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Characterization of non-dominant lethal mutations in the yeast plasma membrane H^+-ATPase gene

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Abstract

Site-directed mutants of yeast ATPase were previously studied after introduction of mutant alleles into a yeast strain where these alleles were constitutively expressed while the expression of the wild-type chromosomal ATPase gene was turned off. As a functional H^+ pump is essential, strong selective pressure leads to the accumulation of revertants during growth of cells harboring variants with low activity. Thus, constitutive expression of the mutant gene can select phenotypes which reflect events such as gene conversion or reversion. We have therefore re-evaluated the phenotypes of non-dominant lethal alleles in an alternative set of conditional expression systems. We show that eight of 11 previously described site-directed mutations behave as recessive lethal alleles.

Keywords: H^+-ATPase/plasma membrane; Non-dominant lethal mutation; (*Saccharomyces cerevisiae*)

1. Introduction

The plasma membrane H^+-ATPase from *Saccharomyces cerevisiae* belongs to the large family of cation ATPases which form a transient aspartyl-phosphate intermediate [1]. The H^+-ATPase, encoded by the *PMA1* gene, generates an electrochemical proton gradient essential for active nutrient uptake and intracellular pH regulation [2–5].

Site-directed mutagenesis has been used to study the role of potentially important residues of the enzyme. The mutant enzymes were tested for physiological activity in a yeast strain where the expression of the chromosomal wild-type ATPase was turned off and the mutant ATPases were constitutively expressed [6–9]. Several significant drawbacks of this expression system have become apparent. (1) Since the mutant gene is co-resident in the cell with a wild-type gene, a dominant lethal mutation can be lost during sequence shuffling by either gene conversion with PMA1 or gene reversion [10–12]. (2) Because a functional ATPase is essential, there is strong selective pressure for revertants to accumulate during growth of cells harboring variants with low activity. (3) When cells are transferred to glucose medium in order to turn off the wild-type chromosomal ATPase, cells expressing inactive mutant ATPases stop growing. This can leave a significant background wild-type enzyme in the plasma membrane. It was therefore important, first, to determine whether any of the site-directed mutants studied was in fact dominant lethal and, second, to characterize the non-dominant lethal mutations using an alternative expression system. Dominant lethal mutations were reported in a

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previous study [13]. Here, we have re-evaluated the phenotype of non-dominant lethal mutant ATPases using an expression system in which the mutant enzymes are conditionally expressed and accumulated in secretory vesicles where they are analyzed [14].

2. Materials and methods

2.1. Yeast strains and growth media

S. cerevisiae strain SY4 is GAL$^+$ *MAT*a *ura3 leu2 his4 sec6 pma1*::*YIpGAL-PMA1* and was used for expression of the ATPase in secretory vesicles [14]. Strain XZ611 is Gal$^+$ *MAT*a *ura3 leu2 trp1* [12] and was used in immunofluorescence experiments. Synthetic media with 2% dextrose (SD), 2% lactic acid (SL) or 2% galactose (SG) and the appropriate requirements were used [15]. Yeast cells were transformed using the lithium acetate procedure [16].

2.2. Expression of the ATPase in secretory vesicles

The construction of the *pma1* alleles used in this study has been described [6–9] and all of them were re-sequenced to ensure that only the desired mutation was present. Site-directed mutations were introduced into plasmid pPMA1.2 [14] by exchange of either a 1.5 kb *Eco*RV-*Bam*HI (*E129Q*→*K474Q*) or a 2.2 kb *Xba*I (*D560N*→*T912A*) fragment with the corresponding fragment in plasmid pPMA1.2. After the fragments were moved into plasmid pPMA1.2, a 3.77 kb *Sac*I-*Hin*dIII fragment containing the entire ATPase coding sequence was cloned into vector YCp2HSE [14] placing the mutant alleles under the control of a heat-shock inducible promoter. The resulting plasmids were transformed into strain SY4 [14]. This strain has the chromosomal *PMA1* gene under the control of the *GAL1* promoter and also carries the *sec6-4* mutation that blocks fusion of secretory vesicles with the plasma membrane. For expression studies, SY4 transformants were grown to mid-exponential phase ($OD_{660}\sim0.5$) on SG and shifted to SD for 3 h to turn off transcription of chromosomal *PMA1*, then shifted to 37°C for 2 h to turn on expression of the plasmid-borne gene and block fusion of secretory vesicles with plasma membrane. Secretory vesicles were then isolated by differential centrifugation as described [14]. Each plasmid was rescued from yeast expressing functional Pma1p and the corresponding *pma1* allele sequenced to ensure that only the desired mutation was present.

