Distinct Functions of Evolutionary Conserved MSF1 and Late Embryogenesis Abundant (LEA)-like Domains in Mitochondria*†

Brandon M. Hall‡, Kjerstin M. Owens§, and Keshav K. Singh†$

From the ‡Department of Genetics, Roswell Park Cancer Institute, Buffalo, New York 14263 and the §Departments of Genetics, Pathology, Environmental Health, Center for Free Radical Biology, Center for Aging and University of Alabama at Birmingham Comprehensive Cancer Center, University of Alabama at Birmingham, Birmingham, Alabama 35294

Background: PRELID1 homologs are highly conserved mitochondrial proteins that are poorly characterized. PRELID1 homologs in yeast (UPS1–3) are essential for maintaining multiple mitochondrial processes. UPS1–3 exhibit distinct contributions to mitochondrial respiration, respiratory competency, and mitochondrial phosphatidylethanolamine metabolism. UPS2, the only late embryogenesis abundant (LEA) domain-containing protein identified in yeast, exerts pleotropic effects on mitochondrial processes through distinct roles of the MSF1 and a newly identified LEA-like domain.

Results: PRELID1 homologs in yeast (UPS1–3) are essential for maintaining multiple mitochondrial processes. UPS2 exerts pleotropic effects on mitochondrial processes through distinct roles of the MSF1 and a newly identified LEA-like domain.

Conclusion: UPS2 exerts pleotropic effects on mitochondrial processes through distinct roles of the MSF1 and a newly identified LEA-like domain.

Significance: UPS2 is the first cytoprotective LEA-like domain-containing protein identified in yeast.

PRELID1, the only late embryogenesis abundant (LEA) domain-containing protein in humans, exerts cytoprotective effects through its LEA domain within the mitochondria. Although PRELID1 homologs in vertebrates contain the LEA domain, homologs in lower eukaryotes are thought to lack this domain. In this study, we identify a novel LEA-like domain in a yeast PRELID1 homolog, Ups2p, which contains sequence conservation with the LEA domain of human PRELID1. PRELID1 homologs, including Ups2p, are known to contain the PRELI/MSF1 domain. Our study reveals that the MSF1 domain of Ups2p maintains proper mitochondrial electron transport chain function, respiratory competency, and mitochondrial phosphatidylethanolamine metabolism. The Ups2p MSF1 domain mediates cardiolipin depletion in the absence of Ups1p. However, the Ups2p LEA-like domain is responsible for cardiolipin depletion resulting from UPS2 overexpression. The regulation of phosphatidylethanolamine levels by the MSF1 domain is antagonized by the Ups2p LEA-like domain. We demonstrate that the yeast LEA-like domain protects cells from oxidative stress and can be functionally replaced by the human LEA domain. Together our studies suggest distinct roles of MSF1 and LEA-like domains in mitochondrial function and resistance to oxidative stress.

Human PRELID1 and yeast PRELID1 homologs (UPS1–3) contain the PRELI/MSF1 domain, a domain implicated in intra-mitochondrial protein sorting (1–3). Although similar in sequence, Ups proteins show functional divergence. Of recent interest, Ups1p and Ups2p have been shown to be crucial regulators of the levels of essential mitochondrial phospholipids cardiolipin (CL)2 and phosphatidylethanolamine (PE). Although inactivation of UPS1 leads to CL depletion, inactivation of UPS2 leads to depletion of mitochondrial PE. Interestingly, the additional deletion of UPS2 reverses the CL depletion in upsΔ cells, indicating that this UPS1 deletion defect is Ups2p-dependent (4–7). In fact, the majority of UPS1 deletion phenotypes occur only in the presence of Ups2p, demonstrating the importance and central role of Ups2p in Ups protein biology and mitochondrial function.

In addition to its MSF1 domain, PRELID1 contains a late embryogenesis abundant (LEA) domain, consensus sequence (A/T)AEKA(K/R)ETKD (8, 9). Although rare among non-plant species, LEA domains are abundant and highly diversified in plants where they play essential roles in protection against various forms of developmental and environmental stress (10–13). Recently our studies revealed LEA-dependent functions for PRELID1 in development and apoptotic resistance (9, 14). To date, only human PRELID1 and its vertebrate homologs are described to contain a LEA domain (8, 9, 14–16). PRELID1 homologs of lower eukaryotes lack the LEA domain (3–5, 17–20).

This study investigates the role of yeast PRELID1 homologs in mitochondrial respiration and respiratory competency, revealing a prominent role for Ups2p. We demonstrate the presence of a LEA-like domain in yeast Ups2p and its functional substitution by the LEA domain of human PRELID1. We elucidate distinct contributions of the Ups2p MSF1 and LEA-like domains to mitochondrial respiration, respiratory competency, and mitochondrial phospholipid metabolism.

