Molecular mechanisms of vesicle transport between the prevacuolar compartment and the vacuole in yeast or the lysosome in mammalian cells are poorly understood. To learn more about the specificity of this intercompartmental step, we have examined the subcellular localization of a SEC1 homologue, Vps33p, a protein implicated in function in transport between the prevacuolar compartment and the vacuole. Following short pulses, 80–90% of newly synthesized Vps33p cofractionated with a cytosolic enzyme marker after making permeabilized yeast cells. However, during a chase, 20–40% of Vps33p fractionated with permeabilized cell membranes in a time-dependent fashion with a half-time of ~40 min. Depletion of cellular ATP increased the association rate to a half-time of ~4 min and caused 80–90% of newly synthesized Vps33p to be associated with permeabilized cell membranes. The association of Vps33p with permeabilized cell membranes was reversible after restoring cells with glucose before permeabilization. The N-ethylmaleimide-sensitive fusion protein homologue, Sec18p, a protein with known ATP binding and hydrolysis activity, displayed the same reversible energy-dependent sedimentation characteristics as Vps33p. We determined that the photosensitive analog, 8-azido-[α-32P]ATP, could bind directly to Vps33p with low affinity. Interestingly, excess unlabeled ATP could enhance photoaffinity labeling of 8-azido-[α-32P]ATP to Vps33p, suggesting cooperative binding, which was not observed with excess GTP. Importantly, we did not detect significant photolabeling after deleting amino acid regions in Vps33p that show similarity to ATP interaction motifs. We visualized these events in living yeast cells after fusing the jellyfish green fluorescent protein (GFP) to the C terminus of full-length Vps33p. In metabolically active cells, the fully functional Vps33p-GFP fusion protein appeared to stain throughout the cytoplasm with one or two very bright fluorescent spots near the vacuole. After depleting cellular ATP, Vps33p-GFP appeared to localize with a punctate morphology, which was also reversible upon restoring cells with glucose. Overall, these data support a model where Vps33p cycles between soluble and particulate forms in an ATP-dependent manner, which may facilitate the specificity of transport vesicle docking or targeting to the yeast lysosome/vacuole.

The compartmentalized nature of eukaryotic cells demands high fidelity mechanisms to ensure maintenance of each organelle’s unique content. Organelles of the secretory and endocytic system rely on vesicle-mediated transport pathways not only to move protein cargo throughout the cell but also to preserve their specific protein and lipid compositions.

The SNARE hypothesis describes fundamental molecular mechanisms that give rise to the specificity of vesicle-mediated protein transport in eukaryotic cells (1). The basic tenet of this notion is that interactions between integral membrane proteins in transport vesicles (v-SNAREs) and in their cognate target organelles (t-SNAREs) direct the specificity of all intercompartmental events (2). Although substantial evidence exists to support the existence of many v- and t-SNARE proteins, precisely how they function in vesicle transport specificity is continually being revised as more details are uncovered for different systems (3, 4). However, a fact remaining clear and central to the SNARE hypothesis is that multimeric interactions of v- and t-SNAREs with cytosolic proteins play an essential role in the precision of vesicle-mediated transport (5, 6).

The robust genetics of the yeast, *Saccharomyces cerevisiae,* has identified four genes whose cytosolic protein products are believed to help specify vesicle docking and/or fusion. Fittingly, one of these, SEC1, not only was the first secretory gene identified through mutant isolation (7) but was also the namesake of this family of proteins that are ubiquitously expressed in eukaryotic organisms (for reviews see Refs. 8–10). The Sec1 group of proteins in yeast consists of Sec1p (11), Vps45p (12, 13), Sly1p (14), and Vps33p (15, 16).

The VPS33 gene was identified in a selection for yeast mutants with defects in vacuolar protein sorting (17). Loss of VPS33 function results in three prominent phenotypes; 1) temperature-sensitive growth (restrictive temperature is 38 °C), 2) severe missorting of both soluble and membrane vacuolar proteins, and 3) abnormal vacuole morphology. Although the vacuole in vps33Δ mutants is very fragmented, it is not as severe as the other class C vps mutants, end1/vps11, vps18, and vps16 (18). The VPS33 gene product, Vps33p, is 691 amino acids long, mostly hydrophilic, and cytoplasmically localized. The primary sequence of Vps33p contains two regions that are similar to the type A and B (also called Walker A and B) nucleotide consensus sequences (15), suggesting that it may bind or hydrolyze ATP, which has never been established.

In this study, we have examined ATP binding in the possible function of Vps33p. Our data indicate that Vps33p can indeed bind ATP and that deletion of a region that includes the type B consensus pattern results in both the loss of ATP binding and a nonfunctional protein. Further, Vps33p reversibly changed its localization from the cytosol to a particulate fraction when ATP was depleted in yeast cells, which was also observed for the NSF1 homologue, Sec18p.
MATERIALS AND METHODS

Media and Strains

Yeast strains were maintained on YPD medium (1% yeast extract, 2% peptone, 2% dextrose, and 2.5% bactoagar). Liquid medium for radiolabeling and plasmid maintenance was Wickerham’s minimal pro- line (19) medium supplemented with 0.5% yeast extract.

Strains

The yeast strains used in this study include TVY3350, LBY317 (15) CFY-Inc-LEU2 (leu2−3,112::BHY11; BGY3300, MAT a ura3−52 leu2−1,112 his3−200 trp1−423, 3.7-kilobase pair fragment from pYcP-KX2 (25) in pJWY33–39. The resulting plasmids, pBG416-GFP-SK, pBG416-GFP-KS (cen/ARS), pBG426-GFP-SK, and pBG426-GFP-KS (2u origin) as vector, pGY33–39; the second uses pRS426 (2u origin) as vector, pBG33–126. The plasmid, pPRP33–100, is a pBluescript KS vector pH/pPS416 (2u origin) as vector, pBG33–116. To delete 57 nucleotides encoding amino acids 423–429 of VPS33, an oligonucleotide (5'-CTATCATATAATGGATC-3') was used to loop out the T3 RNA polymerase promoter.

