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Deletion analysis of yeast plasma membrane H^+-ATPase and identification of a regulatory domain at the carboxyl-terminus

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The function of the amino- and carboxyl-terminal domains of the yeast plasma membrane H^+-ATPase have been investigated by constructing deletions in vitro and selectively expressing the mutant enzymes in vivo. The first 27 amino acids are dispensable but deletion of a further 33 amino acids greatly decreases the appearance of the enzyme in the plasma membrane. Membrane localization is also prevented by carboxyl-terminal deletions which include the last hydrophobic stretch, but the last 46 amino acids of the ATPase are not required. Removal of the last 11 amino acids produces an enzyme in glucose-starved cells with the kinetic parameters of the wild-type ATPase activated by glucose fermentation. This region seems to constitute a regulatory domain.

ATPase, plasma membrane; Regulatory domain; (*Saccharomyces cerevisiae*)

1. INTRODUCTION

The yeast plasma membrane ATPase is a proton pump which belongs to the family of cation-pumping ATPases with a phosphorylated intermediate [1,2]. The N- and C-terminal domains (before the first and after the last hydrophobic stretch, respectively) are the most divergent regions of these homologous enzymes. This suggests that these domains have evolved functions specific to each type of cation pump. These specific functions could include cation binding, signals for assembly or degradation or regulation of activity.

This last possibility is especially attractive because many regulated enzymes such as protein kinases [3], adenylate cyclase [4] and the yeast α-factor receptor [5] contain inhibitory domains at their N- or C-termini. The inhibitory interaction between these domains and the catalytic site is modulated by either binding of effector molecules [3,4] or phosphorylation [5]. Inhibition can also be released by removal of the inhibitory domain by either partial proteolysis [3,4] or truncation at the gene level [5]. An inhibitory domain has been identified at the C-terminus of the Ca^{2+}-ATPase of animal plasma membranes [6]. This domain is involved in binding calmodulin, an activator of the enzyme, and is subject to phosphorylation during activation of the enzyme by protein kinases. The activity of the yeast plasma ATPase is regulated in response to growth conditions [7-9] and glucose metabolism seems to be one of the most important regulatory factors [7]. There are indications that phosphorylation of the enzyme is involved in the regulation of its activity [10-12]. Finally, the activity of the yeast ATPase is increased by partial proteolysis [13], suggesting the existence of a terminal inhibitory domain.

We have performed a deletion analysis of the N- and C-termini of the yeast ATPase and analyzed the level and activity of mutant enzymes in the plasma membrane and their regulation by glucose. Our results indicate that most of the N-terminal domain is required for the functional insertion of the

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enzyme into the plasma membrane and that the last part of the C-terminal domain is involved in the regulation of the enzyme by glucose.

2. MATERIALS AND METHODS

2.1. *Construction of N-terminal deletions*

*Nco*I sites with the ATG codons at amino acid positions 1, 28, 61 and 85 of the plasma membrane ATPase gene of *Saccharomyces cerevisiae* were introduced by site-directed mutagenesis. The mutagenic oligonucleotides (changes underlined) were: ATCACCATGGCTGATAC, GCCTGCCATGGCTTACG, CGAAGCCATTGGATAACG and CCCATCCCATGGTTTAA, respectively. Mutagenesis of the 2.1 kb *Kpn*I fragment of the ATPase gene in M13mp19, sequencing of the mutations and reconstruction of the 5 kb *Hind*III fragment containing the mutant ATPase gene in a modified pUC18 plasmid were performed as described [14]. Deletions were generated by replacing the 1.6 kb *Nco*I-*Bam*HI fragment of the mutant containing the *Nco*I site at the first amino acid with the corresponding fragment of the other mutants. In the case of the larger deletion the reading frame was adjusted by digestion with *Nco*I, filling the ends with the Klenow fragment of DNA polymerase I and religation. The resulting genes had coding regions starting with the following one or two amino acid changes (allele designation between parentheses, see fig.1): Lys 28→Met and Lys 29→Ala (Δpma 1-241); Ser 61→Met (Δpma 1-242); Ser 85→Met and Tyr 86→His (Δpma 1-243).

