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Regulation and pH-dependent expression of a bilaterally truncated yeast plasma membrane H^+-ATPase

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Abstract

Constitutive, chromosomal expression of yeast *pma1* deletion alleles in *Saccharomyces cerevisiae* yielded functional, truncated forms of the plasma membrane H^+-ATPase which were independently capable of supporting wild type yeast growth rates. Deletion of 27 amino-terminal residues affected neither the enzyme's activity nor its responsiveness to changes in glucose metabolism. By contrast, removal of 18 carboxy-terminal amino acids produced an enzyme with a V_{max} that was relatively insensitive to glucose-dependent metabolic status and with a K_m that was significantly lower than that of the wild type enzyme. These effects were exaggerated when the amino- and carboxy-terminal deletions were combined in a bilaterally truncated H^+-ATPase, suggesting that the amino terminus may have a subtle role in modulating ATPase activity. In *pma1*ΔΔ cells cultured at pH 6, plasma membrane H^+-ATPase levels were much lower than those in cells expressing a wild type ATPase. Increased expression levels could be achieved by growing the *pma1*ΔΔ mutant at pH 3, a result that was at least partially due to a sustained, elevated transcription of *pma1*ΔΔ mRNA. Our observations suggest that intracellular proton balance can be maintained by regulation of the activity and/or quantity of H^+-ATPase in the plasma membrane.

Keywords: H^+-ATPase; Plasma membrane; Bilateral truncation; (Yeast)

1. Introduction

The H^+-ATPase of the *Saccharomyces cerevisiae* plasma membrane is a P-type cation transporting ATPase [1,2]. This polytopic integral membrane protein is an essential component of the yeast cell, responsible for intracellular pH regulation and generation of the transmembrane electrochemical gradient that is required for secondary ion transport and the uptake of many nutrients and amino acids [3]. The importance of the H^+-ATPase to cellular homeostasis is underscored by the relationship between ATPase activity and yeast growth rate [4,5]. The amount of active enzyme in the plasma membrane is regulated by both transcriptional [6] and post-translational mechanisms [7–9].

Glucose metabolism causes a functional modification of the ATPase which results in an increased V_{max}, a lowered K_m, an increased sensitivity to vanadate and a shift towards a more neutral pH optimum [4,10]. In the absence of glucose, an inhibitory interaction between the carboxyl terminus of the ATPase and other cytoplasmically located regions of the enzyme, including the ATP binding domain, decreases ATPase activity [11]. Specific residues that affect this

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phenomenon have been identified by site-directed mutagenesis and suppressor mutation analysis [9,11–13]. Recent studies suggest that the YOR137c gene product [14] and the ubiquitin-proteasome pathway [15] are additional factors involved in glucose activation of the H^+-ATPase.

Portillo et al. [4] studied plasmid-encoded *pma1* deletion mutations in an *S. cerevisiae* strain with a wild type, chromosomal copy of *PMA1* under control of the *GAL10* promoter. Limited truncations of the ATPase were made at either the amino (up to 27 amino acids) or the carboxyl (up to 46 amino acids) terminus, apparently without affecting export to the plasma membrane or preventing yeast growth due to inhibition of H^+-ATPase function. Deletion of 11 or more amino acids from the carboxyl terminus of the ATPase eliminated an inhibitory domain, producing a constitutively activated enzyme [4]. The presence of an inhibitory carboxy-terminal domain is a regulatory feature that has been found in numerous enzymes, including the plant H^+-ATPase [16] and the Ca^{2+}-ATPase of animal plasma membranes [17].

Using an alternative method of *S. cerevisiae pma1* mutant construction, we have made homozygous *pma1* deletion mutants whose viability and growth depend exclusively on the functional expression of truncated forms of the ATPase as encoded by chromosomal copies of *pma1* deletion alleles. A bilaterally truncated version of the *S. cerevisiae* H^+-ATPase was engineered to see if both termini could be removed without compromising enzyme function and to test whether or not the terminal domains of the enzyme are interdependent. Mutants expressing this enzyme have plasma membranes with a homogeneous, constitutively activated population of ATPase molecules. The amount of mutant enzyme present in the plasma membrane can be manipulated simply by changing the pH of the growth medium.

2. Materials and methods

2.1. Yeast strains and growth media

S. cerevisiae strain SH122 (*HO*/*HO MATa*/*MAT*α *ade6-1*/*ade6-1 trp5-1*/*trp5-1 leu2-1*/*leu2-1 lys1-1*/*lys1-1 ura3-1*/*ura3-1 pma1*Δ:*LEU2*/*PMA1*), an isogenic derivative of *S. cerevisiae* strain Y55 (*HO gal3 MAL1 SUC1*) [18], was used as the recipient strain for all yeast transformations. Control yeast strain T48 and the *pma1* deletion mutant strains *pma1*Δ1-27, *pma1*Δ873-918, *pma1*Δ901-918 and *pma1*Δ1-27Δ901-918 (see Fig. 1) were isolated as *leu URA* cells after sporulation and tetrad dissection of SH122 transformants as previously described [19]. Yeast strains were grown in either YPD medium (1% yeast extract, 2% peptone, 2% glucose) adjusted to the appropriate pH with HCl or complete synthetic medium lacking uracil (CSM-ura, Bio101). Solid media contained 2% agar (Davis Bacteriological Agar). The in vivo effect of hygromycin B on yeast growth was assessed by including the antibiotic in YPD medium, pH 6, at the concentrations specified.

