexophagy, or the degradation of peroxisomes. Like the Cvt pathway, pexophagy also occurs via a mechanism which shows mechanistic overlaps with classical macroautophagy, but is an inducible, not a constitutive phenomenon (13).

Recent studies in yeast and in mammalian cells have uncovered experimental conditions in which mitochondria are degraded through mitophagy, in response to environmental insults. Specifically, it was shown that when cells are transferred from rich glucose-based medium into a nitrogen starvation medium with lactate as a carbon source, large scale mitophagy is observed. This response appears to depend on Uth1p, a mitochondrial outer membrane protein that is also involved in cell longevity and bax-induced cell death (14, 15). In addition, it was suggested that yeast cells with a temperature-sensitive mitochondrial ATPase undergo mitophagy under anaerobic conditions at restrictive temperature (16). Finally, studies in rat hepatocytes suggest that the mitochondrial permeability transition pore (MPT) regulates mitochondrial autophagy (17).

Intriguing links have recently surfaced between mitochondrial regulation and macroautophagy. Reggiori et al. (18) reported that Atg9p, an integral membrane protein required for autophagy, cycles between mitochondria and the preautophagosomal structure (PAS), which is thought to be an assembly point for autophagosomes (18). In mammalian cells, a short isoform of the tumor suppressor p19 ARF was found to localize to mitochondria and induce macroautophagy (19).

Atg1p is a protein kinase required for both classical, starvation-induced macroautophagy as well as for the selective variants of autophagy such as the Cvt pathway and pexophagy. Atg1p was also shown to be required for the cycling of Atg9p between the PAS and the mitochondria (20). We previously reported (21) that truncation of the C-terminal 18 amino acids of Atg1p abrogates the Cvt pathway, yet still allows autophagic trafficking under nitrogen starvation conditions. In addition, the truncated version of Atg1p (Atg1Δ880) is constitutively hypophosphorylated, whereas wild-type protein was phosphorylated in rich medium and dephosphorylated upon starvation. To better understand the regulation of Atg1p, we searched for protein phoshatase homologs that genetically interact with the Atg1p protein kinase, by searching for phosphatase homolog deletion mutants that would show a synthetic interaction with the atg1Δ880 mutant. One phosphatase homolog identified in this fashion is encoded by the previously uncharacterized open reading frame YCR079w, which we have renamed AUP1. In this report, we present a functional characterization of Aup1p. Aup1p is a highly conserved protein that localizes to the mitochondrial intermembrane space. Surprisingly, while it is not absolutely required for classical starvation-induced macroautophagy, we find that it is required for effective mitophagy in stationary phase yeast cells. These data suggest that AUP1 may be part of a signal transduction mechanism that marks mitochondria for selective degradation through mitophagy.

EXPERIMENTAL PROCEDURES

Yeast Strains and Culture Conditions—The strains used in this study are listed in Table 1. Strains were grown in synthetic dextrose medium (SD; 0.67% (w/v) yeast nitrogen base, 2% (w/v) glucose, and auxotrophic amino acids and vitamins as required) or synthetic lactate medium (SLM; 0.67% (w/v) yeast nitrogen base, 2% (w/v) lactate, and auxotrophic amino acids and vitamins as required). Nitrogen starvation was carried out in SD-N (0.17% (w/v) yeast nitrogen base without ammonium sulfate and amino acids and 2% (w/v) glucose). Yeast transformation was according to (23). To generate strain HAY670, a PCR-amplified cassette was synthesized using plasmid pFA6–3HA–HIS3MX6 (24) as template. This cassette carries the Schizosaccharomyces pombe HIS5 gene, and at its 5′-end contains a coding region for 3 repetitions of the 9 amino acid influenza virus hemagglutinin (HA) epitope followed by a stop codon. The HA codons are fused in-frame to the C terminus of the AUP1 reading frame as encoded by the 5′ PCR primer overhang, while the 3′ PCR primer contains an overhang homologous to 3′-untranslated region of the AUP1 gene (all primer sequences will be provided upon request). Integration of this cassette creates a chromosomal AUP1 gene fused to the HA epitope at its C terminus, as verified by PCR and Western blotting. To generate strain HAY695, strain HAY469 was transformed with a PCR cassette synthesized using pHAB102 (21) as template, to create a truncation/fusion of the ATG1 reading frame with the protein A tag at amino acid 880. Strain HAY805 was created by transforming strain HAY75 with a PCR-amplified cassette carrying the monomeric RFP sequence fused in-frame to the 40 3′-nucleotides of the VPH1 reading frame and the kanamycin resistance gene, synthesized using plasmid pHAB152 as template (see below). Strain HAY809 was generated by integrating a PCR-amplified cassette carrying the S. pombe HIS5 gene flanked by short regions of homology to regions upstream and downstream of the AUP1 gene, to generate an aup1Δ:HIS5 genotype, as verified by diagnostic PCR. Strain HAY831 was generated by transforming strain HAY75 first with a PCR-amplified cassette carrying the RFP open reading frame fused in-frame to the 40 3′-nucleotides of the COX9 reading frame (generated using pHAB152 as template) and carrying the KanR selectable marker to create strain HAY829, which was then transformed with a second PCR-amplified cassette carrying the GFP open reading frame fused in-frame to the 3′-40 nucleotides of the AUP1 reading frame and carrying the HIS5 selectable marker (generated using plasmid pFA6a-GFP(S65T)-HIS3MX6 (24) as template). Both Aup1-HA and Aup1-GFP constructs were functional as assessed by complementation of the rapamycin sensitivity of the aup1Δ mutant (25).

