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Escape of mitochondrial DNA to the nucleus in *yme1* yeast is mediated by vacuolar-dependent turnover of abnormal mitochondrial compartments

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SUMMARY

Inactivation of Yme1p, a mitochondrially-localized ATP-dependent metallo-protease in the yeast *Saccharomyces cerevisiae*, causes a high rate of DNA escape from mitochondria to the nucleus as well as pleiotropic functional and morphological mitochondrial defects. The evidence presented here suggests that the abnormal mitochondria of a *yme1* strain are degraded by the vacuole. First, electron microscopy of Yme1p-deficient strains revealed mitochondria physically associated with the vacuole via electron dense structures. Second, disruption of vacuolar function affected the frequency of mitochondrial DNA escape from *yme1* and wild-type strains. Both *PEP4* or *PRC1* gene disruptions resulted in a lower frequency of mitochondrial DNA escape. Third, an in vivo assay that monitors vacuole-dependent turnover of the mitochondrial compartment demonstrated an increased rate of mitochondrial turnover in *yme1* yeast when compared to the rate found in wild-type yeast. In this assay, vacuolar alkaline phosphatase, encoded by *PHO8*, was targeted to mitochondria in a strain bearing disruption to the genomic *PHO8* locus. Maturation of the mitochondrially localized alkaline phosphatase pro-enzyme requires proteinase A, which is localized in the vacuole. Therefore, alkaline phosphatase activity reflects vacuole-dependent turnover of mitochondria. This assay reveals that mitochondria of a *yme1* strain are taken up by the vacuole more frequently than mitochondria of an isogenic wild-type strain when these yeast are cultured in medium necessitating respiratory growth. Degradation of abnormal mitochondria is one pathway by which mitochondrial DNA escapes and migrates to the nucleus.

Key words: Mitochondrial DNA escape, Vacuole, Autophagy, *Saccharomyces cerevisiae*, Mitochondria

INTRODUCTION

Alteration of the mitochondrial genetic system has had a major impact on the evolution of eucaryotic cells and continues to affect the viability and function of cells and organisms. The transfer of genetic information from mitochondria to the nucleus during the evolution of eucaryotes is evidenced by the presence of DNA sequences homologous to mitochondrial DNA (mtDNA) in a number of nuclear genomes and the presence of nuclear-encoded mitochondrial proteins that are predicted, based on phylogenetic analysis, to have a mitochondrial origin (reviewed by Thorsness and Weber, 1996). It has also been proposed that DNA of organellar origin may act as a mutagenic agent in the nucleus (Richter, 1988; Hadler, 1989; Shay and Werbin, 1992). Integration of escaped mtDNA in nuclear structural genes or regulatory regions could disrupt or alter vital cell processes. A recent report supports this assertion. In primary low-grade brain neoplasms, fluorescent in situ hybridization analysis found mtDNA localized in the nucleus in correlation with an overall increase in mtDNA content in the cell (Liang, 1996). This ontogenically early event is important in the etiology of these tumors. Similarly, in hepatoma cells mtDNA sequences are present in the nuclear genome at a higher copy number than found in normal tissue (Corral et al., 1989). Finally, alteration of mitochondrial function through mutation and rearrangement of mtDNA has been implicated in the pathogenicity of a large number of human diseases (Hatfill et al., 1993; Schapira, 1994).

To understand the process by which mtDNA escapes to the nucleus, a sensitive assay was developed that allows identification and analysis of genes that affect this process in the yeast *Saccharomyces cerevisiae* (Thorsness and Fox, 1990, 1993). In this assay, the normal locus for a nuclear gene was mutated so that the escape and migration of mtDNA containing a wild-type copy of the nuclear gene complements the mutant nuclear phenotype. Inactivation of any of a set of nuclear genes, designated *YME* (yeast mitochondrial escape), alters the frequency of mtDNA escape to the nucleus (Thorsness and Fox, 1993). Some of these mutations also severely impair mitochondrial function. The focus of this study is one such mutant gene, *yme1*.

In addition to a high rate of mtDNA escape to the nucleus, *yme1* yeast exhibit several phenotypes indicative of abnormal mitochondrial function. A *yme1* strain has temperature-sensitive growth defects on non-fermentable carbon sources at 37°C and enriched glucose medium at 14°C (Thorsness et al., 1993). Loss or deletion of the mitochondrial genome in *yme1* strains results in severely retarded growth (Thorsness et al.,

1993; Weber et al., 1995). In addition, for all growth temperatures and carbon sources, a *yme1* strain demonstrates abnormal mitochondrial morphology (Campbell et al., 1994). *YME1* encodes an ATP-dependent metalloprotease, Yme1p, located in the inner mitochondrial membrane (Nakai et al., 1995; Leonard et al., 1996; Weber et al., 1996). Although cytochrome oxidase subunit 2 (Cox2p) is the only confirmed substrate of Yme1p, the variety of *yme1* phenotypes clearly indicates that Yme1p acts on other proteins as well. Despite a relatively extensive understanding of Yme1p function, it has proven difficult to explain the increased rate of mtDNA escape that is observed in *yme1* strains. In this paper, we provide evidence that vacuolar degradation of mitochondria in *yme1* yeast is one pathway by which mtDNA escapes to the nucleus.

MATERIALS AND METHODS

Media

Escherichia coli containing plasmids were grown in LB (10 g/l bactotryptone, 10 g/l NaCl, 5 g/l yeast extract) plus 125 μg/ml ampicillin. Yeast (Table 1) were grown in YPD (20 g/l glucose, 20 g/l bactopeptone, 10 g/l yeast extract and 40 mg/l of tryptophan), YPEG (30 ml/l glycerol, 30 ml/l of ethanol, 20 g/l of bactopeptone, 10 g/l of yeast extract and 40 mg/l of tryptophan) or SD, SGal or SRaf + nutrients (6.7 g/l of yeast nitrogen base without amino acids, 20 g/l of glucose, galactose, or raffinose, respectively). SEG medium contained 6.7 g/l of yeast nitrogen base without amino acids, 30 ml/l of glycerol, and 30 ml/l ethanol. Nutrients included uracil at 40 mg/l, adenine at 40 mg/l, tryptophan at 40 mg/l, lysine at 60 mg/l, histidine at 30 mg/l or leucine at 100 mg/l. For agar plates, bactoagar was added at 20 g/l. Bactoagar, bactopeptone, bactotryptone, yeast extract and yeast nitrogen base without amino acids were obtained from Difco. Ampicillin and nutrients were obtained from Sigma.

Growth curves

PTY52 (*yme1-Δ1::URA3*) and the isogenic wild-type, PTY44, were grown to low density in liquid YPEG at 30°C, then split for culturing at 30°C or 37°C. Aliquots were removed at hourly time points and measured with a Klett colorimeter.

