Mitochondrial DNA Instability Mutants of the Bifunctional Protein Ilv5p Have Altered Organization in Mitochondria and Are Targeted for Degradation by Hsp78 and the Pim1p Protease*

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Ilv5p is a bifunctional mitochondrial protein in Saccharomyces cerevisiae required for branched-chain amino acid biosynthesis and for the stability of wild-type (ρ+) mitochondrial DNA (mtDNA). Mutant forms of Ilv5p defective in mtDNA stability (aD) are present as 5–10 punctate structures in mitochondria, whereas mutants lacking enzymatic function (aD) show a reticular distribution, as does wild-type Ilv5p. aD ilv5 mutations are recessive, and the mutant protein is redistributed to a reticular form when co-expressed with wild-type Ilv5p. Ilv5p proteins that are punctate in vivo are also less soluble in detergent extracts of isolated mitochondria, suggesting that the punctate foci in aD Ilv5p mutants are aggregates of the protein. aD Ilv5p proteins are selectively degraded in cells lacking a functional mitochondrial genome, but only in cells grown under derepressing conditions. The targeted degradation of aD Ilv5p, which occurs even when co-expressed with wild-type Ilv5p, is mediated by the glucose-repressible chaperone, Hsp78, and by the ATP-dependent Pim1p protease, whose activity may be modulated by ρ+ mtDNA.

The stability and inheritance of mitochondrial DNA (mtDNA) in Saccharomyces cerevisiae depends on a surprisingly large number of proteins, some of which would not have been anticipated to be involved in mtDNA transactions (1). One example is Ilv5p, a mitochondrial NADPH-requiring acetohydroxyacid reductoisomerase (11). Mutations of the aD class map to a region buried within the core of the protein at or close to residues known to bind the substrate and cofactors, NADPH and Mg2+ (11). By contrast, the majority of mutations of the aD class map within or adjacent to two α-helices, also present in Ilv5p of yeast, that are on the surface of the spinach protein. These data suggested that the aD mutations could affect intermolecular interactions of the yeast Ilv5p. The mtDNA redistribution function of Ilv5p (9) was also found to be defective in aD mutants (10), suggesting that the stability and organizational state of mtDNA nucleoids are functionally linked. Finally, none of these mtDNA transaction defects is observed in the aD mutants, further confirming that branched-chain amino acid biosynthesis per se is not connected to these mtDNA activities.

To show here that the aD mutant proteins are present as a small number of aggregated, punctate structures within mitochondria and that the extent of aggregation is related to the severity of the mtDNA instability phenotype. In contrast, wild-type Ilv5p and the aD mutants have a distinctly reticular distribution within mitochondria. Surprisingly, we find that the aD mutant proteins are unstable in petite cells, but not in ρ− cells, and that the instability is glucose-repressible. This instability is independent of the organizational state of the aD mutant protein and can be attributed to the Pim1p protease, whose activity may be modulated by ρ+ mtDNA.
protease, whose activity we have found to be regulated by the glucose-repressible chaperone Hsp78.

EXPERIMENTAL PROCEDURES

Strains and Growth Conditions—All strains described were derivatives of either 14C W N( MATa ade2-1 ura3-52 trpl1 leu2-3,112 rho -1) or 14C W N( MATa ade2-1 ura3-52 trpl1 leu2-3,112 rho -1) and were then used to replace the wild-type genes in congenic strains (Research Genetics, Invitrogen, CA). These PCR products were created by transplacing each mutant allele into strain 1 ura3 -1 ade2 -1 ilv5::ura3/H11001 (YNBRaff cas), or 2% raffinose/H11001 grown at 30 °C, confirmed by PCR, Southern analysis, or DNA sequencing. Cells were grown at 25 °C, except that the mitochondrial purification, lysis, and affinity chromatography column (Bio-Rad) for 1 h at 4 °C. The column was then washed with 15 ml of wash buffer (the same as column buffer with the imidazole concentration increased to 20 mM), and the bound protein was eluted with three sequential additions of 0.5 ml of elution buffer (the same as column buffer with the imidazole concentration increased to 250 mM and the addition of 20% glycerol).

Western Blot Analysis—Trichloroacetic acid precipitates of total yeast cell proteins were prepared from OD600 values of 0.6–1.0 cultures as described previously (16). For SDS-PAGE, equal volumes of extract were solubilized in 1× SDS-PAGE loading buffer (see above); samples were loaded onto 10% SDS-PAGE gels and separated using the Ready Gel system (Bio-Rad). Proteins were transferred to nitrocellulose membranes (Schleicher & Schuell) using a Hoefer SemiPhor semidry transmembrane apparatus (Amersham Biosciences). Immunodetection of proteins was carried out using primary rabbit anti-I1v5p (15) and mouse anti-Tifin (Molecular Probes). Anti-rabbit or anti-mouse immunoglobulin G-coupled horseradish peroxidase (Bio-Rad) were used as secondary antibodies and were visualized using the ECL system (Amersham Biosciences).

