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Regulation of Yeast Ectoapyrase Ynd1p Activity by Activator Subunit Vma13p of Vacuolar H^+-ATPase*

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CD39-like ectoapyrases are involved in protein and lipid glycosylation in the Golgi lumen of *Saccharomyces cerevisiae*. By using a two-hybrid screen, we found that an activator subunit (Vma13p) of yeast vacuolar H^+-ATPase (V-ATPase) binds to the cytoplasmic domain of Ynd1p, a yeast ectoapyrase. Interaction of Ynd1p with Vma13p was demonstrated by direct binding and co-immunoprecipitation. Surprisingly, the membrane-bound ADPase activity of Ynd1p in a *vma13*Δ mutant was drastically increased compared with that of Ynd1p in *VMA13* cells. A similar increase in the apyrase activity of Ynd1p was found in a *vma1*Δ mutant, in which the catalytic subunit A of V-ATPase is missing, and the membrane peripheral subunits including Vma13p are dissociated from the membranes. However, the E286Q mutant of *VMA1*, which assembles inactive V-ATPase complex including Vma13p in the membrane, retained wild type levels of Ynd1p activity, demonstrating that the presence of Vma13p rather than the function of V-ATPase in the membrane represses Ynd1p activity. These results suggest that association of Vma13p with the cytoplasmic domain of Ynd1p regulates its apyrase activity in the Golgi lumen.

Ectoapyrases (nucleoside triphosphate diphosphohydrolases (NTPDases),[1] formerly called E-ATPases) are members of a rapidly expanding family of enzymes that hydrolyze a wide range of purine and pyrimidine nucleoside tri- and diphosphates in the presence of divalent cations (usually Ca^{2+} or Mg^{2+}) (1–5). The enzymes have an extremely active nucleotide hydrolysis site located outside the cytoplasm and are insensitive to the classic inhibitors of P-, F-, and V-type ATPases (1). The NTPDases are widely distributed in eukaryotic cells from yeast to mammals (1, 3, 4). It has been suggested that they participate in many biological processes such as modulation of neural cell activity, prevention of intravascular thrombosis, immune response regulation, and purinergic signaling regulation (3, 6–10). The molecular identity of the NTPDases has been recently revealed by the cloning of a soluble apyrase from potato tubers (*Solanum tuberosum*) (2). Amino acid sequence alignment showed that NTPDases possess highly conserved sequences called apyrase conserved region motifs (2). CD39, a human and mouse lymphoid cell antigen, was the first mammalian enzyme identified (3); it is responsible for inhibition of ADP-induced platelet aggregation (8, 11). Despite the recent progress in the molecular characterization of NTPDases (4, 5, 10, 12, 13), little is known about the regulation of these proteins.

In the lumen of the Golgi, specific oligosaccharide modification of proteins and lipids occurs. The substrates for these reactions, nucleotide sugars, are synthesized in the cytosol and are transported into the Golgi lumen via specific carrier proteins (14, 15). After transfer of sugar residues to proteins and lipids by glycosyltransferases, the resulting nucleoside diphosphates are converted to nucleoside monophosphates by the ectoapyrases (16–18). In this way, nucleotide diphosphates that are inhibitors of glycosyltransferases do not accumulate in the lumen of the Golgi (19–21), and the nucleoside monophosphates exit the lumen of Golgi in exchange for sugar nucleotides in the cytosol (14, 15). It has been shown that the NTPDases in *Saccharomyces cerevisiae* are required for *N*- and *O*-linked glycosylation in the Golgi lumen (16, 17). Only two NTPDases (*GDA1* and *YND1*/*APY1*) have been found within the entire yeast genome (16–18). Gda1p has a high activity toward GDP and a low activity toward UDP but no activity toward other nucleotides (16). Deletion of *GDA1* in yeast caused a marked reduction in Golgi glycosylation of proteins and lipids (16) and resulted in a 4-fold lower rate of GDP-mannose entry into the Golgi lumen (22). Ynd1p has a typical apyrase activity with broad substrate preference (17, 18). The *ynd1*Δ mutant was defective in *O*- and *N*-linked glycosylation in the Golgi compartment (17). The *gda1*Δ*ynd1*Δ double deletion had a synthetic effect on cell growth and cell shape (17, 18). The Ynd1p function is partially redundant with that of Gda1p, as *ynd1*Δ cells behaved differently from *gda1*Δ cells in drug sensitivity (17). The precise relationships between Ynd1p, Gda1p, and glycosylation in the Golgi are not understood.

The vacuolar H^+-ATPases (V-ATPases) function to acidify intracellular compartments in eukaryotic cells, including the Golgi apparatus, lysosomes, coated vesicles, chromaffin granules, and the central vacuole of yeast, *Neurospora*, and plants (23–27). V-ATPases consist of two structural domains, V_1 and V_0. The peripheral V_1 domain contains at least eight different subunits responsible for ATP hydrolysis. The integral V_0 domain functions in proton translocation and is composed of at least five different subunits. Organelle acidification and/or membrane energization by the V-ATPases is important for a number of cellular processes such as receptor-mediated endocytosis, protein sorting, zymogen activation, and solute uptake into specific organelles (28–30).

In this study, we investigated the function and regulation of the yeast NTPDase Ynd1p using a two-hybrid screen. Ynd1p contains an unusually long cytoplasmic domain at the COOH

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[1] The abbreviations used are: NTPDases, nucleoside triphosphate diphosphohydrolases; MES, 4-morpholineethanesulfonic acid; V-ATPase, vacuolar H^+-ATPase; PAGE, polyacrylamide gel electrophoresis; GST, glutathione *S*-transferase.

terminus (18), compared with other known NTPDases. We chose this domain as bait for the screen. A peripheral membrane protein Vma13p, the activator subunit of V-ATPases, was found to bind specifically to the cytoplasmic domain of Ynd1p. We provide evidence that the activity of Ynd1p is regulated through the binding of Vma13p to its cytoplasmic domain.