**EXPERIMENTAL PROCEDURES**

Yeast Strains and Media—Yeast strains used in this study are isogenic to wild type BY4741 (Table 1) (21). Double and
triple UPS deletion mutants were created using PCR-mediated one-step gene replacement (22) utilizing the HIS3 and LEU2 auxotrophic markers from the pRS413 and pRS415 vectors (23), respectively. The ups2-Δ181 one-step allelic replacement cassette was constructed by PCR amplification of the ups2-Δ181-p426ADH plasmid. Primers were designed to create UPS2-homologous regions at the 5’ and 3’ ends of the replacement cassette comprised of the UPS2 truncation allele and the URA3 selectable marker (24). The cassette was used for allelic replacement in wild type and ups1Δ cells, resulting in the ups2-Δ181 and ups1Δups2-Δ181 strains, respectively. A similar strategy was implemented for creation of the ups2-Δ210 and ups2-Δ181+ hLEA strains. Primers used for the creation of these strains are listed in supplemental Table S1. Yeast transformations of plasmids and PCR cassette were performed using the Frozen-EZ yeast transformation II kit (Zymo Research, Orange, CA). The strains were grown in 1% yeast extract and 2% peptone with either 2% dextrose (YPD); 2% glycerol, 2% lactic acid, pH 6.0 (YPGL); or 2% glycerol, 2% ethanol (YPGE). Plasmids in transformed yeast were grown in 1% yeast extract and 2% peptone with either 2% dextrose (YPD); 2% glycerol, 2% lactic acid, pH 6.0 (YPGL); or 2% glycerol, 2% ethanol (YPGE). Plasmids in transformed yeast were maintained by growth in selective, synthetic medium lacking uracil (SDC-uracil or SGLC-uracil).

**Plasmid Construction**—PRELID1-p426ADH was constructed by PCR amplification of the open reading frame from PRELID1-pOTB7 (accession number BC007268; ATCC) followed by digestion and ligation into the EcoRI site of UPS2-Δ181 plasmid obtained from Open Biosystems (Huntsville, AL) (26, 27). UPS1 and ups2-Δ181 were cloned into the HindIII and Xhol sites of p426ADH. The ups2-Δ181 truncation allele was constructed by introducing a nonsense mutation of Ser-182, which followed by digestion and ligation into the EcoRI site upstream of PRELID1 in the UPS1-p426ADH plasmid and subsequently removing nucleotides between the Ups2p MSF1 domain and the human PRELID1 LEA domain, resulting in an in-frame yeast/human chimeric gene, as previously described (28). Primers used for plasmid creation are listed in supplemental Table S1.

**Spontaneous Petite Formation**—The frequency of spontaneous petite formation in the BY4741 background was determined as previously described (29). Briefly, overnight YPD cultures of single colonies were plated onto both YPD and YPGL medium and grown for 3 and 5 days, respectively. The percent of respiratory incompetent cells was calculated by quantifying the difference of colony-forming units between the two media. For transformants, the cells were grown under selection in SGLC medium lacking uracil to saturation. These cultures were then inoculated at an A600 of 0.05 in SDC medium lacking uracil, grown for ~7 generations (2 days), diluted, and plated onto both YPD and YPG medium.

**Mitochondrial Electron Transport Chain Complex Activities**—Crude mitochondrial fractions were prepared from yeast grown to midlog phase in YPG medium. The cells were disrupted, and mitochondrial fractions were obtained via differential centrifugation, as previously described (30, 31). The mitochondrial pellets were then resuspended in 20 mM KP, buffer, pH 7.4, followed by three freeze-thaw cycles at −80°C (32). Protein concentration was determined using the Bradford protein assay and then diluted to 250 μg/ml in KP, pH 7.4 buffer. Antimycin A-sensitive NADH-coupled cytochrome c reductase (NCCR) activity was measured as described by Powers et al. (33) with minor modifications. Briefly, 2.5 μg of the mitochondrial fraction were solubilized in complex activity buffer (25 mM KP, buffer, pH 7.2, 5 mM MgCl2, 2 mM KCN, 2.5 mg/ml bovine serum albumin, 0.5 mM n-dodecyl-β-maltoside) supplemented with 1.4 mM NADH. The rate of cytochrome c (50 μM) reduction was measured (ΔAbs550) via complex I oxidation of NADH before and after the addition of 2 μg/ml antimycin A. Ubiquinol-coupled cytochrome c reductase (QCCR), succinate dehydrogenase (SDH), and cytochrome c oxidase (COX) activities were measured as described previously by Birch-Machin et al.
Distinct Roles of MSF1 and LEA-like Domains

RESULTS

Yeast PRELID1 Homologs Maintain Mitochondrial Respiratory Function—Saccharomyces cerevisiae contains three PRELID1 homologs (UPS1–3). The role of Ups proteins in mitochondrial respiratory function has not been investigated. To determine the effects of UPS inactivation on mitochondrial respiration, spectrophotometric assays were performed to measure the activities of mitochondrial electron transport chain (mtETC) complexes. The steady-state activities of NCCR (complex I + III), QCCR (complex III), SDH (complex II), and COX (complex IV) were measured in mitochondria isolated from wild type cells and UPS deletion mutants ups1∆, ups2∆/[r], ups3∆, ups1∆ups2∆, ups1∆ups3∆, and ups1∆ups2∆ups3∆ (Fig. 1). NADH dehydrogenase activity of the mtETC was ascertained by comparing the relative activities of NCCR and QCCR (Fig. 1, A and B). In ups1∆ cells, a decrease was observed in both NCCR and QCCR activity compared with the wild type strain. These results suggest a defect in steady-state levels of complex III activity in ups1∆ cells. The additional inactivation of UPS3 (in ups1∆ups3∆ cells) reverses the defects in both NCCR and QCCR. Notably, the absence of Ups2p function (in ups2∆, ups1∆ups2∆, ups2∆ups3∆, and ups1∆ups2∆ups3∆ cells) decreased NCCR activity by 35–50% in the absence of significant changes in QCCR activity. This indicates that Ups2p function influences the steady-state levels of yeast NADH dehydrogenase activity. NCCR and QCCR activities were unaltered in ups3∆ cells compared with wild type cells.

The activity of SDH was decreased in ups1∆ cells (Fig. 1C). However, deletion of UPS2 or UPS3 (ups1∆ups2∆ and ups1∆ups3∆ cells) reversed this defect. The ups1∆ups2∆ double mutant exhibited a slight increase in SDH activity compared with the wild type strain, whereas ups1∆ups3∆ cells exhibited wild type levels. The SDH activities of other UPS deletion mutants were unaffected.

