Functional complementation of yeast cytosolic pyrophosphatase by bacterial and plant H$^+$-translocating pyrophosphatases

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Two types of proteins that hydrolyze inorganic pyrophosphate (PP$_i$), 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 PP$_i$ releasing heat, whereas H$^+$-PPases, so far unidentified in animal and fungal cells, couple the energy of PP$_i$ hydrolysis to proton movement across biological membranes. The budding yeast *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 (*IPP1*) have been unsuccessful, thus suggesting that this protein is essential for growth. Here, we describe the generation of a conditional *S. cerevisiae* mutant (named YPC-1) whose functional *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 PP$_i$ 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 PP$_i$ *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 (PP$_i$) 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 PP$_i$-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 PP$_i$ yielding heat (1); by contrast, H$^+$-translocating inorganic pyrophosphatases (H$^+$-PPases) are integral membrane proteins that couple the energy liberated by PP$_i$ 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 (vacuo-lysosomal) membranes of higher plants (5, 9, 10) and diverse photosynthetic and nonphotosynthetic protists (11, 12). Two distinct biochemical subclasses of H$^+$-PPases have been characterized to date: K$^+$-stimulated (3, 7, 11, 12) and K$^+$-insensitive (6, 9, 14) H$^+$-PPases. Although all H$^+$-PPases studied so far have been shown to hydrolyze PP$_i$ *in vitro*, some controversy about the actual physiological function of these proteins arises from the

fact that they can also synthesize PP$_i$ at the expense of a previously generated proton gradient (15, 16).

Two highly similar PPases of the soluble type occur in the budding yeast *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 *IPP1* (17) and the mitochondrial enzyme by *PPA2* (18). Previous attempts to disrupt *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 *S. cerevisiae*, in agreement with the situation previously described for metazoan animal and other fungal cells (2, 3, 5).

Heterologous expression of H$^+$-PPases from different sources in *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 to the presence of the H$^+$-PPase was reported. In this communication, we describe the generation of an *S. cerevisiae* mutant whose functional *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 K$^+$-stimulated protein of *Arabidopsis thaliana* and the other for the K$^+$-insensitive H$^+$-PPase of the photosynthetic bacterium *Chloroflexus aurantiacus*, under the control of yeast *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 *IPP1* for yeast growth and provide direct evidence that H$^+$-PPases can mediate net hydrolysis of PP$_i$ *in vivo*, suggesting also that PP$_i$ 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. *S. cerevisiae IPP1* gene was amplified from genomic DNA by high-fidelity PCR using *Taq*Plus DNA poly-

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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 SalI sites at their respective 5′-ends were used as primers. The 863-bp-long DNA fragment obtained was digested with SalI and inserted with the right orientation into the unique XhoI site of the yeast shuttle vector pJR-1 (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 p-GEM-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$^+$-PPase of the higher plant $A.$ $thaliana$ Columbia ($AVP1$) (9) and a putative H$^+$-PPase of the photosynthetic green nonsulfur eubacterium $C.$ $aurantiacus$ (cvp, contig 1051; http://spider.jgi-psf.org/JGI_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 SalI sites were introduced at the 5′-ends of the primers for cloning purposes. The PCR-amplified DNA fragments were digested with SalI 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$^+$-PPases 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 (MAT**a**, $ade2-1$ $can1-100$ $his3-11,15$ $leu2-3,112$ $trp1-1$ $ura3-1$) 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 in culture medium without uracil. The resulting strain was transformed with plasmid pYPC2, previously digested with SacI and SacII. Transformation and selection were carried out as above except that plates were made in glucose-free galactose-containing medium devoid of histidine. Several transformants were checked for growth in glucose, and one of them (mutant strain YPC-1) was unable to do so; therefore, it was further characterized and selected for subsequent experiments. Mutant YPC-1 was transformed with plasmids pRS-1024, pIPP1, pAVP1, and pCVP and extended onto 2% agar plates made in medium containing 2% galactose, 0.17% YNB, 0.5 mM tryptophan, and 0.2 mM adenine. For complementation experiments and isolation of yeast membranes, a rich synthetic culture medium was used; this contained 2% (wt/vol) glucose or galactose, an appropriate mixture of nucleotides and amino acids (26), and 50 mM Tris adjusted to the appropriate pH with Mes buffer.