2.2. *Construction of C-terminal deletions and deletion substitutions*

Three deletions were made introducing termination codons by site directed mutagenesis of the 2.2 kb *Xba*I fragment of the ATPase gene as described above [14]. The mutagenic oligonucleotides (changes underlined) were: CTGCTATGTAAAGAGTC, GGTTTCTAATACGAAAT and GGTGGTCTTAAAACTGG, corresponding to the mutations (allele designation between parentheses, see fig.1) Gln 908→stop (Δpma 1-244), Tyr 873→stop (Δpma 1-246) and Glu 847→stop (Δpma 1-247).

Three deletion substitutions were made utilizing *Xho*I sites artificially introduced along the ATPase gene by partial digestion with either *Alu*I or *Taq*I and insertion of *Xho*I linkers [15]. The oligonucleotide GCTTAATTAATTAAGC (Universal Translation Terminator, Pharmacia) contains stop codons in all reading frames. It was ligated with *Xho*I linkers (CCTCGAGG, Pharmacia), digested with *Xho*I and inserted into *Xho*I sites at positions 3534, 3330 and 3268 of the 5 kb *Hind*III fragment containing the ATPase gene [15]. This creates the following three deletion substitutions: (Glu 901→Ala, Asp 902→Ser, Phe 903→Arg, Met 904→Ala and Ala 905→stop; allele Δpma 1-245), (Asp 833→Ala, Ile 834→Ser, Ile 835→Arg, Ala 836→Ala and Thr 837→stop; allele Δpma 1-248) and (Ala 813→Ser, Gly 814→Ser, Pro 815→Ala and Phe 816→stop; allele Δpma 1-249).

2.3. *Transformation of yeast with the mutant ATPases and growth of the transformants*

The mutant ATPase genes were subcloned on yeast centromeric plasmid pSB32 and introduced into strain RS-72 as previously described [14]. RS-72 is a genetically engineered strain of *Saccharomyces cerevisiae* with the constitutive promoter of the chromosomal wild type ATPase gene replaced by a galactose-dependent promoter [16]. Transformants were selected and propagated on galactose medium, where both the wild type (chromosomal) ATPase and mutant (plasmid) ATPase are expressed. In order to express only the mutant enzymes, the galactose grown cells (absorbance at 660 nm about 3) were diluted 50-fold in glucose medium and growth recorded after 24 h. The synthetic growth medium was as described [14]. After about three duplications the preformed ATPase has been sufficiently diluted and further growth depends on the ATPase expressed by the plasmid. Cultures expressing active ATPase reach a final absorbance of about 1.5 while cultures expressing inactive phenotypes only reach about 0.3 [14,16].

Strains in glucose medium expressing either wild type ATPase or no ATPase have been previously described (RS-303 and RS-357, respectively, see [14]). Purified plasma membranes from glucose-grown cells of RS-357 contain residual wild type ATPase amounting to about 10% of the RS-303 level (Portillo, F., unpublished and [14]).

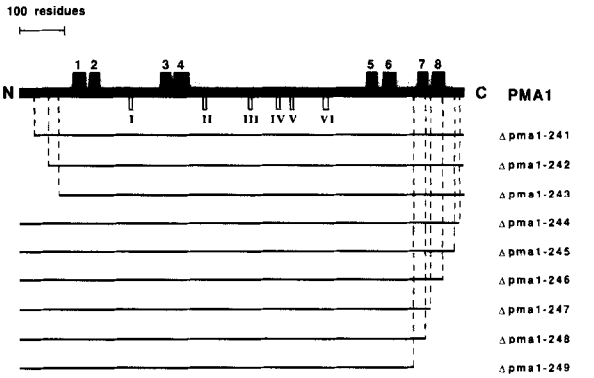

Fig.1. Scheme of the terminal deletions of the yeast plasma membrane ATPase. PMA1 refers to the wild type gene and Δpma1 to the deletion mutants. 1 to 8 are the hydrophobic stretches of the yeast ATPase proposed to constitute transmembrane α-helices (stretch 5 of reference [1], amino acids 692-713, is not considered as transmembrane in this scheme) and I to VI are the conserved motifs within the family of ATPases with a phosphorylated intermediate [1].