Inactivation of UPS1 resulted in a 1.8-fold increase in the steady-state levels of COX activity (Fig. 1D). Similar COX activities were observed between ups1∆ and ups1∆ups3∆ strains. The increased COX activity observed in ups1∆ cells was reversed to wild type levels upon inactivation of UPS2 (ups1∆ups2∆), despite the slight increase in COX activity observed in the single UPS2 deletion mutant (ups2∆). The triple UPS deletion mutant showed an increase in COX activity over wild type levels, comparable with that observed in ups2∆ cells (1.3-fold).

In summary, inactivation of UPS1 or UPS2 led to differential effects on steady-state levels of mtETC activities. In the absence of Ups1p function, mitochondria exhibited Ups2p- and Ups3p-dependent decreases in SDH and QCCR activities and a Ups2p-dependent increase in COX activity. In the absence of Ups2p function, a defect in NCCR activity emerged.

The MSF1 Domain of Ups2p Is Required for Optimal NCCR Activity—To determine whether the MSF1 domain is sufficient to maintain steady-state levels of NCCR activity, we created a UPS2 truncation allele, ups2∆Δ181, which encodes only the MSF1 domain of Ups2p. Expression of either full-length Ups2p (UPS2) or the Ups2p MSF1 domain (ups2∆Δ181) in ups2Δ cells restored NCCR activity to levels observed in wild type cells,
indicating that MSF1 domain function maintains optimal NCCR activity during respiratory growth (Fig. 2A). Expression of human PRELID1 also restored NCCR activity in ups2Δ/H9004 cells.

**FIGURE 2.** The Ups2p MSF1 domain rescues the NCCR defect in ups2Δ cells. A and B, the NCCR (A) and QCCR (B) activities were measured in the mitochondrial fractions of indicated transformants (strain [plasmid]) during midlog growth in selective medium. C, Citrate synthase activity was measured to assess mitochondrial mass across mitochondrial fractions. Specific activities (min⁻¹ mg⁻¹ mitochondrial protein) measured in wild type mitochondria were as follows (mean ± S.E.): NCCR, 0.427 ± 0.042 μmol of cytochrome c reduction; QCCR, 1.50 ± 0.41 μmol of cytochrome c reduction; and citrate synthase, 0.138 ± 0.020 μmol of 5,5-dithio-bis[2-nitrobenzoic acid] reduction. The activities are expressed relative to measurements in the WT [p426ADH] strain. Normalized averages and S.E. are shown (NCCR, n = 3; QCCR, n = 2; citrate synthase, n = 2). Analysis of variance was used. ***, p < 0.001.
petite frequency was elevated to levels observed in the wild type cells. Furthermore, in both double deletion mutants, mutation displayed frequencies led to an increase in petite frequency. The maintenance of respiratory competency, the frequency of spontaneous petite formation was measured in UPS deletion strains (Fig. 3A). Inactivation of the UPS2 gene led to an increase in petite frequency. The ups2Δ single deletion mutant displayed frequencies ~3-fold higher than observed in wild type cells. Furthermore, in both double deletion mutants where UPS2 is inactivated (ups1Δups2Δ and ups2Δups3Δ), the petite frequency was elevated to levels observed in the ups2Δ strain. Inactivation of the UPS1 gene decreased the observed petite frequency. The additional inactivation of UPS2, but not UPS3, reverses the decreased petite frequency in ups1Δ cells, indicating that the observed decrease is dependent upon Ups2p function but not Ups3p function. These results demonstrate that Ups2p maintains respiratory competence. However, inactivation of all UPS genes (in ups1Δups2Δups3Δ cells) caused a synergistic increase in the frequency of petites (Fig. 3A), demonstrating that the Ups proteins are all required for maintaining respiratory competence.

To assess the contribution of the Ups2p MSF1 domain to maintaining respiratory competence, spontaneous petite frequency was measured in ups2Δ cells exogenously expressing either full-length Ups2p (UPS2) or the Ups2p MSF1 domain (ups2-Δ181). The expression of full-length Ups2p was able to reverse the petite formation in the UPS2 deletion strain to wild type levels (Fig. 3B). Expression of the Ups2p MSF1 domain (ups2-Δ181) was also able to rescue this defect, indicating that the maintenance of respiratory competence is dependent upon MSF1 domain function. The rescue of the petite formation defect by expression of the Ups2p MSF1 domain and the inability of human PRELID1 expression to suppress this defect were reconfirmed in a second genetic background (data not shown).

**Evolutionarily Conserved LEA-like Domain and Its Role in Oxidative Stress Resistance**—Human PRELID1 is comprised of two domains, an N-terminal MSF1 domain and a C-terminal LEA domain (8, 9). Although the MSF1 domain is common to all PRELID1 homologs, only vertebrate homologs contain a LEA domain (Fig. 4A). All three yeast PRELID1 homologs (Ups1p, Ups2p, and Ups3p) contain an MSF1 domain (Fig. 4B) (4, 5, 17). Our analysis revealed that, unlike Ups1p and Ups3p, Ups2p contains a unique, uncharacterized C terminus (Fig. 4B). Further analysis of protein sequences revealed that the C terminus of yeast Ups2p and human PRELID1 align, exhibiting 40% sequence identity across the LEA domain (Fig. 4C). Conservation of these residues can also be seen in the Ups2p homologs of other yeast and insect species, comprising a LEA-like domain (Fig. 4D and supplemental Fig. S1).

