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The *VPH1* Gene Encodes a 95-kDa Integral Membrane Polypeptide Required for *in Vivo* Assembly and Activity of the Yeast Vacuolar H^+-ATPase*

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Yeast vacuolar acidification-defective (*vph*) mutants were identified using the pH-sensitive fluorescence of 6-carboxyfluorescein diacetate (Preston, R. A., Murphy, R. F., and Jones, E. W. (1989) *Proc. Natl. Acad. Sci. U. S. A.* 86, 7027–7031). Vacuoles purified from yeast bearing the *vph1-1* mutation had no detectable bafilomycin-sensitive ATPase activity or ATP-dependent proton pumping. The peripherally bound nucleotide-binding subunits of the vacuolar H^+-ATPase (60 and 69 kDa) were no longer associated with vacuolar membranes yet were present in wild type levels in yeast whole cell extracts. The *VPH1* gene was cloned by complementation of the *vph1-1* mutation and independently cloned by screening a λgt11 expression library with antibodies directed against a 95-kDa vacuolar integral membrane protein. Deletion disruption of the *VPH1* gene revealed that the *VPH1* gene is not essential for viability but is required for vacuolar H^+-ATPase assembly and vacuolar acidification. *VPH1* encodes a predicted polypeptide of 840 amino acid residues (molecular mass 95.6 kDa) and contains six putative membrane-spanning regions. Cell fractionation and immunodetection demonstrate that Vph1p is a vacuolar integral membrane protein that co-purifies with vacuolar H^+-ATPase activity. Multiple sequence alignments show extensive homology over the entire lengths of the following four polypeptides: Vph1p, the 116-kDa polypeptide of the rat clathrin-coated vesicles/synaptic vesicle proton pump, the predicted polypeptide encoded by the yeast gene *STV1* (Similar To *VPH1*, identified as an open reading frame next to the *BUB2* gene), and the TJ6 mouse immune suppressor factor.

The yeast vacuole, like the mammalian lysosome, requires an acidic lumen to perform certain of its cellular functions. The pH difference across the vacuolar membrane provides an electrochemical gradient that drives the secondary transport of a variety of basic amino acids (Ohsumi and Anraku, 1981) and ions (Ohsumi and Anraku, 1983). The enzyme responsible for the generation of the transmembrane gradient by the active transport of protons is the vacuolar H^+-ATPase. Mutations in or disruptions of some of the structural genes of the vacuolar H^+-ATPase render yeast cells sensitive to high extracellular Ca^{2+} concentrations (Ohya *et al.*, 1991) and neutral buffered medium (Yamashiro *et al.*, 1990; Foury, 1990; Nelson and Nelson, 1990). This demonstrates both the essential role the vacuole plays in Ca^{2+} homeostasis and the essential role of the vacuolar H^+-ATPase for normal vacuolar function.

The yeast vacuolar H^+-ATPase is a member of the vacuolar-type or V-type H^+-ATPase (EC 3.6.1.3) found in and responsible for the acidification of plant and fungal vacuoles, lysosomes, endosomes, clathrin-coated vesicles, chromaffin granules, synaptic vesicles, and Golgi (most recently reviewed in Forgac, 1989). V-type ATPases are evolutionary related to F_1F_0 H^+-ATPase (Gogarten *et al.*, 1989) and have a similar multimeric bipartite structure consisting of membrane-bound proton channel and a peripherally bound catalytic core. On the basis of co-purification and immunoprecipitation, the yeast vacuolar H^+-ATPase is composed of at least 8 subunits: 100, 69, 60, 42, 36, 32, 27, and 17 kDa (Kane *et al.*, 1989). The structural genes for four of these subunits have been identified redundantly and appear in the literature with the following designations: 69 kDa, *TFP1* (Kane *et al.*, 1990) and *VMA1* (Hirata *et al.*, 1990); 60 kDa, *VMA2* (Anraku *et al.*, 1989), VATP B (Nelson *et al.*, 1989), and *VAT2* (Yamashiro *et al.*, 1990); 31 kDa, *VMA4* (Foury, 1990); 17 kDa, VATPc (Nelson and Nelson, 1989) and *VMA3* (Umemoto *et al.*, 1990). The peripherally bound catalytic domain is composed of the 69- and 60-kDa subunits, both of which contain consensus sequence nucleotide-binding domains (69 kDa: Bowman *et al.*, 1988a; Zimniak *et al.*, 1988; Kane *et al.*, 1990; Hirata *et al.*, 1990; 60 kDa: Bowman *et al.*, 1988b; Manolson *et al.*, 1988; Nelson *et al.*, 1989) and have been shown to bind ATP analogs *in vitro* (Manolson *et al.*, 1985; Randall and Sze, 1987; Uchida *et al.*, 1988). The proton channel through the membrane is

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) M89778.

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thought to be formed by six copies of the DCCD[1]-binding 17-kDa proteolipid (Rea *et al.*, 1987; Sun *et al.*, 1987; Arai *et al.*, 1987; Kaestner *et al.*, 1988).

Early reports on the partial purification of V-type H^+-ATPases from plant and yeast vacuoles, and more recently, the immunoaffinity purification of the bovine kidney V-type H^+-ATPase (Gluck and Caldwell, 1987) did not report the presence of any subunits larger than the 70-kDa nucleotide-binding subunit. Evidence that V-type H^+-ATPases do contain a 95–120-kDa polypeptide are as follows. 1) Removal of the 116- and 38-kDa polypeptides from the purified clathrin-coated vesicle H^+-ATPase prevented Mg^{2+}-supported ATP hydrolysis and proton pumping activity (Xie and Stone, 1988). 2) Immunoprecipitation of the coated vesicle H^+-ATPase (Arai *et al.*, 1988) and the yeast vacuolar H^+-ATPase (Kane *et al.*, 1989) with monoclonal antibodies that recognize the native enzyme co-precipitated a 100-kDa polypeptide. 3) Antibodies specific to a 120-kDa polypeptide that co-purified with the chromaffin-granule V-type H^+-ATPase recognized a 115-kDa subunit that co-purified with the renal tubule V-type H^+-ATPase (Gillespie *et al.*, 1991). 4) Disruption of the structural gene for the 17-kDa DCCD-binding subunit of the yeast vacuolar H^+-ATPase (*VMA3*) results in the absence of the 100-kDa polypeptide in vacuolar membranes and decreased levels in whole cell extracts (Kane *et al.*, 1992). 5) On the basis of immunofluorescence microscopy on yeast cells, the peripherally bound nucleotide-binding subunits (60 and 69 kDa) appear to block epitope sites on the 100-kDa polypeptide (Kane *et al.*, 1992).

In this report we present genetic, biochemical, and sequence data that a vacuolar membrane-bound 95-kDa polypeptide, encoded by the *VPH1* gene, is a subunit of the yeast vacuolar ATPase and is required for *in vivo* enzyme assembly and activity.

EXPERIMENTAL PROCEDURES

Materials—The zwitterionic detergent *N*-tetradecyl-*N,N*-dimethyl-3-ammonio-1-propanesulfonate (ZW3-14) was from Calbiochem, Lyticase L8137 and phenylmethylsulfonyl fluoride from Sigma, ATP and pepstatin A from Boehringer Mannheim, 6-carboxyfluorescein diacetate (C1362) from Molecular Probes, and Acridine Orange from Kodak. Bafilomycin A_1 was a kind gift from Dr. Karlheinz Altendorf (University of Osnabruck). Random Primer Extension Kit, [α-^{32}P]dCTP (3000 Ci/mmmol), and [^{35}S]dATP (1000 Ci/mmol) were from Du Pont-New England Nuclear. Nitrocellulose type HAHY (0.45 μm) was purchased from Millipore and nitrocellulose BA85 and Nytran (0.45 μm) from Schleicher and Schuell. Goat anti-rabbit IgG-horseradish peroxidase conjugate was obtained from Bio-Rad.