EXPERIMENTAL PROCEDURES

Strains, Media, and Reagents—All DNA manipulations were performed using the *Escherichia coli* strain DH5α (*supE44D lacU169* (*f80lacZDM15*) *hsdr17 recA1 endA1 gyrA96 thi-1 relA1*). *S. cerevisiae* strains used are BCY123 (*MATa pep4::HIS3 prb1:: LEU2 bar1::HISG lys2::GAL1/10-GAL4 can1 ade2 trp1 ura3 his3 leu2-3, 112*), PJ69-4A (*MATa trp1-901 leu2-3,112 ura3-52 his3-200 gal4Δ gal80Δ LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ* (31)), YPH500 (*MATα leu2 ura3 trp1 lys2 his3 ade2* (32)), RH302 (Δ*vma13::TRP1* derivative of YPH500 (32)), SF838-5Aα (*MATα leu2-3,112 ura3-52 ade6, vma1Δ::LEU2* (33)), URA-VMA1-WT (*SF838-5Aα MATα leu2-3,112 ura3-52 ade6, vma1Δ::LEU2, URA3::VMA1* (33)), and URA-VMA1-E286Q (*SF838-5Aα MATα leu2-3,112 ura3-52 ade6, vma1Δ::LEU2, URA3::VMA1E286Q* (33)). To create a *trp1* marker in URA-VMA1-WT and URA-VMA1-E286Q, a polymerase chain reaction-based Kan4X module was used to knock out completely the *TRP1* gene as described (34). The primers used are XZ154 (5′-TCTGTTATTAATTTCACAGGTAGTTCTGGTCCATTGGTGAAAGTTAGCTTGCCTCGTCCCCGCCGG-3′), in which the first 45 nucleotides are the sense sequence of the *TRP1* open reading frame (nucleotides 4–48) followed by the 5′-sense sequence of Kan4X module that is underlined; XZ155 (5′-CTTCGCATTTTTGACGAAATTTGCTATTTTGTTAGAGTCTTTTACTCGACACTGGATGGCGGCGTT-3′), in which the first 45 nucleotides are the antisense sequence of the *TRP1* open reading frame (nucleotides 625–669) followed by the 5′-antisense sequence of the Kan4X module that is underlined. Standard rich (YPD) and complete minimal tryptophan drop-out media were used (35). Standard rich medium for *E. coli* was used (36). Nucleoside phosphates were purchased from Sigma. Zymolyase 20T was purchased from ICN (Irvine, CA).

Two-hybrid Analysis—To construct the plasmid (pGZ125) expressing the hybrid bait protein, a *Bam*HI-*Sal*I DNA fragment encoding the COOH-terminal 113 amino acids of Ynd1p was cloned into the yeast expression vector containing the Gal4 DNA-binding domain (pGBDU-C1) and the URA3 marker (31). The pGZ125 plasmid was transformed into the yeast reporter strain PJ69-4A (31). The resulting strain was subsequently transformed with a *S. cerevisiae* genomic library and selected as described (31). The candidate colonies that grew on SD plates-lacking uracil, histidine, and adenine and that turned blue in the 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal) filter assay were examined for the loss of pGZ125 by streaking them on SD plates, lacking leucine and containing 2.5 μg/ml 5′-fluoroorotic acid. Plasmid loss was verified by replica plating on SD plates lacking uracil and leucine. The clones dependent on the presence of the COOH-terminal domain of YND1 were isolated to recover the plasmids, which were then amplified in *E. coli* strain DH5α.

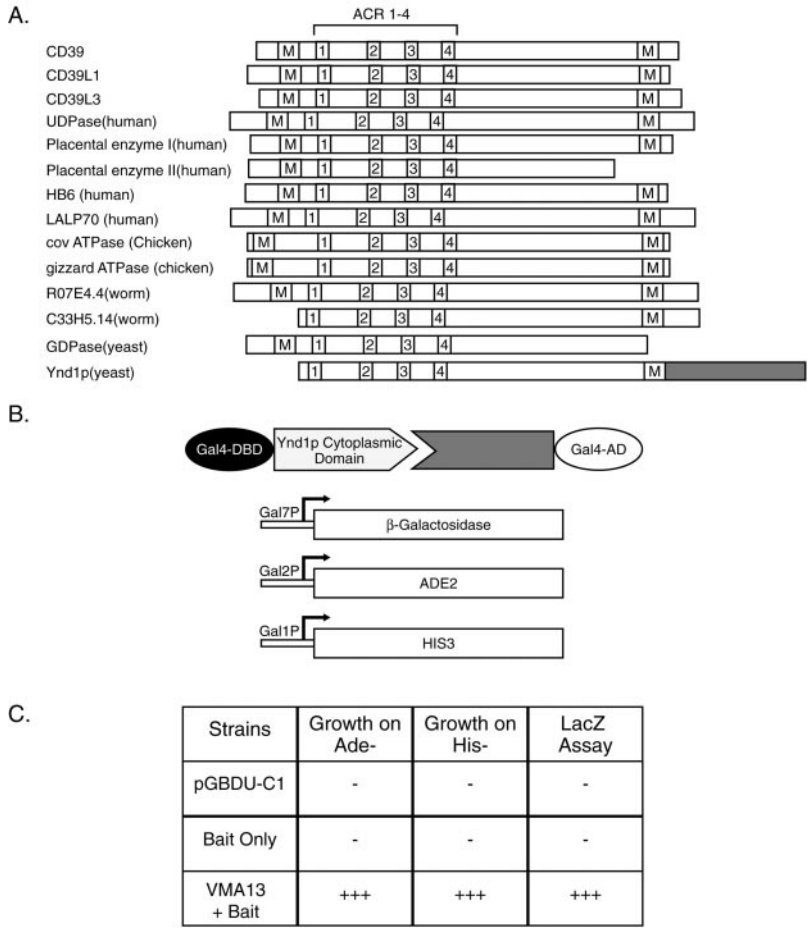
Purification of Recombined Proteins, in Vitro Binding Assay and Immunoprecipitation—A *Bam*HI-*Sal*I DNA fragment encoding Vma13p was cloned in vector pGEX4T-1 (Amersham Pharmacia Biotech) to produce a fusion between GST and Vma13p. Protein expression and purification were performed as described previously (37). Glutathione was removed from purified GST and GST-Vma13p by dialysis. Membrane fractions (100 μg) from BCY123/pGZ105 cells (18) were extracted with 1 ml of binding buffer: 50 mM Tris·HCl, pH 7.5, 100 mM KCl, 0.1% digitonin. The extract was incubated with GST or GST-Vma13p (about 10 μg were used in each reaction) attached to beads at 4 °C with end-over-end rotation for 1 h, followed by three washes with 1 ml of binding buffer. To the beads were added 100 μl of 2× SDS loading buffer; the samples were heated for 5 min in boiling water before SDS-PAGE and immunoblotting. For immunoprecipitation, 5 μl of polyclonal anti-Vmam13p antibody or 12 μg of IgG and 50 μl of 40% (v/v) protein A-Sepharose CL-4B (Amersham Pharmacia Biotech) were added to the extract prepared as described above. The mixtures were incubated at 4 °C with end-over-end rotation overnight, followed by three washes with 1 ml of binding buffer. A 100-μl aliquot of 2× SDS loading buffer was added to elute samples from the beads before SDS-PAGE and immunoblotting. To overexpress a His-tagged Vma13p, a *Bam*HI and *Pst*I polymerase chain reaction fragment encompassing the entire *VMA13* gene was cloned into pTrcHisB (Invitrogen). Primer 144 (5′-GCGGGATCCGATGGGCGCAACCAAAATTTTAATGGAC-3′, containing a *Bam*HI site and a sense sequence of the *VMA13* open reading frame (nucleotides 1–27)) and primer 145 (5′-AAACTGCAGTTATTTGAAGGTATATCCAATGATTGC-3′, containing a *Pst*I site and an antisense sequence of the *VMA13* open reading frame (nucleotides 1407–1434)) were used with chromosomal DNA isolated from YPH500 as the template. The resulting plasmid pGZ152 was transformed into DH5α. To purify His-Vma13p, DH5α/pGZ152 cells were grown in 500 ml of TB medium (200 μg/ml ampicillin, 0.5% glucose) at 37 °C; when A_{600} was 0.6, isopropyl-1-thio-β-D-galactopyranoside was added to a final concentration of 0.8 mM. After 3 h at 37 °C, cells were pelleted and resuspended in binding buffer (50 mM Tris·HCl, pH 7.9, 150 mM NaCl); the cell suspension was passed through a French press (SLM-Amino, Urbana, IL) at 17,000 pounds/square inch. The lysate was ultracentrifuged at 39,000 × *g* for 20 min. The supernatant was saved and used to purify $(His)_6$-Vma13p with a Ni^+ column as described in the Invitrogen manual.