Chemicals and Antisera—Chemicals were purchased from Sigma-Aldrich (Rehovot, Israel) unless otherwise stated. Custom oligonucleotides were from Hy-Labs (Rehovot, Israel) or IDT (Bet Shemesh, Israel). Anti-Ape1p antibodies were as previously described (11). Nycodenz was from Axis-Shield (Oslo, Norway). Anti-Aco1 antibodies were a gift of Dr. O. Pines (Hebrew University). Anti- Tom70 and anti-Cyb2 were generously donated by Dr. Liza Pon (Columbia University). Anti-HA antibodies were from Santa Cruz Biotechnology (Santa Cruz,
CA). Anti-Sed5, anti-Kar2, and anti-Hxk1 antibodies were a gift of Dr. J. E. Gerst.

**Plasmids and Genetic Manipulations**—Plasmid pHAB152 was constructed by excising the PacI-Ascl fragment of plasmid pFA6a–KanMX6 (24), and ligating a PacI-Ascl PCR amplicon of the mRFP reading frame (26) synthesized with PacI and Ascl overhangs, into the linearized plasmid. The resulting insert contains the mRFP sequence preceded by 2 glycines as an optional linker. Plasmid pYX142, encoding a mitochondrial GFP marker (27), was a gift from Dr. Benedict Westermann.

**Cell Fractionation and Membrane Extraction Procedures**—30 A600 units (or as indicated in specific experiments) of exponentially growing cells were collected, washed, and resuspended in SLM supplemented with 1 M sorbitol, 50 mM potassium acetate, 2 mM EDTA, 1 mM phenylmethysulfonyl fluoride, 5 mg/ml antipain, 1 mM/ml leupeptin, 1 mg/ml pepstatin, 10 mg/ml a2-macroglobulin, and 40 mM HEPES-KOH, pH 7. Cells were disrupted by homogenization (15 strokes) in a 7-ml dounce homogenizer (Kimble-Kontes) using a tight fitting pestle. The crude homogenates were clarified by centrifugation at 300 × g for 5 min using a fixed-angle rotor. Clarified lysate (S300) was centrifuged for 5 min at 15000 × g to generate P1500, and the supernatant was re-centrifuged 10 min at 12,500 × g to generate P13 and supernatant (S13) fractions. S300, P1500, P13, and S13 fractions were precipitated with 10% (w/v) trichloroacetic acid, washed twice with cold acetone, and solubilized in SDS-PAGE sample buffer. From each fraction, 2 A600 unit equivalents of cell extract were loaded per lane, separated by SDS-PAGE and analyzed by immunoblotting.

Samples were solubilized in SDS-PAGE sample buffer and analyzed by SDS-PAGE and immunoblotting.

**Protease Protection Assays**—50 A600 units of cells were spheroplasted, and P13 fractions were generated as above. The pellets were resuspended in lysis buffer or in hypotonic buffer (80 mM sorbitol, 20 mM Pipes-KOH, pH 7) at a concentration of 0.3 A600 equivalents/μL. Time 0 samples (12 A equivalents) were withdrawn and precipitated with 10% trichloroacetic acid. Proteinase K was added to the main sample to a final concentration of 0.3 μg/ml, and samples were taken at the indicated time points. Reactions were quenched by precipitation with 10% trichloroacetic acid followed by two washes in cold acetone. Samples were analyzed by SDS-PAGE and immunoblotting as above.

