Vps9p Is a Guanine Nucleotide Exchange Factor Involved in Vesicle-mediated Vacuolar Protein Transport*

(Received for publication, December 15, 1998, and in revised form, March 20, 1999)

Hiroko Hama‡, Gregory G. Tall, and Bruce F. Horazdovsky§

From the Department of Biochemistry, University of Texas Southwestern Medical Center, Dallas, Texas 75235-9038

Vacuolar protein sorting (vps) mutants of Saccharomyces cerevisiae missort and secrete vacuolar hydrolases. The gene affected in one of these mutants, VPS21, encodes a member of the Sec4/Ypt/Rab family of small GTPases. Rab proteins play an essential role in vesicle-mediated protein transport. Using both yeast two-hybrid assays and chemical cross-linking, we have identified another VPS gene product, Vps9p, that preferentially interacts with a mutant form of Vps21p-S21N that binds GDP but not GTP. In vitro purified Vps9p was found to stimulate GDP release from Vps21p in a dose-dependent manner. Vps9p also stimulated GTP association as a result of facilitated GDP release. However, Vps9p did not stimulate guanine nucleotide exchange of GTP-bound Vps21p or GTP hydrolysis. We tested the ability of Vps9p to stimulate the intrinsic guanine nucleotide exchange activity of Rab5, which is a mammalian sequence homologue of Vps21p, and Ypt7p, which is another yeast Rab protein involved in vacuolar protein transport. Rab5, but not Ypt7p was responsive to Vps9p, which indicates that Vps9p recognizes sequence variation among Rab proteins. We conclude that Vps9p is a novel guanine nucleotide exchange factor that is specific for Vps21p/Rab5. Since there are no obvious Vps9p sequence homologues in yeast, Vps9p may also possess unique regulatory functions required for vacuolar protein transport.

Vesicle-mediated protein transport is responsible for executing many intracellular protein trafficking events (1). This process is mediated by complex machinery that is highly conserved from yeast to cells of higher eukaryotes (2, 3). Members of the Sec4/Ypt/Rab family of small GTP-binding proteins are an integral part of this conserved machinery and are thought to participate in the targeting and/or fusion of transport vesicles with the appropriate target membrane (4, 5). Although the exact function(s) of Rab proteins is unknown, vesicle targeting events have been coupled to the cycling of Rab proteins between their GTP-bound and GDP-bound states, leading to the following model (4, 5). GTP-bound Rab proteins associate with transport vesicles derived from the donor compartment. Transport vesicles with this form of the Rab protein are competent for targeting to the acceptor organelle (6). At the acceptor organelle, a GTPase-activating protein or GAP may act on the Rab to stimulate the hydrolysis of Rab bound GTP to GDP (7). The GDP-bound Rab is then recycled back to the vesicle donor membrane in a complex with GDP-dissociation inhibitor (GDI)1 (for review, see Ref. 8). Reloading Rab proteins with GTP is thought to involve two steps. In the first step, the Rab protein is dissociated from GDI by a GDI dissociation factor (GDF) (9). Once separated from GDI, the Rab protein is now accessible to the activity of a guanine nucleotide exchange factor (GEF) that facilitates the exchange of GDP for GTP. The Rab protein in its GTP-bound form is now capable of participating in another round of vesicle targeting and fusion.

An important regulatory step within the Rab cycle is at the stage of guanine nucleotide exchange. Several GEFs have been described for members of the Sec4/Ypt/Rab family of small GTP-binding proteins. Novick and colleagues (10) have demonstrated that Sec2p possesses guanine nucleotide exchange activity for Sec4p. Sec4p is involved in vesicle-mediated transport of secretory proteins from the yeast Golgi to the plasma membrane (11, 12). In addition, a GEF has been purified from rat brain that shows specificity for Rabs 3A, 3C, and 3D (13). Interestingly, primary amino acid sequence comparisons fail to show any obvious sequence similarity among the GEFs that stimulate guanine nucleotide exchange of Sec4p/Ypt/Rab GTP-binding proteins; indicating that each of these GEFs function in distinct vesicular transport pathways.

