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Functional complementation of yeast cytosolic pyrophosphatase by bacterial and plant \( \text{H}^+ \)-translocating pyrophosphatases

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Two types of proteins that hydrolyze inorganic pyrophosphate (PPi), very different in both amino acid sequence and structure, have been characterized to date: soluble and membrane-bound proton-pumping pyrophosphatases (sPPases and H+-PPases, respectively). sPPases are ubiquitous proteins that hydrolyze PPi, releasing heat, whereas H+-PPases, so far unidentified in animal and fungal cells, couple the energy of PPi hydrolysis to proton movement across biological membranes. The budding yeast \( \text{Saccharomyces cerevisiae} \) has two sPPases that are located in the cytosol and in the mitochondria. Previous attempts to knock out the gene coding for a cytosolic sPPase (\( \text{IPP1} \)) have been unsuccessful, thus suggesting that this protein is essential for growth. Here, we describe the generation of a conditional \( \text{S. cerevisiae} \) mutant (named YPC-1) whose functional \( \text{IPP1} \) gene is under the control of a galactose-dependent promoter. Thus, YPC-1 cells become growth arrested in glucose but they regain the ability to grow on this carbon source when transformed with autonomous plasmids bearing diverse foreign H+-PPase genes under the control of a yeast constitutive promoter. The heterologously expressed H+-PPases are distributed among different yeast membranes, including the plasma membrane, functional complementation by these integral membrane proteins being consistently sensitive to external pH. These results demonstrate that hydrolysis of cytosolic PPi is essential for yeast growth and that this function is not substantially affected by the intrinsic characteristics of the PPase protein that accomplishes it. Moreover, this is, to our knowledge, the first direct evidence that H+-PPases can mediate net hydrolysis of PPi in vivo.

YPC-1 mutant strain constitutes a convenient expression system to perform studies aimed at the elucidation of the structure–function relationships of this type of proton pumps.

Inorganic pyrophosphate (PPi) is an abundant byproduct of cellular metabolism whose removal allows the shifting of the equilibrium of anabolic reactions toward biosynthesis (1). Two types of structurally very different PPi-hydrolyzing enzymes have been characterized to date, namely, soluble and membrane-bound inorganic pyrophosphatases (EC 3.6.1.1). Soluble pyrophosphatases (sPPases) are ubiquitous proteins that hydrolyze PPi, yielding heat (1); by contrast, H+-translocating inorganic pyrophosphatases (H+-PPases) are integral membrane proteins that couple the energy liberated by PPi hydrolysis to proton movement across biological membranes (2, 3). sPPases have been identified in prokaryotes, plastids, and mitochondria of diverse eukaryotes and the cytosol of animal and fungal cells (4). H+-PPases have been reported to occur in different groups of eubacteria and archaea (5–8) and in internal (vacuolar-lysosomal) membranes of higher plants (5, 9, 10) and diverse photosynthetic bacteria (4, 5, 8–10, 12). Two distinct biochemical subclasses of H+-PPases have been characterized to date: \( \text{K}^+ \)-stimulated (3, 7, 11, 12) and \( \text{K}^- \)-insensitive (6, 9, 14) H+-PPases. Although all H+-PPases studied so far have been shown to hydrolyze PPi, in vitro, some controversy about the actual physiological function of these proteins arises from the fact that they can also synthesize PPi at the expense of a previously generated proton gradient (15, 16).

Two highly similar PPases of the soluble type occur in the budding yeast \( \text{Saccharomyces cerevisiae} \), one being located in the cytosol and the other within the mitochondria, where it interacts noncovalently with a protein complex of the inner membrane (17, 18). The genes coding for both proteins have been identified, the cytosolic sPPase being encoded by \( \text{IPP1} \) (17) and the mitochondrial enzyme by \( \text{PPA2} \) (18). Previous attempts to disrupt \( \text{IPP1} \) have been unsuccessful, suggesting that the protein encoded by this gene is essential for the viability of the yeast cell (18). No gene coding for a putative H+-PPase has been identified in the genome of \( \text{S. cerevisiae} \), in agreement with the situation previously described for metazoan animal and other fungal cells (2, 3).