LEA-containing proteins were recently described to protect against ROS-producing insults in plant and animal species (9, 39–41). To assess whether the conserved LEA-like domain of Ups2p performs a similar protective role in yeast, wild type, ups2Δ, and ups2-Δ181 strains were exposed to superoxide-producing menadione during growth in respiratory medium. No differences were observed between the growth of wild type and ups2Δ cells under oxidative stress; however, ups2-Δ181 cells showed significant growth impairment (Fig. 5A). Treatment with menadione-inhibited growth of ups2-Δ181 cells, indicating that the LEA-like domain-containing C terminus of Ups2p provides oxidative stress resistance in these cells. To determine whether conserved LEA-like residues (Fig. 4C) play a role in...
Distinct Roles of MSF1 and LEA-like Domains

FIGURE 4. Ups2p possesses a unique C terminus which aligns to the LEA domain of human PRELID1. Protein sequences were obtained from NCBI UniProt database (55). A, schematic representation of human PRELID1 and eukaryotic homologs (drawn to scale). The PRELI/MSF1 domain (dark gray) is conserved in all PRELI homologs. The LEA domain (light gray) is an attribute of PRELID1 homologs in vertebrates (left column) but not invertebrate species (right column). The following sequences were used for analysis: Homo sapiens (Q9Y255), Mus musculus (Q8R107), Rattus norvegicus (Q5M829), Bos taurus (Q32KN9), Galbus gallus (Q9673), Xenopus laevis (Q7ZK3), Drosophila melanogaster (Q9V579), Caenorhabditis elegans (Q17476), Schizosaccharomyces pombe (Q9UT07), S. cerevisiae (UPS1 [Q95776]; UPS2 [P35200]; UPS3 [Q04006]), and Arabidopsis thaliana (Q9FYA3). B, schematic representation of the human PRELID1 and yeast PREL homologs (Ups proteins). H.s., H. sapiens; S.c., S. cerevisiae. C, sequence alignment reveals conservation between the human LEA domain and the C terminus of yeast Ups2p (40% identity and 48% similarity, where indicated). Identical (bold type) and similar (italic type) amino acids are indicated. D, sequence alignment conservation of the LEA-like domain in fungal and animal homologs of Ups2p. The following sequences were used for analysis: S. cerevisiae (P35200), Vanderwaltozyma polyspora (A71122), Candida glabrata (Q6F555), Kluiveromyces lactis (Q6CQ69), Lachancea thermotolerans (CSDK23), Clavispora lusitaniae (C4Y603), Scheffersomyces stipitis (A3LZM0), Pichia pastoris (C4RD2), Debaryomyces Hansenii (Q6BHS4), Pediculus humanus corporis (E0VVK9), Bombyx mori (Q6PTY2), Aedes aegypti (Q16HW5), Tribolium castaneum (D6WM43), Ashbya gossypii (Q751A6), D. melanogaster (Q9V3U9), and H. sapiens (Q9Y255). The degree of shading indicates percentage of sequence conservation across residues. Sequence alignment and shading of conserved residues were carried out in GeneDoc (v2.6.002) (56).

protection against oxidative stress, the non-LEA-like sequence was truncated from the C terminus of Ups2p. This strain, ups2-Δ210, was not sensitive to menadione when compared with the ups2-Δ181 strain (Fig. 5A). These data demonstrate that the LEA-like sequence is required for protection against oxidative stress. The effects of menadione treatment observed during growth in liquid medium were recapitulated on solid medium (Fig. 5B).

To further characterize the effects of menadione on the ups2-Δ181 strain, we measured colony-forming units of treated cultures. We determined that cell death and petite formation did not significantly contribute to the optical density of ups2-Δ181.
cultures (data not shown), revealing that menadione is cytotoxic to \textit{ups2-}/H9004181 cells. On solid medium, the growth rate of \textit{ups2-}/H9004181 cells, not survival, was affected by menadione exposure; this became apparent when plates were incubated for additional time (data not shown). To determine whether this cytostatic effect was superoxide-specific, strains were also treated with tert-butylhydroperoxide (supplemental Fig. S2). In the presence of tert-butylhydroperoxide, the \textit{UPS2} mutant strains exhibited wild type levels of growth, indicating a superoxide-specific sensitivity in \textit{ups2-}/H9004181 cells.

We also tested whether the LEA domain of human PRELID1 could functionally replace the Ups2p LEA-like domain. A strain was generated (\textit{ups2-}/H9004181/hLEA) that expresses a chimeric protein containing the Ups2p MSF1 domain (encoded by the \textit{ups2-}/H9004181 allele) fused to the LEA-containing C terminus of human PRELID1 (+hLEA). This strain exhibited resistance to menadione similar to the \textit{ups2-}/H11011 strain (Fig. 5, A and B). Together, these data suggest that the conserved Ups2p LEA-like domain is responsible for the protective effect conferred by the human LEA domain in the chimeric strain.

\textbf{LEA-like Dependent Regulation of Cardiolipin Levels in \textit{UPS2}-overexpressing Cells—}Osman et al. (5) demonstrated that overexpression of \textit{UPS2} generates a defect in CL metabolism. To assess the contribution of the Ups2p MSF1 and LEA-like domains to CL metabolism, we overexpressed full-length \textit{Ups2p (UPS2)} or the Ups2p MSF1 domain (\textit{ups2-}/H9004181) in \textit{ups2-}/H9004181 cells and measured CL levels. Exogenous expression increased transcripts of the \textit{UPS2} alleles greater than 20-fold over wild type levels (Fig. 6A).

