The Amino-terminal Domain of the Vacuolar Proton-translocating ATPase a Subunit Controls Targeting and in Vivo Dissociation, and the Carboxyl-terminal Domain Affects Coupling of Proton Transport and ATP Hydrolysis*

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The 100-kDa “a” subunit of the vacuolar proton-translocating ATPase (V-ATPase) is encoded by two genes in yeast, VPH1 and STV1. The Vph1p-containing complex localizes to the vacuole, whereas the Stv1p-containing complex resides in some other intracellular compartment, suggesting that the a subunit contains information necessary for the correct targeting of the V-ATPase. We show that Stv1p localizes to a late Golgi compartment at steady state and cycles continuously via a prevacuolar endosome back to the Golgi. V-ATPase complexes containing Vph1p and Stv1p also differ in their assembly properties, coupling of proton transport to ATP hydrolysis, and dissociation in response to glucose depletion. To identify the regions of the a subunit that specify these different properties, chimeras were constructed containing the cytosolic amino-terminal domain of one isoform and the integral membrane, carboxyl-terminal domain from the other isoform. Like the Stv1p-containing complex, the V-ATPase complex containing the chimera with the amino-terminal domain of Stv1p localized to the Golgi and the complex did not dissociate in response to glucose depletion. Like the Vph1p-containing complex, the V-ATPase complex containing the chimera with the amino-terminal domain of Vph1p localized to the vacuole and the complex exhibited normal dissociation upon glucose withdrawal. Interestingly, the V-ATPase complex containing the chimera with the carboxyl-terminal domain of Vph1p exhibited a higher coupling of proton transport to ATP hydrolysis than the chimera containing the carboxyl-terminal domain of Stv1p. Our results suggest that whereas targeting and in vivo dissociation are controlled by sequences located in the amino-terminal domains of the subunit a isoforms, coupling efficiency is controlled by the carboxyl-terminal region.

The V-ATPases¹ are a family of ATP-dependent proton pumps responsible for acidification of intracellular compartments in eukaryotic cells (1–8). Acidification of these compartments is crucial for such processes as receptor-mediated endocytosis, intracellular trafficking, the processing and degradation of macromolecules, and the coupled transport of small molecules. In addition, V-ATPases in the plasma membrane of specialized cells function in such processes as pH homeostasis (9), bone resorption (10), renal acidification (11), potassium transport (12), and tumor metastasis (13). In yeast, the V-ATPase functions to create the driving force for uptake of small molecules and ions into the vacuole (14) and is important for post-Golgi protein trafficking (15–17).

The V-ATPase complex is composed of the following two domains: a soluble V₁ domain responsible for ATP hydrolysis and an integral V₀ domain responsible for proton translocation (1–8). The V₁ domain is a 500-kDa complex composed of eight different subunits (subunits A–H) of molecular masses 70 to 14 kDa, whereas the V₀ domain is a 250-kDa complex containing five different subunits (subunits a, d, c, c’, and c”) of molecular masses 100 to 16 kDa (1–8). The V-ATPases thus resemble in domain structure the F-ATPases (or ATP synthases) that normally function in ATP synthesis (18–23). An evolutionary relationship between these classes has been established by the sequence homology that exists between several subunits in these ATPases (24–26).

The 100-kDa “a” subunit of the V₀ domain has a bipartite structure containing a hydrophilic amino-terminal domain of ~50 kDa and a hydrophobic carboxyl-terminal domain containing multiple transmembrane helices (27–29). Topological studies employing cysteine mutagenesis and chemical labeling have led to a model for the a subunit containing nine transmembrane helices, with the amino-terminal domain on the cytoplasmic side of the membrane and the carboxyl terminus on the luminal side of the membrane (30), although data suggesting a cytoplasmic orientation of the carboxyl terminus have also been reported (31). Mutagenesis studies have identified several buried charged residues in the last two transmembrane helices that appear to play some role in proton transport (32, 33), suggesting that the 100-kDa subunit functions analogously to subunit a of the F-ATPases. It is the interaction between the...
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F-ATPase a subunit and the ring of proteinophilic c subunits that is thought to facilitate proton movement through the F₀ domain (19, 34, 35).

In yeast the a subunit is encoded by two genes (VPH1 and STV1), which show ~50% identity at the amino acid level (28, 29). Vph1p has been shown to associate with V-ATPases targeted to the vacuole, whereas Stv1p is present in V-ATPase complexes localized to some other intracellular compartment (29). The identity of the signal responsible for this differential localization is not known, but the role of the a subunit in targeting is supported by data from mammalian cells. Thus, in vivo dissociation of the V₁ domain is controlled by the carboxyl-terminal domain. The amino-terminal domain of one isoform and the carboxyl-terminal domain of the other. Our results suggest that in vivo dissociation of the V₁ domain is supported by data from mammalian cells. Thus, in vivo dissociation of the V₁ domain is controlled by the carboxyl-terminal domain.

**ExPERIMENTAL PROCEDURES**

**Materials**—Zymolyase 100T was obtained from Seikagaku America (Ijamsville, MD). Concanamycin A was purchased from Fluka Chemical Corp. (Milwaukee, WI). Protease inhibitors were from Roche Molecular Biochemicals. The monoclonal antibodies against the HA epitope, 3F10 and 12C6, were also from Roche Molecular Biochemicals. Monoclonal antibodies against ALP (1D3-A10), Pep12p (24D11-B2), and the Alexa (A594)-conjugated goat anti-rabbit antibody were from Molecular Probes (Eugene, OR). The affinity-purified polyclonal antibody against the HA epitope has been described previously (17). The purified anti-HA monoclonal antibody used for immunofluorescence (HA.11) was purchased from Covance Research Products (Richmond, CA). Biotin-conjugated goat anti-mouse and streptavidin-conjugated fluorescein isothiocyanate (FITC) antibodies were obtained from Jackson ImmunoResearch (West Grove, PA). *Escherichia coli*Life Technologies Inc., or Promega (Madison, WI). Oligonucleotides were synthesized by Keystone Laboratories (Camarillo, CA). ATP, phenylmethylsulfonyl fluoride, and most other chemicals were purchased from Sigma.