Heterologous expression of H+-PPases from different sources in \( \text{S. cerevisiae} \) has been successfully used by several groups to perform biochemical and/or site-directed mutagenesis studies (3, 7, 9, 19–22). In all of these cases, yeast cells were just a source of foreign protein, and no phenotype associated with the presence of the H+-PPase was reported. In this communication, we describe the generation of an \( \text{S. cerevisiae} \) mutant whose functional \( \text{IPP1} \) gene is under the control of a yeast galactose-dependent promoter. In this conditional mutant (named YPC-1), the cytosolic PPase activity can be brought down to negligible levels by transferring cells from galactose to glucose. YPC-1 cells devoid of sPPase activity are expectedly unable to continue growth. Transformation with two plasmid-borne H+-PPase genes, one coding for a \( \text{K}^+ \)-stimulated protein of \( \text{Arabidopsis thaliana} \) and the other for the \( \text{K}^- \)-insensitive H+-PPase of the photosynthetic bacterium \( \text{Chloroflexus aurantiacus} \), under the control of yeast \( \text{PMA1} \) constitutive promoter restored their ability to grow on glucose. Noteworthy, the functionally complemented mutant showed sensitivity to pH, especially when transformed with the plant gene. Cellular localization studies of the heterologously expressed H+-PPases were also performed. These results demonstrate the essential role of \( \text{IPP1} \) for yeast growth and provide direct evidence that H+-PPases can mediate net hydrolysis of PPi, in vivo, suggesting also that PPi, hydrolysis by these membrane proteins may be hindered when pumping protons against external acidic pH (below 5). YPC-1 mutant is a system in which heterologously expressed H+-PPases can produce observable phenotypes, being therefore a valuable tool to perform studies aimed at the elucidation of the structure–function relationships in this class of proton pumps.

Materials and Methods

Plasmid Constructs. \( \text{S. cerevisiae} \) \( \text{IPP1} \) gene was amplified from genomic DNA by high-fidelity PCR using TaqPlus DNA poly-

Abbreviations: ACMA, 9-amino-6-chloro-2-methoxacamidine; PPase, inorganic pyrophosphatase; H+-PPase, proton-translocating PPase; CVP, \( \text{Chloroflexus aurantiacus} \) H+-PPase; sPPase, soluble PPase; PP, inorganic pyrophosphate.

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merase (Stratagene), following the manufacturer's instructions. Oligonucleotides corresponding to positions 1–21 and 845–863 of the coding sequence (17) and containing artificial SacI sites at their respective 5'-ends were used as primers. The 863-bp-long DNA fragment obtained was digested with SacI and inserted with the right orientation into the unique XhoI site of the yeast shuttle vector pJR1-7, thus obtaining plasmid pYPC1. This contained IPP1 under the control of yeast GAL1 promoter and the URA3 gene as a selectable marker. Plasmid pYPC2, bearing a disrupted version of IPP1, was obtained by ligating the PCR-amplified gene to pGEM-T (Promega) and inserting a 1.7-kb BamHI restriction fragment containing the yeast HIS3 gene between the unique BstXI and EcoRV sites of the coding sequence of IPP1 (17). Ligated fragments were previously made blunt at both ends by treatment with T4 DNA polymerase to allow ligation between the incompatible sites involved. The genes coding for the K+-stimulated H+-Pase of the higher plant A. thaliana Columbia (AVP1) (9) and a putative H+-Pase of the photosynthetic green nonsulfur eubacterium C. aurantiacus (cyp, contig 1051; http://spider.jgi-psf.org/FIG_microbial/html/) were amplified by high-fidelity PCR. A cDNA clone containing the full-length A. thaliana AVP1 coding sequence and C. aurantiacus genomic DNA were used as templates, respectively. Artificial SacI sites were introduced at the 5'-ends of the primers for cloning purposes. The PCR-amplified DNA fragments were digested with SacI and ligated in the right orientation to the unique XhoI site of yeast shuttle vector pRS-1024 (23), thus obtaining pAVP1 and pCVP, respectively. These plasmids contained the coding sequences of the H+-Pases inserted between the promoter and the transcription termination regions of the yeast PMA1 gene (24) and LEU2 gene as a selectable marker. S. cerevisiae IPP1 gene was also correctly inserted in pRS-1024, following the same cloning strategy, resulting in plasmid pIPP1.