Subcellular Fractionation, Nucleotide Phosphatase Activity, and Immunoblotting—Subcellular fractionation was done as described (38). Briefly, spheroplasts were lysed by dilution in hypo-osmotic buffer. The lysate was centrifuged at 1,000 × *g* for 10 min to precipitate unbroken cells, and the supernatant was centrifuged at 13,000 × *g* (P13) and 120,000 × *g* (P120) for 20 and 60 min, respectively. The P13 and P120 pellets were resuspended in 0.8 M sorbitol, 10 mM triethanolamine, pH 7.2, 1 mM EDTA. Nucleotide phosphatase activity was assayed as described (18) with 2 mM ADP. Buffers 50 mM MES/NaOH, pH 5.0, 50 mM MES/NaOH, pH 6.0, 50 mM MES/NaOH, pH 6.5, 50 mM Tris·HCl, pH 7.0, 50 mM Tris·HCl, pH 7.5, 50 mM Tris·HCl, pH 8.0, 50 mM Tris·HCl, pH 8.5, 25 mM boric acid/NaOH, pH 9.0, 25 mM boric acid/NaOH, pH 10.0, and 20 mM $NaHCO_3$/NaOH, pH 11.5, were used for nucleotide phosphatase assays. Anti-Myc monoclonal antibody (1:1,000 dilution) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Vma1p monoclonal antibody (1:2,000 dilution) was purchased from Molecular Probes. Polyclonal rabbit anti-Vma13p antibody (1:2,000 dilution) was a kind gift from Dr. Tom H. Stevens (University of Oregon).

DNA Manipulation—DNA manipulations were carried out according to standard protocols (39). Plasmid pGZ105 (18) was digested with *Xba*I and *Hin*dIII, and a 3.6-kilobase pair fragment containing the glycerol-3-phosphate dehydrogenase promoter and the *YND1* gene was purified and cloned into a 2-μm *URA3* marker plasmid YEplac195 (40); the resulting plasmid is pGZ148. To overexpress Vma13p in yeast, a *Bam*HI/*Sal*I polymerase chain reaction product containing the entire *VMA13* gene was cloned into the pG1 vector (pGZ153). Primer 153 (5′-GCGGGATCCGTAACCATGGGCGCAACCAAAATTTTAATGGAC-3′, containing a *Bam*HI site and sense sequence of the *VMA13* open reading frame (nucleotides 1–27)) and primer 137 ((5′-ACGCGTCGACTTATTTGAAGGTATATCCAAT-3′, containing a *Sal*I site and antisense sequence of the *VMA13* open reading frame (nucleotides 1413–1434)) were used with chromosomal DNA isolated from YPH500 as a template.

RESULTS

Identification of a Vacuolar H^+-ATPase Peripheral Subunit That Specifically Interacts with the Cytoplasmic Domain of Ynd1p—Fig. 1*A* shows the line up of all reported membrane-bound NTPDases. These enzymes have an apyrase domain located on the exterior face of the cell membrane and one or two transmembrane domains to anchor the apyrase domain to the membrane. Although most of these membrane-bound NTPDases have short cytoplasmic domains (less than 50 amino acids), Ynd1p has a long carboxyl-terminal cytoplasmic domain (113 amino acids). We suspected that this region might be involved in regulation of the enzyme and therefore searched for potential interacting proteins using its cytoplasmic domain as the bait for a yeast two-hybrid screen (41). As shown in Fig. 1*B*, the cytoplasmic domain of Ynd1p fused to the Gal4 DNA-binding domain was used to find interacting partners obtained from yeast genomic DNAs fused to the activation domain of the *GAL4* gene. To reduce the incidence of false positives, a unique host strain PJ69-4A (31) containing three reporter genes, each driven by three different inducible promoters, was used for the screen (Fig. 1*B*). The first reporter gene was detected by the blue color of the colonies (β-galactosidase), and the second and third reporter genes allowed for the growth of cells harboring positive interacting proteins on adenine-deficient (Ade^-) and



C.

Strains	Growth on Ade-	Growth on His-	LacZ Assay
pGBDU-C1	-	-	-
Bait Only	-	-	-
VMA13 + Bait	+++	+++	+++

FIG. 1. **Identification of Vma13p as an interacting protein for the cytoplasmic domain of Ynd1p.** *A,* schematic representation of membrane-bound ectoapyrases: CD39 (62); CD39L1 and CD39L3 (5); UDPase (4); placental enzymes I, II (63); HB6 (12); LALP70 (64); cov (chicken oviduct) ATPase (65); chicken gizzard ATPase (66); R07E4.4, C33H5.14 (*Caenorhabditis elegans* genes) (4); GDPase (16); and Ynd1p (18). *M* indicates a transmembrane domain. *ACR* stands for apyrase conserved region. *B,* schematic representation of interacting proteins for yeast two-hybrid system. *C,* results of yeast two-hybrid screen using Ynd1p cytoplasmic domain as the bait. "+" indicates the growth of cells in drop-out media or high activity of β-galactosidase. "−" indicates the lack of growth of cells in drop-out media or no detectable activity of β-galactosidase. *PGBDU-C1* is the expression vector containing the Gal4 DNA-binding domain. *Bait Only* represents the construct encoding the fusion of the Gal4 DNA-binding domain with the COOH-terminal 113-amino acid segment of Ynd1p. *VMA13 + Bait* is the construct encoding the fusion of the Gal4 DNA activation domain with the fragment of VMA13 in addition to the bait construct. The plates with the transformed cells were incubated at 30 °C for 3 days.

histidine-deficient (His^-) media, respectively. Six individual recombinant plasmids recovered from 2×10^6 original transformants survived these selections. All the clones expressed a fusion with a fragment of Vma13p (amino acid residues 69–478) (Fig. 1*C*), a 54-kDa peripheral subunit of yeast V-ATPase (32). Vma13p is not required for the assembly of the V-ATPase complex in the membrane of the vacuole; however, in the absence of Vma13p, the complex has no ATPase activity or proton pumping activity (32). The amino-terminal 160 amino acids of Vma13p are not required for its function with the V-ATPase as a cDNA encoding Vma13p lacking the amino-terminal fragment complements *vma13*Δ (32). Since the Gal4 fusion of Vma13p lacking the amino-terminal 68 amino acids interacts with Ynd1p, this region of Vma13p is also not required for the interaction between Vma13p and Ynd1p.