**Plasmid Construction**—The plasmids used in this study are shown in Table I. pDJ48 was generated by the insertion of a triple HA epitope tag into the 2-α vps27 region from pFA6-kanMX2 containing the kanamycin resistance (pSKN34). pSKN11 was generated by recombinant polymerase chain reaction (PCR) using pSKN13 and pSKN14, Vph1p and STV1 ORF. This was ligated with a 1-kilobase pair filled in using Vent DNA polymerase, removing base pairs 163–2341 of the STV1 ORF. This was ligated with a 1-kilobase pair *SacI-EcoRI* fragment from pFA6-kanMX2 containing the kanaycin resistance gene, which had been filled in as above (43), generating pKEB11. Plasmid pSKN11 (STV1-2xHA in pRS316) and pSKN12 (VPH1-2xHA in pRS316) were used to generate constructs expressing Vph1p under the control of the STV1 promoter (pSKN13), Vph1p and Stv1p chimeras (pSKN23, pSKN24, and the Stv1p-Δ165–208 deletion (pSKN34). Plasmid pSKN13 was generated by recombinant polymerase chain reaction using pSKN11 or pSKN12 as a template. pSKN23 and pSKN24 were generated by swapping the region encoding the amino-terminal and carboxyl-terminal domains of each gene at a unique restriction enzyme site, *MluI*, which was generated by site-directed mutagenesis at the position of *Leu⁴³⁶* in *STV1-2xHA* and at *Leu⁴⁰⁶* of *VPH1-2xHA*. The plasmids pSKN14 and pSKN25 were generated by digestion of pSKN11 and pSKN23 with *XbaI* and *BamHI* followed by subcloning of the insert into the 2-α plasmid YEps32. pSKN34 was generated by deletion of the region corresponding to Pro¹⁵⁵—Pro²⁰⁶ of Vph1p.

**Yeast Strains**—Yeast strains used in this study are shown in Table I. Strains were constructed by standard genetic techniques and grown in 50% identity at the amino acid level (28, 29). Vph1p has been shown to associate with V-ATPases targeted to the vacuole, whereas Stv1p is present in V-ATPase complexes localized to some other intracellular compartment (29). The identity of the signal responsible for this differential localization is not known, but the role of the a subunit in targeting is supported by data from mammalian cells. Thus, in vivo dissociation of the V₁ domain is controlled by the carboxyl-terminal domain. The amino-terminal domain of one isoform and the carboxyl-terminal domain of the other. Our results suggest that in vivo dissociation of the V₁ domain is controlled by signals residing within the amino-terminal domain. In contrast, the coupling of ATP hydrolysis to proton translocation is controlled by the carboxyl-terminal domain.

**TABLE I**

| Strain      | Genotype                                      | Ref.   |
|-------------|-----------------------------------------------|--------|
| SF838-1D    | MATα ura3-52 leu2-3,112 his4-519 ade6 gal2 pep4-3 STV1-3XHA | 66     |
| KEBY2       | MATα ura3-52 leu2-3,112 his4-519 ade6 gal2 pep4-3 stv1Δ Kan¹ | This study |
| KEBY4       | MATα ura3-52 leu2-3,112 his4-519 ade6 gal2 pep4-3 stv1Δ Kan¹ | This study |
| KEBY9       | MATα ura3-52 leu2-3,112 his4-519 ade6 gal2 pep4-3 vph1Δ LEU2 stv1Δ Kan¹ | This study |
| SF838-9D    | MATα ura3-52 leu2-3,112 his4-519 ade6 gal2 pep4-3 vph1Δ | 67     |
| NB72        | MATα ura3-52 leu2-3,112 his4-519 ade6 gal2 pep4-3 pho8ΔX | 68     |
| KEBY44      | MATα ura3-52 leu2-3,112 his4-519 ade6 gal2 pep4-3 pho8ΔX STV1-3XHA | This study |
| KEBY45      | MATα ura3-52 leu2-3,112 his4-519 ade6 gal2 pep4-3 pho8ΔX ups27Δ LEU2 STV1-3XHA | This study |