2.2. pma1 deletion mutant construction and yeast transformation

Standard recombinant DNA techniques were performed using the *Escherichia coli* strain XL1-Blue (Stratagene) as host. Bacteria were grown in Luria broth using 0.05 mg/ml ampicillin for plasmid selection. Mutant *pma1*Δ alleles were constructed using either recombinant DNA techniques or site-directed mutagenesis. Thus, *pma1*Δ1-27 (*pma1*NΔ) was made by ligation of a 2.1 kb *Pac*I-*Bgl*II fragment from plasmid pRS611 (kindly provided by R. Serrano) to a 6.9 kb *Pac*I-*Bgl*II fragment from plasmid pDP100 [20], while *pma1*Δ873-918 and *pma1*Δ901-918 (*pma1*CΔ) were made by site-directed mutagenesis [21] of a single-stranded DNA version of plasmid pDP100 using mutagenic oligonucleotides of 17 (5′-GGT TTC TAA TAC GAA AT-3′) and 24 (5′-CAG AAG TGT CTA AGA CTT CAT GGC-3′) nucleotides, respectively. The single nucleotide substitutions (underlined in each oligonucleotide) introduced ochre stop codons in place of Y^{873} and E^{901}. The *pma1*Δ1-27Δ901-918 (*pma1*ΔΔ) construct, a bilaterally truncated version of *PMA1*, was made by ligating *Xho*I-*Bgl*II fragments of 2.6 kb and 6.5 kb from plasmids pma1NΔ and pma1CΔ, respectively.

From each plasmid construct described above, a 6 kb *Hin*dIII fragment incorporating the mutant *pma1* gene and a *URA3* marker gene for selection

was used to transform *S. cerevisiae* strain SH122 by lithium acetate treatment, using single-stranded carrier DNA [22] and commercially available reagents (Bio101). Heterozygous *leu/leu URA/ura* diploid transformants were sporulated and tetrads dissected [19].

2.3. Nucleotide sequence confirmation of S. cerevisiae pma1 deletion mutants

Yeast DNA minipreps were made from 5 ml cultures grown in YPD (pH 5.7) for 16–18 h [23]. These DNA preparations were diluted for use as PCR templates in the amplification of modified regions (usually <1 kb) of the *pma1* gene. PCR products were sequenced using the Sequenase PCR product sequencing kit (USB/Amersham Life Science).

2.4. RNA analysis

Stationary phase cultures of *S. cerevisiae* T48 and *S. cerevisiae pma1*ΔΔ grown in YPD at either pH 6.0 (A_{600nm} approx. 7.0) or pH 3.0 (A_{600nm} approx. 10.0) were used to inoculate fresh 100 ml volumes of YPD (at pH 3.0 or pH 6.0) to a starting $A_{600nm} = 0.1$. Two to 10 ml samples of the growing cultures were taken at appropriate timepoints and total RNA was extracted [24], ethanol precipitated, washed with 70% ethanol, dried, dissolved in DEPC-treated water, and quantitated on the basis of its A_{260nm}. RNA samples (approx. 2.5 μg), including an RNA ladder (Gibco BRL) for size comparison, were separated on 1.2% agarose gels containing 1.1% formaldehyde [25]. A control RNA sample was included on each gel to allow for between-gel comparisons. After electrophoresis, gels were washed extensively with several changes of DEPC-treated water, then dried under vacuum, without heat, for 2 h followed by a further 1 h at 60°C. Direct gel hybridization with a ^{32}P-labelled DNA probe for *PMA1* (2.1 kb *Ava*II DNA fragment) was performed as described previously [26]. Photographs of the ethidium bromide-stained gels and autoradiographs were scanned into Adobe Photoshop in grey scale using a Microtek ScanMaker 600Z flat-bed scanner. Images were analysed using NIH Image version 1.5 on a Macintosh IIVx computer.

2.5. Isolation of yeast plasma membranes and ATP hydrolysis assays

Yeast strains were grown in 500 ml YPD (pH 3.0 or pH 6.0) until they reached an A_{600nm} of 3.0. The cells were harvested by centrifugation, resuspended in approx. 10 ml of ice cold homogenization medium (50 mM Tris pH 7.0, 1 mM EDTA, 0.5 mM PMSF and 2% glucose) and homogenized by vortexing with glass beads. The homogenate was adjusted to pH 7.0 with 1 M Tris. Microsomal membranes were recovered by differential centrifugation and then a plasma membrane-enriched preparation was obtained by sucrose-gradient centrifugation [27]. Cells to be starved of glucose prior to membrane isolation were washed three times with 20 volumes of deionized distilled water at room temperature, resuspended in 30 ml of deionized distilled water and incubated in a shaking incubator at 30°C for either 2 h or 4 h. These cells were disrupted and membranes prepared as described above, using homogenization medium without added glucose. Microscale ATP hydrolysis assays, which included inhibitors of non-plasma membrane H^+-ATPase activity, were performed as described previously [27]. Protein concentration was estimated according to Bradford [28] using the Bio-Rad (Bio-Rad, Richmond, CA) dye reagent and bovine IgG as standard.