Physical Interactions of Vma13p with Ynd1p—To confirm the results of the two-hybrid analysis that suggested a specific interaction between Vma13p and Ynd1p, we prepared a soluble glutathione *S*-transferase fusion protein containing intact Vma13p for an *in vitro* binding assay (Fig. 2*A*). GST and the GST-Vma13p fusion protein bound to glutathione-agarose were used to isolate binding partners from detergent-solubilized membrane proteins made from a yeast strain expressing myc-Ynd1p. As can be seen in Fig. 2*B*, myc-Ynd1p bound efficiently to GST-Vma13p (*lane 2*). Under the same conditions, Ynd1p was not retained by GST alone (*lane 1*). These results mirrored those obtained by the two-hybrid analysis. To investigate further the interaction of Vma13p with Ynd1p, anti-Vma13p antibodies were used to co-immunoprecipitate Ynd1p from a clarified solution of detergent-solubilized cell membranes (Fig. 2*C*). Ynd1p was in fact found in the immunoprecipitate with anti-Vma13p antibody (*lane 2*) but not in that of control IgG (*lane 1*), indicating that Vma13p does interact specifically with Ynd1p.

*Membrane-bound ADPase Activity of Ynd1p in a vma13*Δ *Mutant Is Increased Compared with That of Ynd1p in VMA13 Cells*—To investigate the consequences, if any, of the interaction between Vma13p and Ynd1p, we examined the effects of deleting or overexpressing *VMA13* on Ynd1p activity. Two isogenic strains, YPH500 (*VMA13*) and RH302 (*vma13*Δ), kindly provided by Dr. Tom Stevens (University of Oregon), were transformed with the 2-μm plasmid pGZ148 encoding myc-Ynd1p. The transformants of YPH500 were visible after 2–3 days of incubation at 30 °C, whereas the transformants of RH302 appeared after 5–6 days. The slow growth phenotype of the transformants of the *vma13*Δ cells was associated with Ynd1p expression, because the RH302 cells transformed with vector pVT101U grew after 2–3 days of incubation (Table I). To create a *VMA13*-overexpressing strain, the *VMA13* gene was placed behind a constitutive glycerol-3-phosphate dehydrogenase promoter in plasmid pGZ153, which was used to transform YPH500/pGZ148. Cells of all the relevant strains were subjected to subcellular fractionation by differential centrifugation. Spheroplasts were lysed in a hypo-osmotic buffer, and the lysate was centrifuged sequentially at 1,000, 13,000, and 120,000 × *g*. The 13,000 × *g* pellet (P13) contains most of the endoplasmic reticulum, vacuolar membrane, and plasma membrane (42, 43), whereas the 120,000 × *g* pellet (P120) is a Golgi-enriched fraction (16, 37, 44, 45).

First the subcellular distribution of Vma13p was determined. The subcellular fractions from *VMA13* cells and *vma13*Δ cells, both expressing myc-Ynd1p, were separated by SDS-PAGE and blotted with anti-Vma13p antibody (provided by Dr. Tom Stevens). Fig. 3 shows that a 54-kDa band recognized by anti-Vma13p was visible in the vacuole-containing

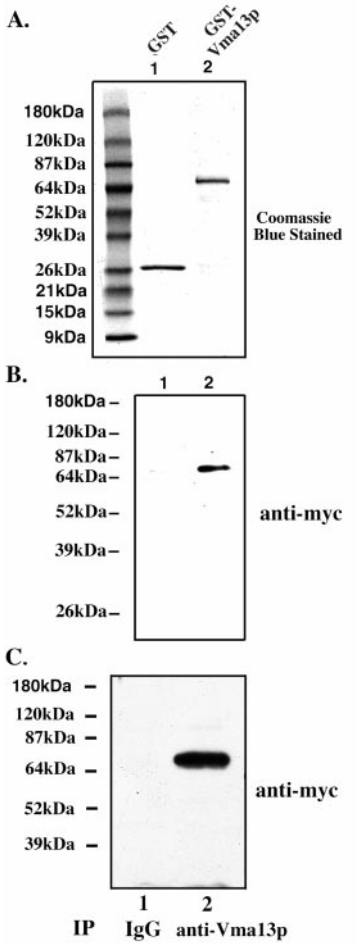


FIG. 2. **Specific binding of Vma13p to Yndlp.** *A,* GST and GST-Vma13p (10 μg/reaction) were purified on glutathione-agarose beads. Protein bound to beads was stained with Coomassie Blue. *B,* digitonin-solubilized P120 membrane (100 μg of protein) was incubated with GST-Vma13p adsorbed to glutathione-agarose. Proteins bound to the beads after extensive washing were separated by SDS-PAGE and subjected to immunoblotting with anti-Myc antibodies. *C,* digitonin-solubilized P120 membrane (100 μg of protein) was immunoprecipitated (*IP*) by anti-Vma13p antibody or nonspecific IgG, as described under "Experimental Procedures." Proteins bound to the protein A-Sepharose CL-4B beads were separated by SDS-PAGE and immunoblotted with anti-Myc antibodies.

TABLE I
Effects of V-ATPase mutations on the growth properties, Yndlp distribution, and ADPase activity of cells expressing Yndlp

Strains	Days required for the appearance of yeast transformants (vector alone/*YNDl*)[a]	Relative amounts of mycYndlp in P13 and P120 membrane fractions[b]	ADPase activity
Wild type	2–3/2–3	−	Low
*vmal3*Δ	2–3/5–6	+	High
*vmal*Δ	2–3/7–8	+	High
VMA13 expression	2–3/2–3	−	Low
URA-VMA1-E286Q	2–3/5–6	+	Low
URA-VMA1	2–3/2–3	+	Low

[a] Time required from plating to observe yeast colonies of 3 mm in size.
[b] − indicates that the P120 and P13 fractions contain approximately equal amounts of Yndlp. + indicates that the P120 fraction contains at least 4 times more Yndlp than does the P13 fraction.

P13 fraction of *VMA13* cells (*lane 6*) but not in that of *vma13*Δ cells (*lane 3*). The 54-kDa Vma13p was also found in the cytosol (*lane 4*), as reported for other peripheral subunits of V-ATPase (23, 46). An 87-kDa band in the cytosolic fraction is probably not Vma13p-specific, because it was visible in both *VMA13* and

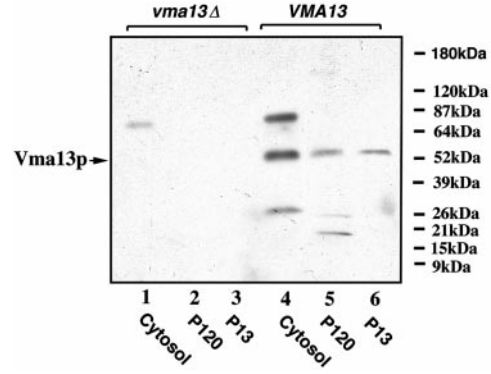


FIG. 3. **Subcellular distribution of Vma13p.** YPH500/pGZ148 (*VMA13*) and RH302/pGZ148 (*vma13*Δ) cells were fractionated to prepare a cytosolic fraction, and P13 and P120 membrane fractions, as described under "Experimental Procedures." Aliquots of these fractions (cytosolic, 100 μg; P13, 50 μg; P120, 50 μg) were subjected to SDS-PAGE and examined by immunoblotting with anti-Vma13p antibodies.