**Yeast Manipulation Procedures.** S. cerevisiae haploid strain W303-1A (MATa, ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1) was used for transformation with plasmid pYPC1 by the LiOAc/PEG method (25). Cells were initially grown at 30°C in synthetic medium containing 2% glucose, 0.17% yeast nitrogen base without amino acids, tryptophan (0.5 mM), adenine (0.2 mM), histidine (0.4 mM), leucine (1 mM), and uracil (0.2 mM). Transformants were selected by growing cells on 2% agar plates made in glucose-free galactose-PEG (25), and finally resuspended and homogenized in 1 ml of the same buffer.

**DNA and RNA Analyses.** Ten micrograms of yeast genomic DNA obtained as described elsewhere (27) was digested with EcoRV, loaded onto 0.7% agarose gels in TBE buffer, transferred to nylon membranes (28), and hybridization was carried out in all cases with 32P-labeled probes at 65°C, and washed with 0.4× SSC at the same temperature. Probes were labeled with a “Ready-to-Go” kit (Amersham Pharmacia) following the manufacturer's instructions.

**Proton Pumping Assay.** Yeast cells were assayed as described (20). Membrane fractions of control yeast cells were assayed in parallel with the samples as negative controls; 0.5 mM NaF was always present in the H+-Pase activity assays to avoid interferences from any remaining yeast cytoplasmic and/or mitochondrial sPPases. Immunoblot (Western blot) assays of protein samples were performed as reported elsewhere (30) by using antibody PABTK (8), generously donated by P. A. Rea (University of Pennsylvania), and a polyclonal antibody raised against the chloroplastic sPPase of the eukaryotic microalga *Chlamydomonas reinhardtii* (M. R. Gómez-García and A.S., unpublished work). Total protein was estimated by the Bradford method (31) with ovalbumin as a standard.

**Protein Estimation.** Total yeast membranes were homogenized by vigorous shaking with glass beads and centrifuged for 10 min at 2,400 rpm (Sorvall SS-34 rotor) to remove beads and debris; the supernatant was centrifuged for 30 min at 40,000 rpm (Beckman 60 Ti rotor) to sediment the “total membrane fraction.” The supernatant from the ultracentrifugation step was the soluble crude extract, and the pellet was resuspended in 20 ml of buffer B, homogenized, and centrifuged again as above. The washed pellet was resuspended in 2 ml of buffer B, homogenized, and applied to a discontinuous sucrose gradient made of 2.5-Ml cushions of 27%, 35%, 43.5%, and 53.5% sucrose in buffer B without glycerol. After at least 6 h of centrifugation at 28,500 rpm (Beckman SW41 rotor), different membrane fractions were collected from the interphases, diluted 20-fold with buffer B, sedimented by ultracentrifugation (30 min, 40,000 rpm Beckman 60 Ti rotor), and finally resuspended and homogenized in 1 ml of the same buffer.

**Protein Determination.** Protein concentrations were determined by the Bradford method (31) with ovalbumin as a standard.

**Determination of Intracellular PP Levels.** Fifty-milliliter cultures of YPC-1 cells transformed with plasmids pRS-1024, pCVP, pAVP1, and pIPP1 were grown in glucose-containing medium, sedimented, and broken as described for isolation of yeast membranes, except that a 10% (w/v) thichloroacetic acid, 4% (v/v) perchloric acid aqueous solution was used instead of buffer A. Beads, debris, and denatured proteins were removed by centrifugation (5 min, 700 × g), and PP, was determined in the supernatant after neutralization with NaOH, by utilizing a PPi enzymatic determination kit following the manufacturer's instructions (Sigma–Aldrich).