2.6. SDS-PAGE and Western blotting

Purified plasma membranes dissolved at room temperature in SDS-lysis buffer (2% SDS, 50 mM Tris-HCl pH 6.7, 10% glycerol, 2.5 mM EDTA, 0.01% PMSF, 1 μg/ml bromophenol blue, 40 mM dithiothreitol) were separated by SDS-PAGE according to Laemmli [29] using a Bio-Rad minigel system. Prestained molecular weight markers (M_r range 14–200 kDa; Gibco BRL) were used as standards. After electrophoresis, proteins were either stained with Coomassie blue R250 or electrotransferred [30] to Amersham Hybond C nitrocellulose membranes (Amersham, UK). Membranes were blocked with 5% non-fat dried milk in phosphate buffered saline (PBS) pH 7.2 for 5 min, washed with three changes of PBS containing 0.2% gelatin and 0.05% Tween 20 and then incubated with either an affinity-purified rabbit antibody to native yeast plasma membrane

YEAST STRAIN	AMINO TERMINUS	CARBOXYL TERMINUS	AAs (total)
	1 10 20 30	890 900 910	
T48 (WT)	MTDTSSSSSSSSASSVSAHQPTQEKPAKTYDD.....	.....KPMKEKKSTRSVEDFMAAMQRVSTQHEKET	918
pma1Δ1-27 *	**MA**YDD.....	.....KPMKEKKSTRSVEDFMAAMQRVSTQHEKET	891
pma1Δ901-918	MTDTSSSSSSSSASSVSAHQPTQEKPAKTYDD.....	.....KPMKEKKSTRSV	900
pma1Δ1-27Δ901-918	**MA**YDD.....	.....KPMKEKKSTRSV	873

Fig. 1. Deduced amino acid sequences of wild type and mutant H^+-ATPases expressed in *S. cerevisiae* SH122. Residues underlined are putative phosphorylation sites for calmodulin-dependent protein kinase (RXXS/T) and a casein kinase II-like phosphorylation site (S/TXXE/D). Residues shown in bold type at the new amino terminus differ from those in the wild type *PMA1* sequence. **pma1*Δ1-27 is equivalent to the Δ*pma1-241* allele described by Portillo et al. [4].

ATPase or an affinity-purified rabbit antibody directed specifically against epitopes at the carboxyl terminus of the ATPase [31]. Both antibody preparations were used at 1/400 dilutions in PBS/gelatin/Tween 20 buffer (PBSGT). A 1/2000 dilution of alkaline phosphatase conjugated goat anti-rabbit IgG (Sigma) in PBSGT was used to identify antigen-antibody complexes and the blot was developed with a BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium) alkaline phosphatase substrate mixture (Sigma). Gels and blots were scanned through an ochre overhead transparency and analysed as described in Section 2.4.

3. Results

S. cerevisiae pma1 deletion mutations (Fig. 1) were introduced into the genome of the diploid yeast strain SH122 by homologous recombination and replacement of a *LEU2*-disrupted copy of the resident *PMA1* allele [19]. Heterozygous diploid transformants that had received mutant H^+-ATPases capable of independently supporting haploid cell growth (i.e. *pma1*NΔ, *pma1*CΔ and *pma1*ΔΔ) gave four viable spores after meiotic segregation analysis. By contrast, the *pma1*Δ873-918 mutant gave only two viable spores (both *PMA1 ura*), indicating a recessive lethal mutation.

Truncation of the plasma membrane H^+-ATPase proteins was confirmed by comparing their electrophoretic mobilities by SDS-PAGE and by Western blot identification using a polyvalent rabbit antiserum against the *S. cerevisiae* ATPase (Fig. 2A). Mobility changes were clearly apparent for the proteins Pma1NΔp and Pma1ΔΔp, which had been shortened by 27 and 45 amino acids, respectively. The deletion of 18 amino acids from the carboxyl terminus was demonstrated by Western blot analysis using an antiserum specific for the carboxy-terminal domain of the wild type ATPase [31]. This antiserum reacted only with Pma1T48p (wild type) and Pma1NΔp proteins even after extended blot development, confirming the absence of carboxy-terminal epitopes from the Pma1CΔp and Pma1ΔΔp proteins (Fig. 2B).

In common with other P-type ATPases, the car-

Table 1
Growth phenotypes of T48 (control) and *pma1* deletion mutants

Phenotype	T48	*pma1*NΔ	*pma1*CΔ	*pma1*ΔΔ
Growth rate (ΔOD_{600nm}/h)[a]	0.65 ± 0.04	0.53 ± 0.05	0.63 ± 0.09	0.65 ± 0.11
Growth yield (OD_{600nm}) at 24 h	6.3 ± 0.4	5.1 ± 0.2	5.8 ± 0.3	6.7 ± 0.8
I_{50} pH	2.68 ± 0.02	2.63 ± 0.04	2.71 ± 0.00	2.73 ± 0.01
I_{50} Hyg B (mM)	0.17 ± 0.01	0.56 ± 0.05	0.25 ± 0.05	0.50 ± 0.11

Values given are means (± S.D. for entire population) derived from at least three separate determinations made on cultures growing in YPD, pH 6.
[a]At mid-exponential phase.