The levels of mitochondrial phospholipids in these strains were determined. The \textit{ups1-}/H9004 strain transformed with empty vector (\textit{ups1-}/H9004[p426ADH]) displayed a characteristic decrease in CL levels (Fig. 6B, panel I). As expected, the \textit{ups2-}/H9004[p426ADH] control strain exhibited wild type levels of CL. However, the decrease in PE levels associated with \textit{UPS2} deletion was not observed in these cells. Although the reason for this is unclear, it may be related to growth in minimal medium (SDC lacking uracil), which was required to maintain the p426ADH plasmids. The overexpression of full-length \textit{Ups2p (ups2-}/H11011) resulted in CL depletion (Fig. 6B, panel II). Depletion of CL was not observed in the strain expressing the Ups2p MSF1 domain (\textit{ups2-}/H11011) (Fig. 6B, panel I and II). In these cells, the loss of the \textit{Ups2p} LEA-like domain alleviates CL depletion caused by \textit{Ups2p} overexpression. These results demonstrate that the LEA-like domain of \textit{Ups2p} negatively regulates CL levels.

To determine the effect of \textit{UPS2} expression on growth, doubling time was calculated in strains that expressed full-length \textit{Ups2p} at endogenous levels (WT [p426ADH]) or at elevated
expression levels ($ups2\Delta$ [UPS2-p426ADH]). The overexpression of Ups2p increased doubling time by ~50 min compared with the wild type strain (Fig. 6C). However, cells overexpressing the Ups2p MSF1 domain ($ups2\Delta$ [ups2-\Delta 181-p426ADH]) exhibited wild type rates of growth. In these cells, the loss of the Ups2p LEA-like domain alleviates growth inhibition caused by Ups2p overexpression. Together, these data demonstrate that the concomitant decreases in CL levels and growth rate are dependent upon the LEA-like domain of Ups2p.

LEA-like Independent Regulation of Cardiolipin Levels in the Absence of Ups1p—The presence of Ups2p is responsible for CL depletion in the absence of Ups1p (4, 5). We sought to determine whether the regulation of CL levels is dependent on the LEA-like domain, as we observed when Ups2p was overex-
pressed. To address this question, mitochondrial CL levels were measured in the wild type strain, UPS deletion mutants lacking Ups1p and/or Ups2p (ups1Δ, ups2Δ, and ups1Δups2Δ strains) and in genomically modified strains where the UPS2 wild type allele was replaced with the ups2Δ181 truncation allele (ups2Δ181 and ups1Δups2Δ181 strains). Consistent with previous studies, ups1Δ cells show a decrease in CL, whereas ups2Δ and ups1Δups2Δ strains contain wild type levels (Fig. 7). Our data demonstrate that the Ups2p LEA-like domain does not affect CL levels in the presence or absence of Ups1p, as observed in ups2Δ181 and ups1Δups2Δ181 strains, respectively. This indicates that the function of the Ups2p MSF1 domain, not the LEA-like domain, is responsible for the loss of CL in the absence of Ups1p.

DISCUSSION

PRELID1 homologs are highly conserved mitochondrial proteins. In this study, the functions of yeast PRELID1 homologs were investigated by determining the effects of UPS gene inactivation on mtETC complex activity and respiratory competence. The MSF1 domain of Ups2p was essential to its role in maintaining these processes. In addition to the MSF1 domain, we identified a LEA-like domain in Ups2p that exhibits a high degree of sequence conservation with the LEA domain of human PRELID1. Additionally, a conserved function of the yeast LEA-like domain in oxidative stress resistance was discovered. We also demonstrate that both the MSF1 and LEA-like domains contribute to mitochondrial phospholipid metabolism.

The three Ups proteins contain MSF1 domains (Fig. 4B), yet their sequences are divergent. The sequence of Ups1p differs more from Ups2p and Ups3p (30 and 25% identical, respectively), which are more similar in sequence (57% identical). Furthermore, Ups2p differs from its other two homologs in that it contains an additional C-terminal domain (Fig. 4B), which we have characterized as a LEA-like domain. Expression of the Ups2p MSF1 domain rescues the NCCR defect (Fig. 2) and petite formation defect (Fig. 3B) in ups2Δ cells. Additionally, cells endogenously expressing the Ups2p MSF1 domain are able to maintain mitochondrial PE to levels slightly higher than in wild type cells (Fig. 7). These results demonstrate that the MSF1 domain is functional in the absence of the LEA-like domain-containing C terminus. Furthermore, it is the MSF1 domain, not the LEA-like domain, that is required for these functions of Ups2p. Thus, whereas all Ups proteins have an MSF1 domain, the sequence variation of this domain provides unique Ups protein functions.

The Ups proteins differentially influence the steady-state activity of mtETC complexes (Fig. 1). In ups1Δ cells, NCCR, QCCR, and SDH activities are negatively affected (Fig. 1, A–C), and COX activity is increased (Fig. 1D). In the absence of Ups2p function, NCCR activity is negatively affected (Fig. 1A). Because NCCR (complex I + III) activity is dependent upon QCCR (complex III) activity, the NCCR defect in ups1Δ cells may result from the underlying defect in QCCR activity (Fig. 1B). In cells lacking Ups2p, the NCCR defect is not associated with a decrease in QCCR activity, revealing a defect in mtETC-associated NADH dehydrogenase activity. The SDH and QCCR defects in ups1Δ cells are dependent upon both Ups2p and Ups3p function. These data suggest that, as previously reported for Ups2p (4), Ups3p may also antagonize Ups1p function. Inactivation of UPS1 also leads to an increase in the steady-state levels of COX activity (Fig. 1D). This alteration in COX activity is reversed in ups1Δ cells upon inactivation of UPS2, but not upon inactivation of UPS3. Because CL synthesis and COX
assembly are interdependent (42), the increase in COX activity in CL-deficient ups1Δ and ups1Δups3Δ cells was unexpected (Fig. 1D). However, a study utilizing a mutant of PG51 (the enzyme catalyzing the rate-limiting step of CL synthesis) in CHO cells (43, 44) recapitulates our CL (Fig. 7) and COX activity (Fig. 1D) data in ups1Δ cells. Ups1p function may influence Pgs1p activity in yeast.