Media and Stock Solutions—YEPD medium contained 1% yeast extract (Difco), 2% Bacto-peptone (Difco), and 2% dextrose, synthetic media for yeast were prepared as described in Zubenko *et al.* (1982). Media for propagation of phage and bacteria were made as described in Maniatis *et al.* (1982). A 200 mM phenylmethylsulfonyl fluoride stock solution was prepared in isopropyl alcohol and kept at 4 °C, pepstatin A was stored at 1 mg/ml in 66% ethanol at −20 °C, 20 mM 6-carboxyfluorescein diacetate was stored in dimethyl sulfoxide at 4 °C, 0.5 mM bafilomycin A_1 in ethanol was stored at −20 °C, and a 10% ZW3-14 solution was stored at room temperature. 50 mM Na_2ATP was immediately titrated to pH 6.9 with Tris base and stored at −20 °C.

Preparation of Whole Cell Yeast Extract—Whole cell extracts were prepared by a Braun homogenizer as described in Jones *et al.* (1982) except that homogenization was done at 4 °C in 100 mM Tris-HCl, pH 7.5, 1 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, and 1 μg/ml pepstatin A. Yeast whole cell extracts were denatured for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 63 mM Tris-HCl, pH 6.8, 1% SDS, 0.6 mM β-mercaptoethanol, and 5% glycerol for 2.5 min at 65 °C. Although these conditions were not optimal for solubilization of most proteins, higher temperatures or longer incubation times resulted in less detectable Vph1p antigen.

Purification of Vacuolar Membranes and Vacuolar H^+-ATPase—Isolation of vacuolar membranes through flotation of intact vacuoles on Ficoll gradients and the subsequent purification of the vacuolar H^+-ATPase through ultracentrifugation on glycerol gradients were done essentially by the method of Uchida *et al.* (1985) with the following modifications. Isolation of vacuolar membranes, assay of their purity, and Na_2CO_3 extraction of the resulting vesicles were done exactly as described in Woolford *et al.* (1990). Purification of the vacuolar H^+-ATPase was done with the modifications described in Kane *et al.* (1989) with the following exceptions as suggested by Dr. Patricia M. Kane (The College of William and Mary). ZW3-14-solubilized membranes were layered on top of an 11.8-ml step gradient (20–25–30–35–40–45–50% (v/v) glycerol) and centrifuged for 16 h at 180,000 × *g* in a Beckman SW41 rotor. 0.6-ml fractions were collected from the top, and ATPase activity was assayed immediately without adding exogenous phospholipids.

Enzyme Assays—Hydrolytic activity of the vacuolar H^+-ATPase was assayed for 30 min at 30 °C in 25 mM Mes/Tris, pH 6.9, 5 mM $MgCl_2$, 25 mM KCl, and 5 mM Na_2ATP with released inorganic phosphate determined by the method of Ames (1966). Acidification of the lumen of purified intact vacuoles by the vacuolar H^+-ATPase was detected by following the rate of fluorescence quenching of acridine orange as described by Rea and Poole (1985) except that buffer and ion concentrations were as described above for hydrolytic activity. For bafilomycin A_1 sensitivity in both proton pumping and hydrolytic assays, the protein samples (which did not exceed 20 μg of protein) were preincubated in 1 μM bafilomycin A_1 for 10 min prior to the assays.

Production of Anti-Vph1p Antisera—Rabbit polyclonal antisera to Vph1p was raised by the method of Vaitukaitis (1981) with the modifications described in Manolson *et al.* (1989). Vacuolar membranes were stripped of peripheral proteins with 100 mM Na_2CO_3, pH 11, as described in Woolford *et al.* (1990), the Na_2CO_3-insoluble proteins were resuspended in 0.5 M Tris-HCl, pH 8.0, at a concentration no greater that 6 mg/ml and homogenized in a tight-fitting Dounce homogenizer until there were no visible particulates (about 10 strokes). 1/4 volume of SDS-PAGE denaturation buffer containing 0.315 M Tris-HCl, pH 6.8, 5% SDS, 3 M β-mercaptoethanol, and 50% glycerol was added, followed by an overnight incubation on ice. The pH was adjusted to neutrality with Tris base, and insoluble matter was removed by centrifugation at 12,000 × *g* for 10 min. 1.5 mg of solubilized protein was loaded on a 1.5-mm-thick, 14.5-cm-long, 7.5% polyacrylamide-preparative SDS-PAGE. Vph1p was visualized through KCl staining, cut out, and prepared for intradermal injections exactly as described in Manolson *et al.* (1989). The rabbit received an initial injection and 4 additional injections spaced 1 month apart; each injection contained approximately 50 μg of Vph1p. Test bleeds were taken 2 days after injection, the rabbit was killed 8 days after the 5th injection.

Affinity Purification of Anti-Vph1p—Raw serum was precipitated by 50% $(NH_4)_2SO_4$, the pellet was resuspended to one-half the original volume and dialyzed extensively against 10 mM Tris-HCl, pH 7.3, 0.9% NaCl (Tris-saline buffer). To remove antibodies directed against carbohydrates moieties, 5 ml of the resulting dialysate was incubated twice with 0.3 g wet weight of intact yeast cells for 1.5 h at 4 °C with gentle rocking, followed by a short 12,000 × *g* centrifugation to remove the yeast cells. 1 mg of Na_2CO_3-insoluble vacuolar membrane proteins was prepared and electrophoresed as described above. The protein was transferred to nitrocellulose by the method of Burnette (1981) and visualized by Ponceau S staining. A small strip (0.2 × 14.5 cm) containing about 40 μg of Vph1p antigen was cut out and blocked in 3% bovine serum albumin in Tris-saline buffer for 1 h at 42 °C. The nitrocellulose strip was washed three times for 10 min in Tris-saline buffer and then incubated with 2 ml of the antiserum (prepared as described above) overnight at 4 °C with constant shaking. The 2-ml aliquot was conserved, as affinity purification did not significantly deplete this fraction of anti-Vph1p antibodies. The nitrocellulose strip was washed four times for 10 min in Tris-saline buffer, and antibodies were eluted by incubating the washed strip in 1 ml of ice-cold 200 mM glycine-HCl, pH 2.3, at 4 °C for 1 h, after which this fraction was immediately neutralized with the addition of 150 μl (15% v/v final) of 1 M Tris. The nitrocellulose strip was washed four times

[1] The abbreviations used are: DCCD, dicyclohexylcarbodiimide; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; kb, kilobase(s); bp, base pair(s); Mes, 2-(*N*-morpholino)ethanesulfonic acid.

TABLE I
Yeast strains

Strain	Genotype
BJ2663	*MATa trp1 ura3-52*
BJ3131	*MATα/MATa +/ade6 +/his1 leu2-1/leu2-1 trp1/+ ura3-52/ura3-52*
BJ4873	*MATα leu2 ura3-52 vph1-1*
BJ4875	*MATα alg6 trp1 vph1-1*
BJ4876	*MATa leu2 ura3-52*
BJ4877	*MATa alg6 trp1 vph1-1*
BJ4878	*MATα leu2 ura3-52*
BJ4879	*MATa alg6 trp1 ura3-52 vph1-1*
BJ4886	*MATα alg6 ura3-52 vph1-1*
BJ4887	*MATα trp1 vph1-1*
BJ4888	*MATa leu2 ura3-52*
BJ5035	*MATa leu2 ura3-52 vph1-1*
BJ6716	*MATα/MATa +/ade6 +/his1 leu2-1/leu2-1 trp1/+ ura3-52/ura3-52 Δvph1::LEU2/VPH1*
BJ6717	*MATa ade6 his1 trp1 ura3-52 Δvph1::LEU2*
BJ6718	*MATα his1 ura3-52 Δvph1::LEU2*
BJ6719	*MATa leu2-1 ura3-52*
BJ6720	*MATα ade1 leu2-1 trp1 ura3-52*
DBY1034	*MATa his4-539 lys2-801 ura3-52*

for 10 min in Tris-saline buffer and conserved for another round of affinity purification.