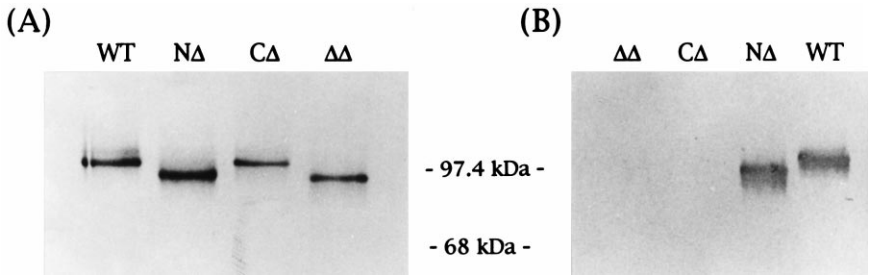

Fig. 2. Western blot detection of full size and truncated H^+-ATPases. Plasma membranes from homogenates of wild type and *pma1*Δ mutants of *S. cerevisiae* were recovered on sucrose gradients. Membrane samples (2 μg protein) were solubilized in SDS-PAGE sample buffer, separated on 10% SDS-PAGE gels and transferred to nitrocellulose as described in Section 2. Blots were analysed for H^+-ATPase proteins using affinity-purified polyvalent rabbit antibodies against either (A) native *S. cerevisiae* H^+-ATPase, or (B) the carboxyl terminus of the *S. cerevisiae* H^+-ATPase [31]. WT, wild type (T48); NΔ, 27 amino acid N-terminal truncation (*pma1*NΔ); CΔ, 18 amino acid C-terminal truncation (*pma1*CΔ); ΔΔ, bilateral truncation totalling 45 amino acids (*pma1*ΔΔ).

boxyl terminus of the *S. cerevisiae* H^+-ATPase is located in the cytoplasm [31]. A consensus P-type ATPase model with ten transmembrane helices [32,33], places E^{901} approx. 20 amino acids after the cytoplasmic end of transmembrane helix 10. Truncation of the ATPase at this position yielded strain *pma1*CΔ. This mutant and strain *pma1*ΔΔ, which had a bilaterally truncated ATPase combining the amino- and carboxy-terminal deletions, had phenotypic characteristics similar to those of the isogenic control strain (*S. cerevisiae* T48) which expressed a wild type enzyme (Table 1). Slight reductions in growth rate and 24 h growth yield were observed for the *pma1*NΔ mutant but not for *pma1*ΔΔ, suggesting that the unilateral truncation of the amino terminus had had a minor effect on ATPase function without severely compromising the proton translocating capacity of the enzyme. This effect was apparently nullified by removal of the carboxyl terminus. The minimal impact of the *pma1*NΔ mutation was also evident from both the lack of significant effect on cell sensitivity to acid pH and the weak (threefold) increase in resistance to hygromycin B. The latter effect appeared to be a consequence of the amino-terminal deletion because weak hygromycin resistance was shown by the bilaterally truncated *pma1*ΔΔ mutant but not by the *pma1*CΔ mutant. Strong hygromycin B resistance has been associated with mutant ATPases that have either lower activity or a reduced capacity to generate the membrane potential believed to be required for the drug's uptake [18,20,34,35].

Cells expressing the bilaterally truncated H^+-ATPase and growing in YPD at pH 6.0 had a comparatively low ATPase content in their plasma membranes as shown by SDS-PAGE and Western blot analysis (see Fig. 3A,B and Table 2). Proteins Pma1CΔp and Pma1ΔΔp were expressed at 66%

Table 2
pH-induced changes in ATPase content of plasma membranes from control and *pma1* deletion mutants

Strain	Culture pH	100 kDa protein (% tpmp)[a]	100 kDa protein (% wild type)	ATPase antigen (% wild type)
T48	6	17.7 ± 0.5	100	100
	3	21.4 ± 0.1	121 ± 4	115 ± 3
NΔ	6	17.3 ± 0.6	97 ± 0	82 ± 6
	3	17.5 ± 0.1	99 ± 3	78 ± 14
CΔ	6	10.5 ± 1.0	59 ± 4	66 ± 4
	3	17.0 ± 0.8	96 ± 2	94 ± 10
ΔΔ	6	7.3 ± 0.2	41 ± 2	35 ± 15
	3	16.5 ± 0.8	93 ± 2	110 ± 11

Values given are means (± S.D. for entire population) for at least two different sucrose gradient-purified plasma membrane preparations.
[a]% total Coomassie-stained plasma membrane protein.

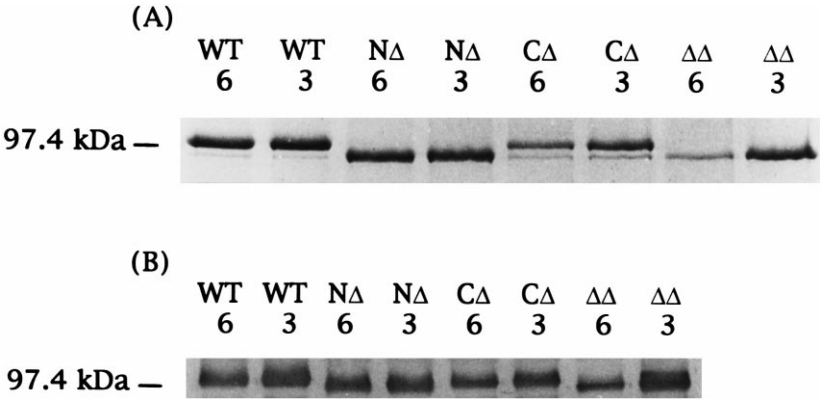

Fig. 3. Effect of pH on expression of truncated ATPases. Relative amounts of ATPase protein in sucrose gradient-purified plasma membranes prepared from wild type (T48) and *pma1*Δ mutant *S. cerevisiae* strains grown in YPD at either pH 6 or pH 3 compared by SDS-PAGE and Western blot (see Section 2). (A) Coomassie-stained ATPase proteins. (B) H^+-ATPase detected on Western blot using affinity-purified polyvalent rabbit antibody to native *S. cerevisiae* H^+-ATPase. Samples designated as defined in Fig. 2.