Oxygen consumption

Oxygen consumption was assayed using a Clarke-type oxygen electrode (Yellow Springs Instruments, Yellow Springs, OH). An equivalent cell density was assayed at each time point using the following method: Strains were initially grown in YPEG at 30°C to a cell density of 26 Klett units. At each time point, cells were diluted back to the Klett 26 density, using pre-warmed YPEG. A 10 ml aliquot was pelleted, resuspended in 10 ml fresh, 37°C YPEG, then assayed in triplicate. Assays were performed at the indicated temperature with 3 ml cells and a magnetic stir bar. The 0-100% scale on a chart recorder was calibrated at 0% with the addition of dithionite to water. The 100% mark was determined by bubbling water with atmospheric oxygen. Rates of oxygen consumption were read from the chart recordings as change in percent saturation per minute.

Oxygen consumption was calculated according to the following formula, using 210 nmol O_2/ml at 100% saturation for 37°C and 236 nmol O_2/ml for 30°C, where,

$$\Delta\%\ \text{saturation} \times \frac{1}{\text{min}} \times \frac{\text{nmol } O_2/\text{ml}}{\text{Klett}} = \text{nmol } O_2/\text{minute, Klett, ml}$$

at 100% saturation (Nakashima et al., 1984).

Electron microscopy

Strains were cultured in YPEG at 30°C and fixed according to methods described by Byers and Goetsch (1990) for transmission electron microscopy.

DNA escape assay

DNA escape from the mitochondria to the nucleus was assayed as described (Thorsness and Fox, 1993). Briefly, a tryptophan nutritional marker, *TRP1*, was inserted into the mitochondrial genome of a strain bearing a *trp1-Δ1* mutation of the nuclear locus. Yeast were first grown on enriched medium (YPD or YPEG), then transferred onto minimal medium lacking tryptophan (Trp^-). When transferred to Trp^- medium, the only yeast that grew were those in which a *TRP1*-bearing mtDNA fragment had escaped to the nucleus. Tryptophan prototrophs appear as papillae on the shadow of transferred cells.

Strain constructions

CCY13 was generated by combining the D273-10B [rho^+, *TRP1*] mitochondrial genome with the nuclear genome of FY251, a S288C derivative. This was achieved by mating FY251 [rho^0] to the karyogamy deficient strain MCC17 [rho^+, *TRP1*], then selecting for FY251 nuclear markers, and thus yielding CCY13. CCY14 was generated by transforming CCY13 with the *Hin*dIII/*Bgl*II fragment of pPT45, containing *yme1-Δ1::URA3* (Thorsness and Fox, 1993), and screening uracil prototrophs for those that displayed *yme1* phenotypes. CCY28 and CCY29 are haploid progeny of a cross between YPH500 [rho^0] and CCY13. CCY30 was derived from CCY28 and CCY29 via a multi-step process. First, the *PHO8* locus was disrupted in CCY29 by transformation with an *Eco*RI fragment bearing *pho8Δ::URA3* from pAR2 (provided by Janet Shaw). Next, *PHO13* was disrupted by transformation of CCY28 with an *Eco*RI fragment bearing *pho13Δ::URA3* from pPH13 (Kaneko et al., 1989). Then, CCY29 *pho8Δ::URA3* was mated to CCY28 *pho13Δ::URA3* and a Ura^+ haploid was chosen from a tetrad bearing 2:2 Ura^+:Ura^- segregants and named CCY30. All *pho8* and *pho13* strains were confirmed by assaying for endogenous ALP activity using a *p*-nitrophenylphosphate (Kaneko et al., 1982) or an α-naphthylphosphate (Kaneko et al., 1985) colony overlay assay. CCY31 was generated by disrupting *YME1* in CCY29, as described above, then mating to CCY30. A haploid, CCY31, was again chosen from a tetrad bearing 2:2 Ura^+:Ura^- segregants.

The *pep4* mutation was introduced into each relevant strain by transforming with a *Bam*HI fragment of pTS17 bearing *pep4Δ::LEU2* (provided by Janet Shaw). The *prc1* mutation was introduced by transformation with an *Eco*RI/*Sal*I fragment from pJL1 bearing *prc1Δ::HIS3* (provided by Janet Shaw). All *pep4* and *prc1* strains were confirmed by a carboxypeptidase Y well test (Jones, 1991).

Recombinant DNA manipulations

The mitochondrially-targeted *PHO8Δ60* (ALP^m) expression vector was constructed by inserting the 0.81 kb *Eco*RV fragment of *ADH1-COXIV* from p*COXIV*wt (Pinkham et al., 1994) into the *Eco*RV site of pRS313 (Sikorski and Hieter, 1989) in which the *Xba*I site had been destroyed. Then a PCR fragment corresponding to *PHO8Δ60* (*PHO8* gene deleted of the first 60 codons) was ligated in-frame to the *COXIV* leader at the *Xba*I and *Bam*HI sites, generating the plasmid pCC4. The 5′ *PHO8Δ60* PCR primer was GGTCT AGA TCT GCA TCA CAC AAG AAG AAG AAT GTC ATA TTC TTC GTG and the 3′ *PHO8Δ60* PCR primer was GGG GAT CCG GGA GAG TTA GAT AGG ATC AG. A plasmid designed to express cytoplasmically-localized ALP, pCC5, was generated by excising the *COXIV* leader from pCC4 by *Eco*RI/*Xba*I cleavage and re-ligating with a linker containing an ATG start codon. Two single-stranded oligos were used for the linker: AATTC ACC ATG G and GTGG TAC CGA TC. Both the pCC4 and pCC5 constructions were confirmed by DNA sequencing.

Cell fractionation

Mitochondria were isolated from strains grown in minimal galactose medium (SGal) (Yaffe, 1991). Vacuoles were isolated from strains grown in YPD or YPEG (Roberts et al., 1991). Protein content of cell