Electron Microscopy and Immunolabeling—The yeast strain DBY1034 was grown in YEPD medium to 5×10^6 cells/ml. A 100-ml culture of cells was harvested and suspended in 25 ml of ice-cold fixative (40 mM potassium phosphate, pH 6.7, 1.25 M sorbitol, 3% formaldehyde, 0.5% glutaraldehyde, 1 mM $MgCl_2$, 1 mM $CaCl_2$) for 30 min. The fixed cells were washed three times in 40 mM potassium phosphate, pH 6.7, buffer containing first 0.75 M sorbitol, then 0.25 M sorbitol, and finally no sorbitol. A more detailed description of the fixation conditions will be presented elsewhere.[2]

The fixed cells were resuspended in 1% sodium metaperiodate and then in 50 mM ammonium chloride as described previously (Van Tuinen and Riezman, 1987). Dehydration was performed on ice by washing the cells in 50% ice-cold ethanol followed by 70%, 80%, 90%, and 95% ethanol, and finally by three incubations in 100% ethanol. The dehydrated cells were infiltrated with L. R. White resin (Polysciences) and polymerized as described by Wright and Rine (1989). Sections measuring 80 to 90 nm (as determined by a gold interference color) were cut using a diamond knife and were mounted on 300-mesh nickel grids.

Anti-Vph1p antibodies were diluted 1:3 in PBST (140 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.05% Tween 20) containing 2% ovalbumin and were incubated with the cell sections. Gold-conjugated secondary antibodies were diluted as suggested by the manufacturer (BioCell, Cardiff, UK). All incubations were performed as described previously (Preuss *et al.*, 1991).

Screening λgt11 Expression Library—Antibodies specific to Vph1p were used to screen a Clontech λgt11 yeast (X2180) genomic expression library by the method of Huynh *et al.* (1985) with the modifications of Manolson *et al.* (1988) except that 25,000 plaque-forming units were used per 90-mm plate. A total of 275,000 insert-containing plaque-forming units were screened (approximately 8 yeast genomes worth), resulting in four identical positives. Expression of microgram quantities of the λgt11 fusion protein was performed by the method of Mitchell (1989).

Strains—All strains were derived from X2180-1B (*MATα gal2 SUC2*) or from crosses between isogenic derivatives of the strain and strains congenic to X2180-1B obtained from D. Botstein (Stanford University). The relevant genotypes of strains used in this work are listed in Table I. Strains BJ4875 to BJ4878 are segregants from a single tetrad resulting from the mating of BJ4887 to BJ4888. Strain BJ6716 resulted from the transformation of BJ3131 with the 4.25-kilobase (kb) *Apa*I-*Bam*HI fragment of the plasmid p*Δvph1::LEU2* (see below for details on plasmids). Strains BJ6717 to BJ6720 are segregants of one tetrad resulting from the sporulation of BJ6716.

DNA Sequencing—The nucleotide sequence of *VPH1* was sequenced to completion on both strands through the following methods: 1) constructing a set of exonuclease III nested deletions, 2) subcloning restriction fragments into a sequencing vector, and 3) custom synthesizing an oligonucleotide. For the exonuclease III nested deletions, the 3.6-kb *Cla*I-*Eco*RI fragment of pVIP1-78 (Fig. 1*a*) was cloned into *Cla*I-*Eco*RI-digested KS M13⁻ (pVIP1-82) and SK M13⁻ (pVIP1-83) Bluescript vectors (Stratagene cloning systems) as shown in Fig. 1*b*. pVIP1-82 was linearized with *Sal*I, filled in with thionucleotides to create an exonuclease III-resistant end, and then cut again with *Cla*I. pVIP1-83 was linearized with *Bam*HI, filled in with thionucleotides, and cut again with *Eco*RI. Both plasmids were then subjected to a exonuclease III digestion using the Double-Stranded Nested Deletion Kit (Pharmacia LKB Biotechnology Inc.) following the manufacturer's instructions. A series of 15 and 14 subclones were constructed from pVIP1-82 and pVIP1-83, respectively; deletion subclones differed successively by approximately 250 bp. To sequence through and past the *Eco*RI site, the 460-bp *Bst*YI-*Sca*I fragment of pVIP1-78 was cloned into *Bam*HI-*Eco*RV-digested Bluescript KS M13⁺ (pBSKS101) and KS M13⁻ (pBSKS102) as shown in Fig. 1*c*. Single-stranded DNA templates were sequenced by the dideoxynucleotide chain termination method of Sanger *et al.* (1977) with the Sequenase™ Kit (United States Biochemical Corp.) using the T3 or SK primers. Finally, the oligonucleotide 5′-ACG TTT GCA CTG GGT TG-3′ (Fig. 2, bp 2880–2896) was synthesized to allow determination of the 3′ end of *VPH1* via double-stranded sequencing on pVIP1-78. To resolve sequencing compressions, dITP was used instead of dGTP in the nucleotide mixture.

Plasmids—The plasmids pVPH1A3-1 and pVIP1-78 are derivatives of YCp50. pVPH1A3-1 contains an 8.8-kb yeast genomic insert that complements the *vph1-1* mutation. pIBEVPH1 contains a 6.9-kb *Bam*HI-*Eag*I fragment from the pVPH1A3-1 insert ligated into the *Bam*HI-*Eag*I sites of YIp5. The disruption plasmid p*Δvph1::LEU2* was constructed via a trimolecular ligation with the following three restriction fragments. 1) A 1.8-kb *Xba*I-*Kpn*I (with the *Xba*I site filled in to form a blunt end) fragment of the nested deletion subclone pVIP1-83-10D (see Fig. 1*d*) containing a sequence from the *Cla*I site in pVIP1-78 to nucleotide 782 (using the numbering in Fig. 2). 2) A 2.2-kb *Sal*I-*Xho*I (with the *Sal*I site filled in to form a blunt end) fragment of YEp13 containing the entire open reading frame of *LEU2*. 3) A 3.2-kb *Kpn*I-*Xho*I fragment of the nested deletion subclone pVIP1-82-16f (see Fig. 1*e*) containing the entire Bluescript KS M13⁻ vector and sequence from the *Eco*RI site in pVIP1-78 to nucleotide 2389 (using the numbering in Fig. 2). The end result is 5′ *VPH1* sequence starting at the *Cla*I site up to nucleotide 782 followed by the coding region of LEU2 (replacing 64% of the coding region of *VPH1*) followed by 3′ *VPH1* sequence starting at nucleotide 2389 and ending with the *Eco*RI site, all of which is inserted into the *Cla*I-*Eco*RI sites of the polylinker of KS M13⁻ (Fig. 1*f*).

Genetic Methods—Sporulation, dissection, and scoring of nutritional markers were performed as described in Hawthorne and Mortimer (1960). The *VPH1* marker was scored on the basis of the pH-sensitive fluorescence of 6-carboxyfluorescein as described in Preston *et al.* (1992).

DNA Manipulations—Most of the techniques for the preparation and analysis of DNA are detailed in Maniatis *et al.* (1982). Yeast transformations were performed as described in Woolford *et al.* (1990).

RESULTS

Cloning VPH1—The *VPH1* gene was cloned twice in our laboratory by two independent methods: firstly by complementation of the *vph1-1* mutation, and secondly by screening a λgt11 expression library with antibodies raised against a 95-kDa vacuolar integral membrane protein.

Cloning through Complementation of the vph1-1 Mutation—The *vph1-1* mutant BJ4886 was transformed with the yeast genomic library in the low copy plasmid YCp50 (a gift from M. Rose (Princeton University), J. Thomas (University of Washington), and P. Novick (Yale University)). Approximately 19,000 Ura⁺ transformants were screened for complementation of the *vph1-1* fluorescence phenotypes. For the three colonies that, upon retest, gave plasmid-associated complementation, potential plasmid candidates were shuttled through *Escherichia coli* and retransformed into BJ4886. Two of the plasmids had overlapping restriction maps. A 6.9-kb *Bam*HI-*Eag*I DNA fragment common to the two inserts was

[2] D. Preuss, J. Mulholland, A. Franzusoff, N. Segev, and D. Botstein, submitted for publication.

subcloned into the integrating vector YIp5 that had been digested with *Bam*HI and *Eag*I, generating the plasmid pIBEVPH1. This chimeric plasmid was linearized within the 6.9-kb insert with *Bgl*II and transformed into BJ4873 (*vph1-1*) and BJ4878 (*VPH1*), selecting for Ura$^+$ transformants.