and 35%, respectively, of the level of the wild type enzyme. Analysis of unfractionated cell homogenates by SDS-PAGE and Western blotting detected no accumulation of improperly processed truncated ATPase (data not shown). Since the expression of an unregulated ATPase at wild type levels could have resulted in an unsustainable depletion of cellular ATP supplies, the reduced plasma membrane levels of enzymes truncated at the carboxyl terminus may have been mitigated by the expected loss of autoregulatory control normally effected through this domain. Although Pma1NΔp was expressed at 82% of the wild type enzyme level, this difference was not as consistent as the decreased expression of C-terminally truncated enzymes at pH 6 and was not considered to constitute a significant decrease in membrane ATPase content.

Increased plasma membrane expression of ATPases lacking the carboxy-terminal regulatory domain was induced by growing cells in YPD adjusted to pH 3.0. This pH neither prevented the growth, nor decreased the growth yield of any of the strains. In terms of their growth characteristics at pH 3.0, only the *pma1*ΔΔ cells differed markedly from the other strains, showing an extended lag phase before entering exponential growth. Relative to expression levels at pH 6.0, growth at pH 3.0 induced increases of 42% and 214%, respectively, in the amounts of Pma1CΔp and Pma1ΔΔp incorporated into the yeast plasma membrane (Table 2). In each case, the plasma membrane ATPase content was elevated to approximately the same level as that seen in T48 cells grown at pH 6. The Pma1NΔp enzyme showed no pH 3.0-induced increase and the significance of the small increase (15%) in wild type ATPase in T48 grown at pH 3.0 is difficult to assess. Given the simplistic assumption that growing cells at pH 3.0 placed an additional burden on the plasma membrane H^+-ATPase to export protons out of the cell, our result for T48 (and *pma1*NΔ) cells grown at pH 6.0 suggests that either (i) the H^+-ATPase operated at submaximal proton-pumping capacity at pH 6.0, or (ii) a proportion of the enzyme population was inactive as a proton pump under these conditions. Hence, in terms of activity and conformation, C-terminally truncated ATPases (particularly the bilaterally truncated enzyme) may present as more homogeneous membrane ATPase populations than the wild type enzyme.

The effect of low pH on H^+-ATPase expression was examined at the RNA level in both wild type (T48) and *pma1*ΔΔ cells shifted from stationary phase cultures at either pH 3.0 or pH 6.0 into fresh YPD at pH 3.0 (Fig. 4). Growth curve profiles were mostly unaffected by the pH shifts, the exception being *pma1*ΔΔ cells transferred from pH 6.0 to pH 3.0. These cells showed an extended lag phase prior to exponential growth. T48 cells entered exponential growth at the same time (6 h) as *PMA1* mRNA transcription reached peak levels. *PMA1* transcript levels in cells transferred from pH 3.0 were induced threefold and returned to starting levels by 8 h, whereas cells shifted from pH 6.0 showed a fivefold induction in *PMA1* message which returned to start-

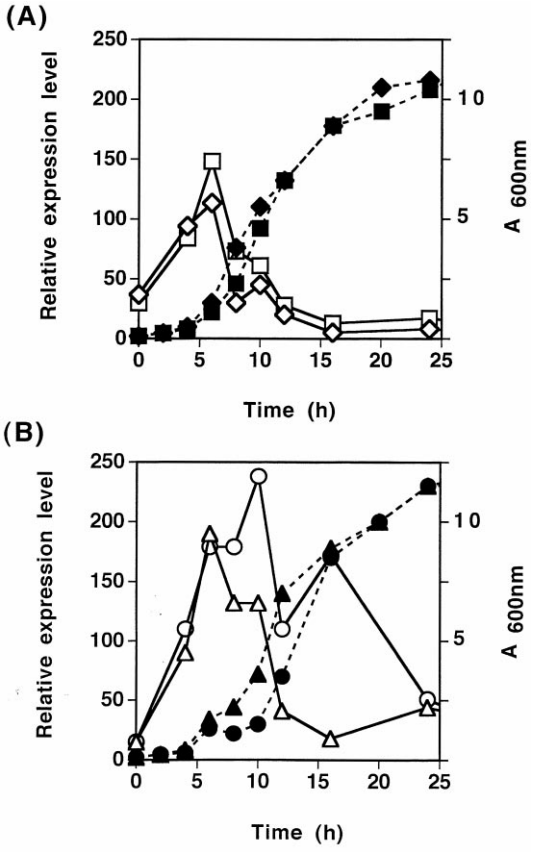

Fig. 4. Relative expression of H^+-ATPase mRNA in wild type and *pma1*ΔΔ cells shifted to YPD at pH 3. Relative amounts of H^+-ATPase mRNA (open symbols with solid lines) at selected timepoints during growth (solid symbols with broken lines) of (A) T48 and (B) *pma1*ΔΔ strains shifted from YPD at either pH 6 or pH 3 to YPD at pH 3. Message levels were estimated from densitometric scans of autoradiographs of RNA gels hybridized directly with a *PMA1* DNA probe (see Section 2) and adjusted according to levels of ethidium bromide-stained 18S ribosomal RNA. □, T48, pH 6→pH 3; ◇, T48, pH 3→pH 3; ○, *pma1*ΔΔ, pH 6→pH 3; △, *pma1*ΔΔ, pH 3→pH 3.