2.3. Construction of HA-tagged pma1 alleles

The HA-tagged *pma1* alleles used in this study were generated by exchange of either a 0.6 kb *Eco*RV-*Bst*EII (*T231G* and *S234A*) or a 3.4 kb *Bst*EII-*Hin*dIII (*K474H*, *K474H*, *D560N*, *D634N* and *D730N*) fragment containing the desired mutation with the corresponding fragment in plasmid pFP302. The plasmid pFP302 is a derivative of the *URA3* single copy plasmid YCp50 [17] and it carries a HA-tagged wild-type *PMA1* under the control of the *GAL1* promoter [13].

2.4. Biochemical methods

Quantitative immunodetection of the ATPase was performed as described [14], using affinity purified rabbit polyclonal antibody against yeast ATPase [18]. ATPase activity was assayed at pH 6.5 with 5 mM ATP [19]. H^+ pumping into secretory vesicles was monitored by fluorescence quenching of acridine orange [14]. Protein concentration was determined by the Bradford method [20] with the Bio-Rad Protein Assay Reagent and bovine IgG as standard. Localization of HA-tagged Pma1 was determined by immunofluorescence as described [13].

3. Results

The site-directed mutations characterized in this study and their topological location in Pma1p are illustrated in Fig. 1. The mutant genes were placed under the control of a heat-shock inducible promoter in the plasmid YCp2HSE [14]. Plasmid YCp2HSE containing the mutant genes were transformed into strain SY4 [14]. This strain has the chromosomal *PMA1* gene under the control of the *GAL1* gene promoter and also carries the temperature-sensitive *sec6-4* mutation that blocks fusion of secretory vesicles with plasma membrane. Thus, when cells were incubated in galactose medium at 23°C the chromosomal wild-type ATPase was produced and

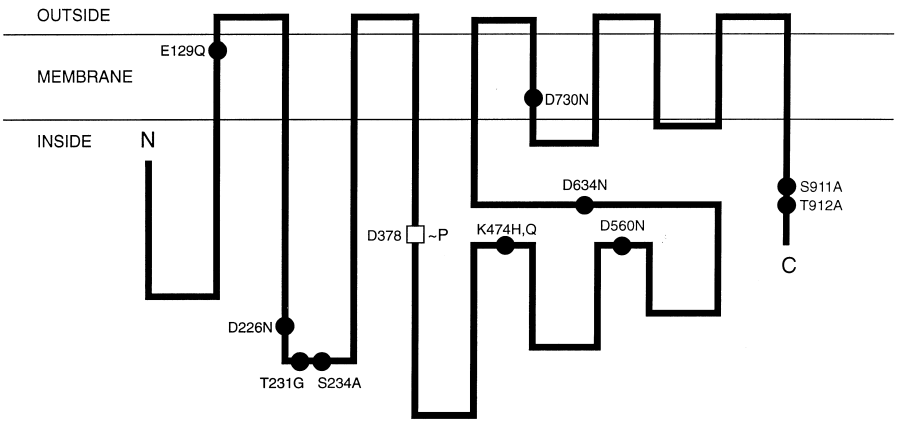

Fig. 1. Location of H^+-ATPase mutations characterized in this study. Phosphorylation site (Asp-378) and positions of the mutations are indicated within the proposed structural topography of the H^+-ATPase as determined from hydropathy analysis.