**Fluorescence Microscopy**—Typically, culture samples were placed on standard microscope slides (3 μl) and viewed using a Nikon E600 upright fluorescence microscope equipped with a ×100 Plan Fluor objective, using a FITC fluorescent filter (for viewing GFP fluorescence) or a Cy3 filter (for viewing mRFP). To achieve statistically significant numbers of cells per viewing field in some pictures, high cell densities were achieved by sedimenting 1 ml of cells for 30 s at 500 × g and resuspending the pellet in 10 μl of medium.

**Quantitative Western Blotting**—To follow protein levels over time with minimal bias, cultures were sampled (10 A600 unit equivalents per sample), and samples were precipitated with TCA followed by acetone washes. Cells were disrupted by a 5-min agitation with 100-μl glass beads (425–600-μm diameter) in 100 μl of cracking buffer (6 mM urea, 1% SDS, 1 mM EDTA, 50 mM Tris-Cl, pH 6.8). An additional 100 μl of cracking buffer was added, and the suspension was mixed and centrifuged 5 min at 14,000 × g. Clarified lysate was transferred to a new tube, and protein levels were determined using the BCA protein assay kit (Pierce). For typical immunoblot analysis 15 μg of protein per sample were mixed with 4× SDS-PAGE sample buffer and double distilled water to a final volume, loaded on SDS-PAGE, and immunooblotted using a C.B.S. semi-dry protein blotting apparatus (C.B.S. Inc., Del Mar, CA). Enhanced chemiluminescence reactions were done using the Supersignal™ kit (Pierce). Visualization of all enhanced chemiluminescence reactions was conducted using a Syngene ChemiGenius™ imaging work station (Synoptics group, Cambridge, UK) to avoid film saturation artifacts.

**RESULTS**

**Aup1 Shows a Synthetic Interaction with the atg1ΔΔ880 Allele**—Previous studies (18) have shown that truncation of 18 amino acids from the C terminus of Aup1 results in abrogation of Cvt

| Strain | Geotype | Source |
|--------|---------|--------|
| HAY75  | MATα, leu2-3,112 ura3-52 his3-Δ200tryp1-Δ901 lys2-801 suc2-Δ9 | Ref. 21 |
| BY4742 | MATα, leu2-3,112 ura3-52 his3-Δ200tryp1-Δ901 lys2-801 suc2-Δ9 pep4Δ | Ref. 22 |
| TVY1   | MATα, leu2-3,112 ura3-52 his3-Δ200tryp1-Δ901 lys2-801 suc2-Δ9 pep4Δ | Ref. 23 |
| HAY395 | HAY75, atg1ΔΔURA3 | Ref. 21 |
| HAY455 | HAY75, atg1ΔΔ880::prA::HIS5 | Ref. 21 |
| HAY649 | BY4742, aup1Δ::KanR | Ref. 23 |
| HAY670 | BY475, AUP1-URA3::HIS5 | This study |
| HAY695 | BY4742, aup1Δ::KanR atg1ΔΔ880::HIS5 | This study |
| HAY805 | BY475, VPH1-RFP::KanR | This study |
| HAY809 | HAY75, aup1Δ::HIS5 | This study |
| HAY831 | HAY75, COX9-RFP::KanR AUP1-GFP::HIS5 | This study |
trafficking but has only a minor effect on autophagic trafficking of prApe1 (21). In mutants of this type, prApe1 accumulates in nutrient rich conditions, because of the disruption of the Cvt

| starvation | prApe1 | mApe1 |
|------------|--------|-------|
| WT         | +      | -     |
| aup1Δ      | -      | +     |
| atg1Δ880   | +      | -     |
| atg1Δ880  aup1Δ | -  | +  |

FIGURE 1. Synthetic interaction between the aup1Δ mutation and the atg1Δ880 allele. Exponentially growing yeast cultures of strains HAY455 (atg1Δ880), HAY469 (aup1Δ), and HAY695 (double mutant) were transferred to nitrogen starvation medium as described under “Experimental Procedures” and incubated for 3 h. Samples were taken prior to starvation (−) or after starvation (+) and analyzed by immunoblotting with anti-Ape1p antibody.