For the radiolabeling and Immunoprecipitations and Permeabilized Cell Fractionation

The preparation and radiolabeling of yeast spheroplasts with Tran35S-label (ICN Radiochemicals, Costa Mesa, CA) used standard methods previously described (26). The preparation of permeabilized yeast spheroplasts was also via a standardized protocol previously described (27). Immunoprecipitations for Vps33p were all treated with IgGsorb (The Enzyme Center, Waltham, MA) for 20 min before the addition of antiserum (15). Extracts were rocked overnight at 4°C and then treated with protein A-Sepharose (Amersham Pharmacia Biotech). The immunoprecipitation was washed twice with two 1-ml portions of 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, and 0.5% Tween 20 (Tween 20 buffer), two 1-ml portions of Tween 20 buffer containing 2 mM urea, and two 1-ml portions of 50 mM Tris-HCl pH 7.5, 150 mM NaCl, and 1 mM EDTA. After drying, the immunoprecipitates were boiled for 4 min in standard Laemmli sample buffer (2X concentration), and SDS-PAGE was performed as described (28). Most gels were dried and subjected to autoradiography using Kodak BioMax film. In some cases, the gels were subjected to phosphor imaging analysis using a STORM PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Fluorescence Microscopy

Cells expressing various GFP fusion constructs were grown in minimal proline medium with 0.5% yeast extract to middle or late log phase. They were concentrated to ~20 A600 units/ml and examined in 6-μm medium. For energy depletion experiments, the cells were washed two or three times in minimal proline medium without glucose and then resuspended in the same medium containing 10 mM NaN3 and 10 mM NaF for periods up to 2 h. To reverse the effects of energy depletion, the cells were washed two or three times in minimal proline medium containing glucose and then incubated for 60–90 min at 30°C. All micrographs were digitally captured on a Zeiss Axioskop epifluorescence microscope using an AxioCam MRC (Zeiss) camera or with Adobe Premiere software (Adobe Systems, Mountain View, CA), a TARGA 2000 video board (Truevision, Inc., Santa Clara, CA), and a PowerPC Macintosh 9500 (Apple Computer, Cupertino, CA). All images were edited with Adobe Photoshop software (Adobe Systems).

Photoaffinity Labeling with 8-Azido-ATP

Fluorescence Microscopy—Cells—Yeast strain TVY3350 containing pBG33–126 (wild-type VPS33), pPRP33–100, or pCT3–202 (VPS33 with a 19-amino acid deletion of the type B-like region) were grown in minimal proline medium to exponential phase. The cells (25–50 A600 units) were harvested and converted to spheroplasts. The spheroplasts were resuspended in YPD plus 1.0 m sorbitol for 60 min at 30°C, harvested again, and washed twice at 4°C in 1.0 m sorbitol, 20 mM HEPES-KOH, pH 7.0, 5.0 mM potassium acetate, and 5 mM magnesium acetate containing sodium azide and sodium fluoride (10 mM each). The washed cells were resuspended in 10 mM Tris, pH 7.6, 5 mM MgCl2, 0.1 mM KPO4, 0.1 mM NaF, 0.1 mM Na3PO4, (lys buffer) by adding 15 μlA600 units, and a protease inhibitor mixture was added, which included α-macroglobulin (125 μl/mg), 1-chloro-3-rosylamido-7-amino-2-phenylamino- (40 μl/mg), N-tert butyloxycarbonyl-L-lysine (1 μl/mg), trypsin inhibitor (50 μg/ml), phosphoramidyl-ylsufonyl fluoride (0.1 mM), and pepstatin A (0.7 μg/ml). The spheroplasts were agitated twice with 0.5-mm glass beads in a bead beater (BioSpec Products, Bartlesville, OK) at 1-min intervals. The lysate was subjected to centrifugation at 4,000 × g for 10 min. The pellet fraction was rinsed with lysine buffer, combined with the first supernatant, and desalted using Bio-Gel P-6DG (Bio-Rad). The desalted extract was subjected to centrifugation at 125,000 × g for 15 min. The pellet fraction was resuspended in lysine buffer at 10–25 mg/ml. All extracts were stored at −70°C.

Photoaffinity Reactions—Reactions were typically 12.5–25 μl in total volume and consisted of ~125 μg of membrane extract, 20–40 μl 8-azido-(α-32P)ATP (ICN Radiochemicals, Costa Mesa, CA, or RPI Corp., Mt. Prospect, IL). All reactions were performed in a 96-well microtiter dish. In general, the final concentrations were 50 mM Tris·HCl pH 7.6, 50 mM MgCl2, 100 μl of reaction was performed for 2–10 min prior to UV irradiation. The microtiter dish was placed ~2 cm below a hand-held 254-nm UV lamp (UVV, Upland, CA, model UVGL-25), and the reactions were irradiated once for 1 min, followed by a 1-min interval of no irradiation and then a second irradiation for 1 min. In some experiments, the filter was removed from the UV light source to increase the radiation. The reactions were stopped by adding

G6PDH, glucose-6-phosphate dehydrogenase; CPY, carboxypeptidase Y; NSF, N-ethylmaleimide-sensitive fusion protein; PAGE, polyacrylamide gel electrophoresis.
an equal volume of 4% SDS and 4 mM dithiothreitol. The samples were transferred to a 1.5-ml tube, and the wells were rinsed with 2% SDS and 2 mM dithiothreitol and pooled with the first portion. The samples were heated (65–100 °C) for 2–5 min. The reactions were then immunoprecipitated for Vps33p, subjected to SDS-PAGE, and subjected to autoradiography or PhosphorImager analysis. Ten percent of every reaction was not immunoprecipitated and was analyzed for the total proteins that were photoaffinity-labeled. Important control reactions included using creatine phosphokinase as a positive control, irradiating the 8-azido-[α-32P]ATP before the addition of the yeast extract, and omitting the irradiation step. Photoaffinity labeling of creatine kinase with 8-azido-[α-32P]ATP was consistently competed greater than 80% with 2 mM ATP. We never observed photoaffinity labeling of Vps33p when the 8-azido-[α-32P]ATP was photolyzed before the addition of extracts, indicating that secondary reactants were not produced. Similarly, we never observed labeling of Vps33p when we did not photolabile the 8-azido-[α-32P]ATP, indicating that the azido group on carbon 8 of the adenine ring was the only reactive moiety. For competition experiments, unlabeled nucleotides were generally added 2–10 min before the addition of the 8-azido-[α-32P]ATP. When augmenting the reactions with unlabeled 8-azido-ATP, a mixture of the cold and radioactive nucleotides was made, and the two were added together.