Vesicle-mediated transport plays an important role in the localization of proteins to the lysosome-like vacuole in yeast. Most vacuolar proteins follow the initial stages of the secretory pathway until they reach a late Golgi compartment. There, vacuolar proteins are actively sorted away from secretory proteins, packaged into transport vesicles and delivered to the vacuole via a prevacuolar endosome (for review, see Ref. 14). Genetic studies of the vacuolar protein sorting (vps) pathway have identified a large number of mutant yeast strains that missort and secrete vacuolar proteins (15–18). These vps mutants (vacuolar protein sorting defective) fall into over 40 complementation groups. One group of vps mutants (termed class D) (19) appears to affect a single stage in the vps pathway, the transport of proteins from the Golgi to the prevacuolar endosome (20). Many of gene products affected in the class D vps mutants have been implicated specifically in the targeting and/or fusion of Golgi-derived transport vesicles and several are members of highly conserved protein families (21–28). One of these, Vps21p, is a small GTP-binding protein of the Sec4/Ypt/Rab family. VPS21 was originally identified by complementation of the vacuolar protein missorting phenotype associated with vps21 mutant cells (25) and by its sequence similarity with mammalian Rab5 (29). A detailed mutational analysis has demonstrated that GTP-binding and membrane association are required for Vps21p function (25). In addition,
cells that lack Vps21p not only missort vacuolar hydrolases, but also accumulate 40–50-nm vesicles (25, 29), indicating a role for Vps21p in vesicle targeting and/or fusion events.

To better understand the role of the Vps21 GTP-binding protein in vesicle targeting, we undertook a study to identify modulators of Vps21p function among the gene products affected in the class D vps mutants. Using in vitro and in vivo techniques, a physical interaction was uncovered between Vps21p and Vp9p. This interaction is potentiated when a mutant form of Vps21p is used that possesses a higher affinity for GDP than GTP. In addition, we show that Vps9p is a GEF that stimulates the intrinsic guanine nucleotide exchange rate of Vps21p. The guanine nucleotide exchange activity of Vps9p is specific for Vps21p and its mammalian sequence homologue, Rab5.

**EXPERIMENTAL PROCEDURES**

**Strains, Media, and Other Reagents**—The Saccharomyces cerevisiae strains used in this study were: L40 (MATa trp1 leu2 his3 lys2::lexAop4 his3 lys2::lexAop4 trp1 [29]), CY1 (Mata leu2–3, 112 ura3–52 his3–200 trp1–901 lys2–801 suc2–124 [17]), CY1 (MATA trp1 ura3–52 his3–200 trp1–901 (27)), G1, G2, G3. Yeast strains were grown on standard medium supplemented with appropriate amino acids as required (34). Polymersases, restriction and modifying enzymes were purchased from New England Biolabs. [35S]Pro Mix, [3H]GTP, peroxidase-conjugated anti-rabbit IgG, and peroxidase-conjugated anti-mouse IgG were purchased from Amersham, Inc. XLI Blue (recA1 endA1 thi-1 hsdR1 supE44) (Stratagene), and M15 (RecA1 strr Rif lacI gal mtl F’ proAB proA10 recA1 endA1 gyrA96 thi-1 hsdR17 supE44 M helper) (33). Yeast strains were grown in LB medium containing ampicillin (50 μg/ml) and/or kanamycin (25 μg/ml) (33). Bacterial strains were grown in LB medium containing ampicillin (50 μg/ml) or and/or kanamycin (25 μg/ml) (33). Yeast strains were grown in 2% peptone, 1% yeast extract, 2% glucose (YPD) or in synthetic medium (SM) supplemented with the appropriate amino acids as required (34). Polymerases, restriction and modifying enzymes were purchased from New England Biolabs. [35S]Pro Mix, [3H]GTP, peroxidase-conjugated anti-rabbit IgG, and peroxidase-conjugated anti-mouse IgG were purchased from Amersham. [3H]GDP was from New England Life Sciences Inc. Monoclonal anti-HA antibody was obtained from Berkeley Antibody Company. Production of antiserum to Vps21p and carboxypeptidase Y has been described previously (25). Canine His-Rab5 protein was a gift from Dr. Robert Schekman.