Meiotic analysis of the cross between the Ura$^+$ transformant of BJ4878 and BJ4879 (*vph1-1*) gave nine tetrads that segregated 2 Ura$^+$Vph$^+$:2 Ura$^-$Vph$^-$, indicating that the plasmid had integrated and that linearization within the insert had directed integration to a site close to the *VPH1* locus. Meiotic analysis of the cross between the Ura$^+$ transformant of BJ4873 (which proved to be of Vph$^+$ phenotype) and BJ2663 (*VPH1*) yielded 18 tetrads that segregated 4 Vph$^+$:0 Vph$^-$ (and 2 Ura$^+$:2 Ura$^-$), indicating tight linkage between the site of plasmid integration and the *vph1-1* allele, and that the integrated gene complemented the *vph1-1* allele. From the data for these two crosses, we concluded that we had cloned the *VPH1* gene. The exceptional tetrads from these crosses, two from the former cross that gave 3 Ura$^+$Vph$^+$:1 Ura$^-$Vph$^-$ and one from the latter cross that showed 3 Vph$^+$:1 Vph$^-$ segregation (the Vph$^-$ segregant was also Ura$^-$), are compatible with the conclusion.

Cloning through a λgt11 Expression Library—Polyclonal antibodies were generated against an abundant 95-kDa vacuolar integral membrane protein as described under "Experimental Procedures." The antibodies were affinity-purified and shown to recognize only one polypeptide of the right size and intracellular location (discussed below). A total of 275,000 insert-containing plaque-forming units from a yeast genomic λgt11 expression library were screened (approximately 8 yeast genomes worth) resulting in four identical positives. The positive clones contained a 3-kb insert and produced an isopropyl β-D-thiogalactopyranoside-inducible fusion protein of about 150 kDa that was recognized by the antibodies used for the screen and by anti-β-galactosidase antibodies (data not shown). To further confirm that the isopropyl β-D-thiogalactopyranoside-induced protein was in-frame and contiguous to β-galactosidase and encoded by a genuine yeast gene, about 300 bp were sequenced starting just before and going through the putative fusion protein's *Eco*RI junction site using the λgt11 forward primer (New England Biolabs, Catalog No. 1218). Sequencing revealed a continuous open reading frame, in-frame and contiguous to *lacZ*, with a codon bias in agreement with the codon bias for yeast genes (data not shown). The β-galactosidase portion of the fusion protein accounts for 114 kDa (Huynh *et al.*, 1985), implying that the insert only encodes a 36-kDa polypeptide. To retrieve a full length clone, the λgt11 insert was radioactively labeled and used in colony hybridization to probe *E. coli* colonies bearing yeast genomic DNA sequences in YCp50. Several positive clones were chosen, which, on the basis of restriction mapping, were found to be completely contained within or overlapping with the largest positive, pVIP1-78. DNA blot hybridization with the radioactively labeled λgt11 insert as a probe demonstrated that the λgt11 insert was contained within the 3.6-kb *Cla*I-*Eco*RI fragment of pVIP1-78 (see Fig. 1*a*).

Similarities were noticed between the restriction maps for DNA encoding *VPH1* and the λgt11-selected DNA contained within plasmids pVIP1-78 and pVIP1-79. Further restriction analysis was performed on pIBEVPH1 and pVIP1-78 with the endonucleases *Eco*RI and *Eco*RV; a 1.8-kb *Eco*RI fragment, a 0.48-kb *Eco*RI-*Eco*RV fragment, and a 0.25-kb *Eco*RV fragment were observed by agarose gel electrophoresis in digests from both plasmid inserts. The radiolabeled 1.8-kb *Eco*RI fragment of the pIBEVPH1 clone proved to hybridize to a 1.8-kb *Eco*RI fragment band from pIBEVPH1 and from

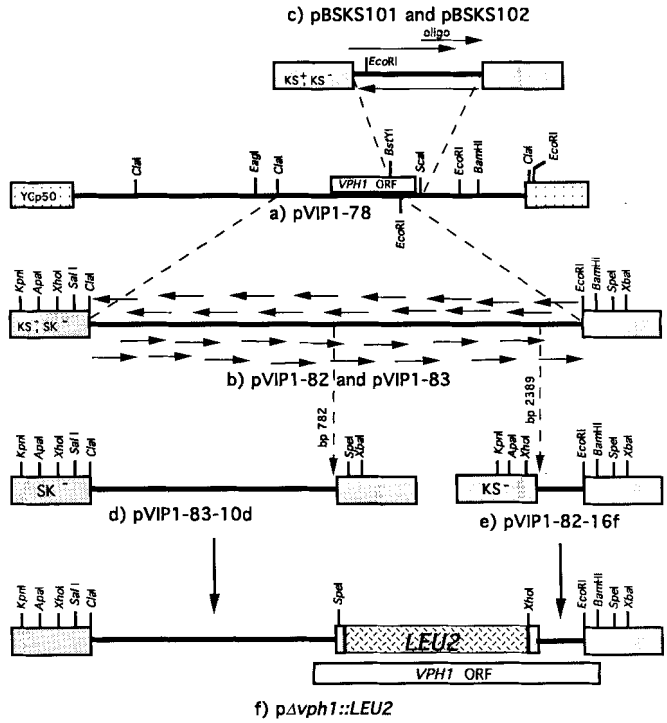

FIG. 1. **Sequencing and gene disruption strategy for *VPH1*.** *Solid lines* denote genomic sequence, *open stippled boxes* denote vector sequence, *open clear boxes* labeled *VPH1 ORF* indicate the extent of the *VPH1* open reading frame, and $KS^{+,-}$ and $SK^{+,-}$ refer to the respective Bluescript M13 vector. This figure is not drawn to scale. See "Experimental Procedures" for details of sequencing and gene disruption.

pVIP1-78 DNA. pVIP1-78, upon transformation into the *vph1-1* mutant strain BJ5035 and the *vph1* deletion-disrupted strain BJ6718, complemented both *vph1* mutations, as assayed by fluorescence. We interpreted these data to mean that pVIP1-78 and pVIP1-79 contain the *VPH1* gene.

Sequence Analysis—The DNA sequence encoding the *VPH1* gene (Fig. 2) was sequenced to completion on both strands, using the strategy described under "Experimental Procedures" and shown in Fig. 1, *b* and *c*. There is an open reading frame of 2521 bp (starting at bp 487 and ending with bp 3007 in Fig. 2) encoding a putative polypeptide of 840 amino acid residues with a predicted molecular mass of 95.6 kDa. The sequence context around the putative ATG start codon is in close agreement with the consensus sequence for highly expressed yeast genes reported by Hamilton *et al.* (1987). Codon usage in the open reading frame of *VPH1* predominantly matches the codon preference statistics for highly expressed yeast genes published by Sharp *et al.* (1986). Vph1p can be essentially divided into two equal size regions, a hydrophilic amino terminus and a hydrophobic carboxyl terminus containing putative membrane-spanning domains. By using the method of Kyte and Doolittle (1982), the calculated grand average of hydropathy value (GRAVY) for residues 1 to 406 is −4.18, for residues 407 to 840, 3.91 (the average GRAVY score for sequenced soluble proteins is −0.4 with hydrophobic values lying above and hydrophilic scores lying below this score). The algorithms of Klein *et al.* (1985), Rao and Argos (1986), and Eisenberg *et al.* (1984) all predict 6 to 7 membrane-spanning regions in the carboxyl-terminal domain when using the parameters and cutoff points suggested in the original papers. The difference in numbers is a result of the algorithms using different minimum and maximum lengths required for a transmembrane region. The putative transmembrane regions have been underlined in Fig. 2.