2.4. *Biochemical methods*

Yeast plasma membrane was purified from washed cells (glucose-starved) by differential and sucrose-gradient centrifugation [17]. When indicated, the cells were treated with glucose before homogenization (glucose-fermenting cells) [7]. ATP hydrolysis was measured [17] in the presence of molybdate, azide and nitrate to inhibit any residual contamination by acid phosphatase, mitochondrial ATPase and vacuolar ATPase, respectively. Under these conditions ATP hydrolysis is fully specific for the plasma membrane ATPase [17]. Electrophoresis in the presence of SDS [18] was as described.

3. RESULTS AND DISCUSSION

Deletion of the first 27 amino acids of the yeast ATPase (Δpma 1-241) affects neither the activity of the enzyme nor its regulation by glucose (table 1) and the ATPase level in the plasma membrane is also normal (fig.2, lane 4). A small change in electrophoretic mobility confirms the deletion at the protein level. The ATPase band, however, is greatly decreased in plasma membranes from strains expressing deletions of the first 60 (Δpma 1-242) or 84 (Δpma 1-243) amino acids. Weak bands with apparent molecular mass of about 94 and 92 kDa are observed in lanes 5 and 6, respectively. They could correspond to the expected truncated ATPases but their levels are only about 20% and 10% of wild type (lane 2), respectively. As a comparison lane 3 shows plasma membranes with about 10% of normal ATPase level.

Deletion alleles 242 and 243 cannot support yeast growth (not shown). The ATPase is essential and rate limiting for growth [15] and growth tests can detect levels of activity greater than 10% of wild type (Portillo, F. and Serrano, R., submitted). The most conserved motif in fungal ATPases between residues 27 and 60 of the yeast enzyme is the sequence DDDIDALIEEL (positions 40-50), with only E/D substitutions in different fungi [1]. It is possible that this sequence is important for the proper assembly of the ATPase into the plasma membrane. Incompletely assembled proteins are known to be retained in the endoplasmic reticulum and not to be transported to the plasma membrane [20]. We have not investigated if mutant ATPases accumulate in some other membrane compartment or are degraded. In addition, if the weak band of allele 242 discussed above corresponds to the truncated ATPase, it seems that the observed lack of growth cannot be completely explained by the decreased amount of ATPase. A reduction in the activity of this mutant enzyme must also be involved. This is in agreement with tryptic cleavage studies of the *Neurospora* ATPase [19]. The first 36 amino acids of this enzyme are dispensable but removal of the succeeding 37 residues, including the conserved acid motif, led to complete loss of activity.

Deletion (or substitution) of the last 11, 18 or 46 amino acids of the ATPase still allows functional insertion of the enzyme into the plasma membrane

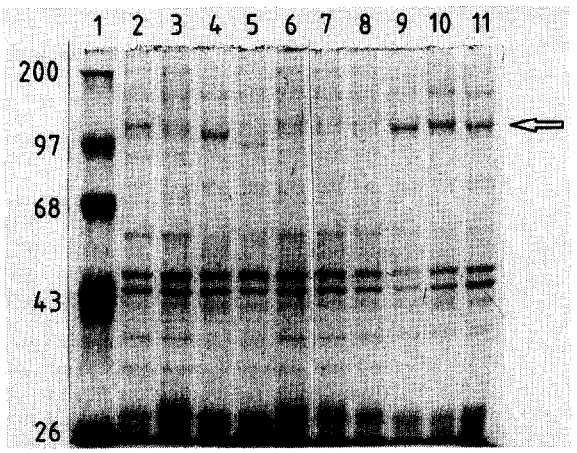

Fig.2. Expression of mutant ATPases at the plasma membrane. Plasma membranes were purified from glucose-grown cells expressing the alleles of the ATPase gene (see fig.1) indicated below. After electrophoresis in SDS the proteins were stained with Coomassie R-250. Lanes: 1, molecular mass standards with their molecular masses indicated in kDa on the left; 2, PMA1 (strain RS-303); 3, no ATPase expressed in glucose medium (strain RS-357); 4, Δpma1-241; 5, Δpma1-242; 6, Δpma1-243; 7, Δpma1-249; 8, Δpma1-247; 9, Δpma1-246; 10, Δpma1-245; 11, Δpma1-244. The arrow indicates the position of the ATPase band of about 100 kDa.