Table 1. Yeast strains

Strain	Genotype	Parent strain	Source
FY251	*MAT**a** ura 3-52 his3-Δ200 leu2Δ1 trp1Δ63*	–	Janet Shaw
MCC17	*MATα ura3-52 ade2-101 kar1-1 [ρ+, TRP1]**	–	Maria Costanzo
YPH500	*MAT**a** ura3-52 lys2-801 ade2-101 trp1Δ63 his 3-Δ200 leu2Δ1*	–	Sikorski and Heiter, 1989
NTY1	*MATα ura3-52 lys2 leu2-3,112 trp1-Δ1 yme1-Δ1:URA3 ynt1-1 [ρ+, TRP1]*	PTY52	Campbell et al., 1994
CCY11	*MATα ura3-52 lys2 leu2-3,112 trp1-Δ1 pep4Δ::LEU2 [ρ+, TRP1]*	PTY44	This study
CCY12	*MATα ura3-52 lys2 leu2-3,112 trp1-Δ1 yme1-Δ1::URA3 pep4Δ::LEU2 [ρ+, TRP1]*	PTY52	This study
CCY13	*MAT**a** ura3-52 his3-Δ200 leu2Δ1 trp1Δ63 [ρ+, TRP1]*	FY251	This study
CCY14	*MAT**a** ura3-52 his3-Δ200 leu2Δ1 trp1Δ63 yme1-Δ1::URA3 [ρ+, TRP1]*	CCY13	This study
CCY15	*MATα ura3-52 lys2 leu2-3,112 trp1-Δ1 yme2-1 pep4Δ::LEU2 [ρ+, TRP1]*	PTY64	This study
CCY16	*MATα ura3-52 lys2 leu2-3,112 trp1-Δ1 yme3-1 pep4Δ::LEU2 [ρ+, TRP1]*	PTY66	This study
CCY17	*MATα ura3-52 lys2 leu2-3,112 trp1-Δ1 yme4-1 pep4Δ::LEU2 [ρ+, TRP1]*	PTY68	This study
CCY18	*MATα ura3-52 lys2 leu2-3,112 trp1-Δ1 yme5-1 pep4Δ::LEU2 [ρ+, TRP1]*	PTY70	This study
CCY19	*MATα ura3-52 lys2 leu2-3,112 trp1-Δ1 yme6-1 pep4Δ::LEU2 [ρ+, TRP1]*	PTY72	This study
CCY20	*MATα ura3-52 lys2 leu2-3,112 trp1-Δ1 yme7-1 pep4Δ::LEU2 [ρ+, TRP1]*	PTY122	This study
CCY21	*MATα ura3-52 lys2 leu2-3,112 trp1-Δ1 yme8-1 pep4Δ::LEU2 [ρ+, TRP1]*	PTY124	This study
CCY22	*MATα ura3-52 lys2 leu2-3,112 trp1-Δ1 yme9-1 pep4Δ::LEU2 [ρ+, TRP1]*	PTY126	This study
CCY23	*MAT**a** ura3-52 his3-Δ200 leu2Δ1 trp1Δ63 pep4Δ::LEU2 [ρ+, TRP1]*	CCY13	This study
CCY24	*MAT**a** ura3-52 his3-Δ200 leu2Δ1 trp1Δ63 yme1-Δ1::URA3 pep4Δ::LEU2 [ρ+, TRP1]*	CCY14	This study
CCY25	*MAT**a** ura3-52 his3-Δ200 leu2Δ1 trp1Δ63 prc1Δ::HIS3 [ρ+, TRP1]*	CCY13	This study
CCY26	*MAT**a** ura3-52 his3-Δ200 leu2Δ1 trp1Δ63 yme1-Δ1::URA3 prc1Δ::HIS3 [ρ+, TRP1]*	CCY14	This study
CCY28	*MAT**a** ura 3-52 ade2-101 his 3-Δ200 leu2Δ1 trp1Δ63 [ρ+, TRP1]*	–	This study
CCY29	*MATα ura 3-52 lys2-801 his 3-Δ200 leu2Δ1 trp1Δ63 [ρ+, TRP1]*	–	This study
CCY30	*MAT**a** ura 3-52 ade2-101 his 3-Δ200 leu2Δ1 trp1Δ63 pho8Δ::URA3 pho13Δ::URA3 [ρ+, TRP1]*	–	This study
CCY31	*MAT**a** ura 3-52 ade2-101 his 3-Δ200 leu2Δ1 trp1Δ63 pho8Δ::URA3 pho13Δ::URA3 yme1-Δ1::URA3 [ρ+, TRP1]*	–	This study
CCY32	*MAT**a** ura 3-52 ade2-101 his 3-Δ200 leu2Δ1 trp1Δ63 pho8Δ::URA3 pho13Δ::URA3 pep4Δ ::LEU2 [ρ+, TRP1]*	CCY30	This study
CCY33	*MAT**a** ura 3-52 ade2-101 his 3-Δ200 leu2Δ1 trp1Δ63 pho8Δ::URA3 pho13Δ::URA3 yme1-Δ1::URA3 pep4Δ ::LEU2 [ρ+, TRP1]*	CCY31	This study
CCY40	*MAT**a** ura 3-52 ade2-101 his 3-Δ200 leu2Δ1 trp1Δ63 pho8Δ::URA3 pho13Δ::URA3 prc1Δ ::LEU2 [ρ+, TRP1]*	CCY30	This study
CCY41	*MAT**a** ura 3-52 ade2-101 his 3-Δ200 leu2Δ1 trp1Δ63 pho8Δ::URA3 pho13Δ::URA3 yme1-Δ1::URA3 prc1Δ ::LEU2 [ρ+, TRP1]*	CCY31	This study
PTY44	*MATα ura3-52 lys2 leu2-3,112 trp1-Δ1 [ρ+, TRP1]*	–	Thorsness and Fox, 1993
PTY52	*MATα ura3-52 lys2 leu2-3,112 trp1-Δ1 yme1-Δ1::URA3 [ρ+, TRP1]*	–	Thorsness and Fox, 1993
PTY64	*MATα ura3-52 lys2 leu2-3,112 trp1-Δ1 yme2-1 [ρ+, TRP1]*	–	Thorsness and Fox, 1993
PTY66	*MATα ura3-52 lys2 leu2-3,112 trp1-Δ1 yme3-1 [ρ+, TRP1]*	–	Thorsness and Fox, 1993
PTY68	*MATα ura3-52 lys2 leu2-3,112 trp1-Δ1 yme4-1 [ρ+, TRP1]*	–	Thorsness and Fox, 1993
PTY70	*MATα ura3-52 lys2 leu2-3,112 trp1-Δ1 yme5-1 [ρ+, TRP1]*	–	Thorsness and Fox, 1993
PTY72	*MATα ura3-52 lys2 leu2-3,112 trp1-Δ1 yme6-1 [ρ+, TRP1]*	–	Thorsness and Fox, 1993
PTY122	*MATα ura3-52 lys2 leu2-3,112 trp1-Δ1 yme7-1 [ρ+, TRP1]*	–	This study
PTY124	*MATα ura3-52 lys2 leu2-3,112 trp1-Δ1 yme8-1 [ρ+, TRP1]*	–	This study
PTY126	*MATα ura3-52 lys2 leu2-3,112 trp1-Δ1 yme9-1 [ρ+, TRP1]*	–	This study

*Mitochondrial genome is shown in brackets.

fractions was determined by Coomassie protein assay (Pierce). 5 μg of post-mitochondrial supernatant or purified mitochondria and 20 μg purified vacuoles were separated on 8% polyacrylamide gels, then transferred to nitrocellulose. Proteins were detected by ECL chemiluminescence (Amersham) using polyclonal rabbit anti-Yme2p, rabbit anti-ALP (Klionsky and Emr, 1990), and mouse anti-carboxypeptidase Y (Molecular Probes).

ALP activity assays

ALP activity assays were performed according to the method of Toh-e et al. (1976) and were expressed as Miller Units:

$$\text{units activity, minute}^{-1}, \text{OD}_{600}^{-1} = 1000*(A_{420}\text{-}1.06A_{550})/0.993*\text{minute}*\text{volume}*\text{OD}_{600}/\text{ml}.$$

RESULTS

yme1 yeast have abnormal mitochondrial compartments

Yeast strains bearing a null mutation of the *YME1* locus have multiple phenotypes indicative of abnormal mitochondrial compartments. These yeasts have a temperature-sensitive growth defect when cultured on a carbon source that requires respiratory growth (Fig. 1A) (Thorsness et al., 1993). In addition, these yeasts show deficient oxygen consumption compared to isogenic wild-type yeast at 37°C (Fig. 1B), indicating compromised mitochondrial function.