Isolation of Yeast Membranes. Yeast cells were liquid-grown up to stationary phase in galactose-containing selective medium; then, 1 ml of stationary culture was used to inoculate 200 ml of glucose-containing rich synthetic medium adjusted to pH 7 as described above. After 18 h of growth, cells were sedimented by centrifugation at 2,000 rpm in a bench centrifuge for 5 min, washed thoroughly with water, resuspended in 5 ml of ice-cold

buffer A (25 mM Tris-HCl, pH 8/10% glycerol/4 mM β-mercaptoethanol/2 mM DTT/2 mM EDTA/10 mM MgCl$_2$/1 mM benzamidine/2 mM ε-aminocaproic acid/1 mM PMSF/1 μg/ml leupeptine), and homogenized by vigorous shaking with glass beads. The homogenate was diluted up to 20 ml with buffer B (10 mM Tris-HCl, pH 7.6/10% glycerol/2 mM DTT/1 mM EDTA) and centrifuged for 10 min at 2,400 rpm (Sorvall SS-34 rotor) to remove beads and debris; the resulting 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.

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 Tris borate EDTA, run, and transferred to nylon membranes (28). Yeast total RNA was isolated according to Carlson and Botstein (29), electrophoresed (20 μg per lane) on 1% agarose gels in the presence of 2% formaldehyde, and transferred to nylon membranes as above. Hybridization was carried out in all cases with ^{32}P-labeled probes at 65°C, and washes were made with 0.4× SSC at the same temperature. Probes were labeled with a "Ready-to-Go" kit (Amersham Pharmacia) following the manufacturer's instructions.

Pyrophosphatase Activity Assay, Western Blot Analyses, and Total Protein Estimation. Pyrophosphatase activity was routinely 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$^+$-PPase 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 PAB$_{TK}$ (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.

Determination of Intracellular PP$_i$ 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) trichloroacetic 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$_i$ was determined in the supernatant after neutralization with NaOH, by utilizing a PP$_i$ 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 PP$_i$.
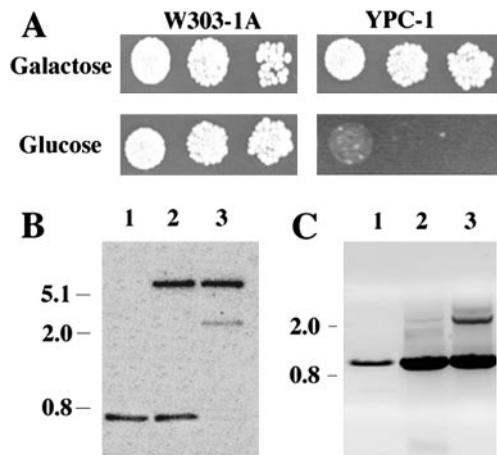
Fig. 1. Drop tests comparing growth of yeast strains W303-1A and YPC-1 on galactose and glucose (A) and genomic analysis of conditional mutant strain YPC-1 by Southern blot (B) and PCR (C). Cells were grown to saturation in galactose medium and transferred to glucose for 18 h to stop the transcription of *IPP1*; then, serial dilutions of the cultures (10^2-, 10^3-, and 10^4-fold) were made in sterile water, and 10 μl of each of the dilutions were spotted onto agar plates containing medium with galactose or glucose as carbon sources. Growth was recorded after 5 days. For Southern blot, DNA preparations from strains W303-1A (lane 1), W303-1A transformed with plasmid pYPC1 (lane 2), and YPC-1 (lane 3) were processed as described in *Materials and Methods*. An *Eco*RV 675-bp-long DNA fragment containing approximately two-thirds of *S. cerevisiae IPP1* coding sequence was used as a probe. PCRs were performed using DNA from the same strains as templates and oligonucleotides corresponding to the full-length *IPP1* coding sequence as primers. Samples were run in a 0.7% agarose gel, and resulting DNA bands were visualized with ethidium bromide.