**Plasmid Construction**—To create the Vps21p, Vps21p-S21N two-hybrid, and the Vps9p, the Vps9p and Vps1p two-hybrid prey, the coding sequences of these genes were amplified by PCR using pGBY21–5, pBHY21–11 (25), and pSG97 (29), respectively, and with low or high copy Vps21p expression plasmids (pGTY1-2 and pGTY2-2, respectively), and/or with low or high copy Vps9p expression plasmids (pGTY1-2 and pGTY2-2, respectively), or a low copy Vps21p-S21N expression plasmid (pGTY2-2). Strains were grown in appropriate SM to an OD600 of 0.8 and spheroplasts were generated, lysed, and treated with the cross-linking agent diithiothreitol (sodium chloride and histidine). β-Galactosidase filter assays were performed as described previously (30).

**Protein Cross-linking—**(GTY1 [vspsD::HIS3 vps21::NEO vps9::NEO] was transformed with low or high copy Vps9HAp expression plasmids (pGTY1-1, pGTY2-1, or pGTY2-2, respectively), and/or with low or high copy Vps21p expression plasmids (pGTY1-2 and pGTY2-2, respectively), or a low copy Vps21p-S21N expression plasmid (pGTY2-2). Strains were grown in appropriate SM to an OD600 of 0.8 and spheroplasts were generated, lysed, and treated with the cross-linking agent diithiothreitol (sodium chloride and histidine). β-Galactosidase filter assays were performed as described previously (30).

**Purification of Vps21p and Ypt7p—**Wild-type Vps21p and Vps21p-S21N were overexpressed from pGTY1 (HIS3) and pGTY2-2 carrying pGBK7 or pGADGH (37), respectively. Recombinant proteins were indented with 1 μl isopropyl-β-d-thiogalactopyranoside for 2 h at 37 °C. The cell pellets were resuspended in lysis buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 150 mM NaCl, 1 mM DTT, 150 μM phenylmethylsulfonyl fluoride), and lysed using a Bead-Buster (BioSpec Products). The lysate was cleared by sequential centrifugation and the supernatant was subjected to ammonium sulfate fractionation. Proteins that precipitated at 40–60% saturation were collected. The desalted proteins were loaded onto a DEAE-Sephalac column pre-equilibrated with buffer A (50 mM Tris-HCl, pH 7.5, 2 mM MgSO4). Proteins that eluted with buffer A containing 100 mM NaCl were precipitated by adding ammonium sulfate to 80%. Dialyzed samples (Buffer A) were loaded onto a Q anion exchange column (Bio-Rad) equilibrated with buffer A. Proteins were eluted with a linear gradient of 0 to 150 mM NaCl in buffer A. Ypt7p was purified from E. coli CW642 carrying pKYP77. Induction and purification was carried out similarly to the Vps21p procedure with minor modifications. Proteins were eluted from a DEAE-Sephalac column with buffer A containing 300 mM NaCl. The eluate was concentrated, dialyzed against buffer B (50 mM Tris-HCl, pH 7.5, 10% glycerol), and loaded onto a Q2 column. Proteins were eluted with a linear gradient of 0–300 mM NaCl in buffer A.

**Purification of His6-Vps9p—**M15 E. coli cells carrying pREP4 and pQEVP59 were grown and induced as described for the Vps1p purification. Cells were harvested, washed, and lysed (His6-Vps9p) was purified from the crude cell extract using Ni-NTA agarose as described by the manufacturer (Qiagen). (His6-Vps9p) was eluted from the Ni-NTA agarose column with buffer A. Proteins were eluted with a linear gradient of 0 to 150 mM NaCl in buffer A. Ypt7p was purified with buffer B.

**Guanine Nucleotide Binding Assay—**Wild-type or Vps21p-S21N was incubated in 50 μl of 50 mM Tris-HCl, pH 7.5, 1 mM MgSO4, 1 mM EDTA, 3 mM DTT, 50 μM [3H]GDP (2.6 × 104 cpm/μl), or [3H]GTP (6.5 × 104 cpm/μl) for 30 min at 30 °C. The reactions were stopped by adding 1 ml of ice-cold buffer C (50 mM Tris-HCl, pH 7.5, 5 mM MgSO4) and subsequently filtered through nitrocellulose membranes (0.45-μm pore). The membrane filters were washed twice with 5 ml of ice-cold buffer C and dried. The amount of radioactivity associated with the filters was determined using a liquid scintillation counter.