RESULTS

Vps33p Fractionates with Permeabilized Yeast Cell Membranes—When the VPS33 gene was first cloned, its protein product, Vps33p, was demonstrated to be a soluble cytoplasmic protein through subcellular fractionation (15). When examining the biochemical function of Vps33p, we used an in vitro reconstitution assay that measures intercompartmental transport of procarboxypeptidase Y to the yeast vacuole in permeabilized cells (27). The vps33-4 allele has a single L646P change and retains partial function for vacuolar protein sorting (15, 29, 30). Spheroplasts from a vps33-4 mutant strain were able to mature 35–40% of carboxypeptidase Y (CPY) in vivo, while the majority was secreted as the Golgi-modified p2 precursor (data not shown). However, even with this capacity for CPY maturation, vps33-4-permeabilized cell membranes were unable to transport p2CPY in vitro when incubated with ATP and a cytosolic extract made from a VPS33 yeast strain (data not shown). In contrast, 40% maturation of p2CPY was achieved in VPS33-permeabilized cell membranes under these same conditions (data not shown). Significant maturation of p2CPY (32%) was still observed if VPS33-permeabilized cell membranes were incubated with a cytosolic extract made from a vps33-3 strain (data not shown). From these results, measuring protein transport to the yeast vacuole, we concluded that the functional fraction of Vps33p was associated with permeabilized cell membranes and not the cytosol. Thus, we examined the fractionation of Vps33p more carefully in wild-type permeabilized cells to determine if any of the protein was associated with a particulate fraction.

After a 5-min pulse with Tran35S-label, ~80% of Vps33p cofractionated with the cytosolic marker protein, glucose-6-phosphate dehydrogenase (G6PDH, Fig. 1A lanes 1 and 2). However, during a 60-min chase, Vps33p fractionated away from the cytosolic supernatant fraction and was progressively recovered more in the permeabilized cell membrane pellet fraction (Fig. 1A, lanes 2–14). Although this separation into a supernatant and pellet was subjected to just 13,000 × g, the Vps33p found in the supernatant continued to remain soluble after sedimentation at >125,000 × g, suggesting that it was indeed soluble. Thus, at steady state, as much as 40% of Vps33p was associated with permeabilized cell membranes in excess of the cytosolic marker G6PDH in this experiment. Subsequent analyses indicated that a more consistent value for the particulate fraction of Vps33p at steady state was 15–20% (see Figs. 2 and 6). The apparent half-time for the association of Vps33p with permeabilized cell membranes was 25–30 min (Fig. 1A).

![Fig. 1. Newly synthesized Vps33p becomes insoluble in a time- and energy-dependent manner.](http://www.jbc.org/)

Loss of Cellular Energy Increases the Rate and Amount of Vps33p Insolubility—We suspected that cellular energy played a role in the association/disassociation of Vps33p with
Depletion of intracellular ATP with Na\textsubscript{3}N\textsubscript{3} and NaF had a pronounced affect on the fractionation behavior of Vps33p. After a 15-min pulse with Tran\textsuperscript{35}S-label, \(-85\%\) of the newly synthesized Vps33p cofractionated with the cytosol (Fig. 1B, lanes 1 and 2) as observed previously (Fig. 1A, lanes 1 and 2). However, if Na\textsubscript{3}N\textsubscript{3} and NaF (10 mM each) were added during the chase, Vps33p rapidly and completely fractionated with permeabilized cell membranes (Fig. 1B, lanes 3–12). The apparent half-time for this association was 3–4 min, which was 8–9-fold faster than the rate observed in the absence of energy poisons \textit{in vivo} (Fig. 1B). The effect of energy depletion was most likely specific to properties associated with Vps33p, because the cytosolic enzyme, G6PDH, remained soluble throughout the course of Na\textsubscript{3}N\textsubscript{3}/NaF treatment. If energy poisons were withheld from the chase, nearly 90\% of the newly synthesized Vps33p remained soluble and cofractionated with G6PDH (Fig. 1B, lanes 13 and 14).

We tested whether the action of Na\textsubscript{3}N\textsubscript{3} and NaF was reversible and extended this analysis to two other proteins, Sec18p and Vps45p. The NSF homolog, Sec18p, was examined because it exists in soluble and particulate forms (34) with putative ATP binding activity like mammalian NSF (35, 36). The Sec1 homolog, Vps45p, also exists in soluble and particulate forms (12) and is 22\% identical to Vps33p (8). However, unlike NSF/Sec18p, Vps45p shows no sequence similarity to proteins that bind or hydrolyze ATP (12, 13). If spheroplasts were fractionated after pulse/chase radiolabeling in glucose medium, \(>80\%\) of Vps33p and Sec18p and \(\leqslant 55\%\) of Vps45p cofractionated with the cytosolic marker G6PDH after 30 min of chase (Fig. 2A, lanes 1 and 2). In contrast, treatment with Na\textsubscript{3}N\textsubscript{3} and NaF for 30 min caused \(>85\%\) of both Vps33p and Sec18p to fractionate with permeabilized cell membranes, but the amount of Vps45p that was membrane-associated did not increase significantly (Fig. 2A, lanes 3 and 4). After the cells were washed several times in medium to remove the Na\textsubscript{3}N\textsubscript{3} and NaF, replenished with fresh glucose, and incubated for another 30 min, \(>90\%\) of Vps33p and \(>70\%\) of Sec18p fractionated again with the cytosol (Fig. 2A, lanes 5 and 6). This suggested that the effects of energy depletion were reversible. Although not shown, the fractionation behavior of Vps33p was the same when cycloheximide (100 \(\mu g/ml\)) was added to the cells during energy regeneration (after Na\textsubscript{3}N\textsubscript{3} and NaF treatment), indicating that new protein synthesis was not required to displace the membrane-associated pool of Vps33p.