*vma13*Δ cells (*lanes 1* and *4*). It has been proposed that yeast V-ATPase acidifies organelles other than vacuoles. We found that the 54-kDa band was also present in the Golgi-enriched P120 fraction of the *VMA13* cells (*lane 5*), but not in that of *vma13*Δ cells (*lane 2*), consistent with the notion that a V-ATPase complex is present in the Golgi (47, 48).

Next the expression of myc-Yndlp in *vma13*Δ cells and *VMA13* cells was compared. As shown in Fig. 4*A*, the *VMA13* cells have slightly more myc-Yndlp in the P120 fraction than in the P13 fraction (*lanes 3* and *4*). Yndlp was shown previously to localize principally in the Golgi fraction, but overexpression of Yndlp caused some of the protein to mislocalize to the vacuole (18), as the latter serves as a default compartment for membrane proteins (49). In the *vma13*Δ cells, roughly 4–5 times more myc-Yndlp was found in fraction P120 than in fraction P13 (*lanes 1* and *2*), suggesting that in the absence of Vma13p there is less mislocalization of Yndlp to the vacuole. Fig. 4*A* also shows the expression of myc-Yndlp was much lower in the YPH500/pGZ153/pGZ148 cells that overexpress Vma13p (*lanes 5* and *6*) than in the *VMA13* cells (*lanes 3* and *4*). However, the distribution of myc-Yndlp in Vma13p-overexpressing cells (*lanes 5* and *6*) was similar to that in the *VMA13* cells (*lanes 3* and *4*), indicating that the overexpression of Vma13p by a factor of 10 did not cause more Yndlp to localize to the vacuole (P13 fraction). Since the absence of Vma13p eliminates V-ATPase activity (32) and inactivation of V-ATPase affects proper sorting of vacuole proteins (50, 51), the decrease of Yndlp in the P13 fraction in the *vma13*Δ cells might be due to the inactivation of V-ATPase in these cells.

To study the effects of Vma13p depletion and Vma13p overexpression on the enzymatic activity of myc-Yndlp, we measured the ADPase activity of P120 and P13 membrane fractions from these strains. As is shown in Fig. 4*B*, only the P120 membrane fraction of VMA13 cells had apyrase activity, although both P13 and P120 had similar amounts of myc-Yndlp. This result is consistent with the previous report that Yndlp activity is only measurable in the Golgi (17, 18). Surprisingly, P120 membranes of the *vma13*Δ cells had approximately 50 times higher activity than the membranes of the *VMA13* cells, although both membrane fractions contained similar amounts of myc-Yndlp. The P13 membranes of the *vma13*Δ cells also had higher ADPase activity than the P13 membranes of VMA13 cells. These results indicate that, in the absence of Vma13p, the ADPase activity of myc-Yndlp was drastically increased and that this activity was associated with both the P120 and P13 fractions. No apyrase activity was observed in either fraction of the cells overexpressing Vma13p, presumably

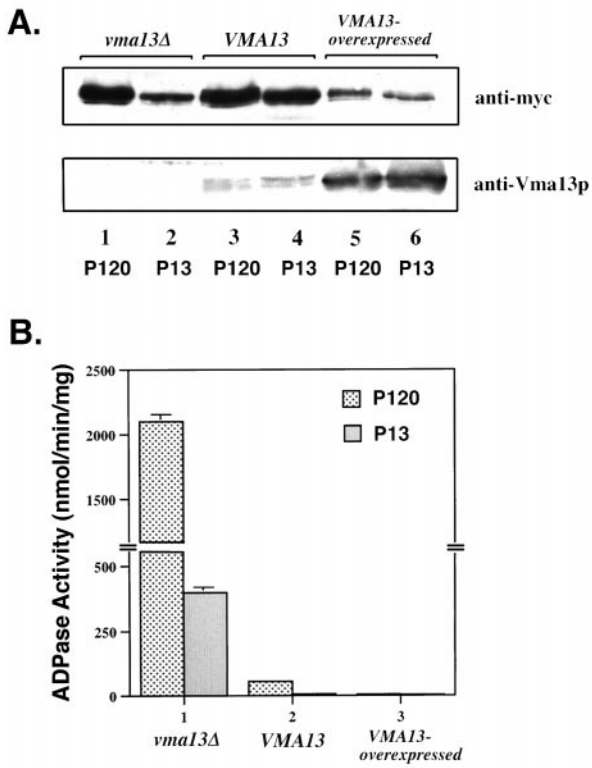


FIG. 4. **Effects of deleting and overexpressing *VMA13* on Ynd1p expression, localization, and activity.** *A,* distribution of Ynd1p in YPH500/pGZ148 (*VMA13*), RH302/pGZ148 (*vma13*Δ), and YPH500/pZ148/pGZ153 (*VMA13*-overexpressed) cells. P120 and P13 membrane fractions (50 μg of protein) were analyzed by SDS-PAGE followed by immunoblotting with anti-Myc and anti-Vma13p antibodies. *B,* ADPase activities of the P120 and P13 membranes of the yeast strains described in *A*. Ca^{2+}-stimulated ADPase activity was determined with 2 mM ADP by subtracting P_i release in the presence of EGTA from that in the presence of 10 mM $CaCl_2$ and EGTA. Assays were done with 2.5 μg of protein of *vma13*Δ cells and with 25 μg of protein of *VMA13* and VMA13-overexpressing cells. All values are means ± S.D. (n = 6).

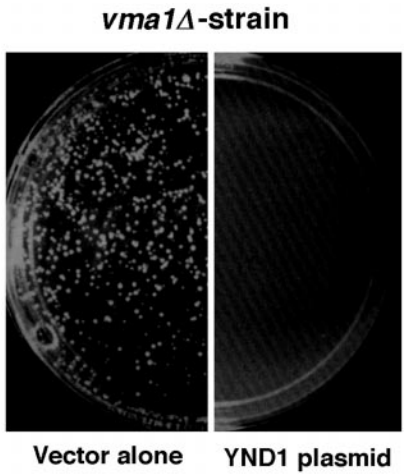


FIG. 5. **Expression of the *YND1* gene slows the growth of transformants of a strain with inactive V-ATPase.** The yeast strain SF838-5Aa (*vma1*Δ) was transformed with equal amounts of either plasmid pGZ148 containing *YND1* or plasmid pVT101U lacking the *YND1* cDNA by the lithium acetate method. Identical aliquots of the transformation reactions were plated on SD plates lacking uracil to select for Ura+ transformants and allowed to grow at 30 °C for 3 days.