| Plasmid     | Description                                      | Ref.     |
|-------------|--------------------------------------------------|----------|
| pKEB11      | stv1Δ Kan¹ in pBluescript II KS⁺                  | This study |
| pBJ6714     | vph1ΔLEU2 in pBluescript II KS⁺                  | 28       |
| pMM1        | STV1 in pBluescript II KS⁺                       | 29       |
| pDJ74       | Integrating plasmid for STV1-3XHA               | This study |
| pDJ48       | STV1-3XHA in pBluescript II KS⁺                  | This study |
| pSN55       | CEN-URA3 plasmid encoding A-ALP                 | 50       |
| pSKN11      | CEN-URA3 plasmid encoding VPH1-2xHA             | 39       |
| pSKN12      | CEN-URA3 plasmid encoding VPH1-2xHA under the STV1 promoter | This study |
| pSKN13      | 2-α URA3 plasmid encoding STV1-2xHA             | 39       |
| pSKN23      | CEN-URA3 plasmid encoding STV1-2xHA-VPH1 chimera | This study |
| pSKN24      | CEN-URA3 plasmid encoding VPH1-2xHA-VPH1 chimera | This study |
| pSKN25      | 2-α URA3 plasmid encoding STV1-2xHA-VPH1 chimera | This study |
| pSKN34      | CEN-URA3 plasmid encoding STV1-2xHA Δ165–208    | This study |
Fig. 1. Stv1p-HA colocalizes with the late Golgi marker A-ALP but shows little overlap with the PVC marker Pep12p. KEBY4 cells (poh8Δ STV1::HA) were transformed with the A-ALP plasmid pSN55. Immunofluorescence was performed as described under “Experimental Procedures,” and cells were permeabilized with 5% SDS for 5 min (upper panels) or 2% SDS for 1 min (lower panels). Stv1p-HA was visualized using a rabbit polyclonal antibody against the HA epitope followed by a secondary Alexa anti-rabbit antibody. The same cells were also stained for A-ALP or Pep12p using monoclonal antibodies against ALP and Pep12p, respectively, followed by anti-mouse biotin and streptavidin-PTC. Confocal micrographs were taken simultaneously of the red and green fluorescence channels, with an 8% bleed through correction, and overlapped to produce the merged image.

Fig. 2. Stv1p-HA colocalizes with A-ALP and Pep12p in the large, aberrant endosome compartment generated in vps27Δ cells. KEBY45 cells (poh8Δ vps27Δ STV1::HA) were transformed with the A-ALP plasmid pSN55 and contained for Stv1p-HA and A-ALP (upper panels) and Stv1p-HA and Pep12p (lower panels). Immunofluorescence was performed as described in the legend to Fig. 1 and under the “Experimental Procedures.” The large white arrows indicate the class E compartment. The small white arrows represent examples of Stv1p-HA and A-ALP colocalization outside the class E compartment (see text for details).

used at a dilution of 1/200 and the anti-HA monoclonal antibody (HA.11) at 1/500. Monoclonal antibodies against ALP and Pep12p were used at dilutions of 1/3 for anti-ALP tissue culture supernatant and 1/1000 for purified anti-Pep12p. Images were generated using a Bio-Rad MRC 1024 confocal microscope.

Analysis of 100-kDa a Subunit Expression and V-ATPase Assembly—Yeast cells transformed with pSKN11 (Stv1p-HA), pSKN13 (Vph1p-HA), pSKN25 (Stv1p-Vph1p-HA), or pSKN24 (Vph1-Stv1p-HA) were grown to log phase at 30°C in selective medium; whole cell lysates were prepared as described previously (39), and the proteins were separated by SDS-PAGE on 8% acrylamide gels. The expression of the 100-kDa subunits was detected by Western blotting using the horseradish peroxidase-conjugated monoclonal antibody 3F10 against HA. To evaluate assembly of the V-ATPase, the complex was detergent-solubilized from whole cell lysates and immunoprecipitated using the monoclonal antibody 8B1-F3 against subunit A of the V1 domain, as described previously (39). Following separation of proteins by SDS-PAGE on 8% acrylamide gels, Western blotting was performed using antibodies against both subunit A and the HA epitope. Blots were developed using a chemiluminescence detection method obtained from Kirkegaard & Perry Laboratories (Gaithersburg, MD). The assembly competence of the chimeric proteins was reflected by their ability to be immunoprecipitated using the anti-A subunit antibody.

Isolation of Vacular Membrane Vesicles—Vacular membrane vesicles were isolated as described previously (39). Yeast transformed with pSKN13 (Vph1p-HA), pSKN14 (Stv1p-HA), pSKN24 (Vph1-Stv1p-HA), or pSKN25 (Stv1-Vph1p-HA) were grown overnight at 30°C to 1 × 10^7 cells/ml in 1 liter of selective medium. Cells were pelleted, washed once with water, and resuspended in 10 mM dithiothreitol and 100 mM Tris-HCl, pH 9.4. After incubation at 30°C for 15 min, cells were pelleted and washed once with YEPD medium containing 0.7 M sorbitol, 2 mM dithiothreitol, and 100 mM Mes-Tris, pH 7.5, and 50 µg/ml of Zymolase 100T and incubated at 30°C with gentle shaking for 60 min. The resulting spheroplasts were osmotically lysed, and the vacuoles were isolated by flotation on two consecutive Ficoll gradients. Protein concentrations were measured by the BCA protein assay (Pierce).

Detection of V-ATPase Subunits Present on Isolated Vacular Membranes—Vacular membranes isolated from each of the mutants were subjected to SDS-PAGE, and Western blots were probed with the mouse monoclonal antibodies 3F10 against HA, 8B1-F3 against the A subunit (Vma1p), and 13D11-B2 against the B subunit (Vma2p). Blots were also probed with rabbit polyclonal antibodies against Vma6p, Vma7p, Vma8p, Vma10p, and Vma13p, as well as Vma4p (a generous gift of Dr. Daniel J. Klionsky), and a mouse monoclonal antibody against Vma5p (a generous gift of Dr. Patricia M. Kane). Following removal of unbound...
primary antibodies by washing, blots were incubated with horseradish peroxidase-conjugated secondary antibodies (Bio-Rad) and developed using chemiluminescence as above.