We also examined the ability of cells to reverse intercompartmental protein transport to the vacuole after energy depletion. After a 15-min pulse with \([\textsuperscript{35}S]\text{Met/Cys}, \sim 18\%\) of CPY was present as the mature vacuolar form, while the remaining \(\sim 85\%\) represented the p1 and p2 precursor forms (Fig. 2B). Nearly complete maturation of CPY occurred in the presence of glucose but was completely blocked when Na\textsubscript{3}N\textsubscript{3} and NaF were added during a 30-min chase period (Fig. 2B). If the energy poisons were washed free of the cells, \(\geqslant 95\%\) maturation of CPY took place during a further 30-min chase in glucose (Fig. 2B). Overall, these results suggest that yeast spheroplasts can withstand the deleterious effects of extreme energy depletion, which argued that the insolubility of Vps33p and Sec18p were not due to irreversible toxicity.

\textbf{Vps33p Aggregates after Energy Depletion but Can Be Dissociated with ATP and Cytosol—}To examine in more detail the biochemical nature of the Vps33p particulate association, various treatments were used to solubilize the protein. After 30 min in Na\textsubscript{3}N\textsubscript{3} and NaF, permeabilized cell membranes were obtained from yeast spheroplasts and treated with KCl (1 M), Triton X-100 (1\%), urea (2 M), and sodium carbonate, pH 11 (0.1 M). Only sodium carbonate was effective at releasing a signifi-
ATP (1 mM plus a regeneration mix) was included in the incubation, ~7% of Vps33p was detected in the supernatant after sedimentation of the membranes (Fig. 3B, lanes 3 and 4). Incubation with a cytosolic protein extract (5 mg/ml) released only ~3% of the particulate Vps33p (Fig. 3B, lanes 5 and 6). However, adding back both ATP and a cytosolic protein extract resulted in the release of 22% of the bound Vps33p (Fig. 3B, lanes 7 and 8). This was a 3-fold increase in dissociation over incubation with ATP alone and a 7-fold increase over incubation with cytosol alone. Moreover, solubilization of Vps33p with ATP and cytosol was much more efficient than treatment with high salt, a nonionic detergent, or a denaturing agent (Fig. 3A). This suggests that activity of a cytosolic protein factor(s) was required to solubilize Vps33p from permeabilized cell membranes in an ATP-dependent fashion.

We further examined the characteristics of the insoluble Vps33p fraction with differential and sucrose gradient centrifugation before and after energy depletion. Permeabilized cell membranes were extracted under iso-osmotic conditions in a low ionic strength buffer (0.8 M sorbitol, 10 mM triethanolamine-acetic acid, pH 7.8, and 1 mM EDTA), which dissociates the membranes into crude organelles (26). The relative distribution of Vps33p during differential centrifugation after extraction of permeabilized cell membranes was very similar between energy-rich and energy-depleted cells (Fig. 4A). Both energy conditions showed ~30% of Vps33p in the low speed P2 pellet and ~10% in the high speed P3 pellet (Fig. 4A, lanes 4 and 6 and lanes 10 and 12). However, a significant difference was observed when these pellet fractions were applied to the top of a sucrose gradient and subjected to equilibrium centrifugation. In pellets from cells labeled in medium free of energy poisons, Vps33p settled to a major peak at fraction 8 (~33% sucrose) and a minor peak at fraction 15 (~46% sucrose, Fig. 4B). In contrast, Vps33p shifted predominantly to the bottom of the gradient (~47% sucrose) in pellets from cells labeled in medium with NaN3 and NaF (Fig. 4B). Along with the inability to solubilize Vps33p with detergent (Fig. 3A), these results suggest that energy deprivation caused aggregation of the protein to an insoluble form.

Vps33p Can Be Labeled with Photoactive ATP—Several possibilities could explain the effect of energy starvation and rapid loss of Vps33p localization from the cytosol to a particulate fraction. One prospect could be that Vps33p is a reversible substrate for a modifying enzyme like a protein kinase or phosphatase. The lack of ATP in the cell may trap the phosphorylation status of Vps33p. A high speed membrane fraction was made from energy-rich and energy-depleted cells (Fig. 4A). Both energy conditions showed ~30% of Vps33p in the low speed P2 pellet and ~10% in the high speed P3 pellet (Fig. 4A, lanes 4 and 6 and lanes 10 and 12). However, a significant difference was observed when these pellet fractions were applied to the top of a sucrose gradient and subjected to equilibrium centrifugation. In pellets from cells labeled in medium free of energy poisons, Vps33p settled to a major peak at fraction 8 (~33% sucrose) and a minor peak at fraction 15 (~46% sucrose, Fig. 4B). In contrast, Vps33p shifted predominantly to the bottom of the gradient (~47% sucrose) in pellets from cells labeled in medium with NaN3 and NaF (Fig. 4B). Along with the inability to solubilize Vps33p with detergent (Fig. 3A), these results suggest that energy deprivation caused aggregation of the protein to an insoluble form.

Covalent cross-linking to a photosensitive ATP analog, 8-azido-ATP was examined to test the simplest model for localization of Vps33p. A high speed membrane fraction was made from a yeast strain deleted for the chromosomal copy of VPS33 but expressing the wild-type gene from a multicopy plasmid (pBG33–126). To demonstrate specificity of the 8-azido-ATP ligand, we examined all of the proteins in the extract. Over a dozen abundant, well resolved polypeptides existed in the high speed pellet fraction (Fig. 5A). However, only three bands were detected after irradiation with 8-azido-[α-32P]ATP, indicating that this ligand reacted with a subset of the many proteins present (Fig. 5A). After immunoprecipitation of extracts for
Vps33p, a radiolabeled band was detectable on SDS-PAGE (Fig. 5C, lane 2). This band was not observed if the vps33Δ strain was transformed with a vector control (pRS426), confirming its identity as Vps33p (Fig. 5C, lane 1). Although not shown here, this band was also not detected if UV irradiation was withheld from the wild-type extracts, demonstrating that photoactivation was required for affinity labeling of Vps33p. As a specific positive control in our experiments, we used creatine kinase, since it is a known ATP-binding protein and has been successfully photolabeled with 8-azido-ATP (37). As expected, creatine kinase, but not CPY, was photolabeled with 8-azido-ATP (Fig. 5B).