when cells were transferred to glucose medium at 37°C the expression of the wild-type ATPase was repressed and the mutant ATPase was expressed. Because strain SY4 carries a temperature-sensitive *sec6-4* mutation the shift to 37°C also led to the accumulation of the newly synthesized mutant ATPase in secretory vesicles. Secretory vesicles were purified by differential centrifugation for analysis of the mutant ATPase [14]. Table 1 summarizes the behavior of the mutant enzymes. In most of the cases (*T231G*, *S234A*, *K474H*, *K474Q*, *D560N*, *D634N*, and *D730N* mutations) quantitative immunoblotting revealed

Table 1
Analysis of mutant ATPase accumulated in secretory vesicles

Allele expressed	Amount of ATPase[a]	ATPase activity[b]		H^+ transport[c]	
		Uncorrected (μmol/min/mg)	Corrected (%)	Uncorrected (% *Q*/mg)	Corrected (%)
Wild-type	100	0.42	100	360	100
None[d]	7	0.04		n.d.	
E129Q	100	0.35	83	278	77
D226N	80	0.04		n.d.	
T231G	5	0.04		n.d.	
S234A	18	0.05		n.d.	
K474H	8	0.04		n.d.	
K474Q	7	0.05		n.d.	
D560N	10	0.06		n.d.	
D634N	9	0.05		n.d.	
D730N	8	0.05		n.d.	
S911A	100	0.50	120	400	110
T912A	85	0.10	30	149	40

n.d., not determined.

[a]Quantified by immunoassay. The value for the wild type was considered 100%.

[b]ATPase activity was assayed at pH 6.5 with 5 mM ATP. The specific activity of each secretory vesicle preparation was measured as the difference between hydrolysis in the absence and in the presence of 250 μM orthovanadate. Values are the average of two independent experiments differing less than 15%. Each mutant value was corrected for the relative amount of mutant protein in the secretory vesicle preparation.

[c]Fluorescence quenching of acridine orange was used to monitor pumping of protons into secretory vesicles. Similar values (within 15%) were obtained with two different secretory vesicle preparations independently isolated. Each mutant value was corrected for the relative amount of mutant protein in the secretory vesicle preparation.

[d]Secretory vesicles were isolated from SY4 transformed with the vector YCp2HSE carrying no *PMA1* gene.