ing levels by 12 h. Transcription of *pma1*ΔΔ mRNA in cells pre-grown at pH 3 peaked at 6 h, was induced 13-fold over starting levels and had returned to starting levels by 12 h. By contrast, *pma1*ΔΔ cells shifted from pH 6.0 to pH 3.0 did not achieve peak *pma1*ΔΔ mRNA levels (induced 16-fold) until approx. 10 h after transfer, a time that coincided with entry into the exponential growth phase. Furthermore, elevated levels of *pma1*ΔΔ mRNA transcription were sustained until at least 24 h after transfer (see Fig. 4).

Patterns of *S. cerevisiae* actin (ACT1) transcriptional expression were very similar to those of *pma1* transcription in the pH-shifted T48 and *pma1*ΔΔ cultures (data not shown). Therefore, the observed increase in *pma1* transcription may not have been an independent prerequisite for exponential growth. Nonetheless, it seems likely that the exaggerated induction of H^+-ATPase message transcription observed in *pma1*ΔΔ cells shifted from pH 6.0 to pH 3.0, compared with the same cells maintained at pH 3.0, was due to a requirement for increased proton pumping capacity in the more acidic medium. This inference is consistent with the effect of growth pH on the levels of ATPase antigen present in the plasma membranes of the *pma1*ΔΔ mutant. When the same strains were shifted to pH 6.0 from either pH 3.0 or pH 6.0, *pma1* mRNA levels peaked 6–8 h after transfer and by 12 h had decreased to stationary phase levels (data not shown).

In glucose-fermenting (GF) cells, amino-terminal truncation of the ATPase had little affect on either its apparent K_m (for Mg^{2+}-ATP) or its apparent V_{max} when compared with the equivalent parameters for the wild type enzyme (Table 3a). Under the same conditions, ATPases truncated at E^{901} showed almost sixfold (Pma1CΔp) and fourfold (Pma1ΔΔp) increases in their apparent V_{max} values and their apparent K_m values (1.11 mM and 0.74 mM, respectively) were lower than that of the wild type enzyme (2.1 mM). It should be noted that the $V_{max(app)}$ values calculated for wild type and mutant ATPases in the present study, while comparable to values reported for the *S. cerevisiae* H^+-ATPase by other researchers [11,20], are several times higher than those reported by Portillo et al. in their conditionally expressed deletion mutant study [4]. These quantitative differences between studies may be attributable to the combined effects of studying different yeast host strains in contrasting expression systems.

Phosphorylation of the yeast plasma membrane proton ATPase [7,9] is thought to play a key role in glucose activation of the enzyme [11]. In particular, phosphorylation(s) at the carboxyl terminus is believed to prevent this domain from negatively regulating ATPase activity via interaction, either direct or indirect, with other elements of the enzyme [11]. By truncating the ATPase at E^{901}, two potential phosphorylation sites implicated in glucose stimula-

Table 3
Kinetic properties of wild type and truncated ATPase enzymes from glucose-starved (GS) or glucose-fermenting (GF) cells[a]

(a) Strain	$K_{m(app)}$ (mM MgATP)			$V_{max(app)}$ (μM P_i/min/mg)		
	GF	GS (2 h)	GS (4 h)	GF	GS (2 h)	GS (4 h)
T48	2.10 ± 0.44	1.92 ± 0.39	2.28 ± 0.98	1.85 ± 0.08	1.09 ± 0.26	1.44 ± 0.01
NΔ	1.83 ± 0.33	2.20 ± 0.30	2.89 ± 0.22	1.94 ± 0.09	0.90 ± 0.08	1.02 ± 0.04
CΔ	1.11 ± 0.01	1.42 ± 0.22	0.78 ± 0.06	10.39 ± 1.91	11.86 ± 1.79	5.63 ± 0.02
ΔΔ	0.74 ± 0.03	0.83 ± 0.10	0.22 ± 0.13	7.47 ± 1.08	8.84 ± 0.74	3.30 ± 0.46

(b) Strain	pH optimum		I_{50} vanadate (μM)		
	GF	GS (4 h)	GF	GS (2 h)	GS (4 h)
T48	6.25	5.70	1.19 ± 0.06	1.88 ± 0.63	2.03 ± 0.15
NΔ	6.25	5.70	1.67 ± 0.06	1.36 ± 0.42	1.74 ± 0.19
CΔ	6.75	6.75	1.13 ± 0.03	0.55 ± 0.03	0.51 ± 0.06
ΔΔ	6.75	6.75	1.08 ± 0.51	0.91 ± 0.45	0.70 ± 0.10

[a]Cells were grown in YPD (pH 6) and harvested at $A_{600nm} = 3.0$. See Section 2 for glucose starvation conditions.
Values given are means (± S.D. for entire population) for at least two different sucrose gradient-purified plasma membrane preparations. Data normalized on the basis of relative ATPase content.

tion of enzyme activity were either partially (S^{899}-D^{902}) or completely (R^{909}-T^{912}) removed.