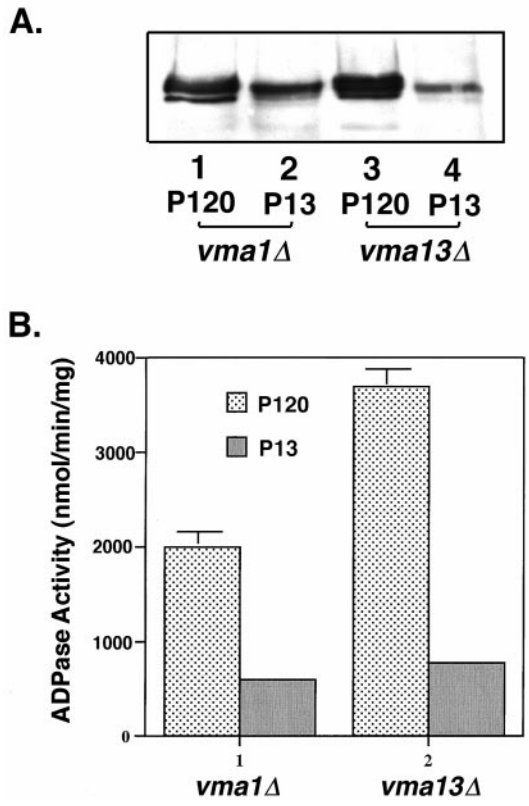


FIG. 6. **Effects of deleting *VMA1* on the distribution and activity of Ynd1p.** *A,* distribution of Ynd1p in SF838-5Aα/pGZ148 (*vma1*Δ) and RH302/pGZ148 (*vma13*Δ) cells. Aliquots (50 μg of protein) of P13 and P120 fractions were subjected to SDS-PAGE followed by immunoblotting with anti-Myc antibody. *B,* ADPase activity of P13 and P120 fractions (2.5 μg of protein per assay) of *vma1*Δ and *vma13*Δ cells. The assays were done as described in the legend of Fig. 4. All values are means ± S.D. (n = 4).

because of the low amount of myc-Ynd1p and the possible inhibitory role of Vma13p.

Mechanism of the Effect of Vma13p on the Activity of Ynd1p—How does Vma13p affect the activity of Ynd1p? One possibility is that Vma13p might directly bind to the cytoplasmic domain of Ynd1p and repress its activity. However, when purified soluble $(His)_6$-Vma13p was incubated with the *vma13*Δ cell membranes, no significant inhibition on the apyrase activity of Ynd1p was observed (data not shown).

Another possibility is that, since *vma13*Δ cells lack V-ATPase and proton pumping activity (32), Ynd1p activity might be regulated by the activity of the vacuolar H^+-ATPase. To test this hypothesis directly, a *vma1*Δ strain SF838–5Aα (52), in which the gene encoding the catalytic subunit A of V-ATPase was deleted, was transformed with pGZ148. Interestingly, as in the case of the *vma13*Δ cells, the transformants were visible only after 7–8 days of incubation at 30 °C, whereas the transformants with vector pVT101U alone appeared after 2–3 days of incubation (Fig. 5), suggesting that increased activity of Ynd1p in the cells lacking V-ATPase activity caused a slow growth phenotype. P120 and P13 membrane fractions were isolated from the SF838–5Aα/pGZ148 cells. Fig. 6*A* shows the subcellular localization of Ynd1p in the *vma1*Δ and *vma13*Δ cells; in both cases, there was more myc-Ynd1p in the P120 Golgi fraction than in the P13 vacuolar fraction, suggesting that inactivation of the V-ATPase influences the mislocalization of myc-Ynd1p to the P13 fraction. The ADPase activity of Ynd1p in both P120 and P13 fractions of the *vma1*Δ cells was high and comparable to that in the fractions of the *vma13*Δ cells (Fig. 6*B*).

These data appear to support the view that Ynd1p is regulated by the V-ATPase activity. However, deletion of *VMA1* affects not only V-ATPase activity but also the assembly of the

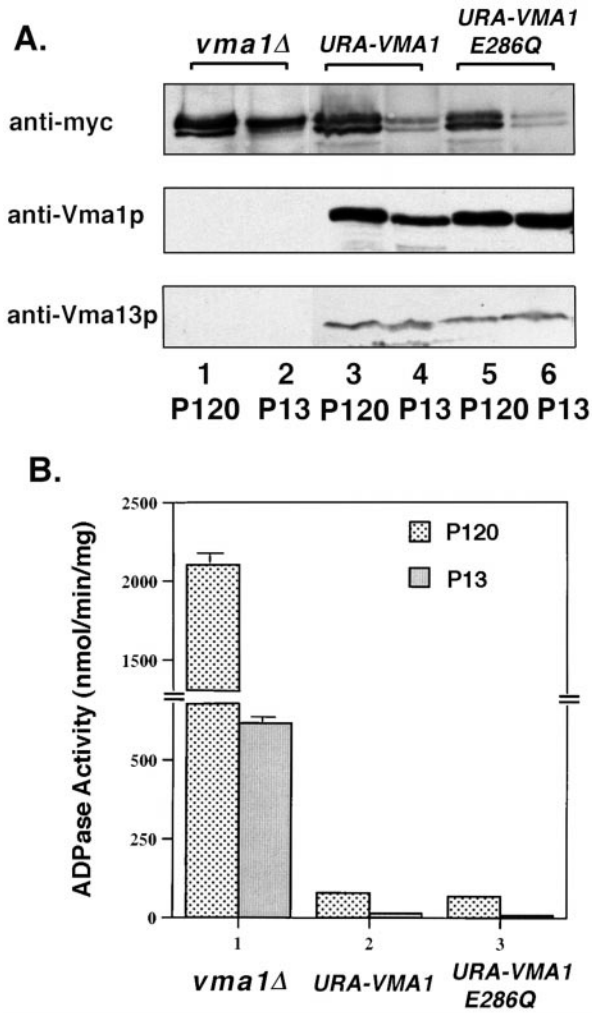


FIG. 7. **The E286Q mutant of VMA1, in which V-ATPase assembles normally but is inactive, retains the wild type level of Ynd1p activity.** *A,* distribution of myc-Ynd1p, Vma1p, and Vma13p in the P13 and P120 membrane fractions of SF838–5Aα/pGZ148 (*vma1*Δ), URA3-VMA1/pGZ105, and URA-VMA1-E286Q/pGZ105 cells. Samples (50 μg of protein) were subjected to SDS-PAGE followed by immunoblotting with anti-Myc, anti-Vma1p, and anti-Vma13p antibodies. *B,* ADPase activity of the P13 and P120 membrane fractions (25 μg of protein per assay) of URA3-VMA1/pGZ105 and URA-VMA1-E286Q/pGZ105 cells. The assays were done as described in the legend of Fig. 4. All values are means ± S.D. ($n = 4$).

other peripheral subunits of the enzyme in the membrane (23, 46, 53). Consequently, it is possible that deletion of *VMA1* resulted in the loss of Vma13p localization to the membrane and that the activity of Ynd1p was increased by lack of contact with Vma13p. Therefore, we examined the membranes of *vma1*Δ cells for the presence of Vma13p by immunoblotting. As shown in Fig. 7*A*, there was no detectable Vma13p in either P120 or P13 membrane fraction of the *vma1*Δ cells (*lanes 1* and *2*), indicating that deletion of *VMA1* did affect membrane assembly of Vma13p. It appears, therefore, that the effect of the *VMA1* deletion on Ynd1p activity might be a consequence of Vma13p absence from the membrane.