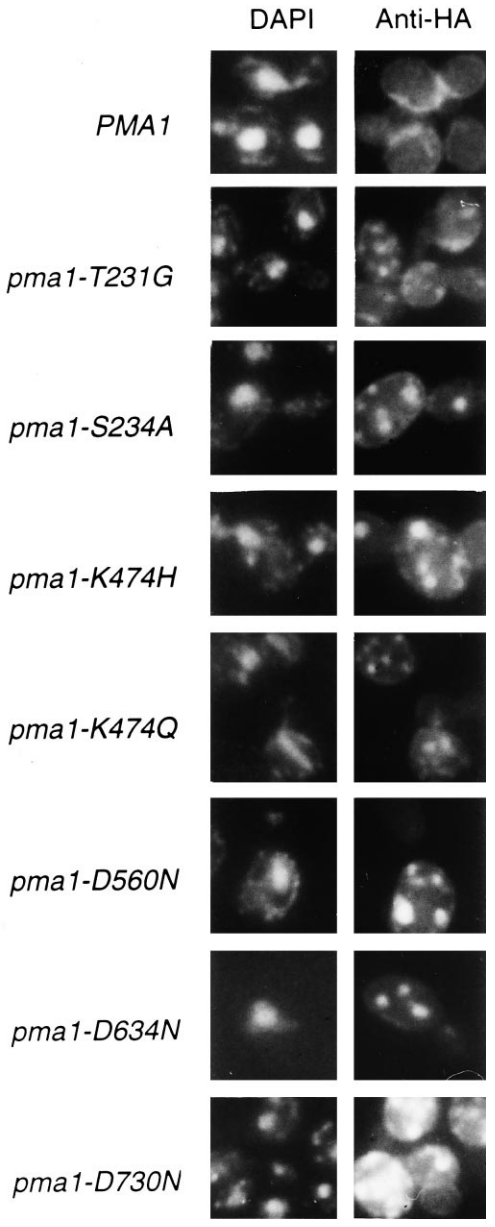

Fig. 2. Accumulation of the HA-tagged mutant protein in cytoplasmic structures. The transformant carried either the HA-tagged *PMA1* or HA-tagged *pma1* genes under the control of the *GAL1* promoter. After galactose induction, cells were stained for the nucleus (DAPI) or immunodecorated for the HA-tagged Pma1p using a rhodamine-conjugated anti-HA antibody (anti-HA).

that mutations led to a substantial decrease in the amount of Pma1p in the secretory vesicles. The amounts of Pma1p antigen detected in these mutants were background or barely above the background (*S234A*) given with the YCp2HSE vector alone. Possible explanations for the failing of mutant enzymes to reach the vesicle would be that these mutations render enzymes abnormally susceptible to degradation or, alternatively, that mutations prevent proper folding or insertion of Pma1p into the membrane and led to retention of Pma1p at an early stage of biogenesis. To test whether these mutations alter the biogenesis of Pma1p, we studied the transport of mutant ATPases to the cell surface. To this end, we introduced the HA epitope in the mutant proteins and HA-tagged proteins were expressed under the control of the *GAL1* promoter in a wild-type strain. We examined the location of these HA-tagged mutant proteins by immunofluorescence decoration after galactose induction. The results are presented in Fig. 2. In all the cases the HA antibody decorated a set of cytoplasmic structures suggesting that mutations led to retention of Pma1p in an early step of the secretory pathway. All of the remaining mutant ATPases (*E129Q*, *D226N*, *S911A* and *T912A*) reached the secretory vesicles at levels ranging from 80% (*D226N*) to 100% (*S911A*). In these cases, secretory vesicle preparations were next assayed for their ability to hydrolyze ATP and to analyze ATP-dependent H^+ transport. In one case (*D226N*), mutation led to an activity value not significantly above background, suggesting that Asp-226 plays an important role in ATPase function. In the remaining mutants (*E129Q*, *S911A* and *T912A*) activity values ranged from 30% hydrolysis/40% transport in *T912A* to 120% hydrolysis/110% transport in *S911A*. In these cases, kinetic analysis (data not shown) gave essentially wild-type values, e.g. K_m for ATP and K_i for orthovanadate.

4. Discussion

A specific role for several amino acids in ATPase function was assigned using site-directed mutagenesis in combination with a constitutive expression system of the mutant ATPases [6–9]. However, gene conversion or reversion allowed the modification of dominant negative phenotypes or inactive proteins. Dominant lethal mutations were previously characterized [13]. Here, we have analyzed the phenotype of non-dominant lethal mutations by expressing the mutant

ATPases in secretory vesicles where they are analyzed.

The present study shows that the previously proposed role of Asp-226, Thr-231, Ser-234, Lys-474, Asp-560, Asp-634, and Asp-730 in ATPase function must be reconsidered. Amino acids Asp-226, Thr-231 and Ser-234 were proposed to be involved in energy transduction as *D226N*, *T231G* and *S234A* mutations caused uncoupling between ATP hydrolysis and H^+ transport [6,7]. The Lys-474 residue was suggested to be essential for enzyme function as mutations at this amino acid greatly decreased ATPase activity [6,7]. Amino acid Asp-560 was considered as part of the ATP binding site as *D560N* mutation altered the nucleotide binding specificity of the enzyme [6]. The Asp-634 residue was proposed to form part of a phosphatase domain, as the *D634N* mutation blocked hydrolysis of the phosphorylated intermediate [7]. Finally, amino acid Asp-730 was described as non-essential as *D730N* mutation had no noticeable effect on ATPase activity [7]. The fact that *D226N* mutation greatly decreases ATPase activity and that *T231G*, *S234A*, *K474H*, *K474Q*, *D560N*, *D634N*, and *D730N* mutations led to retention of Pma1p early in the secretory pathway suggest that these amino acids must be important for protein folding and/or targeting although a specific role cannot be assigned. The observed behavior of these mutations is consistent with a recessive lethal phenotype.