**Guanine Nucleotide Exchange and Displacement Assays—**Displacement was monitored by incubating preloaded [3H]GDP or [3H]GTP, Vps21p in 250 μl of 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 15 μM [3H]GDP (1.5 × 104 cpm/μl), or [3H]GTP (1.3 × 104 cpm/μl) for 30 min at 30 °C. One hundred μl of the preloaded Vps21p was mixed with an equal volume of 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 10 mM MgSO4, 4 mM GTP with or without Vps9p and incubated at 30 °C. At each time point, 25 μl of the mixture was removed and placed in 1 ml of ice-cold buffer C. Precipitation with [3H]GDP or [3H]GTP was determined as in the binding assay described above. The assay was carried out in the same manner for Ypt7p and His-Rab5. Nucleotide exchange activity was monitored by incubating Vps21p in the presence or absence of Vps9p in 200 μl of 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 10 mM MgSO4, 4 mM GTP, and [3H]GTP (100 μCi/ml). Vps21passociated [3H]GTP was determined as described above.
S21N Mutant Form of Vps21p Binds GDP but Not GTP—The activity of small GTP-binding proteins of the Ras family is highly regulated. This regulation is carried out by a growing number of factors that modulate or stabilize the guanine nucleotide associated with the appropriate GTPase. In this study, we undertook a search for factors that associate with Vps21p in its GDP-bound form and in doing so hoped to uncover the factor(s) responsible for exchanging GDP for GTP. An extensive characterization of the nucleotide binding capabilities of mutant Ras proteins (44) have identified a number of amino acid alterations that result in proteins that show great preferences for binding GDP or GTP. One of these, Ras N17, has been shown to bind GDP with a 20–40-fold higher affinity than GTP (44). The equivalent mutant in Vps21p (S21N) has been constructed and was shown to elicit defects in the vacuolar protein sorting pathway, indicating the importance of GTP binding for Vps21p function (25). To characterize the guanine nucleotide binding preferences of recombinant wild-type Vps21p and the Vps21p-S21N mutant form of the protein, these proteins were purified from E. coli as described under “Experimental Procedures” and their abilities to bind GDP and GTP were examined. The estimated purity of these proteins was 75 and 80% for the wild-type and Vps21p-S21N, respectively (Fig. 1A), and no other GTP-binding proteins were detected in these fractions by [32P]GTP blot analysis.2 Purified wild-type and S21N-Vps21p were incubated with [3H]GDP (solid bar) or 50 μM [γ-32P]GTP (hatched bar) for 30 min at 30 °C. The reaction mixture was mixed with ice-cold binding buffer and filtered through nitrocellulose membranes (0.45-μm pore) to separate proteins from unbound nucleotides. The amount of protein-bound nucleotides that remained on the nitrocellulose membranes was determined by scintillation counting.

RESULTS

Immunoprecipitation of CPY—Yeast cells were grown, labeled with [35S]methionine and cysteine and subjected to immunoprecipitation as described (25).

Vps9p Is a Guanine Nucleotide Exchange Factor

into the class D vps morphology group. This group shares a unique subset of phenotypes including, vacuolar protein sorting defects, enlarged vacuolar structures, a temperature-sensitive growth phenotype, as well as defects in mother to daughter vacuole segregation and vacuole acidification. Previous studies have shown that several of the gene products affected in these mutants likely function at the same stage in the vacuolar protein sorting pathway (20, 45) and two (Vps15p and Vps34p) physically interact (46). The yeast two-hybrid system was used to uncover potential interactions between Vps21p and gene products in other class D vps mutants. LexA gene fusions were constructed that contained wild-type or mutant S21N VPS21 coding sequences. A second set of gene fusions was constructed between the activation domain of Gal4p (Gal4AD vector) and LexA-Vps21p-S21N, pGADGH and LexA-Vps21p wild-type, and Gal4AD-Vps9p and pVJL11 (LexA vector) were streaked onto A, a YNB plate lacking tryptophan, leucine, and histidine and onto B, a YNB plate lacking tryptophan and leucine and grown for 72 h at 30 °C. C, the patches were then transferred to a nitrocellulose membranes, the transferred cells were lysed, and subjected to a colorimetric β-galactosidase assay.
expressed both the LexA-Vps21p-S21N fusion and the Gal4AD-Vps9p fusion were prototrophic for histidine. On the contrary, L40 yeast that expressed both the LexA-Vps21p wild-type fusion and the Gal4AD-Vps9p fusion, or the LexA-Vps21p-S21N or Gal4AD-Vps9p fusions alone were not prototrophic for histidine (Fig. 2A). All strains tested were able to grow on synthetic media that contained histidine (Fig. 2B).