**Proton Pumping Assay.** The formation of a pH gradient across vesicle membranes obtained from the gradients was assayed by fluorescence quenching of the acridine dye 9-amino-6-chloro-2-methoxyacridine (ACMA) as described (32) except that ATP was replaced by 1 mM MgATP.
ethidium bromide. Run in a 0.7% agarose gel, and resulting DNA bands were visualized with methylene blue as a loading control.

Immunofluorescence Microscopy. Fixation and immunofluorescence staining of yeast cells were performed as described elsewhere (33). Permeabilization was accomplished by immersion in methanol at −80°C for 6 min and then in acetone at −80°C for 30 s. Cells were treated with an affinity-purified polyclonal antibody against the Rhodospirillum rubrum H⁺-PPase, generously provided by M. Baltscheffsky (University of Stockholm, Stockholm).

Results

Yeast Mutant Strain YPC-1 Has Its Functional IPP1 Gene Under the Control of a Galactose-Dependent Promoter and Becomes Growth Arrested in Glucose. S. cerevisiae haploid strain W303-1A was transformed with a multicopy autonomous plasmid bearing the yeast IPP1 gene under the control of GAL1 promoter. The chromosomal allele of the resulting strain was knocked out by transformation with a linear DNA fragment containing a copy of IPP1 disrupted by insertion of the yeast HIS3 cassette (34). This yielded a conditional IPP1 mutant (YPC-1) that was unable to grow on glucose (Fig. L4), the preferred carbon source for yeast, and was subsequently characterized both at genetic and biochemical levels. Southern blot analysis showed that an IPP1 probe hybridized with a 3-kb-long EcoRV DNA fragment in mutant YPC-1, whereas a smaller more intense band could be observed in control strains. A 6-kb-long band also hybridized with the probe in those strains transformed with plasmid pYPC1 (Fig. 1B). Analysis by PCR resulted in a specific amplification of 0.9-kb-long DNA fragments in all yeast strains tested and an additional band of ~2.3 kb in YPC-1 (Fig. 1C). Northern blot analysis further showed that the level of IPP1 transcript dropped to negligible levels 24 h after transferring YPC-1 cells grown on galactose to glucose-containing medium (Fig. 2), whereas no significant changes could be observed in control cells (not shown). Activity assays as well as Western blot analysis showed that longer periods of time (at least 48 h) were needed to obtain negligible levels of cytosolic sPPase in YPC-1 (Fig. 3A). Mutant cells fully devoid of sPPase failed to grow when transferred to fresh glucose-containing culture medium (Fig. 3B).
Mutant YPC-1 Transformed with pAVP1 and pCVP Regains the Ability to Grow on Glucose at Culture Media pH Levels in the Range 6–7. YPC-1 cells were transformed with plasmids bearing different genes coding for soluble and H⁺-translocating PPases under the control of S. cerevisiae PMA1 promoter, which, although constitutive, is positively regulated by glucose (35). YPC-1 cells transformed with plasmids pAVP1 and pCVP recovered the ability to grow on glucose, as was also the case with pIPP1, unlike cells transformed with control plasmid pRS-1024. Noteworthy, YPC-1 cells transformed with pAVP1 and pCVP were sensitive to the external pH, especially the former, that could hardly grow on glucose at pH 5. By contrast, mutant cells transformed with pIPP1 were pH-insensitive under the same conditions (Fig. 4), and this was also the case for other sPPase genes (not shown). In any case, YPC-1 cells transformed with H⁺-PPase genes grew significantly slower on glucose than those transformed with sPPase genes. Intracellular PP⁻ levels were consistent with the growing abilities exhibited by the different yeast transformants when grown on glucose (at pH 7); thus YPC-1 cells transformed with control plasmid pRS-1024 were estimated to have 0.04 ± 0.01 μmol of PP⁻ per 10⁹ cells (average of two independent experiments), whereas PP⁻ levels of cells transformed with plasmids pCVP (or pAVP1) and pIPP1 were estimated to be 50 and 18%, respectively, of the control value. Northern blot analysis showed the presence of AVP1 mRNA in YPC-1 cells transformed with pAVP1. Consistent with a regulation by the PMA1 promoter, H⁺-PPase transcript levels were significantly higher in glucose in comparison with those observed in galactose. Levels of IPP1 transcript showed the same pattern as in Fig. 2 (Fig. 5). Identical results were obtained in the case of YPC-1 transformed with pCVP (not shown).