Inactivation of UPS1 results in a Ups2p-dependent decrease in the frequency of respiratory incompetence (Fig. 3A). The petite assay performed produces negative values for the calculation of petite frequency in ups1Δ and ups1Δups3Δ mutants, because more colony-forming units appeared on plates possessing a respiratory carbon source (glycerol/lactate) than a fermentable carbon source (glucose). This result demonstrates that these cells are better able to form colonies on medium requiring mitochondrial function for growth. The majority of UPS1 deletion phenotypes (including slowed growth, mitochondrial morphology, intramitochondrial protein import and processing, and mitochondrial membrane potential) occur in fermentable medium but are reversed upon growth in respiratory medium (4). It is known that growth of ups1Δ and ups1Δups3Δ cells exhibit slowed growth on glucose medium but not glycerol medium (4). The extent of this defect is not known, but our data suggest that the fermentable carbon source-dependent mitochondrial dysfunction limits the ability of some cells to form visible colonies.

Inactivation of UPS2 results in an increased frequency of respiratory incompetence (Fig. 3). Aside from nuclear-encoded mitochondrial proteins that exhibit an absolute requirement for mitochondrial respiration (characterized by complete respiratory incompetence in the presence of functional mtDNA), petite formation occurs because of mtDNA instability and/or complete mtDNA loss (producing ρ− or ρ0 cells) (45, 46). The UPS2 deletion mutant was previously described to exhibit an increase in mtDNA loss (5). Thus, Ups2p protects against petite formation through its ability to influence mitochondrial genome maintenance and stability. In both rich and synthetic medium, the loss of Ups2p function resulted in ~3-fold increase in petite frequency (Fig. 3, A and B, respectively). This role of Ups2p is provided by the Ups2p MSF1 domain (Fig. 3B).

Expression of human PRELID1, the UPS1 ortholog, was unable to suppress the petite defect in ups2Δ cells, exhibiting functional divergence of the Ups2p and PRELID1 Ups1p MSF1 domains.

It is known that disruption of mitochondrial PE synthesis, but not CL synthesis, increases the rate of petite formation (47–49). The ability of the Ups2p MSF1 domain to maintain both PE levels (Fig. 7) and respiratory competency (Fig. 3B) suggests that petite formation in cells lacking Ups2p is an indirect effect of PE depletion. The inability of human PRELID1 expression to suppress petite formation in the ups2Δ strain (Fig. 3B) is in accordance with previous work demonstrating that PRELID1 expression failed to suppress PE depletion in the ups2Δ strain (50). The synergistic increase in respiratory incompetence in the UPS triple deletion mutant reveals that, in the absence of Ups2p, Ups1p and Ups3p function protects against petite formation (Fig. 3A). This demonstrates that the mitochondrial functions of the conserved Ups MSF1 domains are required to maintain respiratory competence. The synergistic increase in petite formation may occur by influencing factors required for mtDNA maintenance, which interact synergistically with PE depletion.

We determined that the Ups2p MSF1 domain maintains respiratory competence (Fig. 3), mtETC-associated NADH dehydrogenase activity (Fig. 2), and mitochondrial PE levels (Fig. 7). Although a connection can be made between petite formation and PE levels, the mechanism by which NCCR activity (specifically, the NADH dehydrogenase component) is affected by UPS2 inactivation is less clear (Fig. 1A). Maintenance of NADH dehydrogenase activity appears to be a conserved role of the PRELI/MSF1 family of proteins, because expression of human PRELID1 suppresses the NADH dehydrogenase defect in ups2Δ cells. Indeed, McKeller et al. (9) has shown that expression of PRELID1 in human cells leads to a substantial increase in respiratory function through increased NADH dehydrogenase activity, corresponding to our findings presented here. The ability of human PRELID1 expression to suppress the NADH dehydrogenase defect in ups2Δ cells (Fig. 2) but not suppress the increased petite formation (Fig. 3B) or PE depletion (50) phenotypes suggests that the defect in NADH dehydrogenase activity does not result from PE depletion.

Cells lacking the LEA-like domain (expressing the Ups2p MSF1 domain only) were sensitive to oxidative stress (Fig. 5). However, the UPS2 deletion mutant did not exhibit sensitivity to oxidative stress compared with wild type cells. Thus, in the presence of Ups2p MSF1 domain function, the LEA-like domain of Ups2p is required for protection against oxidative stress (Fig. 5). Although there may be other possible explanations of this data, we propose that the LEA-like domain provides protection against oxidative stress through regulation of the MSF1 domain. Further evidence of MSF1 domain regulation by the LEA-like domain is provided through investigation of the role of Ups2p in mitochondrial phospholipid metabolism. Consistent with published data (4–7), we demonstrate that mitochondrial PE levels are decreased in ups2Δ cells (Fig. 7). However, in strains lacking the Ups2p LEA-like domain (ups1Δups2Δ and ups1Δups2Δups3Δ), PE levels are increased compared with the wild type strain. This indicates that the LEA-like domain negatively regulates maintenance of mitochondrial PE levels by the Ups2p MSF1 domain.