FIG. 3. Schematic illustration of HA-tagged Vph1p, Stv1p, the chimeric proteins, and the Stv1p deletion construct. Sequences derived from Vph1p and Stv1p are indicated as shaded boxes and open boxes, respectively. Positions for the insertion of HA epitope tags, the junction points employed in construction of the chimeras, and the site of deletion (residues 165–208) in Stv1p are indicated by the residue numbers in the original Vph1p or Stv1p sequence.

RESULTS

Stv1p Is Localized at Steady State to the Late Golgi Compartment—The two isoforms of the yeast 100-kDa subunit of the V-ATPase are localized to distinct intracellular compartments. A previous report (29) showed that although Vph1p is localized to the vacuolar membrane at steady state, Stv1p is localized to punctate structures that are non-vacuolar. We sought to identify the intracellular compartment to which Stv1p is targeted by colocalization with known marker proteins. We constructed a version of STVI with a triple HA epitope tag sequence in the same position as described previously (29). STVI-HA was then integrated into the yeast genome, thus creating a strain (KEBY44) in which the sole genomic copy of STVI is epitope-tagged. This tagged version of Stv1p is fully functional as determined by complementation of growth of a vph1Δ strain (39).

In Vivo Dissociation of the V-ATPase in Response to Glucose Deprivation—Dissociation of the V-ATPase in response to glucose depletion was measured as described previously (39). The vph1Δ stv1Δ strain KEBY9 expressing Vph1p-HA, Stv1p-HA, Vph1-Stv1p-HA, or Stv1-Vph1p-HA from the single copy plasmid pRS316 was grown in selective medium overnight to an absorbance at 600 nm of <0.1. The cells were converted to spheroplasts by treatment with Zymolase 100T and incubated in YEP media with or without 2% glucose for 40 min at 30 °C. Spheroplasts were pelleted and lysed in phosphate-buffered saline containing 1% C12E9, protease inhibitors, and 1 mM dithiothreitol(succinimidyl propionate). An aliquot (corresponding to ~6 × 10⁶ cells) was removed to allow analysis of proteins present in the whole cell lysate. The V-ATPase complexes were immunoprecipitated from the remainder of the lysate (corresponding to ~3 × 10⁹ cells) using SB1-F3 against the A subunit and protein G-agarose followed by separation on 8% acrylamide gels and transfer to nitrocellulose. Western blotting was then performed separately using the horseradish peroxidase-conjugated monoclonal antibodies 3F10 against HA to detect the V0 domain or antibody SB1-F3 against the A subunit and protein G-agarose followed by separation on 8% acrylamide gels and transfer to nitrocellulose. Western blotting was then performed using chemiluminescence as above.

FIG. 4. Expression levels of HA-tagged versions of Vph1p, Stv1p, and the chimeric proteins determined by Western blot analysis. a, KEBY9 cells (vph1Δ stv1Δ) were transformed with empty pRS316 plasmid (vector), with pRS316 expressing an HA-tagged version of Vph1p from the VPH1 promoter, or with plasmids expressing HA-tagged versions Vph1p or Stv1p from the STVI promoter. Whole cell lysates were prepared, and proteins were separated by SDS-PAGE on 8% acrylamide gels. The lysate from 5 × 10⁶ cells was loaded in each lane. Western blotting was then performed using the anti-HA antibody as described under "Experimental Procedures." b, KEBY9 cells (vph1Δ stv1Δ) were transformed with empty pRS316 plasmid (vector) or with pRS316 expressing HA-tagged versions of Vph1p, Stv1p, or Vph1-Stv1p, all from the STVI promoter. Whole cell lysates were prepared, and proteins were analyzed as described in a.
meric with the luminal and transmembrane domains of ALP fused to the cytoplasmic domain of dipeptidyl aminopeptidase A (DPAP A, also known as Ste13p). This protein can be detected using a monoclonal antibody raised against the luminal domain of ALP and has been shown to reside in the late Golgi at steady state (49, 50). As shown in Fig. 1, Stv1p-HA showed significant overlap with A-ALP by indirect immunofluorescence visualized with confocal microscopy. Nearly all the structures stained for Stv1p-HA also stained for A-ALP, structures staining for both proteins (Fig. 1, prevacuolar compartment (PVC, 52, 53), shows weak colocalization with Stv1p-HA, with only a small percentage of structures staining for both proteins (Fig. 1, lower panels). These data suggest that Stv1p is predominantly localized to the late Golgi compartment at steady state.

Many yeast late Golgi membrane proteins such as DPAP A, Kex2p (proteases that processes late Golgi compartment at steady state. structures staining for both proteins (Fig. 1, the prevacuolar compartment (PVC, 52, 53), shows weak colocalization with Stv1p-HA, with only a small percentage of structures staining for both proteins (Fig. 1, lower panels). These data suggest that Stv1p is predominantly localized to the late Golgi compartment at steady state.