In the case of the Glu-129 and Ser-911 residues, the results obtained in the present study are essentially coincident with those previously shown [7,9] and suggest that amino acids Glu-129 and Ser-911 are non-essential for ATPase function.

Amino acid Thr-912 was considered to form part of a regulatory domain as *T912A* mutation disturbs the glucose-triggered V_{max} increase of the enzyme [9]. The results shown here are consistent with the proposed role for Thr-912. The *T912A* mutation led to a substantial decrease in activity without altering the K_m of the enzyme for ATP, thus suggesting that the decreased activity could be due to a defect in V_{max}.

Further work which combines new site-directed mutations with the analysis of revertants, expression of the mutant ATPases in secretory vesicles and the transit of mutant ATPases to the cell surface will allow the assignment of specific roles for the essential residues described in this study.

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References

[1] P.L. Pedersen, E. Carafoli, Trends Biochem. Sci. 12 (1987) 146–150.
[2] A. Goffeau, C.W. Slayman, Biochim. Biophys. Acta 639 (1981) 197–223.
[3] R. Serrano, in: J.R. Broach, J. Pringle, E.W. Jones (Eds.), The Molecular and Cellular Biology of the Yeast Saccharomyces, Genome Dynamics, Protein Synthesis and Energetics, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1991, Ch. 9, pp. 523–585.
[4] G.A. Scarborough, in: J.J.H.H.M. dePont (Ed.), Molecular Aspects of Membrane Proteins, Eselvier Science Publishers B.V., Amsterdam, 1992, pp. 117–134.
[5] R. Rao, R.K. Nakamoto, S. Verjovski-Almeida, C.W. Slayman, Ann. NY Acad. Sci. 671 (1993) 195–203.
[6] F. Portillo, R. Serrano, EMBO J. 7 (1988) 1793–1798.
[7] F. Portillo, R. Serrano, Eur. J. Biochem. 186 (1989) 501–507.
[8] F. Portillo, I.F. de Larrinoa, R. Serrano, FEBS Lett. 247 (1989) 381–385.
[9] F. Portillo, P. Eraso, R. Serrano, FEBS Lett. 287 (1991) 71–74.
[10] S.L. Harris, K.S. Rudnicki, J.E. Haber, Genetics 135 (1993) 5–16.
[11] P. Supply, A. de Kerchove d'Exaerde, T. Roganti, A. Goffeau, F. Foury, Mol. Cell. Biol. 15 (1995) 5389–5395.
[12] S.L. Harris, S. Na, X. Zhu, D. Seto-Young, D.S. Perlin, J.H. Teem, J.E. Haber, Proc. Natl. Acad. Sci. USA 91 (1994) 10531–10535.
[13] F. Portillo, FEBS Lett. 402 (1997) 136–140.
[14] R.K. Nakamoto, R. Rao, C.W. Slayman, J. Biol. Chem. 266 (1991) 7940–7949.
[15] M.D. Rose, F. Winston, P. Hieter, Methods in Yeast Genetics. A Laboratory Course Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1990.
[16] H. Ito, Y. Fukuda, K. Murata, A. Kimura, J. Bacteriol. 153 (1983) 163–168.
[17] M.D. Rose, J.R. Broach, Methods Enzymol. 194 (1991) 195–230.
[18] B.C. Monk, C. Montesinos, C. Ferguson, K. Leonard, R. Serrano, J. Biol. Chem. 266 (1991) 18097–18103.
[19] R. Serrano, Methods Enzymol. 157 (1988) 533–534.
[20] M.M. Bradford, Anal. Biochem. 72 (1976) 248–254.