Recently, LEA-containing proteins have been identified as essential cytoprotective proteins in nonplant species (9, 13, 39, 51). In this study, we elucidate a conserved role for the LEA-like domain of Ups2p in oxidative stress resistance in yeast (Fig. 5). Consistent with our findings, recently identified mitochondria-targeted LEA proteins were shown to protect the TCA cycle and mitochondrial respiratory enzymes against reactive oxygen species-inducing water stress in plant and animal models (39, 40). Furthermore, we demonstrate that the LEA domain of human PRELID1 can functionally replace the LEA-like domain in yeast and may provide cytoprotection through mechanisms similar to other mitochondria-targeted LEA-containing proteins. The LEA domain of human PRELID1 promotes mito-ochondrial respiration and protection against apoptosis (9). Because CL is required for mtETC supercomplex formation and provides apoptotic resistance (52, 53), PRELID1 function
may be linked to CL. Recently, a mitochondrial LEA protein was shown to physically interact with CL (54). These data suggest that PRELID1 and possibly other LEA/LEA-like-containing proteins, may exert cytoprotective effects through interaction with mitochondrial phospholipids and, thus, through mitochondrial function.

A direct competition between Ups1p and Ups2p has been proposed, supported by the observations that (i) the CL depletion resulting from the loss of UPS1 is Ups2p-dependent and (ii) UPS2 overexpression results in a decrease in CL, a phenotype similar to UPS1 deletion (5, 6). Our data reveal that the LEA-like domain is responsible for the loss of CL upon Ups2p overexpression (Fig. 6B), yet the MSF1 domain of Ups2p is responsible for the loss of CL in the absence of Ups1p (Fig. 7). Thus, by investigating the contributions of the MSF1 and LEA-like domains of Ups2p in maintenance of mitochondrial function.

In summary, we elucidated roles of the MSF1 and LEA-like domains of Ups2p in maintenance of mitochondrial function. By determining the contributions of the MSF1 and LEA-like domains to the regulation of mitochondrial PE and CL levels, we demonstrate that Ups2p may not directly antagonize Ups1p. Our studies suggest that the LEA/LEA-like domain is functionally conserved in yeast and humans, providing protection against oxidative stress. Further study of the Ups2p LEA-like domain may provide insight into mechanisms of protection conferred by mitochondrial LEA proteins across eukaryotes.

Acknowledgments—We thank William Burhans, Dhyan Chandra, and Kelly Graham for critical reading of the manuscript. We also thank Gregory Wilding and Kristopher Atwood from the University at Buffalo for statistical analysis of our data.