(fig.2, lanes 11, 10 and 9, respectively). A small change in electrophoretic mobility is observed with the last deletion, confirming the mutation at the protein level (fig.2, lane 9). All these truncated enzymes are active and can support normal growth (not shown). On the other hand, larger deletions at the carboxy-terminus starting at positions 847 and 815 greatly decrease the appearance of the ATPase in the plasma membrane (fig.2, lanes 7 and 8) and the enzyme cannot support growth (not shown). Very weak bands, amounting to less than 10% of normal levels, can be observed at the expected positions for these truncated ATPases. Similar results were obtained with a deletion starting at position 837 (Δpma 1-248, not shown). All these deletions remove the last one or two hydrophobic stretches (fig.1). These regions are very conserved in fungal ATPases [1] and they could also be required for assembly into the plasma membrane.

Glucose fermentation before homogenization dramatically changes the kinetic properties of wild type ATPase [7]. The K_m for ATP is decreased 4-fold, the V_{max} increased 3-fold, the sensitivity to vanadate increased 20-fold and the pH optimum

Table 1

Kinetic properties of mutant ATPases from glucose-starved (GS) and glucose-fermenting (GF) cells

ATPase allele	K_m[a]		V_{max}[a]		I_{50} vanadate[b]		Activity ratio pH 5.3/pH 7.5[c]	
	GS	GF	GS	GF	GS	GF	GS	GF
PMA1	0.66	0.15	0.14	0.46	10	0.5	2.6	0.8
Δpma1-241	nd[d]	nd	0.13	0.48	nd	nd	nd	nd
Δpma1-244	0.15	0.15	0.45	0.39	0.8	0.6	0.6	0.7
Δpma1-245	0.14	0.14	0.50	0.40	0.5	0.4	0.7	0.7

[a] ATPase activity was assayed at pH 5.7 with 5 mM $MgSO_4$ and ATP concentrations from 0.2 to 2 mM. The apparent K_m (mM) and V_{max} (μmol Pi·min^{-1}·mg protein^{-1}) were extrapolated from double reciprocal plots fitted by the least-squares method (linear regression coefficients, $r = 0.993$–0.995). Similar values (within 10%) were obtained with three different membrane preparations

[b] Concentration of vanadate (μM) which gives 50% inhibition with 2 mM ATP and pH 5.7

[c] Ratio of activities at the two pH measured with 2 mM ATP

[d] nd, not determined

displaced to more alkaline values (table 1). However, mutant ATPases lacking either the last 11 amino acids (Δpma1-244) or lacking the last 14 and containing substitutions in 4 more amino acids (Δpma1-245) exhibit in the absence of glucose fermentation the properties of the wild type enzyme activated by glucose. Glucose fermentation slightly inhibits these truncated ATPases (table 1). Fig.3 illustrates in more detail the effect of glucose on the activity and pH dependence of wild type and Δpma1-245 ATPases. Allele Δpma1-244 has very similar properties to Δpma1-245 (not shown).

These results suggest that the last 11 amino acids of the yeast ATPase contain an inhibitory domain which could interact with the active site. We have previously identified a mutation (Ala-547→Val) located between active site motifs IV and V (see fig.1) which also results in an ATPase permanently activated in the absence of glucose [21]. The C-terminal inhibitory domain could interact with this part of the active site, although more biochemical and genetic evidence is needed to demonstrate this point. According to this working hypothesis, glucose could activate the wild type enzyme by inducing the phosphorylation [10–12] of either the active site region or the inhibitory domain. This covalent modification could change the conformation of the ATPase and release the interaction between the active site and the inhibitory domain. A corollary of this model is that the C-terminal domain would be located in the cytosol, as suggested for the Ca^{2+}-ATPase of animal plasma membranes [6]. Accordingly, in fig.1 we have depicted 4 hydrophobic stretches in the second half of the ATPase, instead of the 5 stretches previously considered [1].