Many yeast late Golgi membrane proteins such as DPAP A, Kex2p (proteases that processes a-factor), and Vps10p/Pep1p (the receptor for carboxypeptidase Y) cycle via the PVC and are retrieved from this compartment back to the late Golgi (49, 50, 54–58). Thus, these proteins are constantly cycling between the late Golgi and PVC, with the majority concentrated in the late Golgi in a wild type cell. To determine whether Stv1p also cycles via the PVC, we deleted VPS27 in the KEBY44 cells used in the previous experiment. VPS27 is a class E VPS gene and is thus required for the exit of proteins from the PVC (46, 59). Cells lacking Vps27p display a distinct morphological phenotype, with a large aberrant PVC (the “class E compartment”) typically adjacent to the vacuole. Late Golgi proteins that cycle continuously via the PVC, such as Vps10p, Kex2p, and A-ALP as well as the PVC marker Pep12p and proteins en route to the vacuole such as Vph1p, are concentrated in the class E compartment in vps27Δ cells (17, 59). As shown in Fig. 2, Stv1p-HA colocalized with both A-ALP and Pep12p in the class E compartment in vps27Δ cells (large arrows), suggesting that it does cycle via the PVC and back to the late Golgi. Similar to A-ALP, but in contrast to Pep12p, Stv1p-HA also exhibited additional punctate staining in the vps27Δ cells, which is similar to the pattern seen for Stv1p-HA in wild type cells (Fig. 1). This result is consistent with some proportion of Stv1p-HA remaining in a late Golgi compartment. The population of Stv1p-HA that does not accumulate in the class E compartment partially overlaps with A-ALP (small arrows, Fig. 2). Our results show that Stv1p-HA is predominantly localized to the late Golgi compartment but cycles via the PVC and back to the late Golgi in a manner similar to many other late Golgi membrane proteins.

Construction and Expression of Vph1p/Stv1p Chimeras— V-ATPase complexes containing Vph1p and Stv1p differ both in intracellular localization (see above and Ref. 29) and in other properties, including activity, coupling efficiency, and in vivo dissociation in response to glucose depletion (39). To determine which regions of the a subunit are responsible for these differences, chimeras were constructed containing the hydrophilic amino-terminal domain of one isoform and the hydrophobic carboxyl-terminal domain of the other isoform. Fig. 3 shows a schematic illustration of the HA-tagged Vph1p, Stv1p, the chimeric proteins, and a deletion construct of Stv1p in which amino acid residues 165–208 were removed. Chimeras Vph1p-Stv1p-HA contained residues 1–409 of Vph1p-HA and residues 456–890 of Stv1p, whereas chimera Stv1p-Vph1p-HA contained residues 1–455 of Stv1p-HA and residues 410–840 of Vph1p (the difference in residue number for the junction point between Vph1p and Stv1p is due to a 46-residue insertion in the amino-terminal domain of Stv1p). The Vph1p-Stv1p-HA chimera contained a tandem HA tag after residue Asn185, whereas the Stv1p-Vph1p-HA chimera contained a tandem HA tag after residue Leu227. These HA tags, located in the amino-terminal domain, were shown previously not to interfere with the stability or assembly of Vph1p and Stv1p, respectively (39). Both chimeras were expressed using the STV1 promoter, thus ensuring more uniform levels of expression of the chimeric proteins.

Fig. 4 shows the expression levels of the Vph1p-Stv1p-HA and Stv1p-Vph1p-HA chimeras, Vph1p-HA and Stv1p-HA using the STV1 promoter, and Stv1p-HA using the VPH1 promoter. As can be seen, both chimeras were expressed at levels comparable with Vph1p-HA driven from the STV1 promoter (Fig. 4b), which in turn was lower than either Vph1p-HA or Stv1p-HA driven from their own promoters (Fig. 4a). The lower level of Vph1p-HA expressed from the STV1 promoter than from the VPH1 promoter likely reflects the higher transcriptional efficiency of the latter promoter (29). The fact that Stv1p-HA is nevertheless expressed at nearly the same steady state level as Vph1p-HA, when each is driven from its own promoter (Fig. 4a), may be due to a greater stability of Stv1p-HA relative to Vph1p-HA. The presence of two bands differing by 5–6 kDa for the Stv1p-Vph1p-HA chimera (Fig. 4b) may reflect a partial proteolysis of this chimera. Nevertheless, each chimeric gene (STV1-VPH1-HA and VPH1-STV1-HA) is capable of complementing the growth defect of the vph1Δ stv1Δ strain, indicating that the V-ATPase complexes containing these chimeric proteins possess a substantial level of activity.

The Amino Termini of Vph1p and Stv1p Contain the Information Necessary for Their Intracellular Localization—To investigate the localization of Vph1p-HA expressed from the STV1 promoter, and the Stv1p-Vph1p-HA and Vph1p-Stv1p-HA chimeras, we performed immunofluorescence using an anti-HA antibody. As shown in Fig. 5, Vph1p-HA expressed under the control of the STV1 promoter was properly localized to the vacuole membrane. This shows that the differential localization of Vph1p-HA and Stv1p-HA was not due to differences in the expression level of the two proteins but rather was likely due to distinct targeting information within the proteins themselves. As shown with Stv1p-HA expressed from integrated STV1::HA (Fig. 1), Stv1p-HA expressed from the CEN plasmid showed typical Golgi staining (Fig. 5). However, a small amount of Stv1p-HA was seen on the vacuole membrane in cells expressing Stv1p-HA from the low copy, centromere-based plasmid. This is consistent with previous results showing that overexpression of Stv1p-HA causes some vacuolar localization (29, 39), probably due to saturation of the Golgi retention or retrieval machinery.

As shown in Fig. 5, the chimera with the amino terminus of Vph1p-HA and the transmembrane carboxyl-terminal region of Stv1p (Vph1p-Stv1p-HA) was localized to the vacuole membrane.