A second reporter system was utilized to score an interaction between the LexA-Vps21p-S21N fusion and the Gal4AD-Vps9p fusion. The same five strains tested in Fig. 2, A and B, were patched onto an agar plate containing synthetic media (+ histidine). The yeast cells were transferred to nitrocellulose filters, lysed, and the presence of \( \beta \)-galactosidase was determined by an activity assay (see “Experimental Procedures”). Only cells coexpressing the LexA-Vps21p-S21N fusion and the Gal4AD-Vps9p fusion had observable \( \beta \)-galactosidase activity (patch 1, Fig. 2C). Neither the LexA-Vps21p (wild-type) fusion together with the Gal4AD-Vps9p fusion nor the LexA-Vps21p or Gal4AD-Vps9p fusions alone expressed the \( \beta \)-galactosidase reporter (patches 2–5, Fig. 2C). These results were completely consistent with those generated using the HIS3 reporter gene, indicating that the Vps9p may have a preferential binding affinity for a GDP-bound form of Vps21p.

Cross-linking studies were used to confirm the binding of Vps21p-Vps9p two-hybrid results and to examine if the two-hybrid interaction accurately represented an \emph{in vivo} phenomenon. Spheroplasts generated from cells (GTY1; vps21AΔ, vps9Δ2) expressing various combinations of Vps9HAp, Vps21p, and Vps21p-S21N from low or high copy number plasmids, were lysed and the lysates were treated with the homobifunctional cross-linking agent, dithiobis(succinimidylpropionate), or left untreated. The lysates were then subjected to immunoprecipitation with Vps21p antiserum and the immunoprecipitates were resolved by SDS-PAGE. The resolved immunoprecipitates were subjected to Western analysis, using Vps21p antiserum to detect Vps21p or HA monoclonal antibodies to detect Vps9HAp. When extracts generated from strains coexpressing Vps9HAp and Vps21p-S21N from CEN-based (low copy number) vectors were treated with cross-linking agent, two proteins with masses of approximately 64 and 65 kDa were detected in the immunoprecipitates (Fig. 3A, lane 4). These proteins correspond to two forms of Vps9HAp that have increased relative masses due to the covalent addition of cross-linker molecules. Importantly, Vps9HAp was not present in immunoprecipitates when cross-linking agent was omitted (Fig. 3A, lane 5), when Vps9HAp was not expressed (Fig. 3A, lanes 2 and 3), or when Vps21p or Vps21p-S21N was not expressed (Fig. 3A, lane 1). In addition, approximately 10-fold more Vps9HAp was cross-linked to Vps21p-S21N when Vps9HAp was expressed from a multicopy vector (2 \( \mu \mathrm{m} \)) (Fig. 3A, lane 6). A weak interaction between Vps9HAp and wild-type Vps21p was also uncovered. When extracts generated from a strain overexpressing both Vps9HAp and wild-type Vps21p were treated with cross-linking agent, a small but significant amount of Vps9HAp was detected in the immunoprecipitates (Fig. 3A, lane 8) that was not seen in the absence of cross-linking agent (Fig. 3A, lane 9).

The levels of Vps21p in the cell lysates were also determined and compared with the amount of Vps9HAp that was cross-linked. Strains expressing wild-type Vps21p from a high copy number plasmid produced approximately 7-fold more Vps21p (Fig. 3B, lanes 8 and 9) than strains expressing Vps21p-S21N from a low copy number plasmid (Fig. 3B, lanes 4–7) (these immunoblots were exposed to film for different amounts of time as indicated in the figure legend). However, the amount of Vps9HAp that was cross-linked to Vps21p-S21N was approximately 10-fold more than what was cross-linked to wild-type Vps21p despite the fact that wild-type Vps21p was expressed from a high copy number vector (compare Fig. 3A, lanes 6 and 8). It should be noted that when Vps21p was overexpressed, a slower migrating form of the protein (23 kDa) is seen. This larger form represents unprenylated Vps21p (25). These results indicate that Vps9HAp has a higher affinity for Vps21p-S21N than wild-type Vps21p. These observations are consistent with the two-hybrid results which indicated that Vps9p interacts more strongly with Vps21p-S21N.