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pathway. However, upon starvation one observes rapid maturation of the precursor and the appearance of the mature form of Ape1p, mApe1 because of proteolytic processing in the vacuolar lumen. This type of nutrient-induced shift in Ape1p trafficking can be used to assay mechanistic aspects of the induction of macroautophagy (29–32). To identify protein phosphatases that potentially interact with the Atg1 protein kinase, we screened for mutants that, in the genetic background of the atg1Δ880 allele, would cause a synthetic phenotype such that autophagic trafficking of prApe1 would be abolished. To this end we assayed 32 knockouts of protein phosphatase homologs from the yeast deletion library (33) for loss of viability on starvation plates using phloxine B as a vital dye (34). Of these strains, 6 showed characteristic pink coloration because of loss of viability on starvation and were then further analyzed by integration of the atg1Δ880 cassette into the ATG1 locus for analysis of Ape1p trafficking. One of these homologs, deleted for open reading frame YCR079w, showed a clear synthetic effect on prApe1 maturation: Whereas the phosphatase homolog deletion alone had no effect on prApe1 maturation, the atg1Δ880 mutant was able to mature prApe1 under nitrogen starvation, the double mutant was unable to mature Ape1p either in rich medium or nitrogen starvation conditions (Fig. 1). Because YCR079w was not previously characterized, we decided to name it Aup1p, for autophagy-related protein phosphatase.

Aup1p Localizes to Mitochondria—Surprisingly, sequence analysis of Aup1p using the mitoprot II algorithm (35) revealed a high propensity for mitochondrial localization of the protein. Indeed, an Aup1p-GFP fusion strongly co-localizes with a red fluorescent protein fusion of the mitochondrial inner membrane marker cytochrome c oxidase subunit VIIa (Cox9-RFP), when cells are grown in 2% lactate medium (Fig. 2). However, upon long term (3–4 days) incubation, a clear shift in this co-localization was observed. Whereas the GFP fluorescence remained distributed in the cytoplasm, the RFP fluorescence appeared to concentrate at a central location which roughly coincided with the vacuole, although cytoplasmic puncta are still observed as well. This result suggested to us that mitochondria are being delivered to the vacuole under these conditions. Because there are no reports in the literature regarding massive mitophagy under these conditions, we chose to characterize this phenomenon in more detail. To determine whether the observation shown in Fig. 3 was limited to a specific marker protein or fluorophore combination, we used cells expressing a generic mitochondrial GFP marker and a vacuolar Vph1-RFP marker. In addition,
we used this setup to characterize the time course of delivery of the mitochondrial marker to the vacuolar lumen. As shown in Fig. 3, co-localization of Vph1-RFP and mitochondrial GFP begins to be apparent at 3 days of incubation in SLM, and increases through the 5-day incubation period.

**Aup1p Is Required for Efficient Mitophagic Degradation of Aconitase**—To verify that the fluorescent experiments represent bona fide mitophagy, we also followed the levels of aconitase, a protein residing in the mitochondrial matrix, using quantitative Western blotting. As seen in Fig. 4A, aconitase levels drop sharply between the second and third day of incubation, consistent with the fluorescent microscopy data. Furthermore, this decrease was not observed in pep4Δ mutants. Pep4p is the major vacuolar protease and is required for the activation of most other vacuolar hydrolases: in its absence the degradation process occurs in the vacuole, and by definition, therefore, constitutes a form of mitophagy. Because we initially identified Aup1p as a genetic interactor of Atg1p, and because it is therefore, constitutes a form of mitophagy. Because we initially identified Aup1p as a genetic interactor of Atg1p, and because it localizes to mitochondria in a way that correlates with the onset of mitophagy (Fig. 2), we also tested whether aup1Δ and atg1Δ cells are defective in stationary phase mitophagy. **Aup1p** was previously shown to be a nonessential gene (36). As shown in Fig. 4A, **aup1Δ** cells are completely defective in stationary phase mitophagy whereas **atg1Δ** cells also show a reduced level of mitophagy. We then used quantitative Western blotting to ask whether Aup1p protein levels temporally correlate with its point of action during mitophagy. To this end, we C-terminally tagged Aup1p with the HA epitope derived from the influenza virus hemagglutinin protein, and followed its levels using anti-HA antibodies. The Aup1-HA chimera is functional, as assayed by rapamycin sensitivity (see “Experimental Procedures”). As seen in Fig. 4B, Aup1p levels sharply peak during the second day of incubation in SLM, correlating with the onset of mitophagy as observed in Figs. 3 and 4A.