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. 1A), 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 *Eco*RV 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
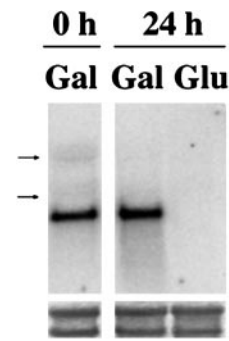

Fig. 2. Analysis by Northern blot of *IPP1* transcription levels in mutant strain YPC-1. Total RNA was obtained from YPC-1 cells grown on galactose to saturation and then transferred to fresh medium containing galactose or glucose for 24 h. Arrows indicate the positions of yeast ribosomal RNAs (3.5 and 1.8 kb). (*Lower*) Staining of the corresponding bands with methylene blue as a loading control.

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).
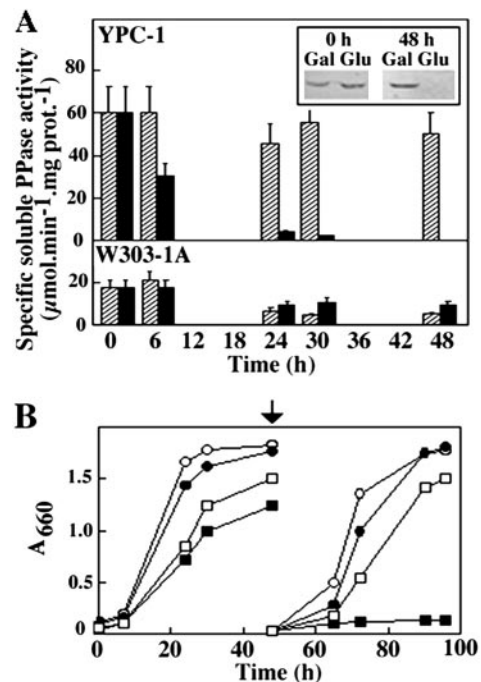

Fig. 3. Time course of sPPase activity (A) and growth curves (B) of strains YPC-1 and W303-1A on different carbon sources. Two milliliters of stationary cultures of W303-1A and YPC-1 grown on galactose were used to inoculate flasks containing 200 ml of selective medium and cultured with agitation at 30°C. Specific PPase activity was assayed at different times in crude soluble extracts of mutant YPC-1 and strain W303-1A grown on galactose (hatched columns) or glucose (black columns). Activity values are means ± SEM obtained in three independent experiments. (*Inset*) Analysis by Western blot of crude soluble extracts obtained from YPC-1 cells at the beginning of inoculation and after 48 h of growth on galactose and glucose using an antibody against the homologous chloroplastic sPPase protein of the microalga *C. reinhardtii*. Growth was monitored by measuring absorbances at 660 nm. After 48 h (indicated by an arrow), 2 ml of stationary cultures were inoculated into 200 ml of fresh medium, and growth was followed for further 48 h. ○, W303-1A in galactose; ●, W303-1A in glucose; □, YPC-1 in galactose; ■, YPC-1 in glucose.
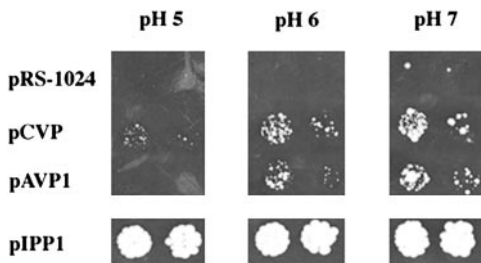
Fig. 4. Growth at different pHs of yeast strain YPC-1 transformed with plasmids bearing the genes coding for the H$^+$-PPases of *A. thaliana* and *C. aurantiacus*. YPC-1 cells were transformed with the indicated plasmids and grown in liquid medium as in Fig. 1. Dilutions (10^3- and 10^4-fold) of the cultures were made in sterile water and spotted onto agar plates made in glucose-containing rich-selective medium at the indicated pH values, adjusted as described in *Materials and Methods*. Growth was recorded after 5 days.