To test this possibility, we used two *vma1*Δ yeast strains, kindly provided by Dr. Michael Forgac (Tufts University), one containing the wild type *VMA1* gene (URA-VMA1-WT) and the other a *VMA1* gene with the E286Q point mutation (URA-VMA1-E286Q) integrated in the *URA3* locus. This mutation abolishes the ATP hydrolysis and proton pumping activity of the V-ATPase complex but does not perturb the assembly of the enzyme complex (33). These two strains were transformed with *YND1*-expression plasmid pGZ105 and with the plasmid pG1 lacking *YND1* cDNA. Transformants of URA-VMA1-WT with pGZ105 appeared after 2–3 days, but the transformants of URA-VMA1-E286Q appeared after 5–6 days. On the other hand, transformants of URA-VMA1-E286Q with pG1 appeared after 2–3 days of incubation (Table I). This *YND1*-dependent slow growth phenotype of URA-VMA1-E286Q cells was similar to those of *vma13*Δ and *vma1*Δ cells. Both URA-VMA1-E286Q/pG1 and URA-VMA1-E286Q/pGZ105 strains were shown to lack V-ATPase activity by their inability to grow in both high $CaCl_2$ and pH 7.6 media (data not shown).

The P120 and P13 membrane fractions of these yeast strains were examined. As is shown in Fig. 7*A*, both Vma1p and Vma13p are present in similar amounts in the membranes from both strain URA-VMA1-WT/pGZ105 and strain URA-VMA1-E286Q/pGZ105 (*lanes 3–6*). The distribution of myc-Ynd1p in these fractions was also similar to those in the *vma1*Δ cells (*lanes 1* and *2* compared with *lanes 3–6*). The fact that there is less myc-Ynd1p in the P13 fractions of URA-VMA1-WT cells (Fig. 7*A, lane 4*) than in that of *VMA1* parental cells (Fig. 4*A, lane 4*) is most likely due to the low V-ATPase activity of these *vma1*Δ cells containing an integrated copy of *VMA1* gene at the URA3 locus relative to the parental *VMA1* strains (33). It has been reported that vacuolar carboxypeptidase Y proteins are also mislocalized to the extracellular medium in URA-VMA1-WT strain presumably for the same reason (33).

The ADPase activities of Ynd1p in these membrane fractions are shown in Fig. 7*B*. The relative specific activities of the P120 fractions from both URA-VMA1-WT/pGZ105 and URA-VMA1-E286Q/pGZ105 were low and similar to that of the P120 fraction of *VMA13* cells (Fig. 4*B*). As expected, the ADPase activities of the P13 fractions of both strains were even lower, consistent with the smaller amount of Ynd1p present in these fractions. Since the apyrase activity of the URA-VMA1-E286Q strain was low even in the absence of V-ATPase activity, we conclude that the apyrase activity of Ynd1p is not principally dependent on the activity of V-ATPase. More relevant is the presence or absence of Vma13p in the membrane, its presence associated with a decrease in Ynd1p apyrase activity. Therefore, we conclude that the activity of Ynd1p is regulated primarily by the presence of Vma13p in the membrane, presumably by its association with the cytoplasmic domain of Ynd1p. The results shown in Fig. 7*A* also indicate that membrane assembly of Vma13p depends on the membrane assembly of other peripheral subunits of V-ATPase.

Effect of pH on Ynd1p Activity—Since the active site of Ynd1p faces the lumina of the Golgi and the vacuole, which are acidified by the V-ATPase, one wonders whether the activity of Ynd1p is regulated by pH. We find that the ADPase activity of Ynd1p decreases sharply with decreasing pH (apparent pK = 6.5) so that at pH 6 there is very little activity (Fig. 8). Although this dependence on pH is not responsible for the *in vitro* activity of Ynd1p in the yeast mutants (Figs. 4, 6, and 7) because the luminal compartments were in contact with the buffered external environment in these assays, it probably has an effect *in vivo* and may explain the peculiar growth characteristics of the mutant cells shown in Table I. Cells that have increased activity of Ynd1p and lack the H^+-pumping activity of the V-ATPase grow slowly compared with the same cells that do not produce Ynd1p. Even in the case of the URA-VMA1-E286Q cells in which the amount of Ynd1p activity is low and similar to that in the URA-VMA1 cells, because both assemble the V-ATPase with Vma13p (Fig. 6), the lack of luminal acidification in the presence of active Ynd1p leads to a slow growth phenotype. We suggest that the high activity of Ynd1p in these cells slows growth through alteration of the control of the glycosylation

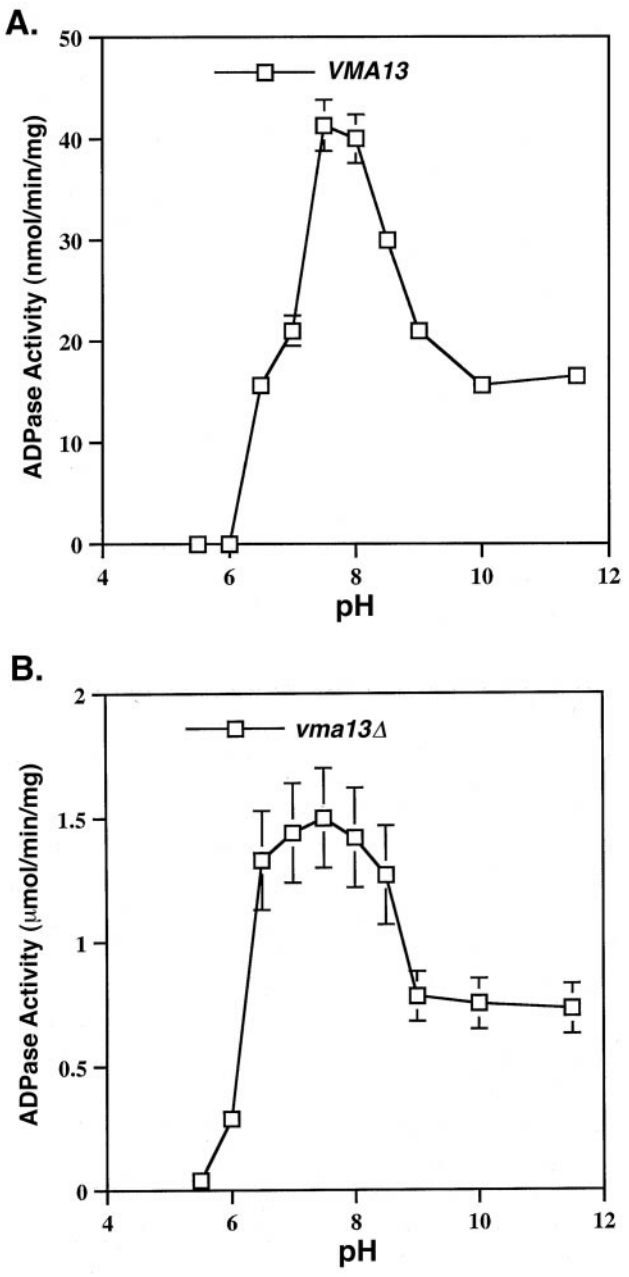


FIG. 8. **Effect of pH on the ADPase activity of Ynd1p.** The pH was varied by using MES, Tris, boric acid, and $NaHCO_3$ as buffers. *A,* ADPase activity of the P120 fraction (2.5 μg of protein per assay) of RH302/pGZ148 (*vma13*Δ) cells. *B,* ADPase activity of the P120 fraction (25 μg of protein per assay) of YPH500/pGZ148 (*VMA13*) cells. All values are means ± S.D. (n = 4).