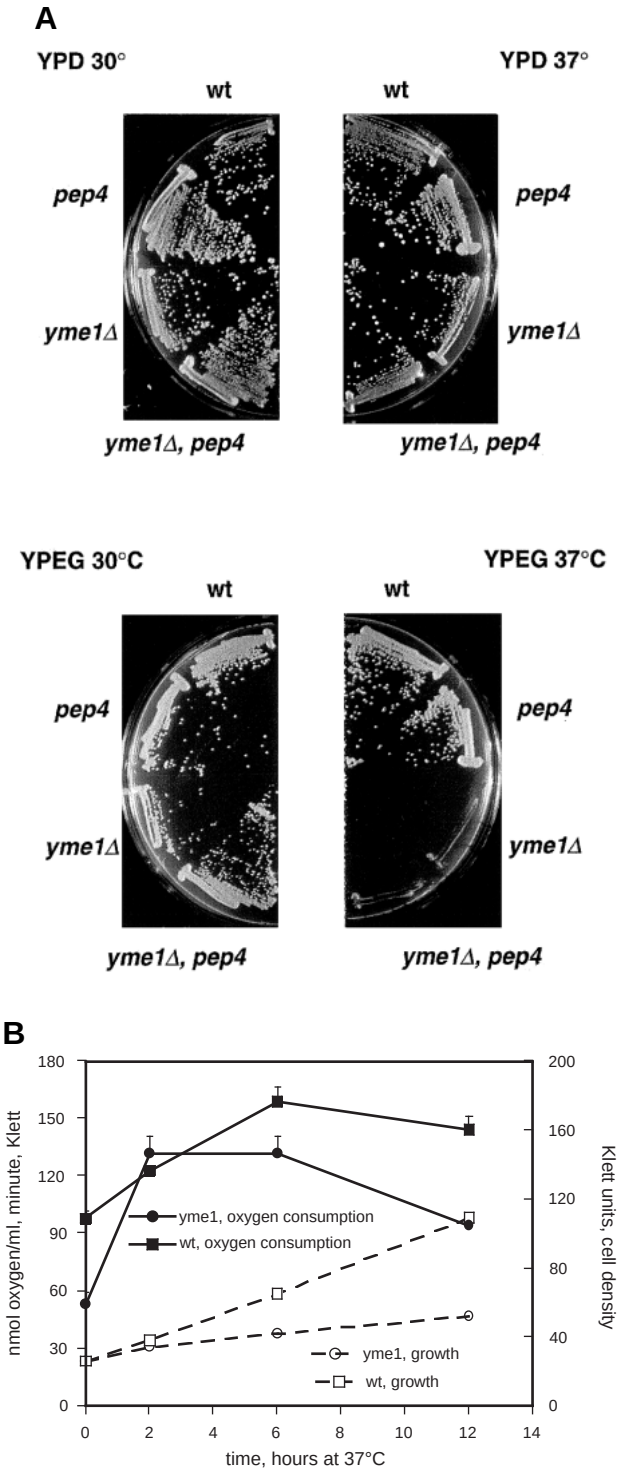

Fig. 1. *yme1* yeast have abnormal mitochondrial function. (A) Temperature-sensitive growth of *yme1* and *yme1 pep4* strains. Strains were streaked on YPEG or YPD , then incubated at 30°C or 37°C. Strains: wt, PTY44; *pep4*, CCY11; *yme1Δ*, PTY52; *yme1Δ pep4*, CCY12. (B) Oxygen consumption is diminished in a *yme1* strain. Strains were grown in YPEG at 30°C to an early logarithmic cell density of Klett=26. Oxygen consumption was determined at 30°C for t=0. Cultures were shifted to 37°C, and oxygen consumption rates were assayed at this temperature for all other time points. Error bar indicates standard deviation. At each time point, cell density was determined by a Klett meter. Strains: wt, PTY44; *yme1*, PTY52.

Yeast strains lacking *YME1* also display mitochondrial morphological abnormalities (Fig. 2) (Campbell et al., 1994). During logarithmic growth in non-fermentable medium, a *yme1* strain has pleiomorphic mitochondria, ranging from abbreviated branched structures to grossly swollen forms, in contrast to the elongated and reticulated network found in wild type. When stained with the cationic dye, DAPSMI (2-(4-dimethylaminostyryl)-1-methylpyridinium iodide), about 12% of *yme1* yeast contain one or two swollen organelles that fluoresce orange-red in contrast to the typical yellowish staining (Campbell et al., 1994), indicating an alteration of protein/lipid composition (Bereiter-Hahn, 1976; Bereiter-Hahn and Voth, 1994).

Abnormal mitochondria in *yme1* strains are associated with the vacuole

Transmission electron micrography shows that mitochondria of a *yme1* strain are at times physically associated with the vacuole, in addition displaying morphological abnormalities (Fig. 2B and C). Electron micrographs of yeast cell cross-sections revealed that mitochondria were found in close proximity to vacuoles in 15% of wild-type yeast and 21% of *yme1* yeast (120 cross-sections of each strain were scored). Although mere proximity of the organelle to the vacuole is not indicative of an uptake event, only mitochondria in *yme1* yeast were observed to have an unusual pinched morphology adjacent to the vacuole that displayed an electron dense structure bridging the two organelles. These mitochondrial/vacuolar structures were observed in 5% of the *yme1* cellular cross-sections. Similar structures were also seen for a double mutant strain, *yme1 ynt1-1* (Fig. 2D). The mutant allele of *YNT1* was originally identified as a bypass suppressor of all *yme1* phenotypes (Campbell et al., 1994). However, the electron micrographic data indicate that this suppressor mutation does not affect association of mitochondrial forms with the vacuole. The possible nature of these vacuole/mitochondrial interactions prompted an investigation of the contribution of vacuolar activity to the increased rate of mtDNA escape in *yme1* yeast.