\textbf{Vps9p Stimulates Nucleotide Exchange Activity of Vps21p in Vitro}—To facilitate the exchange of GDP for GTP, guanine nucleotide exchange factors are likely to first recognize and then associate with Rab proteins in their GDP-bound state. The preferential association of Vps9p with Vps21p in its GDP-bound state (S21N mutant) suggested that Vps9p may function as a guanine nucleotide exchange factor. To test this possibility, guanine nucleotide displacement assays were carried out with purified Vps21p in the presence of wild-type cell extracts or cell extracts from a strain that overexpressed Vps9p approximately 14-fold. Purified Vps21p was preloaded with \(^{3}H\)GDP and then diluted into an exchange buffer that contained a 130-fold excess of unlabeled GDP. Because of the intrinsic nucleotide exchange activity of Vps21p (Fig. 4, triangles), prebound radioactive GDP was slowly exchanged for unlabeled GDP, resulting in a decrease in Vps21p-bound \(^{3}H\) counts with increasing time. This intrinsic exchange was not significantly affected by adding cell extract from wild-type yeast (Fig. 4, circles). However, an extract from cells overproducing Vps9p stimulated this intrinsic exchange 1.8-fold (Fig. 4, squares).
Vps9p Is a Guanine Nucleotide Exchange Factor

This result indicated that a cell lysate containing an increased amount of Vps9p enhanced the intrinsic guanine nucleotide exchange rate of Vps21p.

In order to demonstrate that this stimulatory effect was directly due to the presence of Vps9p, purified recombinant Vps9p was used in the exchange assay. Both bacterial and yeast expression plasmids were constructed that encoded Vps9p with an amino-terminal hexahistidine tag ((His)6-Vps9p). To determine if the His-tagged version of Vps9p was functional in vivo, the yeast expression plasmid was introduced into a strain that lacked Vps9p (CBY20, vps9Δ2) and the ability of His-tagged Vps9p to complement vps9Δ2 mutant phenotypes was analyzed. Cells that lack functional Vps9p missort and secrete soluble vacuolar proteins (27). In the case of the vacuolar hydrolase carboxypeptidase Y (CPY), vps9 mutants secrete the Golgi-modified precursor form of the enzyme (p2CPY), whereas wild-type cells properly localize p2CPY from the Golgi to the vacuole where it is processed to its mature active form (mCPY) (27). In the experiment shown in Fig. 5A, wild-type, vps9Δ2, and vps9Δ2 cells expressing (His)6-Vps9p were pulse-labeled with [35S]methionine and cysteine. Cell lysates were generated, CPY was immunoprecipitated from each lysate and the immunoprecipitates were resolved by SDS-PAGE. In wild-type cells, newly synthesized CPY was delivered to the vacuole as evidenced by the presence of the mature vacuolar form of the enzyme (mCPY) (Fig. 5A, lane 1). In the vps9Δ2 cells CPY delivery to the vacuole was blocked and Golgi-modified p2CPY accumulated (Fig. 5A, lane 2). When recombinant (His)6-Vps9p was expressed in the vps9Δ2 cells, CPY was processed to its mature form (mCPY) (Fig. 5A, lane 3) indicating that vacuolar protein transport was restored. This result demonstrates that the amino-terminal hexahistidine tag does not interfere with the function of Vps9p.

An E. coli strain was transformed with the bacterial expression construct and used to purify (His)6-Vps9p. Although the majority of the overproduced (His)6-Vps9p formed inclusion bodies in E. coli, we were able to purify (His)6-Vps9p from soluble fractions to >90% purity (Fig. 5B). When purified Vps9p was added to the displacement assay, release of pre-bound [3H]GDP was stimulated in a dose-dependent manner (Fig. 6A). In addition, Vps9p acted catalytically. At a molar ratio of 1:4 (Vps9p:Vps21p), Vps9p was able to significantly stimulate nucleotide displacement (Fig. 6A, ●). Stimulation of Vps21p-dependent GTP association was also observed (Fig. 6B). This result clearly demonstrates that Vps9p stimulates the guanine nucleotide exchange of Vps21p. Interestingly, isoprenylation of Rab3 proteins was shown to be necessary for the action of the Rab3 guanine nucleotide exchange factor (Rab3 GEF) (13). However, isoprenylation of Vps21p is not required for Vps9p-dependent nucleotide exchange.