**Aup1p Localizes to the Mitochondrial Intermembrane Space**—One interpretation of these data is that Aup1p functions directly in mediating mitophagy. To better understand the potential ways in which Aup1p may be involved in such a mechanism, we decided to investigate the manner of its association with the mitochondrion. Aup1p may be a peripheral membrane protein adhering to the outer mitochondrial membrane, a peripheral membrane protein of the intermembrane space, a soluble protein of the intermembrane space, or a luminal protein. To analyze the different possibilities, we conducted fractionation studies, coupled with extraction and protease protection protocols. As seen in Fig. 5A, Aup1-HA co-fractionates with Tom70p, a mitochondrial outer membrane protein, in differential centrifugation experiments. Both proteins sediment at...
Aup1p is localized to a protease-resistant compartment that is permeabilized by hypotonic treatment. Strain HAY670 was grown to log phase and lysed as in the legend to Fig. 5. P13 fractions were resuspended in lysis buffer (upper panel) or hypotonic buffer (lower panel) and digested with proteinase K for the indicated times as described under “Experimental Procedures.” After the reactions were quenched, Samples were analyzed by immunoblotting with anti-HA (for both treatments), anti-Tom70 (upper panel, as positive control for proteinase K digestion in whole mitochondria), anti-cytochrome b, (lower panel, as positive control for mitochondrial outer membrane permeabilization), or anti-aconitase (lower panel, as control for mitochondrial inner membrane integrity).

12,500 × g, as expected for mitochondrial proteins, while Kar2 (endoplasmic reticulum) Sed5 (Golgi) and hexokinase (cytosol) are either underrepresented in this fraction or completely absent. Not surprisingly, the vacuolar marker Pho8 shows a similar distribution. To resolve vacuolar and mitochondrial components we resuspended the P13 fraction in lysis buffer and loaded it on a 5–25% Nycodenz gradient. As seen in Fig. 6, Aup1-HA co-fractionates with Tom70 in this gradient and well away from Pho8. These results corroborate the fluorescent microscopy data of Fig. 2. To understand how Aup1p associates with the mitochondrion, we tested whether Aup1-HA can be extracted from the 13,000 × g pellet by treatment with 100 mM Na2CO3 or under hypotonic conditions. Na2CO3 treatment strips peripheral membrane proteins and soluble luminal proteins (37), while hypotonic conditions disrupt the outer mitochondrial membrane, releasing soluble intermembrane proteins as well as loosely associated peripheral membrane proteins (38) (Fig. 6). As seen in Fig. 6, Aup1-HA is stripped by Na2CO3, but not by hypotonic treatment although cytochrome b2, a soluble intermembrane protein, is clearly released by hypotonic treatment. This implies that Aup1p is not a soluble intermembrane protein or an integral membrane protein, but does not indicate which submitochondrial compartment it associates with. To address this issue, we treated resuspended 13,000 × g pellets with proteinase K either in lysis buffer or under hypotonic conditions. As seen in Fig. 7, Aup1-HA is not sensitive to proteinase K when the resuspended 13,000 × g pellet is challenged with proteinase K in lysis buffer, in contrast to Tom70p, an integral outer membrane protein that faces the cytosol. This makes it very unlikely that Aup1p is peripherally associated with the cytoplasmic leaflet of the outer membrane of the mitochondrion. However when the 13,000 × g pellet is resuspended in hypotonic buffer and challenged with proteinase K, Aup1p becomes sensitive to exogenous proteinase K, as is cytochrome b2, a soluble intermembrane protein. As a control, one can see that aconitase, a luminal protein, is not degraded under these conditions, indicating that the inner membrane is not disrupted and that the sensitivity of Aup1-HA to proteinase K reflects the disruption of the outer membrane alone. In summary, because Aup1-HA is not released from the pellet by hypotonic treatment alone, yet becomes sensitive to exogenous proteinase K under these conditions, the simplest explanation is that Aup1p is a peripheral membrane protein associated with one of the two membranes that face the intermembrane space.