It should be noted that the Vph1p-Stv1p chimera migrates faster on SDS-PAGE than Vph1p (Fig. 4), despite the fact that they contain (including the HA epitope tags) 862 and 858 amino acid residues, respectively (Fig. 3). This aberrant migration appears to be due to differences in amino acid composition of the amino- and carboxyl-terminal domains of Vph1p and Stv1p such that the chimeras do not migrate as expected based upon the number of amino acid residues. This can also be seen by comparing the migration of Stv1p and the Stv1p-Vph1p chimera in Figs. 6–8. Thus, although Stv1p and the Stv1p-Vph1p chimera have 908 and 904 amino acid residues, respectively, and would therefore be predicted to comigrate on SDS-PAGE, the Stv1p-Vph1p chimera migrates more slowly than Stv1p. It thus appears as though the amino-terminal domain of Vph1p causes the chimera to migrate faster than expected, whereas the amino-terminal domain of Stv1p causes the chimera to migrate more slowly, although it is also possible that the observed differences are due to the carboxyl-terminal domains.

3 K. Bowers and T. H. Stevens, unpublished results.
of subunit a immunoprecipitated using the antibody against subunit A. As can be seen from Fig. 7, the Vph1-Stv1p-HA chimera showed somewhat lower assembly than the Stv1-Vph1p-HA chimera, which in turn showed almost the same level of assembly as Stv1p-HA itself. These data indicate that both chimeric forms of the a subunit are competent to assemble with the other subunits of the V-ATPase complex.

As a further measure of the assembly competence of the chimeric proteins, vacuoles were isolated from the strains expressing each of the a subunit constructs, and Western blotting was performed using antibodies against subunits A–H of the V₁ domain as well as subunits a and d of the V₅ domain. It has been shown previously that disruption of assembly of the V-ATPase complex results in the disappearance from the vacuolar membrane of multiple V-ATPase subunits (60). To ensure the presence of Stv1p-HA and the Stv1-Vph1p-HA chimera in the vacuolar membrane, these two proteins were overexpressed using the 2-μm plasmid YEp352 as described previously (39). As can be seen in Fig. 8, the chimeras show reduced assembly relative to the native 100-kDa subunits, with the Vph1-Stv1p-HA chimera showing lower assembly than the Stv1-Vph1p-HA chimera. Stv1p-HA also showed reduced assembly relative to Vph1p-HA, consistent with our previous observations (39). Although vacuoles from cells expressing the Stv1-Vph1p-HA chimera showed lower levels of V₁ subunits than vacuoles from cells expressing Stv1p-HA, this does not appear to reflect reduced assembly of the chimera because the chimeric protein itself is also present at reduced levels in the vacuole. It is also notable that subunit B (Vma2p) association with the vacuole appears to be less affected by the switch in 100-kDa subunits than other V₁ subunits. Nevertheless, the data in Fig. 8 confirm that both chimeric forms of the a subunit are competent to assemble with the remaining V-ATPase subunits, although assembly is reduced relative to the native a subunit isoforms.

Vph1p/Stv1p Chimeras Differ in the Coupling Efficiency of Proton Transport and ATP Hydrolysis—We have shown previously that V-ATPase complexes containing Stv1p-HA have a 4–5-fold lower ratio of coupling of proton transport to ATP hydrolysis than complexes containing Vph1p-HA (39). Table II shows proton transport (as measured by ACMA quenching) and concanamycin-sensitive ATPase activities measured for vacuoles isolated from strains expressing Vph1p-HA or the Vph1-Stv1p-HA chimera in pRS316 or Stv1p-HA or the Stv1-Vph1p-HA chimera in YEp352. As can be seen, vacuoles isolated from the strain expressing the Stv1-Vph1p-HA chimera are more active for both proton transport and ATPase activity than vacuoles isolated from the strain expressing the Vph1-Stv1p-HA chimera, consistent with the lower degree of assembly observed for the Vph1-Stv1p-HA chimera (Figs. 7 and 8). Comparison of the ratio of proton transport to ATP hydrolysis (Table II, 3rd column) reveals that the 3–4-fold difference in coupling ratio between Vph1p-HA and Stv1p-HA is also detected in comparison with the Stv1-Vph1p-HA and Vph1-Stv1p-HA chimeras. Thus, the tighter coupling of Vph1p-HA relative to Stv1p-HA appears to be due to differences in the carboxyl-terminal domain. It should be noted that because of the low proton transport activity of complexes containing the Vph1-Stv1p-HA chimera relative to the error of these measurements, there is considerable uncertainty in the calculation of the coupling ratio. Nevertheless, even the highest ratio calculated from the extreme values of the fluorescence quenching and ATPase activities permissible from the error bars for the Vph1-Stv1p-HA chimera (0.45/0.02 = 22.5) is lower than the ratio observed for the Stv1-Vph1p-HA chimera.

The Amino-terminal Domain of the a Subunit Controls in
Vivo Dissociation of the V-ATPase in Response to Glucose Depletion—We have shown previously that V-ATPase complexes containing Vph1p-HA that are localized to the vacuole show in vivo dissociation in response to glucose depletion, whereas complexes containing Stv1p-HA that are localized to its normal compartment do not (39). Stv1p-containing complexes that have been targeted to the vacuole through overexpression, on the other hand, do show dissociation in response to glucose withdrawal. These results suggest that in vivo dissociation of the V-ATPase is primarily controlled through the membrane environment in which the V-ATPase resides. However, we have shown previously (39) that V-ATPase complexes containing Vph1p that have been blocked in transport to the vacuole by disruption of the VPS21 or the VPS27 gene still show glucose-dependent dissociation, although to a somewhat reduced degree. Thus, V-ATPase complexes present in compartments other than the central vacuole can still undergo dissociation in response to glucose withdrawal.