Vps9p Does Not Stimulate GTP Release from Vps21p—It has been shown that nucleotide exchange factors can associate with both GDP- and GTP-bound GTPases and stimulate nucleotide release by stabilizing the nucleotide-free form of the GTPases (21, 47–49). In these cases, release of either bound GDP or GTP can be stimulated by nucleotide exchange factors. To examine whether Vps9p behaves similarly, the displacement assay was carried out using Vps21p preloaded with either [3H]GDP or
Vps9p Is a Guanine Nucleotide Exchange Factor

Vps9p Stimulates Nucleotide Exchange of Vps21p and Rab5 but Not of Ypt7p—

Rab nucleotide exchange factors have been shown to act on distinct sets of Rab proteins (10, 13, 49–51). In order to determine if Vps9p recognized structural variations among Rab proteins, the ability of Vps9p to stimulate the intrinsic nucleotide exchange of mammalian Rab5 and yeast Ypt7p was examined. Each GTPase was preloaded with [3H]GDP and their intrinsic guanine nucleotide exchange activities were determined using the [3H]GDP release assay described in the legend to Fig. 4. As seen in Fig. 8, each GTPase possessed intrinsic exchange activity of different magnitudes (Fig. 8, filled triangles). Addition of Vps9p did not alter the intrinsic nucleotide exchange rate of Ypt7p (Fig. 8C). However, the presence of Vps9p stimulated the intrinsic nucleotide exchange rates of Vps21p (Fig. 8A) and mammalian Rab5 (Fig. 8B). These results demonstrate that Vps9p recognizes sequence variations among different Rab proteins.

**DISCUSSION**

This study describes physical and functional interactions between Vps9p and a Rab protein involved in vacuolar protein transport, Vps21p. The yeast two-hybrid system was used to uncover an interaction between Vps9p and a mutant Vps21p that binds GDP but not GTP. Purified recombinant Vps9p stimulated GDP release and GTP association of Vps21p. Vps9p also stimulated GDP release of the Vps21p mammalian sequence homologue, Rab5 in vitro. These data demonstrate that Vps9p possesses all the hallmarks of a guanine nucleotide exchange factor. Several other observations indicate that the guanine nucleotide exchange activity of Vps9p is physiologically relevant in vesicle-mediated protein transport to the yeast vacuole. First, deletion of the VPS9 gene causes severe defects in vacuolar protein transport and leads to the accumulation of 40–50 nm vesicles (27); these phenotypes are very similar to those of vps21 or vps9 mutants (25). Second, purified Vps9p possesses specific guanine nucleotide exchange activity for Vps21p/Rab5. Vps9p did not stimulate nucleotide exchange of yeast Ypt7p, which is another Rab involved in later stages of vacuolar protein transport. Consistent with the inability of Vps9p to stimulate nucleotide exchange of yeast Ypt7p, which is another Rab involved in later stages of vacuolar protein transport. Consistent with the inability of Vps9p to stimulate nucleotide exchange of yeast Ypt7p, which is another Rab involved in later stages of vacuolar protein transport. Consistent with the inability of Vps9p to stimulate nucleotide exchange of yeast Ypt7p, which is another Rab involved in later stages of vacuolar protein transport.
Vps9p Is a Guanine Nucleotide Exchange Factor

Vps9p is a guanine nucleotide exchange factor. It is involved in the activation of Rab proteins by exchanging GTP for GDP. This process is crucial for the proper regulation of membrane trafficking.