Fluorescence Microscopy—Log-phase cells were observed live using a Leica microscope (model DMRXe) equipped with an HBO 100 W2 mercury arc lamp, and a 100× Plan-Apochromat objective lens. Differential interference contrast and green fluorescent protein were visualized using a filter set described previously (17). Images were captured with a color-chilled three-charged-coupled device camera system (model C5810; Hamamatsu Photonics) and processed using Adobe Photoshop (Adobe Systems, Inc.). Cells were stained with Mitotracker Red CM-H2XRos (Molecular Probes), by adding Mitotracker to log-phase growing cells to a final concentration of 0.5 µl and incubating for 20 min at 30 °C, and pelleting the cells and washing three times in distilled water, before microscopy.

Pette Assay—To measure pete formation in the various strains, mid-log-phase cultures of strains grown in YNBg + cas medium were transferred to YNBg + cas medium and grown for at least thirty generations at various time points. At various time points, the cells were plated onto YNBg + cas plates and grown for 3 days at 30 °C. After 3 days of growth, the cells were stained with 2,3,5-triphenyltetrazolium chloride (TTC) overlay (17), using 0.2% TTC (Sigma) with 0.8% agarose. Between fifty and three hundred colonies were counted for each time point.

RESULTS

A Δ’ ilv5 mutants have variable mtDNA Instability Phenotypes and Form Punctate Structures within Mitochondria—To obtain additional insight into the bifunctional nature of I1v5p, we analyzed three I1v5p mutants: two a Δ’ mutants, I267F and W327R, defective in p+ mtDNA stability, and one a Δ’ mutant, D255E, which has no effect on mtDNA stability but is defective in branched-chain amino acid biosynthesis. Centromeric vectors containing these mutant alleles, wild-type I1v5p, or no insert were transformed into yeast cells in glycerol medium (YNBGly + cas) and plated onto YNBg + cas plates and grown for 3 days at 30 °C. ρ- and pete colonies were distinguished by 2,3,5-triphenyltetrazolium chloride (TTC) overlay (17), using 0.2% TTC (Sigma) with 0.8% agarose. Between fifty and three hundred colonies were counted for each time point.

"a Δ’ ilv5 mutants have variable mtDNA instability phenotypes and form punctate structures within mitochondria—"To obtain additional insight into the bifunctional nature of I1v5p, we analyzed three I1v5p mutants: two a Δ’ mutants, I267F and W327R, defective in p+ mtDNA stability, and one a Δ’ mutant, D255E, which has no effect on mtDNA stability but is defective in branched-chain amino acid biosynthesis. Centromeric vectors containing these mutant alleles, wild-type I1v5p, or no insert were transformed into i1v5α cells. These transformants were pre-growing on glycerol medium (YNBGly+cas) to maintain p+ mtDNA and then transferred to glucose medium (YNBD+cas) and grown for at least thirty generations. At various time points, samples of each culture were plated onto YNBd+cas medium to determine the percentage of p+ cells in the population. In accordance with our previous results (10), p- mtDNA was unstable in i1v5α cells containing the empty vector, whereas it was completely stable in cells containing wild-type I1v5p, or the a Δ’ mutant D255E allele (Fig. 1). Although p- mtDNA was unstable in both of the a Δ’ mutants, the
degree of mtDNA instability was significantly different between them and ilv5Δ cells containing the control plasmid. Specifically, a Δ− mutant I267F has a weaker mtDNA-instability phenotype than ilv5Δ cells, whereas a Δ− mutant W327R has a much stronger mtDNA-instability phenotype than ilv5Δ cells (Fig. 1). We refer to these a Δ− mutant alleles as weak and strong, respectively.

To determine how these mutant forms of Ilv5p are distributed in mitochondria, we constructed C-terminal GFP fusion genes of wild-type Ilv5p and each of the mutant proteins and expressed these under control of the ILV5 promoter from centromeric plasmids transformed into ρ− cells of strain 14CWWΔilv5u−. The wild-type yeast GFP fusion protein complemented all of the phenotypes of ilv5Δ cells, whereas the mutant derivatives reproduced the phenotypes of their respective untagged forms (data not shown). Although all of the Ilv5p-GFP fusions localized to mitochondria when visualized by fluorescence microscopy, there was a dramatic difference in the distribution of these proteins, which correlated with their effects on mtDNA stability: both the wild-type and the a Δ− mutant fusion protein, D255E, had a largely reticular morphology typical of a mitochondrial matrix protein (Fig. 2A). By contrast, both the strong (W327R) and the weak (I267F) a Δ− GFP-tagged alleles were mainly localized to just a few foci in mitochondria (on average between 5 and 10 when all sections throughout the cells were examined). Closer inspection of the distribution of these GFP-tagged proteins in mitochondria also indicated that some of the I267F a Δ− Ilv5p-GFP was also reticular. The punctate distribution of the a Δ− Ilv5p-GFP mutant proteins is not due to an alteration in mitochondrial morphology, because strains expressing these proteins exhibit normal mitochondrial structures as revealed by staining with MitoTracker (Fig. 2B). This reticular structure persists in wild-type and a Δ− mutant ρ− and ρ+ petite cells (data not shown).