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$_i$ 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$_i$ per 10^9 cells (average of two independent experiments), whereas PP$_i$ 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
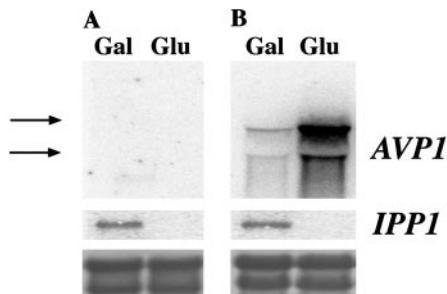

Fig. 5. Analysis by Northern blot of *AVP1* and *IPP1* transcription levels in mutant strain YPC-1 transformed with plasmids pRS-1024 (*A*) and pAVP1 (*B*). Cells were grown on galactose to saturation and then transferred to fresh medium containing galactose or glucose for 24 h. Total RNA was obtained, electrophoresed, and transferred to nylon membranes as described in the main text. Filters were hybridized with DNA probes corresponding to the coding sequences of *AVP1* and *S. cerevisiae IPP1*, respectively. Arrows indicate the positions of yeast ribosomal RNAs (3.5 and 1.8 kb). (*Lower*) Staining of the bands with methylene blue as a loading control.
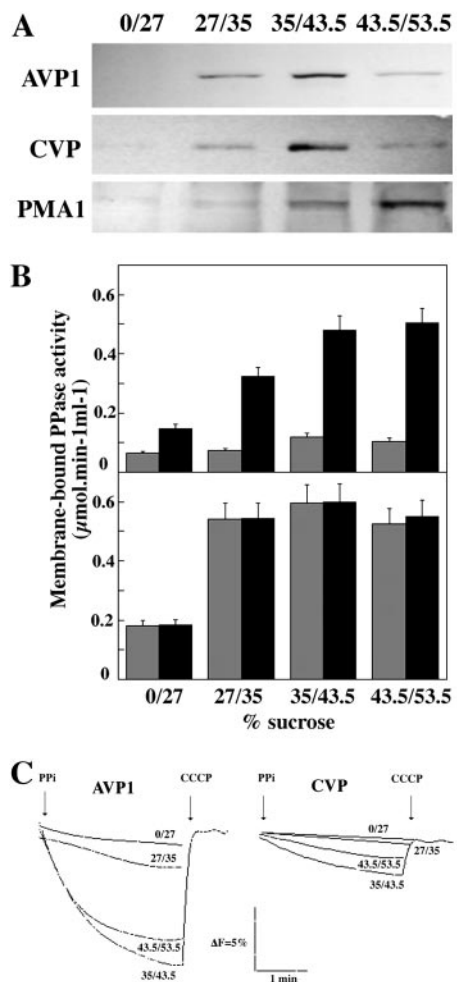

Fig. 6. Subcellular localization of AVP and CVP. Total membranes were obtained from YPC-1 cells transformed with plasmids pAVP1 and pCVP, loaded onto sucrose step gradients, and centrifuged as described in *Materials and Methods*. Membrane fractions were collected from the different interphases and analyzed by Western blot using antibody PAB$_{TK}$. Yeast P-type H$^+$-ATPase (PMA1p) was used as a marker of the plasma membrane (*A*). PP$_i$ 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.