Two important criteria in affinity labeling with photoaffinity nucleotides are to demonstrate competitive binding and saturable binding. Surprisingly, ATP did not appear to compete the binding, but rather it enhanced the ability to photoaffinity-label Vps33p with 8-azido-ATP. An approximately 7- and 20-fold increase was observed with 1 and 5 mM unlabeled ATP, respectively (Fig. 5C, lanes 3 and 5). When the same reactions were performed with creatine kinase, >90% inhibition of photofinity labeling was observed (data not shown). This apparent enhancement of photoaffinity labeling was specific to ATP, since 1 or 5 mM GTP had no effect on radiolabeling of Vps33p (Fig. 5C, lanes 4 and 6).

The stimulation of photoaffinity labeling of Vps33p with 8-azido-[α-32P]ATP by unlabeled ATP most likely reflected either an improvement in cross-linking efficiency or cooperative binding. One approach to improve photocross-linking efficiency is to increase the concentration of the azido nucleotide. For example, supplementing photoaffinity reactions with unlabeled 8-azido-ATP enhances cross-linking to oligoadenylate synthetase (39). A proportional increase in photoaffinity labeling of Vps33p with 8-azido-[α-32P]ATP was observed in the presence of unlabeled 8-azido-ATP (Fig. 5D). This increase in binding was nearly linear between 0.1 and 1 mM 8-azido-ATP and appeared to show half-maximal saturation at ~0.5 mM (Fig. 5D). This suggested that Vps33p exhibited a relatively low affinity for ATP binding under these conditions. Importantly, if the extract was first illuminated with UV light in the presence of 2.0 mM 8-azido-ATP followed by a second photoreaction with 8-azido-[α-32P]ATP, the labeling of Vps33p was decreased nearly 4-fold (Fig. 5D, lane 6). This suggested that low affinity ATP binding of Vps33p could be saturated. Creatine kinase did not show this behavior and was significantly inhibited with 1 mM unlabeled 8-azido-ATP (Fig. 5B). Creatine kinase displays half-maximal saturation of 8-azido ATP binding at 12 μM (37), ~40-fold higher affinity than what we observed in Vps33p (Fig. 5D). With this apparent affinity difference in mind, photolabeling of Vps33p was competed >90% with ATP (1 mM) when we performed the experiment in the presence of 1 mM 8-azido-ATP (Fig. 5E, lane 2). Using ADP as a competitor was ineffective until the concentration reached 10 mM (Fig. 5E, lane 9). This suggests that Vps33p has a greater affinity for ATP than ADP.

**Deleting the Putative Nucleotide Hydrolysis Region from Vps33p Abolishes ATP Binding, Energy-dependent Cytosol Distribution, and Vacuolar Protein Sorting**—We examined if the regions of Vps33p that show similarity to proteins that bind or hydrolyze ATP were involved in photoaffinity labeling with 8-azido-ATP. Using site-directed mutagenesis to change conserved amino acid residues within these motifs was avoided because Vps33p does not strictly conform to the known consensus sequence motifs. Moreover, changing aspartate 678 to asparagine (D678N) did not show defective phenotypes (data not shown). Therefore, we deleted 19 amino acids, residues 661–679, because this encompasses all of the region that shows strong similarity to proteins with demonstrated ATP binding or hydrolysis activity like NSF/Sec18p (36), Vps4p (40), Escherichia coli SecA (41), adenylate kinase (42), Hsp70 (43), and Hsp90 (44). A Vps33p mutant lacking these 19 amino acids showed an 18-fold decrease in binding to 8-azido-ATP, which was unchanged in the presence of excess ATP (Fig. 6A, lanes 3 and 4). This suggested that these 19 amino acids were directly or indirectly critical for forming an ATP binding site or were important in maintaining the stability of Vps33p (see below). Removing the 19-amino acid putative ATP binding region in Vps33p also abolished the ability of the protein to function in vacuolar protein sorting. Maturation of carboxypeptidase Y was not detectable even after a 90-min chase (Fig. 6B).

We further examined the characteristics of Vps33p deleted for amino acids 661–679 in more detail to determine its stability and behavior during energy depletion. After a 15-min pulse with Tran35S-label, greater than 75% of the mutant protein was recovered in the cytosolic supernatant fraction of permeabilized cells, which was very similar to wild-type Vps33p (Fig. 7A, lanes 1 and 2). However, after a 30- and 90-min chase, the majority of the deletion mutant protein was recovered in the
membrane pellet fraction of permeabilized cells, whereas wild-type Vps33p maintained its cytosolic distribution (Fig. 7, C and D, lanes 3 and 4 and lanes 7 and 8). Moreover, the amount of mutant Vps33p in the pellet after 90 min of chase in glucose (80%) was very similar to the amount of wild-type Vps33p in the pellet after 30 min of energy depletion (83%; compare Fig. 7A, lanes 7 and 8 to Fig. 7B, lanes 5 and 6). This observation indicates that the 19-amino acid region of Vps33p, that shows strong similarity to ATP binding proteins and ATPases, was required for maintaining its predominantly cytosolic distribution. Additionally, this region may play a role in efficient localization of Vps33p to a membrane during rapid loss of cellular ATP. After a 30-min treatment with NaN₃ and NaF, twice as much of the deletion mutant protein was recovered in the cytosolic supernatant fraction of permeabilized cells compared with wild-type Vps33p (Fig. 7, A and B, lanes 5 and 6). Another observation from this analysis was that overexpression of Vps33p did not significantly change its distribution in the cytosol or membrane fractions. Using a multicopy plasmid with the endogenous VPS33 promoter gave 10–15-fold greater levels of Vps33p in cells. Even after energy depletion, the ratio of cytosolic to membrane-associated Vps33p with increased pro-
tein levels was the same as when the protein was examined at chromosomal levels, which is estimated to be as low as 0.005% of total cellular protein (15). Overall, these analyses suggested that deletion of the putative ATP hydrolysis region in Vps33p abolished ATP binding and intercompartmental transport to the vacuole and impaired cytosolic localization.