To begin to address whether the signals that control dissociation of the V-ATPase are distinct from those that control targeting of the complex, the in vivo dissociation behavior of complexes containing the two chimeric forms of the α subunit were compared. Spheroplasts were incubated in media with or without glucose for 40 min followed by detergent solubilization and immunoprecipitation using the anti-A subunit antibody. Western blotting was then performed using antibodies against both subunit A and the HA epitope tag. Dissociation of the V-ATPase is reflected as a decrease in the amount of subunit A immunoprecipitated using the antibody against subunit A. Consistent with our previous observations (39), V-ATPase complexes containing wild type Vph1p-HA showed dissociation in response to glucose withdrawal (Fig. 9b), whereas those containing Stv1p-HA did not (Fig. 9a). Interestingly, the Vph1p-Stv1p-HA chimera, like Vph1p-HA, also exhibited glucose-dependent dissociation, whereas the Stv1p-Vph1p-HA chimera (like Stv1p-HA), did not (Fig. 9a). Thus, the dissociation...
Vph1p, Vma1p, and Vma2p blots, 1/H9262 was employed; otherwise 10 Western blot analysis was performed using antibodies against both HA following separation of the proteins by SDS-PAGE on 8% acrylamide gels, subunit (Vma1p) as described under immunoprecipitated using the monoclonal antibody 8B1-F3 against the A subunit as described under “Experimental Procedures.” Following separation of the proteins by SDS-PAGE on 8% acrylamide gels, Western blot analysis was performed using antibodies against both HA and subunit A as described.

**FIG. 7.** Analysis of assembly competence of chimeric proteins by immunoprecipitation and Western blot. Whole cell lysates were prepared from KEBY9 (vph1Δ stv1Δ) cells expressing HA-tagged versions Vph1p, Stv1p, or the Stv1-Vph1p or Vph1-Stv1p chimeras using the plasmids pSKN13, pSKN11, pSKN23, and pSKN24, respectively. Lysates were detergent-solubilized, and V-ATPase complexes were immunoprecipitated using the monoclonal antibody 8B1-F3 against the A subunit (Vma1p) as described under “Experimental Procedures.” Following separation of the proteins by SDS-PAGE on 8% acrylamide gels, Western blot analysis was performed using antibodies against both HA and subunit A as described.

**FIG. 8.** Presence of V-ATPase subunits on vacuolar membranes isolated from strains expressing HA-tagged versions of Vph1p, Stv1p, and the chimeric proteins. Vacuolar membranes were isolated from the vph1Δ stv1Δ strain (KEBY9) transformed with HA-tagged versions of Vph1p or Vph1-Stv1p in pRS316 or Stv1p or Stv1-Vph1p in YEp332. Aliquots were separated by SDS-PAGE followed by Western blotting using the indicated subunit-specific antibody as described under “Experimental Procedures.” For Vph1p, Vma1p, and Vma2p blots, 1 μg of vacuolar membrane protein was employed; otherwise 10 μg of vacuolar membrane protein was used.

behavior of the chimeras correlates with the source of the amino-terminal domain.

**DISCUSSION**

**Targeting Information for the Yeast V-ATPase Complexes Is Contained within the Amino-terminal, Cytosolic Domains of the Subunit a Isoforms—** Differential targeting of subunit a isoforms of the V-ATPase may provide a means of localizing V-ATPase complexes to different subcellular sites. In this study, we show that the V-ATPase complex containing the 100-kDa subunit a isoform Stv1p resides in the late Golgi at steady state, where it colocalizes with the chimeric protein A-ALP (see Fig. 1). Amino acid sequence comparison has shown that Stv1p is 54% identical and 71% similar to the yeast vacuolar a subunit isoform Vph1p (29). Given this significant amount of sequence similarity, these proteins provide interesting tools for the study of targeting signals in yeast and for the analysis of different subunit a isoforms. The greatest sequence divergence is within the amino-terminal domains of the proteins, before the first putative transmembrane domain, suggesting that information for their differential targeting might be contained within this domain. In fact, constructing chimeric proteins in which the amino-terminal domains of Vph1p and Stv1p have been exchanged does indeed change the localization of these proteins (see Fig. 5) but still results in assembled and functional V-ATPase complexes (Figs. 7 and 8). This demonstrates that the localization signals are contained within the amino-terminal, cytosolic domains of the a subunits. Many cytosolic domains of membrane proteins have been shown to contain targeting signals, often short amino acid sequences required for interaction with the cytosolic sorting machinery (for review see Ref. 61). Because previous evidence (54, 62) suggests that targeting to the vacuole in yeast may require no specific signals, it seems more likely that the Stv1p isoform contains Golgi retention or retrieval motifs within its amino-terminal domain.

Although Stv1p- HA is localized to the late Golgi at steady state, it accumulates in the large, aberrant PVC of vps27Δ cells (Fig. 2). These cells have defects in the retrieval of proteins of the PVC and back to the Golgi, as well as protein transport out of the PVC to the vacuole (46, 59). In addition to proteins en

### Table II

| ATPase activity | Initial rate of fluorescent quenching | Coupling ratio |
|----------------|---------------------------------------|----------------|
| μmol ATP/min/g | ΔF/min | fluorescent quenching/ATPase activity |
| Vph1p          | 0.221 ± 0.038 | 9.54 ± 0.71 | 43.2 |
| Stv1p          | 0.082 ± 0.026 | 1.13 ± 0.29 | 13.8 |
| Stv1-Vph1p     | 0.059 ± 0.019 | 1.93 ± 0.70 | 32.6 |
| Vph1-Stv1p     | 0.024 ± 0.004 | 0.25 ± 0.20 | 10.4 |

*ATPase activities were measured on isolated vacuolar membranes (5 μg of membrane protein) prepared as described under “Experimental Procedures.” The activities were measured in the presence of 1 mM ATP and the absence or presence of 1 μM concanamycin A. The results shown represent the concanamycin-sensitive portion of the activity.