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 PP$_i$ Hydrolysis and PP$_i$-Dependent H$^+$ Translocation Activities. Analysis by Western blot of samples obtained from sucrose step gradients using antibody PAB$_{TK}$, raised against a hydrophilic domain common to all H$^+$-PPases characterized so far (8), recognized polypeptides of around 67 kDa in those membrane preparations obtained from YPC-1 cells transformed with plasmids pAVP1 and pCVP. These bands were mainly associated to membrane fractions that equilibrate between 35% and 53.5% sucrose. The use of a polyclonal antibody against yeast P-type H$^+$-ATPase (PMA1) further suggested that a fraction of the membrane-bound polypeptides were located at the plasma membrane (Fig. 6*A*). Consistently, most fluoride-insensitive PPase activity was found in these samples; moreover, activity was
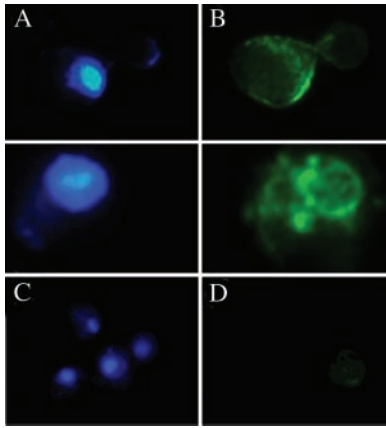
Fig. 7. Immunofluorescence microscopy analyses of YPC-1 cells transformed with plasmid pAVP1. (*A* and *C*) DAPI fluorescence of the nuclei. (*B* and *D*) Fluorescence patterns of fluorescein 5-isothiocyanate-second antibody conjugates in the same cells. The first antibody was an affinity-purified polyclonal antibody against *R. rubrum* H$^+$-PPase that gave better results than antibody PAB$_{TK}$ used in Western blots. (*B*) Detail of two typical fluorescence patterns in YPC-1 cells expressing the *A. thaliana* H$^+$-PPase. (*D*) Staining in control cells 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. 6*B*). PP$_i$-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. 6*C*). PAB$_{TK}$ antibody did not recognize any protein in membrane fractions obtained from YPC-1 cells transformed with plasmids pRS-1024 or pIPP1; consistently, no PPase activity or PP$_i$-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* (*IPP1*) had been unsuccessful (18), suggesting that this protein might play an essential role in this organism by removing most of the PP$_i$ formed by anabolic reactions in the cytosol. One way of circumventing this situation was to have *IPP1* under the control of an inducible-repressable promoter such as that of yeast galactokinase (*GAL1*), which gives a quite high level of transcription in galactose and virtually no transcript in the presence of glucose (36). Initial attempts of obtaining the mutant by an "integrative replacement/disruption" strategy, as previously described for the yeast *PMA1* gene (37), were unsuccessful. Our second strategy involved transformation of yeast haploid strain W303–1A with a multicopy plasmid bearing *IPP1* under the control of *GAL*1 promoter and subsequent insertion of a *HIS3* cassette into the chromosomal

allele. Although this approach is not very efficient, it eventually turned out to be successful, and a clone with the expected phenotype, that is, able to grow on galactose but not on glucose, was obtained (Fig. 1*A*). The new strain, named YPC-1, was characterized at different levels, and Southern blot and PCR analyses performed with the mutant and appropriate controls were consistent with the presence of two types of *IPP1* genes in mutant YPC-1, namely, plasmid-borne full-length copies and a disrupted partially deleted version in the chromosome (Fig. 1 *B* and *C*). Northern blot analysis further indicated that the plasmid-borne copies of *IPP1*, controlled by *GAL1* promoter, were the functional genes in mutant YPC-1. Soluble PPase activity also decreased during growth on glucose, although at a lower rate than the mRNA, suggesting that this protein may have a relatively low turnover rate in yeast. The essential role of the cytosolic sPPase in *S. cerevisiae* was confirmed by the fact that, after all of the soluble activity had disappeared from the cells, these were unable to continue growth on glucose (Fig. 3*B*).

Transformation of mutant strain YPC-1 with genes coding for different soluble and membrane-bound PPases under the control of a constitutive promoter resulted in the recovery of the ability to grow on glucose (Fig. 4). This was expected for *IPP1*, but it was also the case for bacterial sPPases, such as those from the photosynthetic purple bacteria *R. rubrum* and *Rhodopseudomonas palustris* (not shown) and, more strikingly, for the genes coding for the structurally very different membrane-bound 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$_i$ 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$_i$-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).

As far as the subcellular localization is concerned, immuno-localization performed in yeast cells expressing AVP1 typically showed accumulation of foreign protein mostly in membranous structures surrounding the cell nucleus. These structures have been previously observed when expressing other integral membrane proteins like P-type Ca^{2+}- and H^+-ATPases in yeast and referred to as *perinuclear cisternae* (39), *karmellae* (40), or ER-derived membranes (41). However, immunofluorescence staining as well as the distribution of the protein in sucrose gradients showed that the H^+-PPases could also be delivered to the plasma membrane and to small prevacuolar vesicles, consistent with previous reports obtained for the sarcoplasmic reticulum Ca^{2+}-ATPase and AVP1 expressed in yeast (42, 43). No immunolocalization of CVP could be performed because this protein is not easily recognizable by the antibodies available in our laboratory; however, the fact that CVP showed similar distribution as that of AVP1 in sucrose gradients suggested that the intracellular location of both proteins should be similar.

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 a 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 immuno-fluorescence 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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