**VPS33p Is Localized to Punctate Structures after Energy Depletion in Vivo**—To examine the intracellular location of Vps33p, fusion proteins were constructed with the jellyfish green fluorescent protein. This approach was chosen over immunofluorescence because it allowed for a dynamic assessment of intracellular localization in living cells. Three GFP fusion constructs were made to the VPS33 gene all under control of the VPS33 promoter. One was made to the promoter region alone with no coding sequence (VPS33p
\textsuperscript{r}-GFP), a second was made to the first 423 amino acids (Vps33p
\textsuperscript{423}-GFP), and a third was made to the full length protein (Vps33p
\textsuperscript{691}-GFP). Both hybrid proteins had GFP at their C termini. Importantly, the Vps33p
\textsuperscript{691}-GFP construct complemented all phenotypes associated with loss of VPS33 function, including growth at 38 °C, proper sorting of vacuolar proteins, and normal vacuole morphology. The truncated fusion, Vps33p
\textsuperscript{423}-GFP, did not complement any of these phenotypes when expressed in a yeast strain deleted for the entire VPS33 coding region. This suggested that a functionally important polypeptide tract of Vps33p was in the C-terminal third of the protein between amino acids 423 and 691.

**Fig. 6.** Deletion of a putative ATP hydrolysis sequence from Vps33p abolishes photoaffinity labeling with 8-azido-ATP and vacuolar protein sorting. A, a yeast strain with a vps33 null allele (BGY3300) was transformed with multicycpl plasmids encoding the complete VPS33 gene (WT) or a 19-amino acid deletion amino acids 661–679 (pCT202, designated 202). The cells were lysed by vigorous agitation with glass beads, and a 125,000 \( \times \) g pellet was obtained. Approximately 125 \( \mu \)g of protein was incubated on ice with \( \text{-40} \) \( \mu \)M 8-azido-\([\alpha\text{-}^{32}\text{P}]\)ATP with and without 10 mM unlabeled ATP. The samples were irradiated (1 min) twice with UV light, immunoprecipitated for Vps33p, and subjected to SDS-PAGE and PhosphorImager analysis. Vps33p\textsuperscript{*} designates mobility of the 19-amino acid deletion mutant (202), which migrated faster on SDS-PAGE, indicative of an \(-2\)-kDa loss in mass. B, before fractionating the spheroplasts from the 90-min time points in A (WT) and B (202), they were harvested via centrifugation for 5 min, and the extracellular medium (E) and cell pellet (I) fractions were immunoprecipitated for the vacuolar protein CPY.

**Fig. 7.** Deletion of a putative ATP hydrolysis sequence from Vps33p abolishes energy-dependent cytosol localization. In both A (wild-type Vps33p) and B (Vps33p deletion mutant), the cells were converted to spheroplasts, pulse-labeled with Tran\textsuperscript{35}S-label for 15 min, and chased for the indicated time periods with unlabeled Met/Cys. For the 30-min time points, the cells were transferred to fresh glucose (2%) or NaN\(_3\) and NaF (10 mM each). For the 90-min time point, the cells were maintained in the original chase medium. At each time point, the cells were treated under conditions that permeabilize the plasma membrane (freeze/thaw and four hypotonic washes) to generate a cytosolic fraction (S) and permeabilized cell pellet (P). Each fraction was immunoprecipitated for Vps33p and the cytosolic marker G6PDH and subjected to SDS-PAGE and autoradiography. Vps33p\textsuperscript{*} designates mobility of the 19-amino acid deletion mutant (202), which migrated faster on SDS-PAGE, indicative of an \(-2\)-kDa loss in mass.

The Vps33p
\textsuperscript{691}-GFP fusion protein was localized as a haze throughout the cytoplasm of cells in glucose medium (Fig. 8, Vps33p
\textsuperscript{691}-GFP panel). This pattern was consistent with the fractionation experiments described earlier (cf. Figs. 1 and 2). In addition to the cytoplasmic haze, the Vps33p
\textsuperscript{691}-GFP fusion...
protein was also localized to one or two bright spots per cell in glucose, which were usually near the vacuole (Fig. 8, Vps33p691-GFP panel). Significantly, these perivacuolar spots were not observed in cells expressing the truncated Vps33p423-GFP fusion protein or GFP alone (Fig. 8, Vps33p423-GFP and VPS33p-r-GFP panels). The exclusive cytoplasmic localization of these two control constructs argued that the C-terminal third of Vps33p might contain information that caused the protein to associate with a specific subcellular organelle or structural complex. Furthermore, quantitation of the fluorescence in several cells with image analysis software (NIH Image) revealed that the cytoplasmic haze accounted for ~80–
85% of the total signal. The remaining 15–20% of the signal comprised the one or two fluorescent spots. These values correlate well with the fractionation data of Vps33p, where 80–85% of the protein was cytosolic and 15–20% was particulate under steady state conditions.

Energy depletion of cells with NaN₃ and NaF treatment changed the localization pattern of Vps33p691-GFP. The fluorescence was no longer a predominant cytoplasmic haze but instead consisted of 10–20 punctate dots scattered throughout the cytoplasm (Fig. 8). This pattern was again compatible with the fractionation data where Vps33p was predominantly particulate after energy depletion (Figs. 1 and 2). Cells expressing the control constructs, Vps33p423-GFP and VPS33pΔ7–19-GFP, did not show these numerous fluorescent spots during energy depletion and maintained their fluorescent haze in the cytoplasm (Fig. 8). Importantly, this behavior was completely reversible after washing away the NaN₃/NaF and supplying the cells with fresh glucose. The punctate dots were no longer apparent, and the fluorescence was again predominantly cytoplasmic with the one or two perivacuolar spots per cell (Fig. 8).