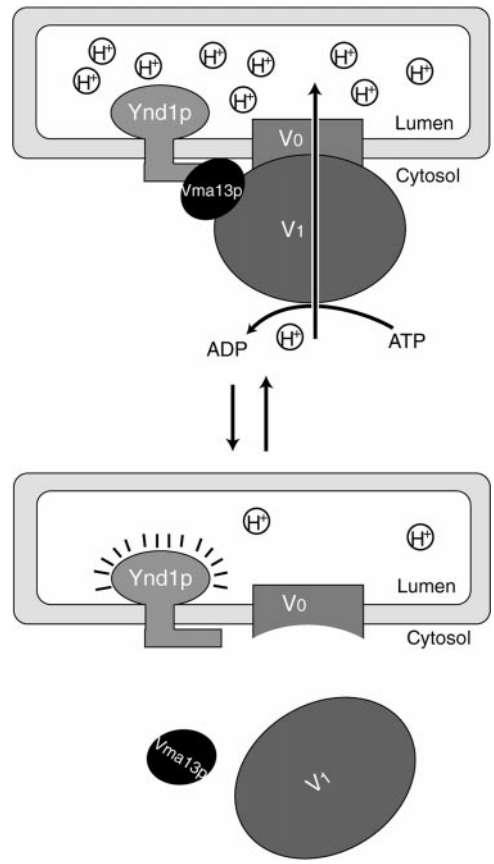


FIG. 9. **Proposed model for the regulation of Ynd1p by Vma13p.** V_1 is the peripheral domain of V-ATPase, and V_o is the integral membrane domain. In yeast intracellular compartments (Golgi stacks and vacuoles), Vma13p associates with the cytoplasmic domain of membrane-bound Ynd1p, and it is also a part of the V-ATPase complex. When Vma13p is present on the membrane, the activity of Ynd1p is repressed. When Vma13p is absent from the membrane, the activity of Ynd1p is dramatically increased.

reactions. We conclude that *in vivo* the activity of Ynd1p is inhibited both by interaction with Vma13p and by V-ATPase acidification of the lumen.

DISCUSSION

In this work we have described two unusual features of a member of the NTPDase family as follows: interaction with a cytoplasmic protein and regulation of the lumenal enzymatic activity through this interaction.

Vma13p has been identified as an interacting partner of Ynd1p, a yeast Golgi ectoapyrase, by a two-hybrid screen directed by the cytoplasmic domain of Ynd1p (Figs. 1–3). To our knowledge, this is the first identification of an interacting protein of a member of the NTPDase family. We have also found that interaction of membrane-bound Vma13p with the cytoplasmic domain of Ynd1p represses its luminal apyrase activity (Figs. 4 and 6). As far as we know, this is the first example of the regulation of the activity of an ectoenzyme by a cytoplasmic interaction. The salient feature of this interaction is that Vma13p must be part of the V-ATPase complex in the membrane, whether or not the complex has H^+-pumping activity, in order to affect the activity of Ynd1p (Fig. 7).

These results are summarized in Fig. 9 that shows a model of the interaction and the regulation. In yeast intracellular compartments (Golgi stacks and vacuole), Vma13p binds to the cytoplasmic domain of Ynd1p, while Vma13p is also part of the V-ATPase complex. We have no evidence that Ynd1p forms a complex with V-ATPase through Vma13p, but the presence of Vma13p on the membrane clearly depends on the membrane assembly of the V-ATPase complex (Fig. 7). Since membrane assembly of V-ATPase occurs by a complex process involving the coordinated association of subunits synthesized in the cytosol with subunits entering the secretory pathway upon synthesis (54–56), it becomes clear why addition of exogenous purified Vma13p to the membranes of *vma13*Δ cells had no effect on the apyrase activity of Ynd1p *in vitro*. As indicated in Fig. 9, when Vma13p is present in the membrane, the apyrase activity of Ynd1p is low; on the other hand, when Vma13p is absent from the membrane, the activity of Ynd1p is drastically increased.

Since the apyrase activity of Ynd1p is highly pH-dependent in the range between pH 6 and 7 (Fig. 8), the absence of Vma13p or of the assembly of the V-ATPase will result *in vivo*

in an extremely elevated activity of Ynd1p through the combined effects of the lack of interaction with Vma13p and the rise of the luminal pH. The high and unregulated activity of Ynd1p may be responsible for the slow growth phenotype through aberrant glycosylation and phosphorylation of proteins and lipids in the Golgi (16–18).

In yeast cells, the V_1 domain of V-ATPase has been found to dissociate rapidly from the membranes in response to substitution of galactose for glucose or depletion of the carbon source from the culture medium (57). That study also showed that addition of glucose-induced rapid reassembly of the enzyme from the previously synthesized V_1 and V_0 domains. Thus, rapid disassembly and reassembly of V-ATPases *in vivo* is a way of changing the acidification in yeast intracellular organelles (56–58). Given that this process of disassembly and reassembly of V-ATPase affects the presence of Vma13p on the membrane, this might also be a way to regulate the apyrase activity of Ynd1p *in vivo*.

It will be interesting to know whether other NTPDases also can be regulated by an interacting subunit. The cytoplasmic domains of most NTPDases are short compared with that of Ynd1p but are sufficient for protein binding. Since NTPDases like CD39 have extremely high ATPase activities (13), one wonders how they manage to transit through the endoplasmic reticulum and the Golgi on their way to the plasma membrane without hydrolyzing the organellar ATP required for chaperone function (59, 60) and phosphorylation of luminal proteins (15); also, it is not obvious why Ynd1p does not hydrolyze all the ATP in the Golgi. Association with a regulatory subunit like Vma13p together with luminal acidification could be a way to control apyrase activity.

The finding that Vma13p, subunit H of the yeast V-ATPase (23), has more than one function is not without precedent. NBP1, subunit H of human V-ATPase, has been reported to interact with the HIV-1 Nef protein, which is a soluble viral protein involved in decreasing the expression of CD4 on the surface of infected cells (61). The interaction between Nef and NBP1 has been proposed to facilitate the internalization of CD4 by Nef.

Although the bifunctional role of Vma13p is now established, the exact mechanism used by Vma13p in the activation of V-ATPase while down-regulating the ectoapyrase activity of Ynd1p remains to be uncovered.

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Regulation of Yeast Ectoapyrase Ynd1p Activity by Activator Subunit Vma13p of Vacuolar H^+-ATPase

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