REFERENCES

1. Nakai, M., Takada, T., and Endo, T. (1993) Biochim. Biophys. Acta 1174, 282–284
2. Anantharaman, V., and Aravind, L. (2002) Genome Biol. 3, research0023
3. Dee, C. T., and Moffat, K. G. (2005) Biochim. Biophys. Acta 174, 51–56
4. Draghici, S., and Bonchev, T. (2006) J. Cell Biol. 174, 375–386
5. Ohtsuka, T., Iijima, M., and Sesaki, H. (2009) Dev. Genes Evol. 219, 1071–1076
6. Potting, C., Wilmes, C., Engmann, T., Osman, M., and Langer, T. (2009) EMBO J. 28, 2888–2898
7. Timmers, T., and Sørensen, P. (2007) J. Cell Biol. 178, 461–472
8. Guzman-Rojas, L., Sims, J. C., Rangel, R., Guret, C., Sun, Y., Alcocer, J. M., and Martinez-Valdez, H. (2001) Int. Immunol. 12, 607–612
9. McKellar, M. R., Herrera-Rodriguez, S., Ma, W., Ortiz-Quintero, B., Rangel, R., Candé, C., Sims-Mouratada, J. C., Melnikova, V., Kashi, C., Phan, L. M., Chen, Z., Huang, P., Dunner, K., Jr., Kroemer, G., Singh, K. K., and Martinez-Valdez, H. (2010) Cell Death Disease 1, e21
10. Hughes, D. W., and Galau, G. A. (1991) Plant Cell 3, 605–618
11. Wise, M. J. (2003) BMC Bioinformatics 4, 52
12. Wise, M. J., and Tunncliffe, A. (2004) Trends Plant Sci. 9, 13–17
13. Hand, S. C., Menze, M. A., Toner, M., Boswell, L., and Moore, D. (2011) Annu. Rev. Physiol. 73, 115–134
14. Tahvanainen, J., Kallonen, T., Lääteenmäki, H., Heiskanen, K. M., Westermarck, J., Rao, K. V., and Lahesmaa, R. (2009) Blood 113, 1268–1277
15. Fox, E. J., Stubbs, S. A., Kyaw Tun, J., Leek, J. P., Markham, A. F., and Wright, S. C. (2004) Biochem. J. 378, 817–825
16. Niu, S., Antin, P. B., and Morkin, E. (1996) Gene 175, 187–191
17. Sasaki, H., Dunn, C. D., Iijima, M., Shepard, K. A., Yaffe, M. P., Machamer, C. E., and Jensen, R. E. (2006) J. Cell Biol. 173, 651–658
18. Carhan, A., Reeve, S., Dee, C. T., Baines, R. A., and Moffat, K. G. (2004) Invert. Neurosci. 5, 65–75
19. Reeve, S., Carhan, A., Dee, C. T., and Moffat, K. G. (2007) Genesis 45, 66–75
20. Tsoubouchi, A., Tsuyama, T., Fujimori, M., Kohda, H., Okamoto-Furuta, K., Aigaki, T., and Uemura, T. (2009) Development 136, 3757–3766
21. Brachmann, C. B., Davies, A., Cost, G. J., Caputo, E., Li, J., Hieter, P., and Boeke, J. D. (1998) Yeast 14, 113–132
22. Baudin, A., Ozier-Kalogeropoulos, O., Demoule, A., Lacroix, F., and Culin, C. (1993) Nucleic Acids Res. 21, 3329–3330
23. Sikorski, R. S., and Hieter, P. (1989) Genetics 122, 19–27
24. Longtine, M. S., McKenze, A., 3rd, Demarini, D. J., Shah, N. G., Wach, A., Brachat, A., Philippsen, P., and Pringle, J. R. (1998) Yeast 14, 953–961
25. Mumberg, D., Müller, R., and Funk, M. (1995) Gene 156, 119–122
26. Zhu, H., Bilgin, M., Bangham, R., Hall, D., Casamayor, A., Bertone, P., Lan, N., Janssen, R., Bidlingmaier, S., Houtek, T., Mitchell, T., Miller, P., Dean, R. A., Gerstein, M., and Snyder, M. (2001) Science 293, 2101–2105
27. Mitchell, D. A., Marshall, T. K., and Deschenes, R. J. (1993) Yeast 9, 715–722
28. Imay, Y., Matsushima, Y., Sugimura, T., and Terada, M. (1991) Nucleic Acids Res. 19, 2785
29. O’Rourke, T. W., Doudican, N. A., Mackereth, M. D., Doetsch, P. W., and Shadel, G. S. (2002) Mol. Cell. Biol. 22, 4086–4093
30. Diekert, K., de Kroon, A. I., Kispal, G., and Lill, R. (2001) Methods Cell Biol. 65, 37–51
31. Leister, D., and Herrmann, J. M. (eds.). (2007) Methods in Molecular Biology: Mitochondria, Practical Protocols, Vol. 372, pp. 87, Humana Press, Totowa, NJ
32. Birch-Machin, M. A., Briggs, H. L., Saborido, A. A., Bindoff, L. A., and Turnbull, D. M. (1994) Biochem. Med. Metab. Biol. 51, 35–42
33. Powers, W. J., Haas, R. H., Le, T., Videen, T. O., Hershey, T., McGee-Minnich, L., and Perlmuter, J. S. (2007) Neurobiol. Dis. 27, 99–101
34. Barrientos, A., Kenyon, L., and Moraes, C. T. (1998) J. Biol. Chem. 273, 14210–14217
35. Kim, I. S., Yun, H. S., Kwak, S. H., and Jin, I. N. (2007) J. Microbiol. 45, 326–332
36. Claypool, S. M., McCaffery, J. M., and Koehler, C. M. (2006) J. Cell Biol. 174, 379–390
37. Stewart, J. C. (1980) J. Biol. Chem. 255, 156–164
38. Menze, M. A., Boswell, L., Toner, M., and Hand, S. C. (2009) J. Biol. Chem. 284, 10714–10719
39. Grelet, J., Benamar, A., Teyssier, E., Avelange-Macherel, M. H., Grunwald, D., and Macherel, D. (2005) Plant Physiol. 137, 157–167
40. Mowla, S. B., Cuypers, A., Driscoll, S. P., Kiddle, G., Thomson, J., Foyer, C. H., and Theodoulou, F. L. (2006) Plant J. 48, 743–756
41. Gohil, V. M., Hayes, P., Matsuyama, S., Schägger, H., Schild, M., and Greenberg, M. L. (2004) J. Biol. Chem. 279, 42612–42618
42. Ohtsuka, T., Nishijima, M., Suzuki, K., and Akamatsu, Y. (1993) J. Biol. Chem. 268, 22914–22919
43. Ohtsuka, T., Nishijima, M., and Akamatsu, Y. (1993) J. Biol. Chem. 268, 22908–22913
44. Merz, S., and Westermann, B. (2009) Genome Biol. 10, R95
Distinct Roles of MSF1 and LEA-like Domains

46. Contamine, V., and Picard, M. (2000) Microbiol. Mol. Biol. Rev. 64, 281–315
47. Birner, R., Bürgermeister, M., Schneiter, R., and Daum, G. (2001) Mol. Biol. Cell 12, 997–1007
48. Zhang, M., Su, X., Mileykovskaya, E., Amoscato, A. A., and Dowhan, W. (2003) J. Biol. Chem. 278, 35204–35210
49. Zhong, Q., Gohil, V. M., Ma, L., and Greenberg, M. L. (2004) J. Biol. Chem. 279, 32294–32300
50. Osman, C. (2008) Characterization of the genetic interactome of prohibitins in S. cerevisiae. University of Cologne, Cologne, Germany
51. Hand, S. C., Jones, D., Menze, M. A., and Witt, T. L. (2007) J. Exp. Zool. A Ecol. Genet. Physiol. 307, 62–66
52. Zhang, M., Mileykovskaya, E., and Dowhan, W. (2005) J. Biol. Chem. 280, 29403–29408
53. Choi, S. Y., Gonzalvez, F., Jenkins, G. M., Slomianny, C., Chretien, D., Arnoult, D., Petit, P. X., and Frohman, M. A. (2007) Cell Death Differ. 14, 597–606
54. Tolleter, D., Hincha, D. K., and Macherel, D. (2010) Biochim. Biophys. Acta 1798, 1926–1933
55. Alvaro, D., Lisby, M., and Rothstein, R. (2007) PLoS Genet. 3, e228
56. Treger, J. M., and McEntee, K. (1990) Mol. Cell. Biol. 10, 3174–3184