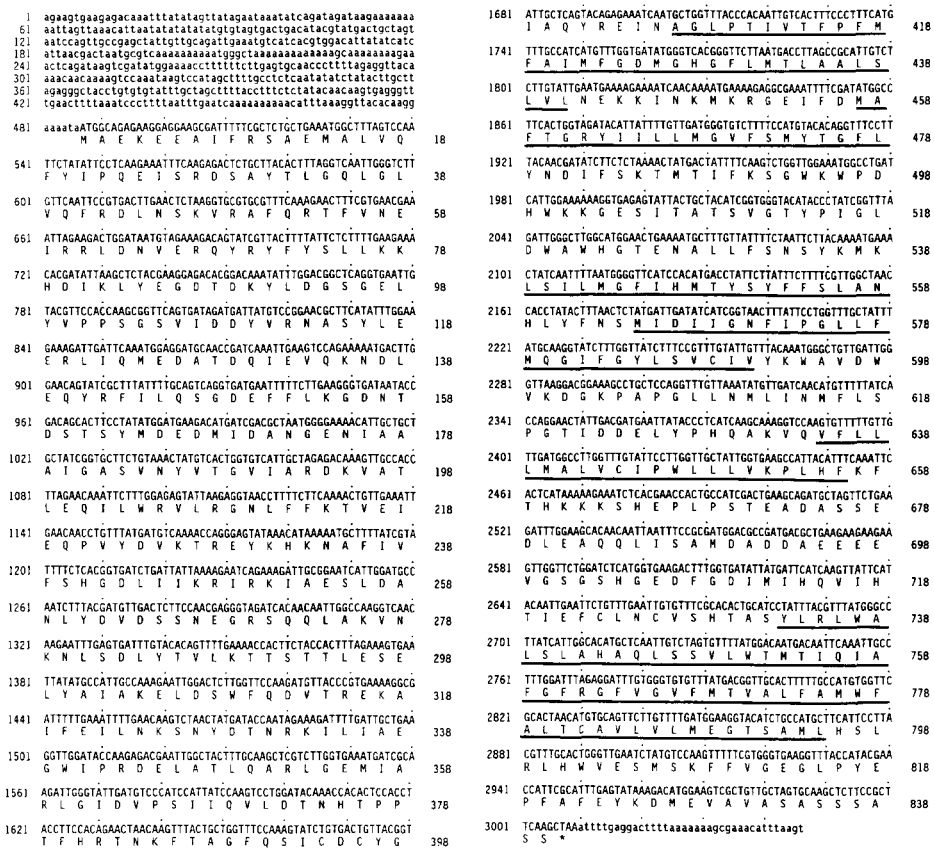

FIG. 2. **Nucleotide and deduced amino acid sequence of *VPH1*.** Nucleotide and translated amino acid sequences (entered above in *single-letter code*) are numbered to the *left* and *right*, respectively. Putative membrane-spanning regions (see "Results" for details) are *underlined*.

The algorithms of both Eisenberg *et al.* (1984) and Klein *et al.* (1985) predict one membrane-spanning region in the hydrophilic amino-terminal domain from residues 172 to 192. The algorithms scored this region right on and just above the cutoff values for predicting a membrane-spanning domain. Thus, the classification of this region as a transmembrane domain is questionable. There are 3 potential N-*X*-S type asparagine-linked glycosylation sites in the hydrophilic domain at amino acid residues 113, 280, and 324.

Sequence Homologies—Using alignments created by the program BESTFIT (Genetics Computer Group Sequence Analysis Software Package), Vph1p has 54% identity (71% similarity) with the yeast gene product Stv1p (Similar To *VPH1*), 42% identity (63.5% similarity) with the 116-kDa subunit of the rat clathrin-coated vesicle/synaptic vesicle H^+-ATPase, and 42% identity (60% similarity) with the TJ6 mouse immune suppressor factor. The evolutionary distance between the two mammalian proteins is almost the same as that between the two yeast gene products (54–55% identity, 71–74% similarity), while each individual yeast gene product has about the same distance to each mammalian protein (38–42% identity, 60–64% similarity). A multiple sequence alignment of the four sequences is shown in Fig. 3. All four sequences have hydrophilic amino-terminal domains and hydrophobic carboxyl-terminal domains containing 6–8 membrane-spanning regions. The major differences between them appear to be several large insertions in Stv1p (in particular residues 155–180 and residues 225–238 in Stv1p) and an insertion in the TJ6 mouse immune suppressor factor (residues 498–508). The small 8-residue homology between the rat 116-kDa subunit and the human von Willebrand factor noted by Perin *et al.* (1991) is almost perfectly conserved among all four sequences (see Fig. 3) with the following consensus sequence GRY I/V I/L LL L/M G. This region is contained within the quadruplicated D3 repeat domain of the human von Willebrand factor (Verweij *et al.*, 1986). The 8 homologous residues are poorly conserved among the four repeat domains D1-D4 in the von Willebrand factor (3 out of 8 residues), and there is no speculation as to the function of this domain in the literature.

Phenotypes Resulting from the vph1-1 Mutation—A recessive nuclear mutation, *vph1-1*, was isolated in a screen for vacuolar acidification-defective mutants using the pH-sensitive fluorescence of 6-carboxyfluorescein (Preston *et al.*, 1989, 1992). The *vph1-1* mutation results in a vacuolar pH of 6.9 (as compared to the wild type pH of 6.2) and eliminates vacuolar accumulation of the weak base quinacrine (Preston *et al.*, 1989). Purified vacuoles (prepared as described under "Experimental Procedures") from strains bearing the *vph1-1* mutation have no detectable ATP-dependent proton-pumping or bafilomycin-sensitive ATPase activity (data not shown). Barely detectable levels of the two peripherally bound nucleotide-binding subunits (69- and 60-kDa subunits) are found associated with vacuoles from a *vph1-1* strain, yet, at least for the 69-kDa subunit, are at wild type levels in whole cell extracts from the same strain (Fig. 4). On the basis of immunoblotting, there is no detectable Vph1p antigen in whole cell extracts from strains bearing the *vph1-1* mutation.

Disruption of the VPH1 Gene—The *VPH1* gene was disrupted by transforming the Leu^- diploid BJ3131 to Leu^+ with the 4.25-kb *Apa*I-*Bam*HI fragment of pΔ*vph1::LEU2*, in which the *LEU2* gene replaces 64% of the *VPH1* open reading frame (described in detail under "Experimental Procedures" and Fig. 1). The resulting heterozygote (BJ6716) was sporulated, and 27 tetrads were dissected, all of which had four spore viability and all of which segregated 2 Vph^+Leu^-:2 Vph^-Leu^+. To confirm that the genomic copy of *VPH1* was replaced with *LEU2*, genomic DNA from the parent recipient