We further examined the nature of the many tiny spots observed after energy depletion with the Vps33p691-GFP fusion protein. One possibility for their identity was the Golgi complex, since this organelle often has punctate morphology when viewed with fluorescence microscopy in yeast (45). We constructed a GFP fusion protein to the cytoplasmic C terminus of Kex2p, a marker for the late Golgi complex in yeast. In glucose medium, the Kex2p-GFP protein presented a pattern very reminiscent of immunofluorescence studies (46) with 5–10 punctate structures throughout the cytoplasm (Fig. 8). After energy depletion, the morphology of the Kex2p-GFP protein was distinct from that observed for Vps33p691-GFP. Rather than dispersing into numerous spots, the Kex2p-GFP appeared to cluster in one or two larger structures per cell (Fig. 8). This suggests that the identity of the small spots seen with the Vps33p691-GFP protein may not be the Golgi complex. Furthermore, the localization of an alkaline phosphatase-GFP (Pho8p-GFP) fusion protein (kindly provided by Greg Odorizzi and Scott Emr), a vacuole marker, suggested that the identities of energy-depleted Vps33p691-GFP spots were most likely not related to the vacuole. After energy depletion, the vacuoles in general did not fragment into smaller structures but instead enlarged and took on a swollen appearance, a common morphology when cells are starved for nutrients (33).

**Discussion**

In these studies, we have presented data to provide evidence for a new biochemical function of a previously identified gene product. Our work establishes Vps33p as a low affinity ATP binding protein that shows energy-dependent cytosolic localization. This conclusion rests on several different types of experimental data. Depleting cellular ATP results in a rapid change of Vps33p from a cytosolic protein to one that fractionates as a particulate protein. Restoring cellular energy with glucose reverses this effect. In glucose medium, a full-length Vps33p-GFP fusion protein was distributed throughout the cytoplasm. After energy depletion, the GFP fluorescence was localized to punctate structures, which returned to the cytoplasm with fresh glucose. The particulate form of Vps33p after energy depletion required both ATP and a cytosolic extract for efficient release to a soluble form in vitro. The ATP analog, 8-azido-ATP, could bind directly to Vps33p in a saturable manner. Deletion of a 19-amino acid region in Vps33p, similar to many ATP binding proteins and ATPases, prevented photoaffinity labeling with 8-azido-ATP. The deletion mutant protein was also particulate even under energy rich conditions and did not have the ability to support vacuolar delivery of CPY. Overall, the data suggest a model where Vps33p may function in vesicle traffic by cycling between soluble and particulate forms dependent on ATP binding or hydrolysis.

The first clue that Vps33p may interact with ATP was its sequence similarity to proteins that bind or hydrolyze this nucleotide (15). The most common and easily identified sequences are the type A (putative triphosphate-binding), and type B (adenine-binding), consensus regions (47, 48). Frequent renditions of the type A and B sequences are (AG)ₓXXXGK(T/S) and (H/R/K)ₓ₅₋₈HyXHyHyHy(H/D/E) (where Hy is hydrophobic), respectively (49). Although Vps33p does not precisely match the type A sequence (15), the putative type B sequence in Vps33p is 668KKGINKRFIIAD678 and matches the consensus pattern more closely except for an alanine residue. The 19-amino acid deletion (residues 661–679) encompassing the type B-like sequence, prevented Vps33p from efficiently binding to 8-azido-ATP and resulted in a nonfunctional protein. This suggests that ATP binding may be necessary for Vps33p function. Furthermore, the deletion of the type B region causes Vps33p to aggregate in ATP-rich conditions, arguing that ATP binding or hydrolysis is required for solubility. Reconstituting the release of Vps33p from membranes with ATP and cytosol in vitro supports this conclusion and suggests that other protein factors contribute to cytosolic localization.

The binding characteristics of Vps33p indicate that it has a low affinity for ATP under our experimental conditions. From the data, we estimate that half-maximal saturation occurs at ~0.5 μM, which is well within the physiological range of 2–5 μM (50). With this low affinity, the binding site would most likely not be constitutively occupied, and this affinity suggests that binding to ATP may serve a regulatory role in Vps33p function. Multimeric interactions with other proteins or itself may give rise to a conformation of Vps33p with higher affinity for ATP. In support of this possibility, photoaffinity labeling of Vps33p was significantly more efficient in particulate extracts compared with cytosolic extracts. These binding properties of Vps33p imply that it does not interact with ATP in the same manner as proteins like the AAA-type ATPases. For instance, Cdc48p (51), NSF/Sec18p (34, 52), and Vps4p (40) all have 50–60 amino acids between the conserved lysine and aspartate residues of their type A and B consensus sequences. The 183-residue stretch separating lysine 494 and aspartate 678 in Vps33p is considerably longer, suggesting a different structure for the functional nucleotide binding fold. Although deletion of 19 residues encompassing the type A-like sequence also reduced binding of 8-azido-ATP to Vps33p, the resulting protein had nearly wild-type function.

The ATP-specific enhancement in photoaffinity labeling of Vps33p with 8-azido-ATP insinuates a component of positive cooperativity in its nucleotide binding. This is best illustrated with the data demonstrating a 96% decrease in 8-azido-ATP with 1 mM ATP, a slight increase in 8-azido-ATP binding with 2.5 mM and 5.0 mM ATP, and an 80% decrease with 10 mM ATP (Fig. 5E). Although one possibility for this property would be differences in binding affinity between ATP and 8-azido-ATP, another explanation is positive cooperativity. For example, the activity of cAMP phosphodiesterase is stimulated over 10-fold with 1–10 μM cGMP but inhibited nearly 10-fold with 100–1000 μM cGMP (53). Since we are measuring only nucleotide binding and not enzymatic activity in our assays, a cooperative interaction is difficult to establish. At present, we do not know if Vps33p is an ATPase, and this issue is best reconciled with purified protein. The ATP binding characteristics of Vps33p are

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very atypical, especially if viewed from the standpoint of a single protein or nucleotide binding site. The extracts that we used for ATP binding were very crude and most likely contain other proteins that could interact with Vps33p. Positive cooperativity in ATP binding may happen if a second site on another protein needed to be saturated or occupied causing a conformational change in Vps33p to form its ATP binding site. This type of cooperative binding occurs in the ribosome activase complex (54, 55), CTP synthetase (56), and E. coli GroEL-GroES chaperone complex (57, 58). This idea gives rise to an intriguing possibility that the type B-like sequence in Vps33p interacts with another protein in such a way as to form an ATP binding site. The heteromultimeric formation of a specific nucleotide binding pocket would provide unique specificity to a vesicle docking or transport reaction.