Aup1p Is Required for Efficient Survival in Long Term Stationary Phase Cultures—The role of autophagy and mitophagy in cell survival has been a point of some debate in the literature. Whereas some authors contend that autophagy is strictly a cell survival mechanism related to starvation stress, others have suggested a role for autophagy in programmed cell death (6, 39–46). Indeed, under some conditions it has been argued that mitophagy in S. cerevisiae correlates with cell death phenomena (14, 16). To determine whether the role of Aup1p in mitophagy reflects a cell survival mechanism or a cell death mechanism under our conditions, we compared the viability of wild-type and aup1Δ cells incubated in SLM over the time scales in which mitophagy occurs. As shown in Fig. 8, wild-type cells slowly lose viability over a 9-day incubation period in SLM, to −30% of the initial viable cell counts. In contrast, viable counts of the aup1Δ mutant reached saturation later than wild type (on day 2) and rapidly lost viability between days 4 and 6, reaching less than 1% of the initial counts by day 6. Interestingly, the onset of the rapid loss of viability in the aup1Δ cells correlates with the onset of mitophagy (days 2 and 3). These results are consistent with a cell survival role for Aup1p, and potentially for mitophagy, under our working conditions.

Aup1p Is the S. cerevisiae Ortholog of a Highly Conserved Family of PP1K Homologs—Aup1p is a mitochondrial protein with significant homology to the protein phosphatase 2C family, and analysis on the NCBI DART program clearly indicates the existence of a PP2C catalytic motif. However the similarity between Aup1p and the other PP2C family members in S. cerevisiae is weak. S. cerevisiae Ptc1, Ptc2, and Ptc3 are not found
in an unbiased BLAST search (47) of all nonredundant protein databases using Aup1p as a query. In pairwise BLAST analysis, one observes respective % identity (and expect) values for alignment of Aup1p with Ptc1, Ptc2, and Ptc3 as being 24% (expect value 3.0), 29% (expect value 5.2), and no significant similarity (for Ptc3). In contrast, human, canine, and murine mitochondrial protein phosphatase 1K, (PP1K, PP2C domain containing, all functionally uncharacterized proteins) are all identified in an unbiased BLAST search using Aup1p as query, with % identity and expect values for these comparisons being 31% (expect value of $5 \times 10^{-11}$), 29% (expect value of $4 \times 10^{-10}$), and 29% (expect value $3 \times 10^{-10}$), respectively. This implies that Aup1p is a S. cerevisiae PP1K ortholog. The lack of additional S. cerevisiae proteins with significant homology to Aup1p (beyond their common PP2C motif) renders it unlikely that it is functionally redundant with the other members of the PP2C family in yeast. Hence, these comparisons define a conserved eukaryotic mitochondrial protein phosphatase family that may share a functional role in mitophagy.

**DISCUSSION**

The clearance of damaged mitochondria is an important household chore in eukaryotic cells (1, 4). This is even more pronounced in cells utilizing oxidative phosphorylation as the main mode for production of energy, as is the case for yeast growing on lactate medium. Damaged mitochondria can generate reactive oxygen species, release cell death-inducing factors such as cytochrome c into the cytosol, or generally burden the metabolic machinery of the cell by decreasing the efficiency of ATP generation. Numerous inherited diseases caused by mitochondrial mutations have been described, and somatic variation and mutation of mitochondria is suggested to underlie additional pathologies (2, 48).

The cellular ability to recognize and delete damaged mitochondria is therefore a vital aspect of aerobic life. It is generally assumed that such clearance occurs by sequestration of mitochondria by the lytic compartment, i.e. the lysosome in animal cells or the vacuole in yeast. Such sequestration, by definition, constitutes a form of specific autophagy, although the precise mechanisms involved are currently obscure.

Pioneering studies of mitophagy in yeast (15, 16) utilized experimental scenarios that are unlikely to be encountered by free living cells in their natural environment. In contrast, this study represents an effort to analyze mitophagy during prolonged stationary phase incubation of aerobic yeast cultures, in the absence of additional manipulation.