When the effects of glucose activation/starvation on the *pma1* deletion mutants were considered, the Pma1NΔp enzyme behaved much like the wild type ATPase, showing a decrease in activity but little change in $K_{m(app)}$ following glucose starvation (GS) for 2 or 4 h at 30°C (Table 3a). Thus, removal of the first 27 amino acids of the ATPase did not appear to affect glucose-mediated regulation of the enzyme's activity. The consistency in the behaviour of wild type and Pma1NΔp enzymes, even after glucose starvation, contrasts with the rapid, substantial (three- to fourfold) increase in $K_{m(app)}$ and greater than 90% decrease in V_{max} previously associated with a glucose-starved wild type enzyme [4,10,11]. While the use of yeast strains with different genetic backgrounds may have contributed to this apparent disparity between studies, the enzyme kinetic data reported herein were probably most influenced by the fact that cells were not subjected to a change in temperature during glucose starvation. Indeed, when T48 and *pma1*NΔ cells were glucose-starved at 0°C for 4 h, dramatic decreases in both ATPase activity and substrate affinities were noted. The glucose-activated wild type enzyme had a $K_{m(app)}$ of 0.71 ± 0.26 and a $V_{max(app)}$ of 3.00 ± 0.26 μM/min/mg, whereas the wild type ATPase from cells starved for 4 h at 0°C had a $K_{m(app)}$ of 1.89 ± 0.79 and a $V_{max(app)}$ of 0.52 ± 0.09 μM/min/mg. Similarly, the glucose-activated Pma1NΔp ATPase had a $K_{m(app)}$ of 1.18 ± 0.20 and a $V_{max(app)}$ of 2.27 ± 0.12 μM/ min/ mg, while the same enzyme from cells starved at 0°C for 4 h had a $K_{m(app)}$ of 2.80 ± 0.20 and a $V_{max(app)}$ of 0.41 ± 0.02 μM/min/mg.

Yeast *pma1*Δ mutants truncated at either A^{905} or Q^{908} have been reported to show starvation-induced increases in ATPase activity of 25% and 15%, respectively, over ATPases from GF cells [4]. In the present study, ATPases truncated at E^{901} retained high levels of ATPase activity even after 2 h of glucose starvation and showed slight but not statistically significant increases in ATPase activity compared to GF cells. After 4 h glucose starvation at 30°C, however, the Pma1CΔp and Pma1ΔΔp enzymes had lost about half of their activity (Table 3a). Extended (4 h) starvation also resulted in a 30% reduction in the $K_{m(app)}$ of the enzyme truncated at the carboxyl terminus. This increase in substrate affinity occurred despite the removal of residues E^{901} and D^{902}, components of a putative casein kinase II phosphorylation site (Fig. 1) implicated in the glucose-triggered decrease in K_m that is regarded as a normal feature of the wild type enzyme [11]. While the α-carboxyl group of V^{900} might have effectively substituted for phosphorylation of S^{899} in the production of an enzyme with a low K_m, at this time we do not know what role, if any, the remaining elements of the phosphorylation

motif might have played in increasing substrate affinity in the absence of glucose. Bilateral truncation of the ATPase exacerbated the effect of glucose metabolism/starvation on the enzyme's substrate affinity, resulting in a 3.4-fold decrease in $K_{m(app)}$ after 4 h of glucose starvation. Thus, in the context of the glucose-starved, C-terminally truncated ATPase, the amino terminus had a negative effect on substrate binding affinity. Perhaps this effect was a consequence of the mutant enzyme's conformation, or it may have been due to external modification of the enzyme. For example, phosphorylation of the ATPase at a site(s) other than the carboxyl terminus has been shown to have a 'down-regulating' effect on ATPase activity at the plasma membrane [9].

ATPases from glucose-starved and glucose-fermenting cells were contrasted on the basis of their pH (pH range 4.0–8.0) and vanadate inhibition profiles. The wild type ATPase from strain T48 showed a glucose-stimulated increase in both pH optimum and vanadate sensitivity, whereas the Pma1NΔp enzyme showed an equivalent alkaline shift in pH optimum but no significant increase in vanadate sensitivity (Table 3b). This result indicates that the amino terminus of the wild type ATPase may be involved in a subtle, glucose-responsive change in the vanadate (Pi) binding site. In contrast, both C-terminally deleted enzymes were at least as sensitive to vanadate as the glucose-activated wild type ATPase. Thus, in glucose-activated cells, deletion of the carboxyl terminus obscured any effects of N-terminal truncation on sensitivity of the ATPase to vanadate. Further evidence of the subtle influence of the amino-terminal domain can be seen by comparing the Pma1CΔp and Pma1ΔΔp enzymes from glucose-starved cells. While the Pma1CΔp enzyme's vanadate sensitivity doubled after glucose starvation, the I_{50} (vanadate) of the doubly deleted enzyme was not significantly altered (Table 3b). Proton ATPases from strains *pma1*CΔ and *pma1*ΔΔ showed glucose-independent pH optima that were closer to neutrality (pH 6.75) than those of ATPases with an intact carboxyl terminus. Glucose-independent, alkaline pH optima were previously noted for ATPases truncated at A^{905} and Q^{908} [4].