The above findings raise the possibility that the mtDNA instability phenotype of the ilv5Δ a Δ− mutants is related to their propensity to form punctate structures in mitochondria. Previous genetic studies established that all of the ilv5Δ a Δ− mutants are recessive (10). This includes not only the mtDNA instability phenotype, but also the temperature-sensitive growth phenotype of ilv5 a Δ− cells grown in medium containing a non-fermentable carbon source. Thus it was of interest to determine whether the punctate morphology of the a Δ− mutant Ilv5p-GFP persists when co-expressed with wild-type Ilv5p. To address this, we introduced a centromeric plasmid containing the a Δ− mutant, W327R Ilv5p-GFP, into ILV5 14CWW wild-type cells expressing untagged Ilv5p and compared the mutant Ilv5p-GFP morphology to that in strain 14CWWΔilv5u− transformed with the same plasmid. The results of this experiment (Fig. 2C) show that there was a dramatic redistribution of W327R Ilv5p-GFP from tight, punctate spots to a large, reticular network of at least 10 foci per mitochondrion, indicating a strong propensity to form punctate structures in mitochondria. This result is consistent with the observation that a Δ− mutant Ilv5p-GFP was also seen in a strain containing an integrated copy of W327R Ilv5p-GFP transformed with pRS-ILV5 encoding wild-type Ilv5p (data not shown). Taken together, these data suggest that the usual punctate distribution of mutant a Δ− Ilv5p is related to the defects in mtDNA transactions.

Although wild-type Ilv5p shows a largely reticular distribution within mitochondria, Ilv5p has been recovered as a component of the mtDNA nucleoid (15), suggesting some functional partitioning of the protein. Because there are fewer a Δ− Ilv5p-GFP punctate structures than ρ+ mtDNA nucleoids, it has been difficult to assess with certainty whether these punctate structures co-localize with ρ+ mtDNA. However, we can ask whether the punctate morphology of a Δ− Ilv5p-GFP depends on the presence of mtDNA. To this end, we transformed the centromeric plasmid containing W327R Ilv5p-GFP into a ρ− derivative of strain 14CWWΔilv5u− and examined the distribu-

![Figure 1](http://www.jbc.org/)

**Fig. 1.** Ilv5p mutants have different mtDNA-instability phenotypes. 14CWWΔilv5u− cells transformed with either wild-type ILV5 or a Δ− mutant allele were pre-grown in YNBGly+cas then shifted to YNBGly+cas medium. Aliquots were removed at the time points indicated and scored by TTC overlay (17) for the fraction of petites in the population.

![Figure 2A](http://www.jbc.org/)

**Fig. 2A.** a Δ− mutant Ilv5p-GFP fusion proteins localize as punctate structures within mitochondria. A, Ilv5p cells were transformed with plasmids containing GFP fusions of wild-type ILV5, a Δ− mutants W327R and I267F, and a Δ− mutant D255E. Representative cells are shown, using differential interference contrast and fluorescence microscopy to visualize GFP. B, Ilv5p distribution was visualized in strain 14CWWΔilv5u− expressing either wild-type Ilv5p-GFP, or a Δ− W327R Ilv5p-GFP, and mitochondrial morphology was visualized by staining with MitoTracker (MT). C, a plasmid containing a Δ− ilv5 mutant W327R Ilv5p-GFP was transformed into either the ILV5 wild-type strain 14CWW or into strain 14CWWΔilv5u−. D, ρ− (HS40) or ρ+ cells of strain 14CWWΔilv5u− were transformed with a plasmid containing a Δ− ilv5 mutant W327R Ilv5p-GFP. All strains were grown to mid-log phase in YNBGly+cas medium. Bar = 3 μm.
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Fig. 3. α′D′-ilv5 mutant proteins have decreased detergent solubility, which can be reversed by co-expression of wild-type Ilv5p. A, mitoplasts were isolated from cells expressing wild-type Ilv5p (ILV5), a D′ mutant (D255E), strong (W327R), or weak (I267F) α′D′ mutant derivatives, solubilized by treatment with n-dodecylmaltoside, separated into supernatant (S) and pellet (P) fractions, and the distribution of Ilv5p was analyzed by Western blotting. All cells were grown in YPG medium. To confirm that mitochondria had been efficiently solubilized, the distribution of the outer membrane protein porin was also analyzed. B, mitochondria isolated from diploid strains (303V5/14V5G, 303W327R/14V5G, and 303W327R/14V5G) expressing either wild-type Ilv5p and wild-type Ilv5p-GFP (ILV5/ILV5-GFP), a D′ W327R mutant Ilv5p, and wild-type Ilv5p-GFP (α′D′/ILV5-GFP), or another weak mutant W327R Ilv5p and a D′ W327R mutant Ilv5p-GFP (α′D′/α′D′-GFP) grown in YPG medium were solubilized with n-dodecylmaltoside, separated into supernatant (S) and pellet (P) fractions, and analyzed as in A.