**Aup1p Is a Protein Phosphatase Homolog Required for Efficient Mitophagy**—We describe the identification and characterization of Aup1p, a yeast protein phosphatase homolog that functionally interacts with the autophagy-dedicated protein kinase Atg1p. Whereas deletion of AUP1 does not cause a defect in starvation-induced macroautophagy (Fig. 1 and data not shown), the mitochondrial localization of the protein and the loss of this localization during mitophagy prompted us to analyze its potential role in mitophagy. Our experiments indicate that Aup1p is required for efficient mitophagy and that its function is required for survival of cells through prolonged stationary phase incubation in a medium containing lactate as carbon source. These results imply a pro-survival role for mitophagy, under our working conditions.

Several recent studies have pointed to a potential connection between mitochondrial function and autophagy, including classical macroautophagy. Reggiori et al. (18, 20) report that Atg9p, a membrane protein that is essential for starvation-induced macroautophagy, cycles between a mitochondrial localization and an autophagy-specific structure called the PAS. A mechanistic involvement of mitochondrial metabolism in the regulation of macroautophagy is reasonable, given the central importance of the mitochondrion to amino acid metabolism, through anaplerotic functions of the tricarboxylic acid cycle.

Uth1p, a peripheral membrane protein of the cytoplasmic face of the mitochondrial outer membrane, was recently shown to be required for selective mitophagy in cells challenged with rapamycin, concomitantly with a switch between fermentative and gluconeogenic culture conditions (15). While both Uth1p and Aup1p are required for mitophagy, their effects on the physiological role of the process appear to be different: knock-out of UTH1 results in increased viability of cells transferred from nitrogen replete medium containing glucose to nitrogen starvation medium containing lactate as carbon source, while we find that knock-out of AUP1 results in decreased viability of cells in prolonged stationary phase incubation under sustained gluconeogenic conditions.

This difference between Aup1p and Uth1p may reflect the differences in experimental protocol, or they may reflect a complex regulation of mitophagy through these proteins. Intriguingly, Uth1p is thought to be regulated by phosphorylation, raising the possibility that its function is regulated by Aup1p. Although direct dephosphorylation of Uth1p by Aup1p is unlikely since Uth1p is projected to be localized at the cytoplasmic leaflet of the outer membrane (49) whereas Aup1p is localized to the intermembrane space (Fig. 6), it is possible that Aup1p is part of a signal transduction network that relays a signal from the mitochondrial matrix to the cytoplasmic interface and which then regulates mitochondrial targeting for vacuolar degradation. Further work on the possible interactions between Aup1p and Uth1p are required to parse these possibilities.

**Aup1p Is a Conserved Protein with Homologs in Higher Eukaryotic Cells**—Aup1p lacks clear paralogs in the yeast proteome, despite the presence of a PP2C motif. Aup1p appears to be a unique yeast representative of a highly conserved yet previously unstudied eukaryotic PP1K family that is represented in all the genomes sequenced and annotated to date. All of the PP1K family members contain a PP2C motif and are predicted to be mitochondrial. It remains to be seen whether the role of Aup1p in regulating mitophagy is evolutionarily conserved. In any event the high degree of conservation between these proteins, their apparently conserved mitochondrial localization, and the lack of paralogs within the S. cerevisiae genome all strongly suggest a conserved function. In addition, Ruan et al. (50) recently showed that recombinant Aup1p has PP2C-type phosphatase activity. Taken together, these results argue that this fam-

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3 S. Manon, personal communication.
ily of mitochondrial proteins function as protein phosphatases, with a conserved role in regulating mitochondrial turnover.

The Relationship between Aup1p Function, Atg1p Function, and Mitophagy—While Aup1p shows a synthetic interaction with the atg1ΔΔ880 allele, the mechanistic significance of this interaction is unclear because we were unable to show a clear-cutoff requirement for Aup1p in classical starvation-induced macroautophagy. Nonetheless, it remains possible that a number of signaling inputs impinge on Atg1p, and that the Atg1ΔΔ880 mutant is defective for some of these inputs, leaving the autophagic machinery dependent on Aup1p signaling. An additional aspect of the relative roles of Atg1p and Aup1p is that while Aup1p appears to be completely required for degradation of aconitase during stationary phase in lactate medium, atg1Δ cells appear to be only partly defective in the degradation of aconitase (Fig. 4A). Several different mechanisms have been proposed to mediate mitophagy, including nonspecific macroautophagy (51), specific macroautophagy (16), and specific microautophagy (52). It is conceivable that the cell uses redundant pathways that are activated by a common cue displayed on the mitochondrial surface, and that Aup1p is required for the generation of this signal. Further studies are required to test this hypothesis.

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