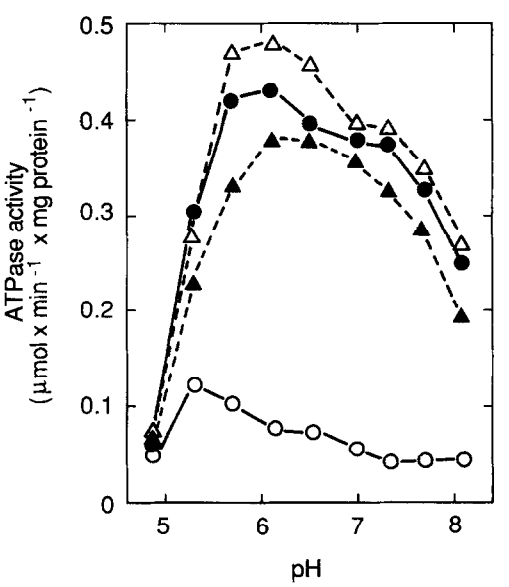

Fig.3. Effect of pH on ATPase activity. Glucose-grown cells expressing either the wild type ATPase (circles, continuous line) or the Δpma1-245 mutant (triangles, discontinuous line) were washed with water and homogenized after incubation in the absence (starved cells, open symbols) or presence (fermenting cells, closed symbols) of glucose. Plasma membranes were purified and the ATPase activity determined as described in section 2.

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REFERENCES

[1] Serrano, R. (1989) Annu. Rev. Plant Physiol. Plant Mol. Biol. 40, 61–94.
[2] Jorgensen, P.L. and Andersen, J.P. (1988) J. Membr. Biol. 103, 95–120.
[3] Hardie, G. (1988) Nature 335, 592–593.
[4] Heideman, W., Casperson, G.F. and Bourne, H.R. (1987) J. Biol. Chem. 262, 7087–7091.
[5] Reneke, J.E., Blumer, K.J., Courchesne, W.E. and Thorner, J. (1988) Cell 55, 221–234.
[6] Verma, A.K., Filoteo, A.G., Stanford, D.R., Wieben, E.D., Penniston, J.T., Strehler, E.E., Fischer, R., Heim, R., Vogel, G., Mathews, S., Strehler-Page, M.A., James, P., Vorherr, T., Krebs, J. and Carafoli, E. (1988) J. Biol. Chem. 263, 14152–14159.
[7] Serrano, R. (1983) FEBS Lett. 156, 11–14.
[8] Tuduri, P., Nso, E., Dufour, J.P. and Goffeau, A. (1985) Biochem. Biophys. Res. Commun. 133, 917–922.
[9] Eraso, P. and Gancedo, C. (1987) FEBS Lett. 224, 187–192.
[10] McDonough, J.P. and Mahler, H.P. (1982) J. Biol. Chem. 257, 14579–14581.
[11] Portillo, F. and Mazon, M.J. (1985) FEBS Lett. 192, 95–98.
[12] Kolarov, J., Kulpa, J., Baijot, M. and Goffeau, A. (1988) J. Biol. Chem. 263, 10613–10619.
[13] Perlin, D.S. and Brown, C.L. (1987) J. Biol. Chem. 262, 6788–6794.
[14] Portillo, F. and Serrano, R. (1988) EMBO J. 7, 1793–1798.
[15] Serrano, R., Kielland-Brandt, M.C. and Fink, G.R. (1986) Nature 319, 689–693.
[16] Cid, A., Perona, R. and Serrano, R. (1987) Curr. Genet. 12, 105–110.
[17] Serrano, R. (1988) Methods Enzymol. 157, 533–544.
[18] Laemmli, U.K. (1970) Nature 227, 680–685.
[19] Mandala, S.M. and Slayman, C.W. (1988) J. Biol. Chem. 263, 15122–15128.
[20] Rothman, J.E. (1987) Cell 50, 521–522.
[21] Cid, A. and Serrano, R. (1988) J. Biol. Chem. 263, 14134–14139.