Mutant YPC-1 Transformed with pAVP1 and pCVP Showed Membrane-Bound PPase Activity Values. Membrane fractions were collected from the different inter-phases and analyzed by Western blot using antibody PAB, Yeast P-type H⁺-ATPase (PMA1p) was used as a marker of the plasma membrane (A). PP⁻, hydrolysis (B) and H⁺ translocation (C) activities were assayed in all fractions collected from the gradients. PPase activity assays were carried out in the absence (gray columns) or presence (black columns) of 100 mM KCl. PPase activity values are means ± SEM obtained in three independent experiments.
YPC-1 cells expressing the *A. thaliana* cells expressing than the corresponding fraction obtained from pCVP-activity. All membrane preparations from pAVP1-transformed assayed in all gradient fractions that exhibited significant PPase nuclear Cisternae. 15918/H11545 pCVP-transformed cells showed K transforming with pAVP1, whereas fractions obtained from plasmid pAVP1-transformed with plasmid pRS-1024. No antibody produced clear staining in cells expressing *C. aurantiacus* H-PPase (not shown).

stimulated more than 4-fold by potassium in the case of cells transformed with pAVP1, whereas fractions obtained from pCVP-transformed cells showed K+-insensitive PPase activity (Fig. 6B). PP$_D$-dependent proton-translocation activity could be assayed in all gradient fractions that exhibited significant PPase activity. All membrane preparations from pAVP1-transformed cells produced higher extents of ACMA fluorescence quenching than the corresponding fraction obtained from pCVP-transformed cells (Fig. 6C). PABTK antibody did not recognize any protein in membrane fractions obtained from YPC-1 cells transformed with plasmids pRS-1024 or pIPPI; consistently, no PPase activity or PP$_D$-dependent H$^+$ translocation could be observed in these preparations (not shown).

**Heterologously Expressed H$^+$-PPases Are Mainly Located at Perinuclear Cisternae.** The localization of *A. thaliana* H$^+$-PPase in internal membranes of yeast cells was observed by immunofluorescence detection of formaldehyde-fixed yeast spheroplasts permeabilized with methanol and acetone. The fluorescence pattern of the stained cells was clearly indicative of a location at perinuclear cisternae, although some immunodecoration could also be observed at the so-called prevacuolar vesicles and, consistent with the results from sucrose gradients, at the plasma membrane (Fig. 7).