Fig. 4. Interaction between wild-type Ilv5p and α′D′ mutant Ilv5p. Plasmids containing wild-type ILV5-GFP or a D′ mutant W327R-GFP were transformed into strain ILV5his and W327Rhis, expressing His6-tagged isoforms of the wild-type or α′D′ mutant W327R Ilv5p, respectively. As a control, the plasmid containing ILV5-GFP was also transformed into strain 14CW expressing untagged, wild-type Ilv5p as a control. Soluble mitochondrial extract (S) from these strains, grown in YNBGly+cas medium, was then chromatographed on Ni2+ resin. The bound (B) material was eluted by addition of 250 mM imidazole, and the bound and unbound (UB) fractions were analyzed for the presence of Ilv5p by Western blotting. A, strain ILV5his co-expressing wild-type wild-type Ilv5p-GFP, B, strain W327Rhis co-expressing wild-type Ilv5p-GFP, C, strain W327Rhis co-expressing a D′ mutant W327R Ilv5p-GFP. D, strain 14CW co-expressing wild-type Ilv5p-GFP.
the wild-type or a mutant allele was complemented by a wild-type copy of ILV5.

Given that a mutant Ilv5p is selectively unstable in petite cells, and the instability of these mutant proteins. To do this we used a diploid strain (W327R/ILV5-GFP), in which the W327R mutant allele was complemented by a wild-type copy of ILV5-GFP. Given that a mutant Ilv5p is selectively unstable in petite cells (Fig. 5A). As expected, the mutant Ilv5p was stable in W327R/ILV5-GFP ρ° cells grown on dextrose (data not shown). By contrast, in W327R/ILV5-GFP ρ° cells grown on raffinose medium, the mutant Ilv5p was stable (Fig. 5B). These findings exclude the possibility that the instability of the a mutant Ilv5p is glucose-repressible in petite cells is simply a consequence of growth on raffinose medium. Importantly, these data also suggest that the instability of the a mutant Ilv5p is not related to the in vivo distribution of the protein, but rather, that the instability is an intrinsic property of the a proteins. Finally, we have also observed that a mutant Ilv5p is stable in ρ° W327R/ILV5-GFP cells grown on raffinose medium in the presence of the respiratory chain inhibitor antimycin A or the ATP synthase inhibitor oligomycin (data not shown). We conclude that the instability of the a mutant Ilv5p in petites is a consequence of the absence of a functional mitochondrial genome rather than simply the loss of oxidative phosphorylation capacity.

The instability of a mutant Ilv5p does not depend on its aberrant organization within mitochondria. A, ρ° derivatives of diploid strains 14V5/14V5G and 14W327R/14V5G expressing either wild-type Ilv5p and wild-type Ilv5p-GFP (ILV5/ILV5-GFP) or a mutant Ilv5p were grown to mid-log phase in raffinose medium. The relative amount of Ilv5p in cell extracts from each strain was then analyzed by Western blotting, using porin as a loading control. B, ρ° parent strains 14V5/14V5G (ILV5/ILV5-GFP) and 14W327R/14V5G (a mutant Ilv5p-GFP) were grown and analyzed as in A.

a + D− Mutant Ilv5p Is Unstable in Petite Cells, and the Instability Is Glucose-repressible—Although microscopy experiments indicated that the punctate morphology of the strong a D− mutant protein is independent of mtDNA (Fig. 2D), we noticed that there was a significant decrease in the abundance of a D− mutant W327R Ilv5p in ρ° petite cells grown in raffinose medium. To investigate this further, we compared the abundance of Ilv5p in wild-type, the a D− mutant, and both the strong and weak a D− Ilv5p mutants in ρ°, ρ°, and ρ° petite cells grown in media containing different carbon sources. Because ρ° mtDNA is unstable in a D− cells grown on fermentable carbon sources, we carried out our initial analysis of the stability of the various forms of Ilv5p in ρ° cells grown in glycerol medium. Under these conditions, wild-type and mutant forms of Ilv5p were present at comparable abundance (Fig. 5A). However, in either a ρ° or a ρ° petite strain grown on raffinose, a non-repressing, fermentable carbon source, both of the a D− mutant proteins were unstable, whereas neither wild-type Ilv5p nor the a D− mutant Ilv5p were affected (Fig. 5, B and D). By contrast, when these petite strains were grown in glucose medium, the a D− mutant proteins were as stable as the wild-type or a D− mutant protein (Fig. 5, C and E). These data suggest that one or more factors accounting for the instability of the a D− mutant proteins in petites is glucose-repressible. The extent of the instability of Ilv5p in the strong and weak a D− mutants (Fig. 5, B and D) also correlated with the severity of the mtDNA instability phenotype associated with these mutations (Fig. 1). Fig. 5 (B and D) show that the level of Ilv5p in the strong mutant, W327R, was severalfold lower than that of the weak mutant, 1267F. Northern blot analysis revealed that there was no difference in the mRNA abundance of wild-type and mutant ilv5 transcripts in cells grown in the different media noted above (data not shown), indicating that the cause of the instability of the a D− mutant proteins is post-transcriptional.