Inactivation of vacuolar proteases affects the rate of mtDNA escape

To investigate the authenticity of the mitochondrial-vacuolar interactions in *yme1* yeast, mutations were introduced that were expected to interfere with the ability of the yeast vacuole to degrade mitochondria. Proteinase A, encoded by *PEP4*, is a major hydrolase of the yeast vacuole and is required for maturation of several vacuolar enzymes. Strains lacking proteinase A have reduced activity of carboxypeptidase Y, proteinase B, vacuolar RNase, alkaline phosphatase, and trehalase (Mechler et al., 1988; Klionsky and Emr, 1989; Sorensen et al., 1994). A *pep4* disruption lowered the frequency of mtDNA escape in a *yme1* strain (Fig. 3A). Similarly, disruption of *PRC1*, which encodes carboxypeptidase Y, also decreased the rate of mtDNA escape in *yme1* yeast. In contrast, inactivation of proteinase B by disruption of its structural gene, *PRB1*, did not affect the rate of mtDNA escape in the PTY44 wild-type strain background or in the isogenic *yme1* strain (data not shown). Interestingly, the suppression of mtDNA escape by *pep4* and *prc1* mutations in *yme1* yeast was only evident when cells were first grown on

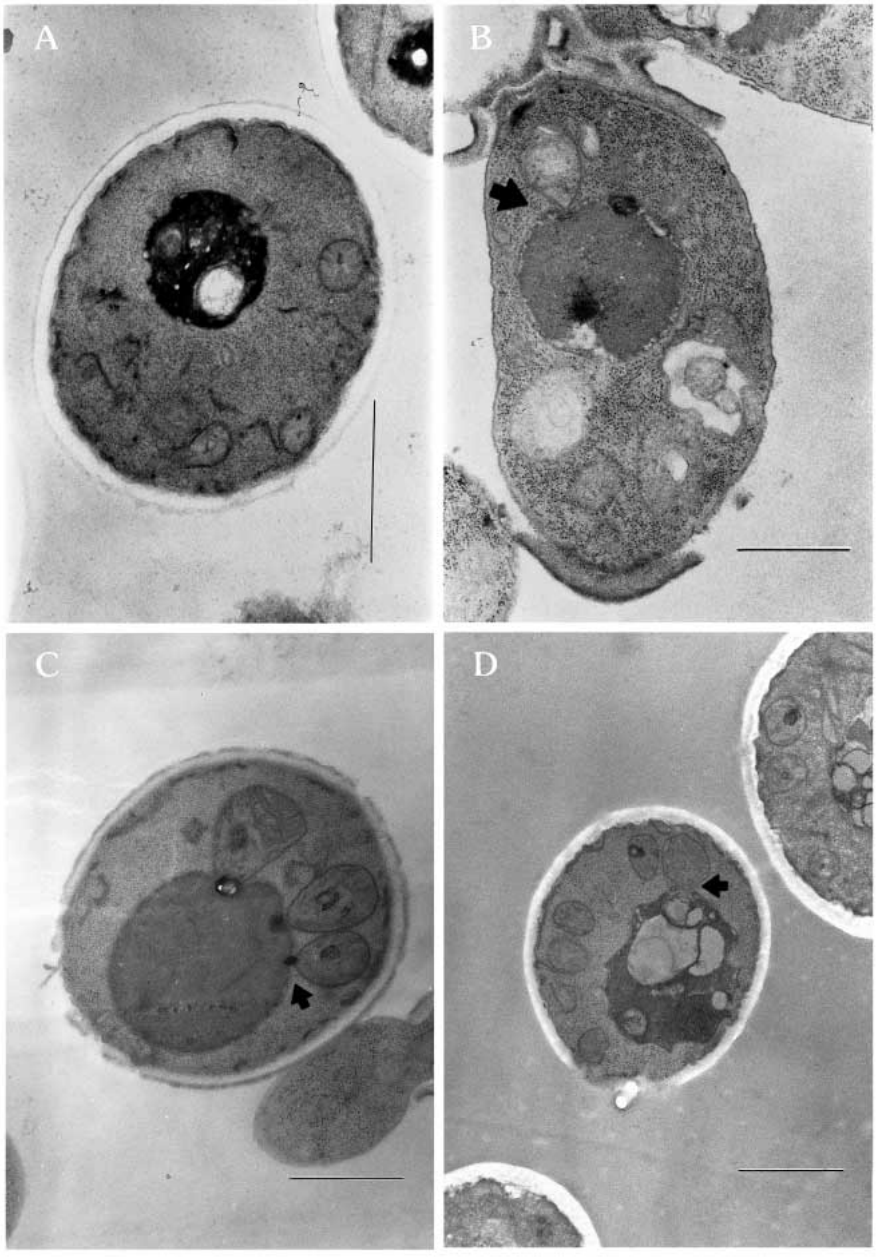

Fig. 2. Transmission electron micrographs of wild-type, *yme1*, and *yme1 ynt1* strains. (A) The wild-type strain, PTY44. (B and C) The *yme1* strain, PTY52. (D) The *yme1 ynt1* strain, NTY1. All strains are isogenic. Arrows denote pinched mitochondria adjacent to the vacuole. Bars, 1 μm.

ethanol/glycerol medium prior to transfer to minimal medium lacking tryptophan. *yme1* and *yme1 pep4* strains that are grown on glucose prior to transfer to minimal medium lacking tryptophan have a similar rate of mtDNA escape (Fig. 3B). Growth of *yme1* strains on medium that necessitates respiration increases the rate of mtDNA escape beyond the already elevated rate, as compared to wild type, that is seen when yeast are cultivated on glucose (data not shown). This stimulation of mtDNA escape in *yme1* strains when grown on nonfermentable carbon sources is apparently dependent upon vacuolar proteases.

Inactivation of *PEP4* did not suppress the collateral growth phenotypes of *yme1*, which include an inability to utilize nonfermentable carbon sources at 37°C (Fig. 1A), cold sensitive growth on rich glucose medium, and slow growth when mtDNA is completely absent from the cell (data not shown). Inactivation of *PEP4* does not alter the rate of mtDNA escape in an isogenic wild-type strain (Fig. 3A) or in strains bearing mutation of *yme2* through *yme9* (Fig. 3C).

Vacuolar-dependent activation of a recombinant mitochondrial protein in *yme1* yeast

Several different plasma membrane proteins are internalized to the vacuole under certain conditions (Egner et al., 1995; Riballo et al., 1995; Egner and Kuchler, 1996; Robinson et al., 1996). These proteins were detected in vacuole fractions of *pep4* strains using immunological techniques. Inactivation of proteinase A decreases the protease activity in vacuoles, subsequently stabilizing the proteins and allowing their detection. Similar efforts to detect mitochondrial proteins in vacuole fractions were undertaken for wild-type, *pep4*, *yme1*, as well as *yme1 pep4* strains. Despite extensive fractionation