Although the precise molecular details of ATP binding to Vps33p are unknown, the reliance on sequence specificity conventions or “consensus” sequences should not be overestimated in establishing these features. The case of Hsp90 is an excellent example where it was concluded not to bind ATP after using ATP-agarose chromatography, 8-azido-ATP photoaffinity labeling, fluorescence nucleotide analogs, and sequence analyses (49). However, the recent structure of an Hsp90 N-terminal domain co-crystallized with ATP and/or ADP refutes these biochemical results (44). Moreover, the first 220 amino acids of Hsp90 used for the crystallization do not contain a type A or B consensus sequence. Likewise, synapsins I and II were recently shown to bind ATP, and both of these proteins also lack the typical consensus sequences (59). This indicates that many different ATP binding folds exist and that primary amino acid sequence data are often over- or misinterpreted (60). The interaction of Vps33p with ATP does not conform to characteristics of a conventional nucleotide binding site. Molecular details of the interaction may be resolved when putative protein partners of Vps33p are identified.

The energy-dependent localization of Vps33p most likely reflects an ATP-driven conformational change in the protein. Such alterations in protein structure are well documented in oligomeric proteins that bind or hydrolyze ATP such as heat shock chaperones (61) and the N-ethylmaleimide sensitive fusion protein, NSF (36, 62). The oligomeric nature of Vps33p is unknown. The fractionation behavior of both Vps33p and the NSF homolog, Sec18p, but not Vps45p, most likely reflects an energy-driven cycle that continually localizes these proteins between soluble and particulate forms with homeostatic ATP concentrations.

The behavior of Vps33p in living yeast cells under ATP-rich and ATP-depleted conditions provides new observations for interpretation of its cellular location. As predicted from permeabilized cell preparations, 75–85% of Vps33p is cytosolic, with the remaining portion localizing to fluorescent spots, which appeared perivacuolar. This localization pattern is reminiscent of the prevacuolar compartment in yeast, which is aberrantly exaggerated in class E VPS mutants (63–65). However, our data with equilibrium sucrose density gradients suggest that the 15–20% of Vps33p that is particulate does not cofractionate with the prevacuolar compartment as defined by the presence of pro-CPY (26). Therefore, the perivacuolar fluorescent spots may be a novel organelle or subcellular structure. Recently, a comprehensive study has shown genetic and physical interactions among the four yeast VPS class C genes and gene products (30). The VPS18 and VPS11 gene products form a major hetero-oligomer with minor amounts of associated Vps16p and Vps33p. This complex fractionates as both a dense insoluble protein complex and as a peripheral component of the vacuole (30). The sedimentation characteristics of the Vps11p-Vps18p insoluble fraction are very similar to what we have observed with Vps33p after energy depletion. This suggests that ATP is required to dissociate Vps33p from the Vps11p-Vps18p complex.

The many Sec1 family members suggest that these proteins function at every vesicle-mediated step in eukaryotic cells (8, 10). However, the precise molecular mechanism by which Sec1-like proteins execute their role in vesicle docking/fusion is unknown. The most recent findings suggest that Sec1-like proteins play a negative regulatory role in SNARE complex interactions. The Drosophila Sec1 homologue, Rop1, can negatively regulate neurotransmitter release (66), and yeast Sly1p can prevent v-SNARE/t-SNARE interactions in endoplasmic reticulum to Golgi transport (67). On the other hand, a rat Sly1 homologue, rSly1p, can positively influence transport between the endoplasmic reticulum and Golgi (68), so it seems likely that Sec1-like proteins do not perform a common function at different vesicle-mediated steps along the secretory and endocytic pathways. From this perspective, Vps33p may display a unique activity not provided by any of the other Sec1 family members, since they are not predicted to bind or hydrolyze ATP.

Future studies on Vps33p will focus on establishing the details of ATP binding in the molecular mechanism of transport between the prevacuolar compartment and lysosome/vacuole. In particular, we will determine if Vps33p can hydrolyze ATP after purification of a recombinant protein. The possible multimeric nature of Vps33p will be resolved with gel filtration analysis and sedimentation through glycerol gradients. The recently described genetic interaction between VPS33 and the vacuolar t-SNARE, VAM3, suggests that their protein products may directly interact (29). We will test this with protein cross-linking to determine if Vam3p is associated with Vps33p after energy depletion. Examination of the full-length Vps33p-GFP fusion protein in a class E VPS mutant background stained with the vital endocytosis marker, FM 4–64, should further clarify whether or not Vps33p localizes to the prevacuolar compartment. Reconstitution of intercompartmental transport to the vacuole will be the definitive system to dissect the molecular details of ATP binding and Vps33p function. We are poised to exploit this system with new temperature-sensitive VPS33 alleles that show a rapid block in vacuolar protein transport at elevated temperatures. Biochemical complementation of vps33 mutants will aid in proving or disproving if ATP binding or hydrolysis functions to cycle Vps33p from the cytosol to a membrane or membrane complex, allowing vesicular transfer to the yeast vacuole.

Acknowledgments—We thank Xiaolan Ma and William Margolin for providing the M2 GFP mutant; Kurt Eakle, William Wickner, and Bruce Horazdovsky for antisera; Bob Fuller for plasmids; and Greg Odorizzi and Scott Emr for the alkaline phosphatase-GFP construct. We also thank Ashok Chavan for valuable advice on photoaffinity labeling with 8-azido-ATP, Sidney (Wally) Whiteheart for sharing results prior to publication, and Roger Barber for helpful discussions of ligand binding.

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The Vesicle Transport Protein Vps33p Is an ATP-binding Protein That Localizes to the Cytosol in an Energy-dependent Manner

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J. Biol. Chem. 1998, 273:15818-15829.
doi: 10.1074/jbc.273.25.15818

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