We wanted to compare the stability of the wild-type and mutant forms of Ilv5p in ρ° and petite cells grown on identical carbon sources and to determine whether the organizational state of a D− mutant Ilv5p proteins is an underlying factor in the instability of these mutant proteins. To do this we used a diploid strain (W327R/ILV5-GFP), in which the W327R a D− mutant allele was complemented by a wild-type copy of ILV5-GFP. Given that a D− ilv5 mutations are recessive, ρ° mtDNA would be stable in those cells grown in raffinose or glucose medium. As a control, we used the diploid strain ILV5/ILV5-GFP containing untagged and GFP-tagged wild-type ILV5 alleles. In W327R/ILV5-GFP ρ° cells, the mutant Ilv5p was unstable when those cells were grown on raffinose medium, exactly as we had observed in cells expressing only the mutant Ilv5p (Fig. 6A). As expected, the mutant Ilv5p was stable in W327R/ILV5-GFP ρ° cells grown on dextrose (data not shown).

The instability of a D− mutant Ilv5p in petites is simply a consequence of growth on raffinose medium. Importantly, these data also suggest that the instability of the a D− mutant Ilv5p is not related to the in vivo distribution of the protein, but rather, that the instability is an intrinsic property of the a D− proteins. Finally, we have also observed that a D− mutant Ilv5p is stable in ρ° W327R/ILV5-GFP cells grown on raffinose medium in the presence of the respiratory chain inhibitor antimycin A or the ATP synthase inhibitor oligomycin (data not shown). We conclude that the instability of the a D− mutant Ilv5p in petites is a consequence of the absence of a functional mitochondrial genome rather than simply the loss of oxidative phosphorylation capacity.

a D− Mutant Ilv5p Is Degraded by the Pim1p Protease, and the Activity Is Regulated by the Glucose-repressible Chaperone Hsp78—The results presented thus far indicate that the a D− mutant Ilv5p is unstable in derepressed cells lacking a functional mitochondrial genome. Examination of microarray data from cells undergoing the diauxic shift (18) did not reveal any known mitochondrial protease in which expression was significantly repressed by glucose. However, the expression of Hsp78, a mitochondrial chaperone that has significant sequence similarity to Escherichia coli ClpB, as well as to ClpA and ClpX—polypeptides that form a complex with the proteolytic subunit ClpP to yield an oligomeric ATP-dependent protease in E. coli (19, 20)—was increased by 6-fold on depletion of glucose from the medium. This observation concurs with an earlier report showing that expression of HSP78 was repressed 3- to 5-fold in cells grown on glucose medium (21). Although there is no known protein with significant sequence similarity to E. coli ClpP in yeast, we nevertheless investigated the possibility that Hsp78 might function in concert with other pro-
Fig. 7. Hsp78 activates the degradation of a $D^\prime$ mutant Ilv5p by the Pim1p protease. A, $\rho^0$ derivatives of wild-type strain 14CWW (WT), a $D^\prime$ mutant W327R, or isogenic strains carrying a null-mutation in the HSP78 gene (hsp78A), were grown to mid-log phase in raffinose medium and the relative amount of Ilv5p in cell extracts from each strain was then analyzed by Western blotting using porin as a loading control. B, a plasmid containing Hsp78 whose expression is under the constitutive TEF promoter (pTEF-HSP78) or a control, empty plasmid (pRS416), were transformed into $\rho^0$ derivatives of wild-type strain 14CWW (WT), or a $D^\prime$ mutant W327R and grown to mid-log phase in glucose medium. The levels of Ilv5p were determined as in A. C, $\rho^0$ derivatives of wild-type strain 14CWW (WT), a $D^\prime$ mutant W327R, or isogenic strains carrying a null-mutation of the PIM1 gene (pim1A) were grown to mid-log phase in raffinose medium and analyzed as in A.