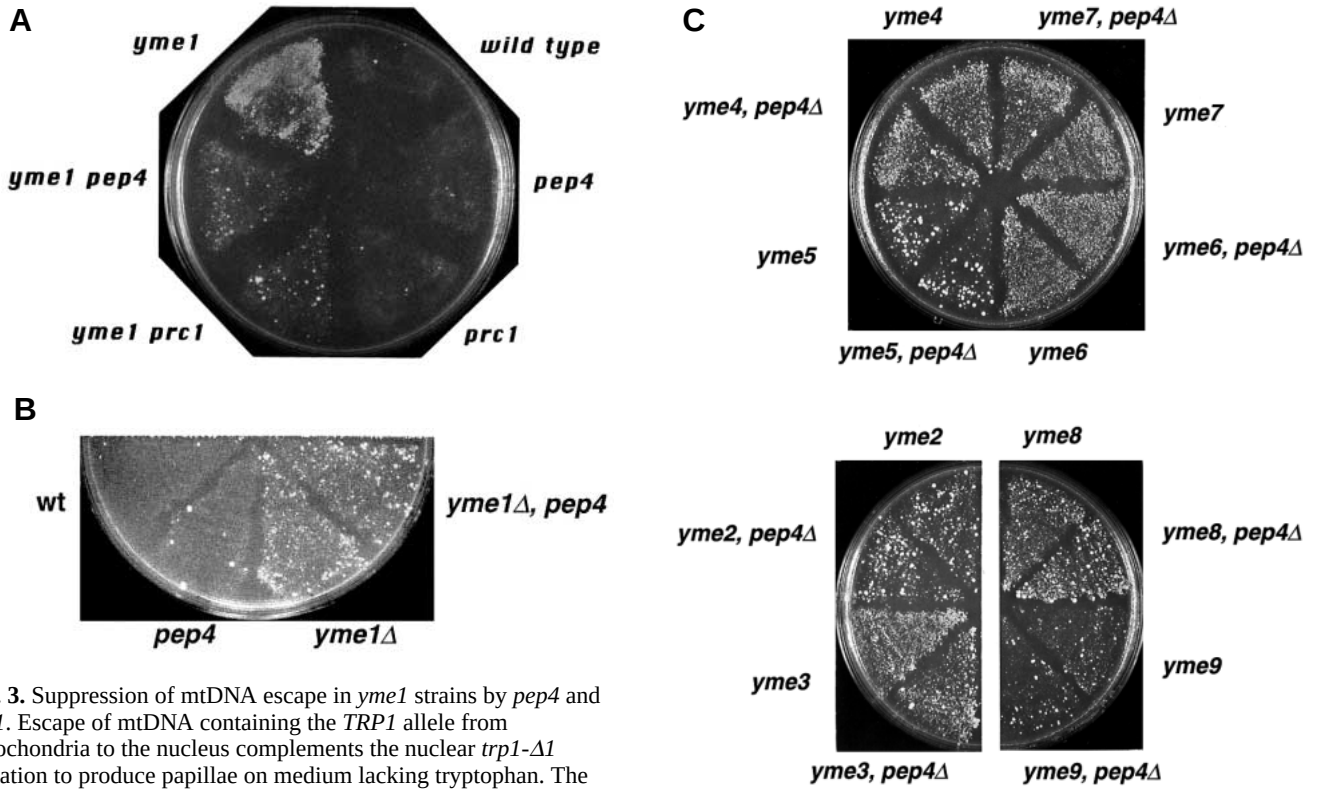

Fig. 3. Suppression of mtDNA escape in *yme1* strains by *pep4* and *prc1*. Escape of mtDNA containing the *TRP1* allele from mitochondria to the nucleus complements the nuclear *trp1-Δ1* mutation to produce papillae on medium lacking tryptophan. The high rate of mtDNA escape for a *yme1* strain is evidenced by many papillae on medium lacking tryptophan. (A) The effect of *yme1*, *pep4*, and *prc1* mutations on mtDNA escape. Strains were grown first on YPEG, then transferred to minimal medium lacking tryptophan. Strains: wt, PTY44; *pep4*, CCY11; *prc1*, CCY25; *yme1 prc1*, CCY26; *yme1 pep4*, CCY12; *yme1*, PTY52. (B) *pep4* does not suppress the high rate of mtDNA escape of *yme1* yeast when they are cultured on glucose (YPD) prior to replica-plating to synthetic glucose medium that lacks tryptophan. Strains: wild-type, PTY44; *pep4*, CCY11; *yme1 pep4*, CCY12; *yme1*, PTY52. (C) *pep4* does not suppress the high rate of mtDNA escape of *yme2-1* through *yme9-1* mutant alleles. Cells were first cultured on YPEG medium and then replica plated to synthetic glucose medium that lacks tryptophan. Strains on top plate: *yme7 pep4*, CCY20; *yme7*, PTY122; *yme6 pep4*, CCY19; *yme6*, PTY72; *yme5 pep4*, CCY18; *yme5*, PTY70; *yme4 pep4*, CCY17; *yme4*, PTY68. Strains on bottom plate: *yme8*, PTY124; *yme8 pep4*, CCY21; *yme9*, PTY126; *yme9 pep4*, CCY22; *yme3 pep4*, CCY16; *yme3*, PTY66; *yme2 pep4*, CCY15; *yme2*, PTY64.

efforts, these strains had equivalent amounts of material that crossreacted with antisera directed against the mitochondrial proteins Cox2p, Aac2p, and Yme2p (data not shown). This may indicate that there is not a significant difference in the amount of mitochondrial proteins associating with vacuoles in wild-type and *yme1* yeast strains. Alternatively, contamination of vacuolar fractions by mitochondria may mask differences in the association of mitochondria with vacuoles in these strains. Hence, it became necessary to develop a sensitive assay for vacuole-dependent turnover of mitochondria that was not subject to the technical limitations inherent to cell fractionation and immunological detection.

To measure mitochondrial degradation by the vacuole we modified an in vivo assay for the detection of autophagy in yeast that was initially described by Noda et al. (1995). Our goal was to determine whether a *yme1* strain has a higher rate of vacuole-dependent mitochondrial turnover than does an isogenic wild-type strain. *PHO8*, encoding the vacuolar alkaline phosphatase (ALP), is translated as a pro-enzyme and matured to its active form in the vacuole upon cleavage of the C-terminus by proteinase A (Jones et al., 1982). For our assay, *PHO8* was modified to direct the pro-enzyme ALP to the mitochondrial compartment (ALP^m) rather than the vacuole by deleting the first 60 codons bearing the vacuolar targeting signal and substituting the mitochondrial targeting signal from *COXIV* (Fig. 4). Cell fractionation confirmed mitochondrial localization of the recombinant protein for strains grown in media that do not necessitate respiration (Fig. 5). Thus, in a yeast strain bearing a deletion of the genomic alkaline phosphatase loci, *pho8* and *pho13*, the only significant source of ALP activity is ALP^m that has been delivered to the vacuole via mitochondria and activated by a vacuolar protease. By assaying ALP^m activity in various genetic backgrounds and growth conditions, the relative rate of vacuole-dependent mitochondrial turnover can be assessed.

ALP activity was assayed in isogenic *yme1* and wild-type strains and expressed as proteinase A-dependent values, corresponding to vacuole-mediated ALP activation. ALP^m activities of *yme1* and wild-type yeast were not significantly different when cells were grown in raffinose or glucose media (Fig. 6A). In contrast, ALP^m activity was higher for the *yme1* strain than for the wild-type strain when cells were grown in ethanol/glycerol medium (Fig. 6A). Thus, *yme1* yeast have an increased rate of vacuole-dependent mitochondrial turnover compared to wild-type yeast under this growth condition. The increase in ALP^m activity for a *yme1* strain cultured in