**FIG. 9.** In vivo dissociation of V-ATPase complexes in response to glucose depletion in cells expressing Vph1p, Stv1p, or the chimeric proteins. a) Spheroplasts were prepared from KEBY9 (vph1Δ stv1Δ) cells expressing HA-tagged versions of Vph1p, Stv1p, the Stv1-Vph1p chimera, or the Vph1-Stv1p chimera (all expressed from the STV1 promoter using pRS316). The spheroplasts were incubated in YEP media in the absence or presence of 2% glucose (as indicated) for 40 min followed by detergent solubilization and immunoprecipitation using the monoclonal antibody 8B1-F3 against the A subunit (Vma1p) as described under “Experimental Procedures.” Following separation of the proteins by SDS-PAGE on 8% acrylamide gels, Western blot analysis was performed using antibodies against both HA and subunit A as described. b) A shorter exposure of the gel shown in a to allow the glucose-dependent decrease in Vph1p immunoprecipitated with the anti-A subunit antibody to be more readily visualized.
Control of V-ATPase Targeting, Dissociation, and Coupling

Although both the Vph1-Stv1p-HA and Stv1p-Vph1p-HA chimeras show better assembly than does the Vph1-Stv1p-HA chimera (Fig. 8), suggesting that the steady state difference in assembly between Vph1p and Stv1p may be attributable to differences in the carboxyl-terminal domain. Similarly, complexes containing the Stv1p-Vph1p-HA chimeras show a 3-fold higher ratio of proton transport to ATP hydrolysis than complexes containing the Vph1p-Vph1p-HA chimera (Table II), suggesting that the tightness of coupling of proton transport and ATP hydrolysis is also affected by the carboxyl-terminal domain of the protein. These results are consistent with our previous identification of mutations in the carboxyl-terminal domain that affect both assembly and activity of the V-ATPase (32, 33). It should be noted, however, that because of the reduced assembly of both chimeric forms of the a subunit (Fig. 8), the observed differences in coupling efficiency may be due to the loss of one or more V\textsubscript{1} subunits from the corresponding complex.

**In Vivo Dissociation of the V-ATPase in Response to Glucose Deprivation Is Controlled by the Amino-terminal Domains of Vph1p and Stv1p**—By contrast with the assembly and coupling properties of the V-ATPases, the *in vivo* dissociation, like the targeting information, appears to reside in the amino-terminal domain of the a subunit. The Kane laboratory has shown (40) that dissociation of the V-ATPase into separate V\textsubscript{1} and V\textsubscript{0} domains represents an important mechanism for regulating the yeast V-ATPase *in vivo*, and a similar mechanism has been proposed to control V-ATPase activity in insects during molting (41). We have shown previously that whereas Vph1p-containing complexes present in their normal intracellular site (the vacuole) undergo dissociation in response to glucose depletion, Stv1p-containing complexes present in the Golgi do not (39). When Stv1p-containing complexes are forced (through overexpression of Stv1p) to localize to the vacuole, *in vivo* dissociation is now observed (39). These results suggest that the cellular localization of the V-ATPase is the primary determinant in controlling dissociation in response to glucose depletion. We would then predict that chimeras localized to the vacuole should show glucose-dependent dissociation, whereas those that localized to the Golgi would not. In fact, this prediction is supported by the observation that complexes containing the Vph1p-Stv1p-HA chimera dissociated in response to glucose depletion, whereas those containing the Stv1p-Vph1p-HA chimera did not (Fig. 9). These results also indicate that there is no signal controlling *in vivo* dissociation of the V-ATPase located in the carboxyl-terminal domain of the a subunit that overrides the targeting information located in the amino terminus.

The amino-terminal domain of the a subunit has been shown previously to interact with both subunit A and subunit H of the V\textsubscript{1} domain (64) and has been suggested to form part of the peripheral stator connecting V\textsubscript{1} and V\textsubscript{0} (64, 65). Based upon these results, it might have been predicted that the amino-terminal domain would play a more important role in controlling interactions between the V\textsubscript{1} and V\textsubscript{0} domains, including assembly, coupling, and regulation of dissociation. The fact that only the latter process appears to be dependent upon signals in the amino-terminal domain suggests that the a subunit may also make contact with the V\textsubscript{1} domain through the carboxyl-terminal region. Alternatively, conformational differences between the carboxyl-terminal regions may be transmitted to the rest of the molecule through the amino terminus of subunit a. It is interesting, in this regard, that a subunit mutations affecting assembly of the V-ATPase appear to cluster on the luminal side of the membrane (30, 32, 33), suggesting a luminal domain that may regulate assembly of the complex. With respect to the tightness of coupling of proton transport

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5 S. Kawasaki-Nishi and K. Bowers, unpublished observations.
and ATP hydrolysis, the dependence on the hydrophobic carboxyl-terminal domain is easier to understand because this coupling is likely to be critically dependent upon the interaction of the a subunit with the proteolipid subunits, as has been shown to be true for the F-ATPases (19, 34, 35). Additional work will be required to identify residues within each of the a subunit domains that directly participate in the multitude of functions served by this subunit.

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The Amino-terminal Domain of the Vacuolar Proton-translocating ATPase a Subunit Controls Targeting and \emph{in Vivo} Dissociation, and the Carboxyl-terminal Domain Affects Coupling of Proton Transport and ATP Hydrolysis
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