**Discussion**

Previous attempts to disrupt the gene coding for the cytosolic soluble PPase of the budding yeast *S. cerevisiae* (*IPPI*) had been unsuccessful (18), suggesting that this protein might play an essential role in this organism by removing most of the PPi formed by the external pH, especially in the first case where hardly any growth was observed on glucose at pH 5 (see below for further discussion). Biochemical analyses demonstrated that yeast cells transformed with plasmids pAVP1 and pCVP indeed produced the H$^+$-PPases of *A. thaliana* and the green nonsulfur bacterium *C. aurantiacus*. However, mutant cells transformed with pAVP1 and pCVP grew significantly slower than cells expressing sPPases, in accordance with the fact that the former had one order of magnitude less of total PPase activity than the latter (not shown). In connection with this, it is interesting to note that YPC-1 cells transformed with control plasmid pRS-1024 exhibited significantly higher levels of intracellular PP, compared with cells transformed with plasmids bearing genes coding for either H$^+$-PPases or sPPases. On the other hand, growth of pAVP1- and pCVP-transformed YPC-1 cells were significantly affected by the external pH, especially in the first case where hardly any growth was observed on glucose at pH 5 (see below for further discussion). Biochemical analyses demonstrated that yeast cells transformed with plasmids pAVP1 and pCVP indeed produced the H$^+$-PPases of *A. thaliana* (AVP1) and *C. aurantiacus* (CVP), respectively; thus, fluoride-insensitive membrane-associated PPase activities were assayed in these cells. Moreover, AVP1 activity was stimulated by potassium, as previously described for this plant protein (3, 8, 9), whereas CVP activity was shown to be potassium-insensitive, as expected from molecular phylogenetic studies performed in our laboratory (11). PP$_D$-dependent H$^+$-translocation activities correlated well with PPase activities in those samples collected from the 35/43.5 and 43.5/53.5 interphases of sucrose density gradients, unlike preparations obtained from the 27/35 interphase, suggesting that the lipid compositions of the membranes that equilibrate at this point of the gradient are unsuitable for the formation of reasonably well sealed vesicles required for the H$^+$ translocation assay. That AVP1 produced higher extents of ACMA fluorescence quenching than CVP suggests that the latter may be comparatively uncoupled under the conditions of the assay. This might reflect differences either in intrinsic properties or in the conformations acquired by each protein in yeast; thus, the bacterial CVP may not fold optimally in this heterologous system and/or its interaction with yeast membrane lipids might be worse than that attained by AVP1. On the other hand, it must be noted that *C. aurantiacus* is a thermophilic bacterium (38); actually, CVP showed maximal PPase activity at 60°C (not shown). Therefore, optimal coupling may be achieved at this temperature. Unfortunately, above 40°C, the high passive conductance of yeast membranes to protons makes it impossible to perform the ACMA assay (7, 8).
Although H^+-PPases have been demonstrated to hydrolyze PP_i in vitro, our results give evidence that these proteins perform this activity in vivo, thus allowing the growth of yeast cells with negligible levels of their own cytosolic sPPase. This resembles the scenario occurring in plant photosynthetic tissues where, due to the absence of cytosolic sPPase, the removal of the cytosolic PP_i is presumably carried out by H^+-PPases (9, 10), albeit in a versatile and easy-to-manipulate organism such as yeast. It is also worthwhile pointing out that the removal of PP_i in YPC-1 cells can be accomplished by two biochemically different types of H^+-PPases, namely a K^+-stimulated protein from an eukaryotic source and a K^+-insensitive protein from a prokaryote. Another point worth consideration is related to the other concomitant activity exhibited by H^+-PPases: proton translocation (2, 3). The sensitivity to external acidic pH exhibited by mutant YPC-1 expressing AVP1 and CVP when growing on glucose might be due to differences in the subcellular localization of the proteins induced by acidification of the culture medium, as it has been suggested for plant H^+-ATPases expressed in yeast (40). In our case, no significant differences have been observed in immunofluorescence staining or in the distribution of H^+-PPases in sucrose gradients performed with cells grown at pH between 5 and 7 (not shown). Alternatively, the sensitivity to pH could also be explained by assuming that the contribution of the proteins localized in the plasma membrane is crucial to maintain the PP_i levels of yeast cytosol at sustainable levels. Acidification of the external medium might hinder the extrusion of protons and, thus, hydrolysis of PP_i, especially in the case of AVP1, which apparently has a tighter coupling between PP_i hydrolysis and H^+ translocation than CVP in this heterologous system. A limited capacity by AVP1 to pump protons against an acidic pH would be consistent with previous reports that suggest that H^+-PPases may be operating near steady state in vivo, when working in parallel with the plant tonoplast H^+-ATPase in photosynthetic tissues (16). If our hypothesis were correct, an intrinsically uncoupled H^+-PPase would be able to support growth of mutant YPC-1 in a pH-independent manner; hence, this system would also allow the search for mutations that affect the coupling mechanism of these proton pumps. Random mutagenesis of H^+-PPase genes and screening of mutations in residues or domains involved in PPase activity or in the coupling between PP_i hydrolysis and H^+ translocation by using mutant YPC-1 are already underway in our laboratory.

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