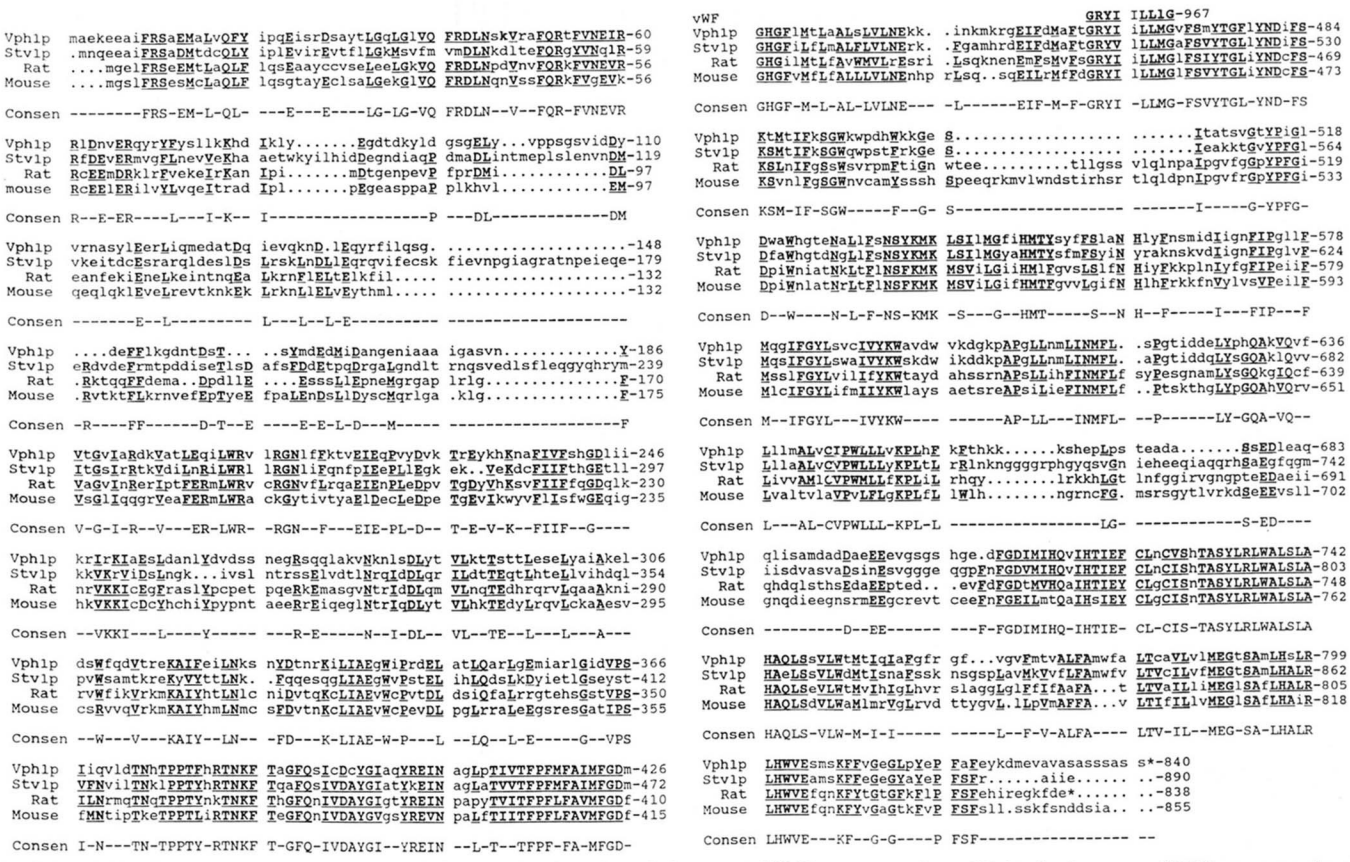

FIG. 3. **Multiple sequence alignment.** The entire lengths of the yeast *VPH1* gene product (*Vph1p*), the yeast *STV1* gene product (*Stv1p*), the 116-kDa polypeptide of the rat clathrin-coated vesicle/synaptic vesicle H⁺-ATPase (*Rat*), the mouse TJ6 mouse immune suppressor factor (*Mouse*), and 9 residues of the human von Willibrand factor (*vWF*) have been aligned using the PILEUP and PRETTY programs (Genetics Computer Group Sequence Analysis Software Package). The alignment and consensus sequences were created using the default setting except that a plurality of 3 was used for calculating the consensus sequence. Amino acid members of the winning coalition are in *upper case* and *underlined*, the consensus sequence is shown directly below the alignment (*Consen*).

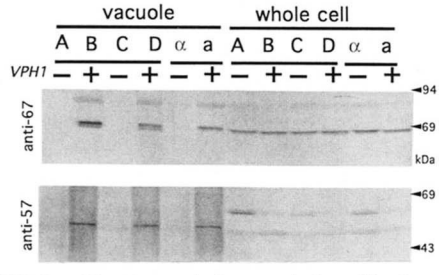

FIG. 4. **Mislocalization of the peripherally bound nucleotide-binding subunits of the vacuolar H⁺-ATPase co-segregates with the *vph1-1* mutation.** Purified vacuoles (5 μg) and whole cell extracts (40 μg) from a *vph1-1* and a wild type parent (BJ4887, α, BJ4888, *a*) and segregants from a single tetrad resulting from their mating (BJ4875, *A*, BJ4876, *B*, BJ4877, *C*, BJ4878, *D*) were electrophoresed and subjected to immunoblot detection using antibodies directed against the plant 67-kDa (*anti-67, top panel*) and 57-kDa (*anti-57, bottom panel*) nucleotide-binding subunits. The *VPH1* genotype is shown above the lanes.

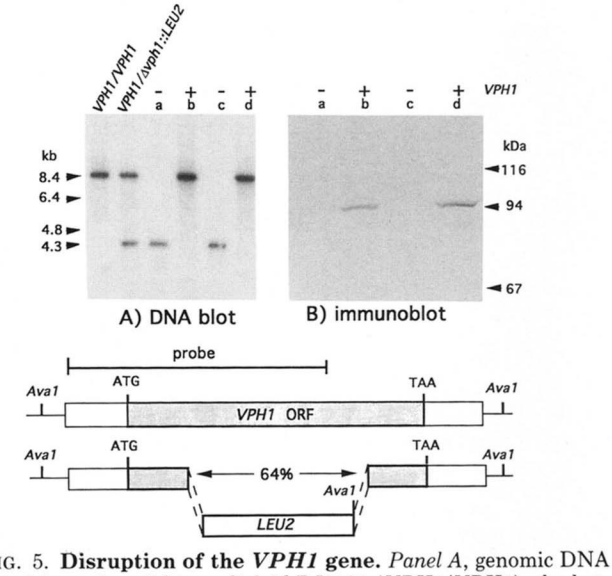

FIG. 5. **Disruption of the *VPH1* gene.** *Panel A*, genomic DNA isolated from the wild type diploid BJ3131 (*VPH1/VPH1*), the heterozygote BJ6716 (*VPH1/Δvph1::LEU2*), and segregants from a single tetrad resulting from sporulation of BJ6716, BJ6717 (*a*), BJ6718 (*c*), BJ6719 (*b*), and BJ6720 (*d*) were digested with *Ava*I and subjected to DNA blot analysis using the probe diagrammed in the figure. *Panel B*, whole cell extract from the segregants in *panel A* were subjected to immunoblot analysis using a 1:350 dilution of the affinity-purified anti-Vph1p antibodies. *VPH1* genotypes for the segregants are shown above both panels.

diploid (BJ3131), the resulting transformant diploid (BJ6716), and segregants from one tetrad (BJ6717 to BJ6720) were digested with *Ava*I and analyzed by DNA blot hybridization. Insertion of the *LEU2* gene results in the addition of an *Ava*I site that reduces a wild type 8.4-kb *Ava*I fragment to 4.3 kb. Fig. 5, *panel A*, shows the wild type 8.4-kb fragment in the diploid parent recipient, both the 8.4- and the 4.3-kb fragment in the heterozygous diploid (BJ6716), and the presence of the 4.3-kb fragment in the Vph⁻ segregants of a tetrad. Further confirmation of the disruption is shown in Fig. 5,

panel b, in which immunoblotting reveals the absence of Vph1p antigen in the Vph⁻Leu⁺ segregants. This result also demonstrates the specificity of the antibodies raised against the vacuolar 95-kDa polypeptide for the gene product of *VPH1*. Henceforth, the antibodies will be referred to as anti-Vph1p. All segregants bearing the *Δvph1::LEU2* allele were viable but acidification-defective, demonstrating that the *VPH1* gene is essential for vacuolar acidification but not essential for growth or viability.

Vph1p Is Localized to the Vacuolar Membrane—The enrichment of Vph1p in the vacuolar membrane is evidenced in the immunoblot in Fig. 6. At a high dilution of anti-Vph1p, Vph1p antigen was just visible in 100 μg of whole cell extract (Fig. 6, whole cell extract) yet was abundant in only 0.2 μg of purified vacuoles (Fig. 6, *vacuoles*). Furthermore, when intact vacuoles were disrupted through osmotic lysis, the Vph1p antigen remained attached to the vacuolar membranes (Fig. 6, *vacuolar membranes*). The specificity of Vph1p for the vacuolar membranes is evidenced in the immunogold electron micrographs shown in Fig. 7. Vph1p antigen (as visualized by the gold particles) is localized specifically over the vacuolar membranes. The identity of these membrane-bound organelles as vacuoles was confirmed by the detection of the vacuolar protease carboxypeptidase Y within the lumen by immunogold electron microscopy (data not shown).

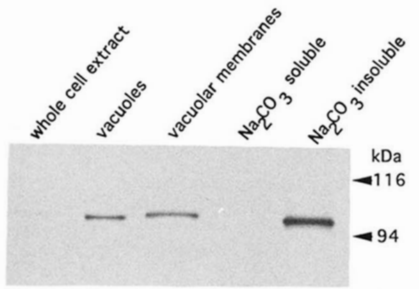

FIG. 6. **Localization and alkaline Na_2CO_3 extraction of Vph1p.** 100 μg of whole cell extracts (whole cell extract), 0.2 μg of purified vacuoles (vacuoles), 0.2 μg of vacuolar membranes (vacuolar membranes), 20 μg of protein extracted from the vacuolar membrane by treatment with alkaline Na_2CO_3 (*Na_2CO_3-soluble*), and 0.2 μg of vacuolar membrane proteins not extracted by the alkaline Na_2CO_3 (*Na_2CO_3-insoluble*) were prepared as described under "Experimental Procedures" and subject to immunoblot analysis with a 1:1000 dilution of the affinity-purified anti-Vph1p antibodies.