Degradation of Ilv5p Mutants by Hsp78 and Pim1p

A major protease of S. cerevisiae mitochondrial matrix proteins is the Pim1p protease, which is an ortholog of the Lon protease in E. coli (22). Besides its protease function, Pim1p is also required for the maintenance of $\rho^0$ mtDNA (23). To determine whether Pim1p is involved in the degradation of a $D^\prime$ mutant Ilv5p, we deleted the PIM1 gene in wild-type and a $D^\prime$ mutant W327R $\rho^0$ strains and compared the abundance of the wild-type and mutant Ilv5p by Western blotting of extracts from cells grown in raffinose medium. This experiment (Fig. 7C) shows that deletion of PIM1 completely reverses the instability of the a $D^\prime$ mutant Ilv5p in $\rho^0$ cells grown in raffinose medium, indicating that Pim1p is responsible for the proteolytic degradation of a $D^\prime$ mutant Ilv5p. Because PIM1 expression is not glucose-repressible (data not shown), these data further suggest that Hsp78 cooperates with Pim1p for protease activity.

DISCUSSION

In this study, we show that a $D^\prime$ Ilv5p mutant proteins, which give rise to a $\rho^0$ mtDNA instability phenotype, have a dramatically altered localization within mitochondria compared with wild-type and a $D^\prime$ proteins. We also show that the a $D^\prime$ Ilv5p mutants are selectively degraded in $\rho^0$ and $\rho^+$ petite cells grown on a non-repressing, fermentable carbon source. This selective degradation is determined by the ClpB ortholog, Hsp78, a glucose-repressible mitochondrial chaperone, and the Pim1p protease, an ortholog of the E. coli Lon protease. Our results suggest that Pim1p works in concert with Hsp78 and implicate a role for wild-type mtDNA in modulating Pim1p protease activity.

A Relation between a $D^\prime$ Mutant Ilv5p Organization and mtDNA Stability—The a $D^\prime$ Ilv5p-GFP's are distributed in mitochondria as a few (5–10) punctate structures, in striking contrast to the largely reticular distribution of wild-type and a $D^\prime$ Ilv5p-GFPs. A number of observations support the conclusion that the $\rho^0$ mtDNA instability phenotype associated with the a $D^\prime$ Ilv5p mutants is a direct consequence of the unusual organization of these proteins within mitochondria. First, the punctate structures are not observed for wild-type or a $D^\prime$ mutant forms of Ilv5p in which $\rho^0$ mtDNA is stable. Second, the weak a $D^\prime$ mutant Ilv5p-GFP has a more reticular distribution underlying its punctate foci than does the strong a $D^\prime$ mutant. Finally, co-expression of wild-type and a $D^\prime$ mutant proteins, which results in a suppression of the $\rho^0$ mtDNA instability phenotype and temperature sensitivity of a $D^\prime$ cells grown on a non-fermentable carbon source (10), also results in a redistribution of the mutant protein from a punctate to a largely reticular structure. These observations correlate well with the difference in biochemical fractionation between the a $D^\prime$ class of Ilv5p mutant proteins and that of wild-type and the a $D^\prime$ Ilv5p mutant: both of the a $D^\prime$ mutant proteins have a marked decrease in detergent solubility compared with wild-type and a $D^\prime$ mutant Ilv5p. The extent of the insolubility of Ilv5p in the strong and weak mutants correlates with the observed differences in their punctate distribution in mitochondria and their mtDNA instability phenotypes. These effects can be explained in part by the propensity of the a $D^\prime$ mutant proteins to self-associate, probably into aggregates, which may be disrupted by interaction with wild-type Ilv5p. Finally, in cells expressing the a $D^\prime$ mutant proteins, mitochondrial morphology appears normal, making it unlikely that the mtDNA instability phenotype associated with these mutant proteins is caused by the known mtDNA instability observed in cells with gross defects in mitochondrial morphology (24).

What is the mechanism by which the aberrant organization of a $D^\prime$ mutant proteins leads to the instability of $\rho^0$ mtDNA? It is unlikely that the presence of a $D^\prime$ mutant Ilv5p in mito-
chondria leads to a complete block in the segregation of nucleoids because a "D" mutant Ilv5p is in a fermentable carbon source and produces stable ρ− petites (10). We showed previously that the affected residues in a "D" ilv5 mutants lie on the surface of the protein and proposed that this might affect intermolecular interactions of the mutant proteins (10). In preliminary experiments, we have identified several proteins present in mtDNA nucleoids that are also associated with wild-type and a "D" mutant forms of Ilv5p. Moreover, the relative abundance of these nucleoids in the Ilv5p complexes differs depending on whether the complex was isolated from wild-type or a "D" mutant strains. Experiments are currently in progress to characterize further the composition and properties of these complexes. These altered interactions of the a "D" mutant forms of Ilv5p with other nucleoid proteins combined with its tendency to aggregate could reflect differences in mtDNA packing that might, for example, affect intramolecular mtDNA recombination events leading to the formation of ρ− mtDNAs. In this connection, we know from previous studies that when overexpressed, Ilv5p effectively suppresses the loss of ρ− mtDNA in cells lacking the mtDNA packaging protein, Abf2p (4), thus implicating a role for Ilv5p in the organization of mtDNA.