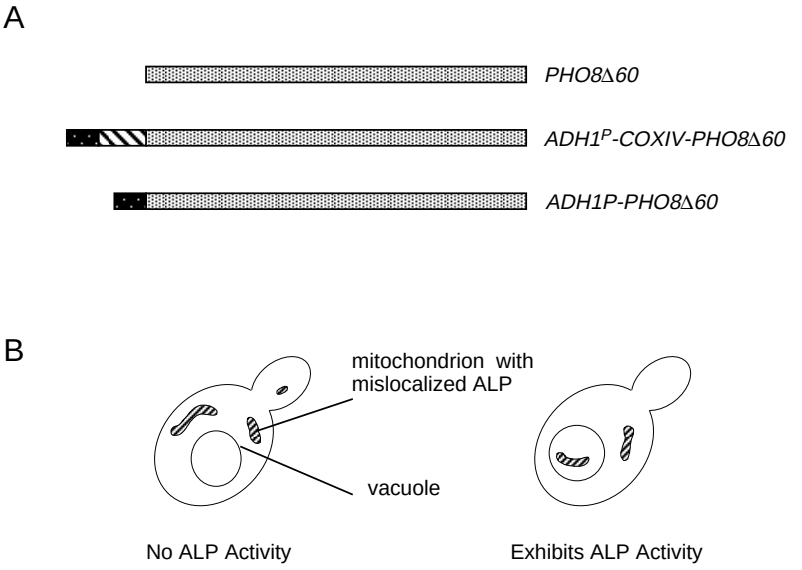

Fig. 4. (A) Modified *PHO8* genes designed to target alkaline phosphatase to mitochondria or to the cytoplasm. *PHO8Δ60* is a 60 amino acid deletion of the *PHO8* gene which removes the vacuolar targeting signal and a transmembrane domain. *ADH1^P-COXIV-PHO8Δ60* is an expression cassette in which the *PHO8Δ60* gene is fused to the *COXIV* mitochondrial targeting sequence and expressed via the *ADH1* promoter. It encodes an alkaline phosphatase that is targeted to mitochondria. *ADH1^P-PHO8Δ60* encodes a cytoplasmically localized alkaline phosphatase. (B) The ALP^m assay system. The strains utilized in this assay have the nuclear loci encoding alkaline phosphatase activity (*pho8*, *pho13*) disrupted and carry a *CEN* plasmid-borne *ADH1^P-COXIV-PHO8Δ60* expression cassette. In the absence of mitochondrial turnover, the strain does not exhibit significant ALP activity. When mitochondria are degraded by the vacuole, ALP^m is matured by a vacuolar protease and ALP activity can be detected.

ethanol/glycerol medium is consistent with the observation that mtDNA escape is suppressed in *yme1 pep4* and *yme1 prc1* for this growth condition (Fig. 3). The ALP activity differences between *yme1* and wild-type yeast were reflective of selective mitochondrial degradation and not of a general increase in autophagy of bulk cytoplasm since wild-type and *yme1* strains bearing a cytoplasmically-targeted *PHO8* gene product showed no significant differences in ALP activity during growth in any media (Fig. 6B). Furthermore, since the cytoplasmically-targeted PHO8 gene product is not activated in *yme1* yeast an alternative method of ALP^m activation via endocytosis of material from lysed cells is unlikely. Scavenging of cellular debris via endocytosis with subsequent delivery to the vacuole would activate the cytoplasmically-targeted *PHO8* gene product as well as the mitochondrially-targeted *PHO8* gene product, and that is not observed (Fig. 6B).

Inactivation of the gene encoding vacuolar carboxypeptidase

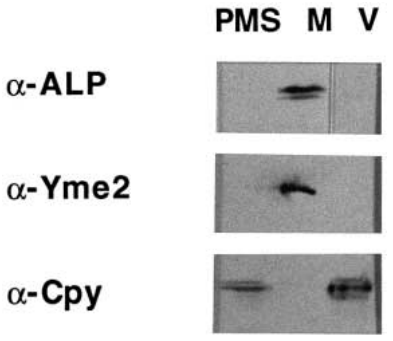

Fig. 5. Localization of ALP^m in yeast cultured in fermentable growth medium. Protein immunoblot analysis of cellular fractions prepared from galactose grown cells demonstrated that ALP^m is localized to the purified mitochondrial fraction (M), not to the crude cytoplasmic fraction (PMS) or the vacuolar fraction (V). Fractions were probed with antisera directed against alkaline phosphatase (α-ALP), a mitochondrial protein (α-Yme2p), and a soluble vacuolar protein, carboxypeptidase Y (α-CPY).

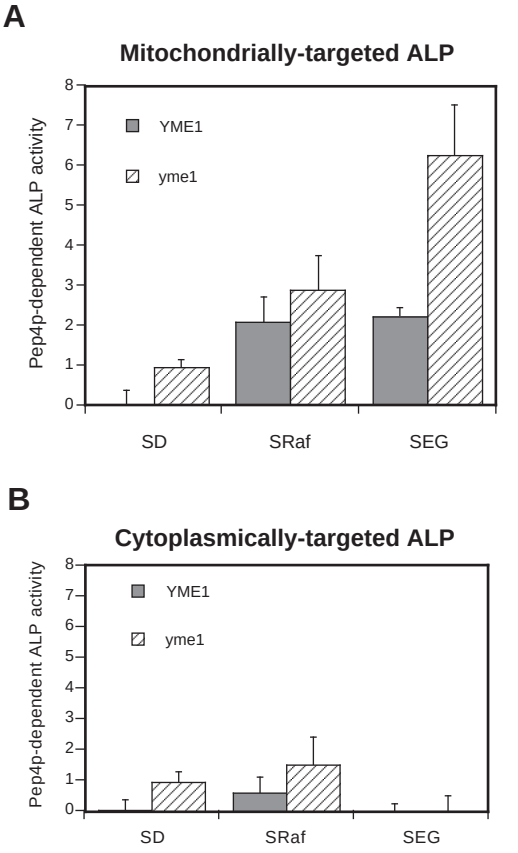

Fig. 6. ALP activity in wild-type and *yme1* strains. ALP activity assays were performed according to the method of Toh-e et al. (1976). Activities were normalized to OD_{600}. To negate incidental maturation of ALP^m in the absence of vacuolar activity, all ALP activities have been expressed as proteinase A-dependent values by subtracting ALP activity values for *PEP4* strains from activity values for isogenic strains bearing *pep4* disruptions. (A) Mitochondrial-ALP (ALP^m) activities for isogenic wild-type and *yme1* strains. (B) Cytoplasmic ALP activities for isogenic wild-type and *yme1* strains.

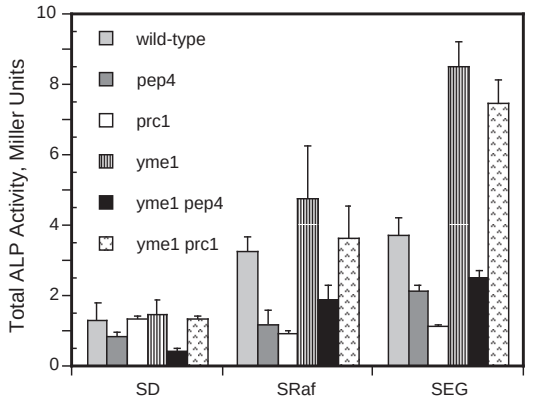

Fig. 7. Mutation of *PRC1* does not alter vacuole-dependent uptake of mitochondria. Total ALP^m activities were determined for strains bearing mutation of the indicated vacuolar protease structural genes. Strains: wild-type, CCY30; *yme1-Δ1*, CCY31; *pep4*, CCY32; *yme1-Δ1 pep4*, CCY33; *prc1Δ::LEU2*, CCY40; *yme1-Δ1 prc1Δ::LEU2*, CCY41.