Vph1p Is an Integral Membrane Protein—Vacuolar vesicles were treated with Na_2CO_3, pH 11.0, exactly as described by Woolford *et al.* (1990), after which the membranes were pelleted at 100,000 × *g*. This treatment has been shown to effectively remove the peripherally bound yeast vacuolar proteins encoded by *PEP5* (Woolford *et al.*, 1990) and *PEP3* (Preston *et al.*, 1991) from the vacuolar membrane. In Fig. 6, no Vph1p antigen is detected in the lane containing the Na_2CO_3-soluble proteins (Na_2CO_3-soluble); all of the signal is recovered in the lane containing the pelleted membranes (Na_2CO_3-insoluble).

Vph1p Is a Vacuolar H⁺-ATPase Subunit—Both the phenotype of strains bearing the *vph1* mutations and the homology of Vph1p with the rat 116-kDa clathrin-coated H⁺-ATPase subunit suggest that the *VPH1* gene encodes a subunit of the yeast vacuolar H⁺-ATPase. To confirm this possibility, the vacuolar H⁺-ATPase was partially purified through solubilization of vacuolar membranes with the detergent ZW3-14 and ultracentrifugation through a glycerol gradient (as described under "Experimental Procedures"). Fractions withdrawn from the glycerol gradient were assayed for protein content, bafilomycin A_1-sensitive ATPase activity, and the Vph1p antigen. Fig. 8 is an immunoblot of the resulting fractions probed with anti-Vph1p antibodies; the specific activity of bafilomycin A_1-sensitive ATP hydrolysis is reported under the corresponding fraction. The Vph1p antigen is seen enriching and co-purifying with the peak of ATPase activity (Fig. 8, *fraction 8*).

DISCUSSION

VPH1 Encodes a Vacuolar H⁺-ATPase Subunit—Here we present genetic, biochemical, and sequence data that a 95-kDa polypeptide, encoded by the *VPH1* gene, is a subunit of the vacuolar ATPase and is required for its *in vivo* assembly and enzyme activity. Mutations, including a deletion, of the *VPH1* gene result in an acidification-defective vacuole (Preston *et al.*, 1989), mislocalization of the peripherally bound H⁺-ATPase nucleotide-binding subunits (Fig. 4), and absence of any detectable bafilomycin A_1-sensitive ATP hydrolysis or ATP-dependent proton-pumping in purified vacuoles. Biochemically, Vph1p was shown to be highly enriched (Fig. 6) and specifically localized (Fig. 7) to the vacuolar membrane

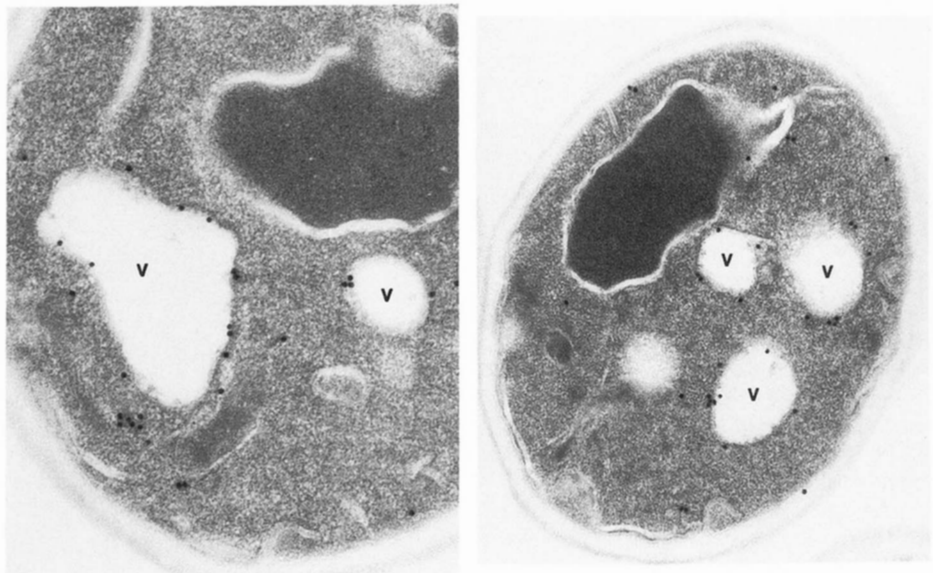

FIG. 7. **Localization of Vph1p through immunogold electron microscopy.** Thin sections of whole yeast cells were prepared, probed with a 1:3 dilution of affinity-purified anti-Vph1p antibodies, and subjected to immunogold electron microscopy as described under "Experimental Procedures." Vacuoles are identified in the micrographs by a *V*.

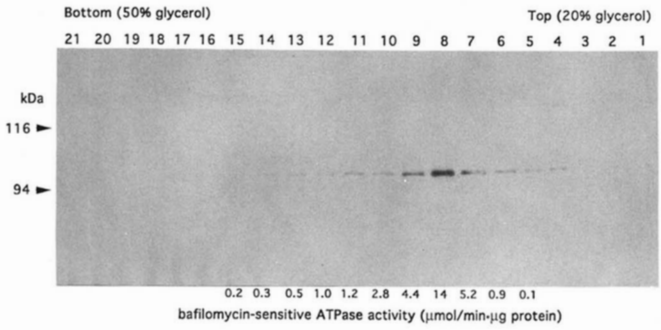

FIG. 8. **Co-purification of Vph1p with the vacuolar H⁺-ATPase.** 1.5 mg of vacuolar membranes were solubilized with the detergent ZW3-14, layered on a 20–50% glycerol gradient, and subjected to ultracentrifugation as described under "Experimental Procedures." 0.6-ml fractions were collected and assayed for bafilomycin A_1-sensitive ATP hydrolysis and subjected to SDS-PAGE followed by immunodetection with a 1:4000 dilution of affinity-purified anti-Vph1p antibodies. *Lanes 1* (*Top (20% glycerol)*) to *21* (*Bottom (50% glycerol)*) contain 1.0 μg of protein from consecutive fractions off the gradient. The *numbers* under the lanes represent the specific activity of bafilomycin-sensitive ATP hydrolysis for the corresponding fraction. *Lanes without numbers* on the bottom had no detectable ATPase activity.

and to co-purify with the vacuolar H⁺-ATPase enzyme on a glycerol gradient (Fig. 8). Furthermore, the deduced amino acid sequence of *VPH1* has extensive homology to the 116-kDa subunit of the clathrin-coated vesicle/synaptic vesicle H⁺-ATPase (Fig. 3) and has approximately the same molecular mass as the 100-kDa subunit reported by Kane *et al.* (1989). The difference between the 95-kDa subunit calculated in this report and the 100-kDa subunit calculated by Kane *et al.* (1989) reflects the difference in molecular mass reported in different molecular size marker kits for phosphorylase *b* (94 kDa *versus* 97.4 kDa), rather than any difference or strain variation in the *VPH1* gene product.

Cloning the VPH1 Gene—The *VPH1* gene was cloned by two independent methods: firstly, through complementation of an acidification-defective mutant using 6-carboxyfluorescein diacetate, and, secondly, through a reverse genetic approach of screening expression libraries with antibodies raised against specific vacuolar proteins. Although different in approach, both strategies were designed with the same goal of identifying yeast genes involved in vacuolar biogenesis and function. That two such different methods converged on a gene essential for normal vacuolar function validates both approaches as viable ways of dissecting vacuolar function. Comparison of restriction maps first suggested that the same gene had been cloned twice. This was confirmed through DNA blot hybridization and finally by showing that the Vph⁻ phenotype of *vph1* mutant strains could be complemented by the plasmid pVIP1-78. Immunoblot analysis of whole cell extracts from strains bearing the *Δvph1::LEU2* allele (Fig. 5) show that the antibodies raised to the 95-kDa protein are indeed specific for and a valid reagent to study the *VPH1* gene product.