Factors Affecting the Stability of a "D" Mutant Ilv5p—We found unexpectedly that a "D" mutant Ilv5p forms are unstable in petite cells grown under derepressing conditions. The instability of a "D" mutant Ilv5p is not just a consequence of respiratory deficiency because the mutant protein was stable in ρ− cells grown in the presence of antimycin A or oligomycin. Interestingly, unlike the mtDNA-instability phenotype, the instability of a "D" mutant Ilv5p appeared to be unrelated to the punctate organization of the protein, because it was equally unstable when dispersed in mitochondria by co-expression with wild-type Ilv5p.

The degradation of a "D" mutant Ilv5p in petite cells is activated by Hsp78, a member of the Clp/Hsp100 family of heat shock proteins (21). We suspected that Hsp78 might be involved in the degradation of a "D" mutant Ilv5p because of its high degree of similarity to ClpA, a regulatory ATP-dependant subunit of the E. coli Clp protease, and because the expression of Hsp78 is glucose-repressible (18, 21). Before this study, the only known in vivo mitochondrial functions for Hsp78 were its requirement for mitochondrial thermotolerance under extreme heat stress (25) and the observation that it could substitute for a defective mt-Hsp70 in an sac1-3 mutant (26). Although the a "D" Ilv5p mutant protein is only marginally stabilized in petite cells with an hsp78Δ mutation, constitutive expression of HSP78 in glucose-repressed petite cells results in the clear destabilization of the mutant Ilv5p. These data provide direct evidence for a role of HpsΔ in the degradation of a "D" Ilv5p and suggest that under derepressing conditions, one or more other chaperones can substitute for Hsp78. Cooperation of Hsp78 with other heat-induced chaperones was previously suggested in its role in reactivation of the mitochondrial protein synthesis apparatus after heat stress (25). Hsp78 has also been shown to be capable of substituting for Hsp104 in mediating cellular thermotolerance when misexpressed in the cytosol (25), suggesting that, like Hsp104, Hsp78 may act to disassemble insoluble protein complexes (27). However, Hsp78 is not acting in this way for a "D" mutant Ilv5p, because Hsp78 is still necessary for degradation of the mutant protein when it is largely disaggregated when co-expressed with wild-type Ilv5p. A likely scenario is that the chaperone activity of Hsp78 alters the conformation of a "D" mutant Ilv5p, so that it becomes a target for Pim1p protease activity.

Deletion of PIM1 completely eliminated the instability of a "D" mutant Ilv5p in petite cells grown in raffinose medium. This finding establishes that Pim1p can degrade an abnormal isoform of an endogenous protein in vivo. Pim1p has been shown to be involved in the turnover of some unassembled subunits of mitochondrial complexes such as the F1 ATPase and mitochondrial ribosomal proteins (23, 28), and in vitro studies have shown that Pim1p degrades chimeric and foreign misfolded proteins (29, 30). With a "D" mutant Ilv5p, Pim1p is likely to be recognizing a subtle alteration in the mutant protein rather than an aggregated population, because it is still degraded when largely disaggregated by the wild-type protein. Indeed, it is unlikely that the a "D" mutant Ilv5p is grossly misfolded, because it retains its enzymatic activity in branched chain amino acid biosynthesis (10) and interacts with wild-type Ilv5p when co-expressed. Aside from the turnover of unassembled or aberrant proteins, the proteolytic activity of Pim1p is required for the stability of ρ− mtDNA (pim1Δ cells produce ρ− petites (22, 23, 31) and for the expression of the mitochondrial COX1 and COB genes (32, 33). In most yeast strains, these genes contain introns, some of which encode maturases that function in the splicing of the intron that encodes them. The requirement for Pim1p in the expression of these genes occurs at multiple levels, including maturase-dependent splicing, RNA stability, and translation (32). Although it is not clear how Pim1p functions in these diverse activities, or in maintaining the integrity of ρ− mtDNA, these findings suggest that there is a significant degree of control over the substrate specificity and activity of the Pim1p protease. Our findings add new dimensions to this control that include the participation of Hsp78 and ρ− mtDNA in regulating Pim1p activity. Additional studies will be required to determine whether these effects occur by direct modulation of Pim1p protease activity or by substrate presentation. Finally, it is intriguing that, like the Lon protease in E. coli (34, 35), the human ortholog of PIM1 has been demonstrated to bind DNA, specifically to single-stranded sequences in mtDNA (36). These observations have led to speculation that Lon proteases might use DNA binding to control mtDNA metabolism by degrading regulatory proteins at sites adjacent to promotors (33). It is reasonable to imagine therefore that an interaction between mtDNA and Pim1p could modulate its activity and substrate specificity.