Y (*PRC1*) suppresses the high rate of mtDNA escape found in *yme1* yeast strains when they are first cultured on nonfermentable carbon sources (Fig. 3). We tested whether disruption of *PRC1* affected the turnover of mitochondria in wild-type and *yme1* yeast. Inactivation of *PRC1* had no affect on the rate of mitochondrial turnover as measured by ALP^m activation in either *yme1* or otherwise wild-type yeast (Fig. 7). Consequently, we conclude that *PRC1* suppresses mtDNA escape in a *yme1* strain not by blocking the uptake of mitochondria, but rather by a defect in vacuolar function that is subsequent to the uptake step. As proteinase A is necessary for the activation of ALP^m, it is not possible to assess the requirement of proteinase A in the uptake of mitochondria by vacuoles in a similar manner.

DISCUSSION

The experiments presented here lead us to propose that *yme1* mitochondria are targeted for degradation by the vacuole during respiratory growth, and that this organellar degradation leads to an increase in mtDNA escape. This hypothesis was suggested by a genetic experiment, in which it was shown that mtDNA escape was reduced in *yme1 pep4* and *yme1 prc1* strains with impaired vacuolar function (Fig. 3). The reduction of mtDNA escape may reflect a reduction in organellar degradation due to the absence of proteinase A or carboxypeptidase Y activity. Alternatively, the effect of the *pep4* mutation may be indirect. Activation of some other vacuolar function required for mtDNA escape may be blocked in the absence of proteinase A or carboxypeptidase Y activity.

To further test whether *yme1* mitochondria are degraded by the vacuole, we devised an in vivo assay for vacuole-dependent turnover of the recombinant mitochondrial protein ALP^m. This assay showed that mitochondria in *yme1* yeast are degraded by the vacuole at an increased rate relative to mitochondria of wild-type yeast when cells are grown on a non-fermentable carbon source (Fig. 6). This turnover does not require the *PRC1* gene product carboxypeptidase Y (Fig. 7), indicating that the suppression of mtDNA escape in *yme1* strains by the inactivation of *PRC1* occurs subsequent to mitochondrial uptake by the vacuole. Because our assay depends on the *PEP4* gene product's activation of ALP^m, we cannot determine whether mitochondrial turnover requires Pep4p.

At least one Yme1p substrate, the inner mitochondrial membrane protein Cox2p, accumulates in the mitochondrial membrane in a *yme1* mutant (Nakai et al., 1995; Pearce and Sherman, 1995; Weber et al., 1996). Accumulation of such incompletely processed or assembled proteins may lead to an imbalanced protein/lipid ratio in the mitochondrial membrane, changing the fluidity of the membrane, and contributing to the swollen shape of the organelle (Walenga and Lands, 1975; Tung et al., 1991). These variations in fluidity and/or morphology may be used as signals for organellar degradation in *yme1* yeast. This model is supported by the observation that carbon source utilization is linked to mitochondrial membrane fluidity. Growth on glycerol requires more fluid membranes than growth on other carbon sources (Walenga and Lands, 1975). It is during growth on glycerol that increased mitochondrial turnover and temperature sensitive growth are observed in *yme1* mutants. It is important to note, however, that alterations in mitochondrial morphology per se do not induce organellar turnover. Both *yme4-1* and *yme5-1* yeast have unusual mitochondrial morphologies (data not shown), but do not show genetic interaction of *PEP4*-dependent vacuole functions and mtDNA escape. *yme1* yeast may have other defects, such as an inability to repair oxidative damage inherent to respiratory growth (Moradas-Ferreira et al., 1996; Costa et al., 1997) that also signal a need for organellar turnover. A lysosomal storage disease in mammals that leads to neurodegeneration is characterized by the accumulation of protein subunit 9 of the mitochondrial ATP synthase (Palmer et al., 1992). In cultured lymphoblasts from diseased individuals, there is a reversible increase in lysosomal storage material as the result of induced mitochondrial damage (Boriack et al., 1995). Hence, it is likely that mitochondrial damage in mammalian cells is dealt with in a manner analagous to damaged mitochondrial compartments in yeast: uptake and turnover by the cell's degradative organelle.

The lysosome-mediated degradation of cellular components has been well characterized in mammalian systems (Dunn, 1990a,b). This phenomenon has been described as two distinct processes, micro- and macroautophagy. Starvation-induced macroautophagy is typified by the uptake of bulk cytoplasmic components through an endosomal intermediate, followed by delivery to the degradative organelle (Knecht et al., 1988; Dunn, 1990a,b; Takeshige et al., 1992; Egner et al., 1993; Baba et al., 1994). Takeshige et al. (1992) reported that tryptophan starvation induces autophagic vesicle formation in *S. cerevisiae*. Therefore, the mtDNA escape assay itself may trigger amino acid starvation-induced macroautophagy since the genetic assay requires the yeast to be starved for tryptophan. This may account for the background level of mtDNA escape seen in wild-type yeast. In contrast, microautophagy involves direct uptake of constituents into the degradative organelle, bypassing the endosomal intermediate. Although macroautophagy in yeast has been well described (Baba et al., 1994; Thumm et al., 1994), reports of

microautophagy are limited. Microautophagy of peroxisomes, following glucose addition, occurs in *S. cerevisiae* (Chiang et al., 1996). *Hansenula polymorpha* and *Pichia pastoris* undergo microautophagy of peroxisomes upon transfer from methanol to an alternative carbon source (Veenhuis et al., 1983; Tuttle et al., 1993). Similarly, the vacuole-associated mitochondria of a *yme1* strain were not contained within autophagic vesicles as has been described for nutrient-starved yeast (Takeshige et al., 1992), suggesting that the mitochondrial degradation described here occurs by microautophagy.

Directed turnover of mitochondrial compartments by an autophagic organelle, the vacuole or lysosome, likely occurs for several reasons. In a similar fashion to that noted above for peroxisomes in methylotrophic yeast (Veenhuis et al., 1983; Tuttle et al., 1993), alteration of growth conditions may initiate reprogramming of cellular metabolism via turnover of a portion of the mitochondrial complement. In cells containing damaged mitochondrial compartments it would also be important to selectively remove these organelles. First, defective mitochondrial compartments may alter a number of cellular metabolic activities and removal of those compartments via a salvage pathway would help optimize cellular metabolism. Second, damaged mitochondrial compartments are also more likely to generate reactive oxygen species that can damage DNA, lipids, and proteins (Halliwell and Gutteridge, 1989). Finally, in higher eucaryotes damaged mitochondrial compartments could inappropriately trigger cell death in a susceptible cell through the untimely loss of cytochrome c, a positive effector of apoptosis (Liu et al., 1996; Kluck et al., 1997; Yang et al., 1997).

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