Sequence Analysis versus Biochemical Data—Computer analysis on the nucleic and deduced amino acid sequence of *VPH1* agrees with the biochemical observations of Vph1p. On the basis of Coomassie Blue staining of polyacrylamide gels, Vph1p is visually the most abundant integral vacuolar membrane protein. This observation is in agreement with the codon usage analysis of the *VPH1* open reading frame which suggests that the *VPH1* gene is highly expressed. The *VPH1* open reading frame encodes an 840-amino acid polypeptide with a predicted molecular mass of 95.6 kDa, almost identical with the 95 kDa calculated for Vph1p on the basis of migration on polyacrylamide gels. The inability of alkaline Na_2CO_3 to strip Vph1p from the vacuolar membrane (Fig. 5) correlates with the 6 predicted transmembrane regions in the carboxyl-terminal domain (Fig. 2) in classifying the *VPH1* gene product as an integral membrane protein. This agrees with the results of similar experiments performed by Adachi *et al.* (1990) and Kane *et al.* (1992) and by the labeling of the 100-kDa subunit of the coated vesicle proton pump by the hydrophobic reagent 3-(trifluoromethyl)-3-(*m*-iodophenyl)diazirine (Arai *et al.*, 1988).

Homologies—The multiple sequence alignment in Fig. 3 demonstrates the extensive homology over the entire lengths of the following: 1) the 95-kDa subunit of the yeast vacuolar H⁺-ATPase, Vph1p, 2) the yeast gene product of *STV1*, 3) the 116-kDa subunit of the clathrin-coated vesicle/synaptic vesicle proton pump, 4) the TJ6 mouse immune suppressor factor. The similarity of their primary sequences is also reflected in predicted secondary structure. All four sequences have the same bipartite structure with a hydrophilic amino-terminal and a hydrophobic carboxyl-terminal region containing the membrane-spanning domains. The extent of similarity suggests that all four proteins are functional homologues, performing the same, as yet unknown, function in their respective V-type H⁺-ATPase.

The homology between the yeast Vph1p and the mammalian 116-kDa clathrin-coated vesicle/synaptic vesicle H⁺-ATPase concurs with the current hypothesis that vacuolar H⁺-ATPases form a separate class of proton pumping enzymes with a common evolutionary origin (Gogarten *et al.*, 1989). The similarity between the DCCD-binding proteolipids and the peripherally bound nucleotide-binding subunits of the V-type (17, 60, and 69 kDa) compared to the F_1F_0 (8 kDa, α and β) H⁺-ATPases suggest a common evolutionary origin between these two classes of enzymes. The absence of any Vph1p-like subunit (either in size or sequence homology) described for the F_1F_0 H⁺-ATPase implies an early evolutionary split between the F_1F_O and vacuolar H⁺-ATPases.

The homology to the TJ6 mouse immune suppressor factor suggests that this factor is a mammalian vacuolar-type H⁺-ATPase subunit. The TJ6 mouse immune suppressor factor was cloned by screening a mouse λgt11 expression library with antibodies directed against secreted immune regulatory proteins (Lee *et al.*, 1990). Lee *et al.* (1990) suggest that the first 20 amino-terminal amino acids of the TJ6 sequence are hydrophobic and characteristic of a signal peptide. We are unable to detect any evidence of an amino-terminal secretory signal sequence using the algorithm of Heijne (1986) in any of the sequences listed in Fig. 3. The significance of selecting a membrane-bound ATPase subunit with antibodies against secreted immune regulatory proteins or *in vitro* immune suppression by TJ6 is as yet unknown.

Sequence homology has identified a yeast gene product Similar To *VPH1*, *STV1*. The existence of the *STV1* gene could be explained as follows. 1) *STV1* may be a pseudogene, an unlikely possibility since pseudogenes in yeast are rare. Furthermore, there are regions of conserved predicted peptide sequence between *VPH1* and *STV1* that are not conserved at the nucleotide level, implying that there has been selection to maintain the peptide but not the nucleic acid sequence (data not shown). 2) *STV1* and *VPH1* are duplicated genes that encode functionally interchangeable proteins. This too seems unlikely as most functionally interchangeable proteins encoded by duplicated genes in yeast (α-tubulin, ribosomal proteins, elongation factor 1α, histones, ubiquitin) are greater than 90% identical, most with only a few amino acid changes.

Stv1p and Vph1p are only 54% identical with long non-homologous insertions in Stv1p (Fig. 3). 3) Stv1p and Vph1p may be differentially expressed subunits of the same vacuolar H^+-ATPase enzyme, the differences between the two polypeptides conferring alternative regulation once assembled into an enzyme. This scenario has been suggested for the 72% homologous γ subunits (*atpC1* and *atpC2*) of the *Arabidopsis thaliana* chloroplast F_1F_0 ATPase by Inohara *et al.* (1991). Although the 80% identical *Schizosaccharomyces pombe* plasma membrane H^+-ATPases, *PMA1* and *PMA2*, are functionally interchangeable, Ghislain and Goffeau (1991) present evidence that the 20% difference between two H^+-ATPases confers differences in phosphorylation and thus regulation of the enzymes. 4) Stv1p and Vph1p are equivalent subunits for specific V-type H^+-ATPases located on different endomembrane organelles, the divergent domains between Stv1p and Vph1p being responsible for the differential targeting and regulation of the enzyme for a specific organelle. Yeast has several endomembrane organelles that require transmembrane electrochemical gradients for their specific functions (vacuole, clathrin-coated vesicles, peroxisomes, and Golgi complex) and thus one expects there to exist unique V-ATPases bound to different endomembrane organelles. These V-type ATPases must be targeted and assembled on different membranes and must be differentially regulated according to the needs of the organelle. Lai *et al.* (1991) have found that, at least in higher plants, the 17-kDa proteolipid subunit is encoded by a small multigene family and suggest that multiple genes provide isoforms for organelle-specific V-ATPases. To address the *in vivo* function of Stv1p, we are studying the effect of overexpressing and disrupting the *STV1* gene, attempting to localize the gene product, and we are searching for additional *STV* genes. This work, together with the details of cloning and sequencing of *STV1*, will be published in a subsequent report.

Role of the 95-kDa Subunit—There is evidence to suggest that the 95-kDa subunit of vacuolar H^+-ATPases may be involved in assembly rather than the catalysis reaction of the enzyme. Xie and Stone (1988) have shown that both the membrane-bound 116- and 16-kDa subunits of the clathrin-coated vesicle H^+-ATPase are dispensable for Ca^{2+}-activated ATP hydrolysis but not for Mg^{2+}-activated ATP hydrolysis or proton pumping. Radiation inactivation analysis of vacuolar H^+-ATPases consistently predicts functional molecular masses that are smaller than one would predict by the addition of the molecular masses of associated subunits (Sarafian *et al.*, 1992). In *S. cerevisiae* specifically, the functional molecular mass for DCCD-sensitive multiple-cycle ATP hydrolysis was calculated by radiation inactivation to be between 410 and 530 kDa (Hirata *et al.*, 1989). Addition of the associated *S. cerevisiae* H^+-ATPase subunits reported by Kane *et al.* (1989) using the subunit stoichiometry suggested by Arai *et al.* (1988) calculates a molecular mass of 721 kDa (95 kDa, 3 × 69 kDa, 3 × 60 kDa, 42 kDa, 36 kDa, 32 kDa, 27 kDa, 6 × 17 kDa). The difference between functional and total molecular mass implies that not all the associated V-type ATPase subunits are involved in the hydrolysis of ATP or translocation of protons.

Kane *et al.* (1992) show that in the absence of either the 69- or 60-kDa nucleotide-binding subunits, the 95-kDa subunit is still correctly and stably inserted into the vacuolar membrane as long as the membrane-bound 17-kDa subunit is present. In the absence of the membrane-bound 95-kDa subunit, the peripherally bound nucleotide-binding subunits (69 and 60 kDa) are no longer correctly targeted to the vacuolar membrane (Fig. 4). These results suggest that the 95- and 17-kDa subunits are epistatic to the 69- and 60-kDa subunits for vacuolar H^+-ATPase assembly. Here we have shown that the 95-kDa subunit of vacuolar H^+-ATPases is required for assembly and hypothesize that it is necessary for targeting of the enzymes to specific organelles.

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