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REFERENCES

1. Contamine, V., and Picard, M. (2000) Microbiol. Mol. Biol. Rev. 64, 281–315
2. Kakar, S. N., and Wagner, R. P. (1964) Genetics 59, 213–222
3. Petersen, J. G. L., and Holmberg, S. (1986) Nucleic Acids Res. 14, 9631–9651
4. Zelenaya-Troitskaya, O., Perlman, P. S., and Butow, R. A. (1995) EMBO J. 14, 3286–3276
5. Difflay, J. F., and Stillman, B. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 7984–7988
6. Megrav, T. L., and Chae, C. B. (1993) J. Biol. Chem. 268, 12725–12763
7. Newman, S. M., Zelenaya-Troitskaya, O., Perlman, P. S., and Butow, R. A. (1995) Nucleic Acids Res. 23, 386–393
8. Zelenaya-Troitskaya, O., Newman, S. M., Okamoto, K., Perlman, P. S., and Butow, R. A. (1998) Genetics 148, 1763–1776
9. MacAlpine, D. M., Perlman, P. S., and Butow, R. A. (2000) EMBO J. 19, 767–775
10. Bateman, J. M., Perlman, P. S., and Butow, R. A. (2002) Genetics 161, 1043–1052
11. Bieza, V., Dumas, R., Cohen-Addad, C., Deuce, R., Job, D., and Pelay-Peyroula, E. (1997) EMBO J. 16, 3405–3415
12. Herskovitz, I., and Jensen, R. E. (1991) Methods Enzymol. 194, 132–146
13. Okamoto, K., Perlman, P. S., and Butow, R. A. (1998) J. Cell Biol. 142, 613–623
14. Mumberg, D., Muller, R., and Funk, M. (1995) Gene 156, 119–122
15. Kaufman, B. A., Newman, S. M., Hallberg, R. L., Sluiter, C. A., Perlman, P. S., and Butow, R. A. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 7772–7777
16. Rothermel, B. A., Shyam, A. W., Etheridge, J. L., and Butow, R. A. (1995) J. Biol. Chem. 270, 28476–28482
17. Osg, M., St. John, R., and Nagai, S. (1957) Science 125, 928–929
18. DeRisi, J. L., Iyer, V. R., and Brown, P. O. (1997) Science 278, 680–686
19. Hwang, B. J., Woo, K. M., Goldberg, A. L., and Chung, C. H. (1988) J. Biol.
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20. Kandror, O., Busconi, L., Sherman, M., and Goldberg, A. L. (1994) J. Biol. Chem. 269, 23575–23582
21. Leonhardt, S. A., Pearson, K., Danese, P. N., and Mason, T. L. (1993) Mol. Cell. Biol. 13, 6304–6313
22. Van Dyck, L., Pearce, D. A., and Sherman, F. (1994) J. Biol. Chem. 269, 23575–23582
23. Suzuki, C. K., Suda, K., Wang, N., and Schatz, G. (1994) Science 264, 273–276
24. Hermann, G. J., and Shaw, J. M. (1998) Annu. Rev. Cell Dev. Biol. 14, 265–303
25. Schmitt, M., Neupert, W., and Langer, T. (1996) J. Cell Biol. 134, 1375–1386
26. Schmitt, M., Neupert, W., and Langer, T. (1995) EMBO J. 14, 3434–3444
27. Suzuki, C. K., Rep, M., van Dijl, J. M., Suda, K., Grivell, L. A., and Schatz, G. (1997) Trends Biochem. Sci. 22, 118–123
28. van Dijl, J. M., Kutejova, E., Suda, K., Perecko, D., Schatz, G., and Suzuki, C. K. (1998) Proc. Natl Acad. Sci. U. S. A. 95, 10584–10589
29. Wagner, I., Arlt, H., van Dyck, L., Langer, T., and Neupert, W. (1994) EMBO J. 13, 5135–5145
30. Saveliev, A. S., Novikova, L. A., Kovaleva, I. E., Luzikov, V. N., Neupert, W., and Langer, T. (1998) J. Biol. Chem. 273, 20596–20602
31. Wagner, I., van Dyck, L., Saveliev, A. S., Neupert, W., and Langer, T. (1997) EMBO J. 16, 7317–7325
32. van Dyck, L., Neupert, W., and Langer, T. (1998) Genes Dev. 12, 1515–1524
33. Van Dyck, L., and Langer, T. (1999) Cell Mol. Life Sci. 56, 825–842
34. Chung, C. H., and Goldberg, A. L. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 795–799
35. Fu, G. K., Smith, M. J., and Markowitz, D. M. (1997) J. Biol. Chem. 272, 531–534
36. Fu, G. K., and Markowitz, D. M. (1998) Biochemistry 37, 1905–1909
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