4. Discussion

The viability of *S. cerevisiae* strains expressing terminally truncated forms of plasma membrane H^+-ATPase, in the absence of a wild type *PMA1* gene, provided unequivocal evidence that limited unilateral or bilateral truncations of the H^+-ATPase do not affect the enzyme's ability to support yeast growth under standard culture conditions. The lethality associated with a *pma1*Δ873-918 mutation was surprising, given that the same mutation was previously reported to support yeast growth and replication, albeit in a plasmid-based conditional expression system [4]. This discrepancy probably reflects the onus that our genetic system for generating yeast mutants places on the mutant enzyme to function during spore formation and outgrowth from spore to vegetative cell. Since a consensus model for P-type ATPases [32,33] places Y^{873} within transmembrane segment 10, the lethality of the *pma1*Δ873-918 mutation may have resulted from either the generation of an unstable, functionally inadequate enzyme and/or insufficient incorporation of the truncated ATPase into the plasma membrane. This finding emphasizes the potential influence of the expression system on the characterization of mutant enzyme structure/function in vivo, particularly when the cells are under intense selective pressure to produce a functional H^+-ATPase. The recent phenotypic re-assignment of 12 out of 25 site-directed *pma1* mutations formerly classified as either wild type or recessive lethal to dominant lethal supports this contention [36].

The in vivo phenotype of the *pma1*NΔ mutant was consistent with the result of Portillo et al. [4], who showed that the same deletion mutation (Δ*pma1-241*) was well tolerated when conditionally expressed from a centromeric plasmid. This unilateral truncation yielded an essentially normal ATPase with in vitro characteristics that were comparable to those of a wild type enzyme. At the whole cell level, the only modified phenotypes were small but consistent reductions in growth rate and growth yield and weak hygromycin resistance. Bilateral truncation of the enzyme suppressed the growth-related phenotypes but not the aminoglycoside resistance.

Truncating the ATPase at its carboxyl terminus had significant effects on both the activity and biogenesis of the enzyme. The large increase in specific activity of the ATPase and the decrease in the $K_{m(app)}$ of the enzyme were probably due to the elimination of autoregulatory elements at the carboxyl terminus. Bilateral truncation of the enzyme seemed to exaggerate the effects of carboxy-terminal truncation alone. This included the pronounced influence of pH on the amounts of plasma membrane H^+-ATPase made. By simply lowering the pH of the growth medium to 3.0, it was possible to increase both the duration and the amount of *pma1*ΔΔ transcription. This manipulation effectively restored the apparent membrane loading of truncated ATPase to near wild type levels. Despite changes in the amount and activity of plasma membrane H^+-ATPase in both strains expressing C-terminally truncated enzymes, there was no evidence of compensatory overexpression of PMA2 or any other protein(s) on SDS-PAGE gels (data not shown). Thus, the constitutively activated, bilaterally truncated enzyme appeared to be produced in just the amounts required to maintain proton economy across the plasma membrane. The situation was different for cells expressing a wild type ATPase, since constitutive but limited overproduction of the enzyme was compensated by post-translational regulatory mechanisms which restricted overall enzyme activity. At this time we cannot exclude the possibility that growth in medium at an acidic pH induced a proliferation of internal membranes containing additional ATPase which then contaminated plasma membrane preparations. However, since the specific activity of the enzyme was unaffected by growth at pH 3.0 (data not shown), we can conclude that any additional ATPase produced was functionally equivalent to that resident in the plasma membrane. In the future, the ability to vary the membrane content of the ATPase target may be used to advantage in screens designed to identify ATPase-specific inhibitors.

Our observations as to the effects of glucose starvation on $V_{max(app)}$ and $K_{m(app)}$ values for the wild type ATPase differed somewhat from the original observations made by Serrano [10]. In the present study, glucose starvation was performed at 30°C in an effort to keep the temperature constant. Furthermore, a protracted starvation period (by comparison with Serrano's protocol) was required in order to demonstrate any significant decrease in ATPase activity. In the absence of a marked temperature change, enzyme behaviour was relatively stable over the period of starvation tested, with total activity falling less than 50% and the $K_{m(app)}$ remaining essentially unchanged. Prolonged (4 h) incubation of cells in the presence of glucose prior to membrane preparation did not significantly modify the kinetic characteristics of the ATPase by comparison with those of the enzyme prepared from cells processed directly (data not shown). These findings suggested that placing cells on ice for an extended period may exacerbate the effects of glucose starvation on H^+-ATPase kinetics. Additional experiments confirmed that this was the case for our yeast strain. The potential for modification of enzyme behaviour as a result of temperature change adds a further parameter to the analysis of wild type and mutant enzymes alike. Further elucidation of the role played by heat shock protein Hsp30 in regulating the activity of the stress-induced ATPase may add to our understanding of this process [37]. The low K_m values seen for C-terminally truncated ATPases from glucose-starved cells lend further support to the idea that these enzymes are rendered catalytically hyperactive without the regulatory influence of the carboxyl terminus. It is also tempting to speculate that the decrease in activity observed for these mutant enzymes after 4 h of glucose starvation is a consequence of the limited supply of intracellular ATP.

As a major consumer of intracellular energy, the plasma membrane proton pump is subject to tight post-translational regulation and probably exists as a partially active or conformationally heterogeneous enzyme population in the plasma membrane. Using the well-expressed, fully functional, bilaterally truncated ATPase, which lacks much of the wild type enzyme's inherent autoregulatory capacity, we are exploring the idea that the mutant can provide a homogeneous population of highly active enzyme molecules better suited to crystallographic study.

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