**REFERENCES**

1. Palade, G. (1975) *Science* 189, 347–357
2. Rothman, J. E., and Orci, L. (1992) *Nature* 355, 409–415
3. Bothwell, M. K., and Scheller, R. H. (1993) *Proc. Natl. Acad. Sci. U. S. A.* 90, 2559–2563
4. Pfeffer, S. R. (1994) *Curr. Opin. Cell Biol.* 6, 522–526
5. Novick, P., and Brenwald, P. (1993) *Cell* 73, 597–601
6. Novick, P., and Balch, W. E. (1984) *Annu. Rev. Biochem.* 63, 949–990
7. Strom, M., Vollmer, P., Tan, T. J., and Gallwitz, D. (1993) *Nature* 361, 736–739
8. Pfeffer, S. R., Dirac-Svejstrup, A. B., and Soldati, T. (1995) *J. Biol. Chem.* 270, 17057–17060
9. Dirac-Svejstrup, A. B., Sumizawa, T., and Pfeffer, S. R. (1997) *EMBO J.* 16, 465–472
10. Walsh-Solimena, C., Collins, R. N., and Novick, P. J. (1997) *J. Cell Biol.* 137, 1495–1509
11. Goud, B., Salminen, A., Walworth, N. C., and Novick, P. J. (1988) *Cell* 53, 753–768
12. Novick, P., Field, C., and Schekman, R. (1980) *Cell* 10, 205–215
13. Wada, M., Nakashiki, H., Satoh, A., Hiranou, H., Ohashi, H., Matsuyama, Y., and Takai, Y. (1997) *J. Biol. Chem.* 272, 3875–3878
14. Horazdovsky, B. F., DeWald, D. B., and Emr, S. D. (1995) *Curr. Opin. Cell Biol.* 7, 544–551
15. Bankaitis, V. A., Johnson, L. M., and Emr, S. D. (1986) *Proc. Natl. Acad. Sci. U. S. A.* 83, 9075–9079
16. Rothman, J. H., and Stevens, T. H. (1986) *Cell* 47, 1041–1051
17. Robinson, J. S., Klionsky, D. J., Banta, L. M., and Emr, S. D. (1988) *Mol. Cell. Biol.* 8, 4938–4948
18. Rothman, J. H., Howald, L., and Stevens, T. H. (1989) *EMBO J.* 8, 2057–2065
19. Raymond, C. K., Howald-Stevenson, I., Vater, C. A., and Stevens, T. H. (1992) *Mol. Biol. Cell* 3, 1389–1402
20. Stack, J. H., Horazdovsky, B., and Emr, S. D. (1995) *Annu. Rev. Cell Dev. Biol.* 11, 1–33
21. Bischoff, F. R., and Ponstingl, H. (1991) *Proc. Natl. Acad. Sci. U. S. A.* 88, 10830–10834
22. Cowles, C. R., Emr, S. D., and Horazdovsky, B. F. (1994) *J. Cell Sci.* 107, 3449–3459
23. Piper, R. C., Whitters, E. A., and Stevens, T. H. (1994) *Eur. J. Cell Biol.* 65, 305–318
24. Becherer, K. A., Rieder, S. E., Emr, S. D., and Jones, E. W. (1996) *Mol. Cell. Biol.* 7, 579–594
25. Horazdovsky, B. F., Busch, G. R., and Emr, S. D. (1994) *EMBO J.* 13, 1287–1309
26. Chen, Y.-J., and Stevens, T. H. (1996) *Eur. J. Cell Biol.* 70, 289–297
27. Burd, C., Mustol, P., Schu, P., and Emr, S. (1996) *Mol. Cell. Biol.* 16, 2589–2577
28. Burd, C. G., Peterson, M., Cowles, C. R., and Emr, S. D. (1997) *Mol. Cell. Biol.* 8, 1089–1104
29. Singer-Kruger, B., Stenmark, H., Dusterhoft, A., Philpippet, P., Yoo, J. S., and Gallwitz, D. (1994) *J. Cell Biol.* 125, 293–298
30. Vojtek, A. B., Hollygen, S. M., and Cooper, J. A. (1993) *Cell* 74, 205–214
31. Horazdovsky, B. F., and Hogg, R. W. (1989) *J. Bacteriol.* 171, 3653–3659
32. Casabianca, M. J., Martinea-Arias, A., Sharipa, S. K., and Chou, J. (1983) *Methods Enzymol.* 100, 293–308
33. Miller, J. (1972) *Experiments in Molecular Genetics*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
34. Sherman, F., Fink, G. R., and Lawrence, L. W. (1979) *Methods in Yeast Genetics: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
35. Alexandrov, K., Horiuchi, H., Steele-Mortimer, O., Seabra, M., and Zerial, M. (1994) *EMBO J.* 13, 5262–5273
36. Jullien-Flores, V., Dorseuil, O., Romero, F., Letourneur, F., Saragosti, S., Berger, R., Tavitian, A., Gucon, G., and Camonis, J. H. (1995) *J. Biol. Chem.* 270, 22473–22477
37. Hannon, G. J., Demetric, D., and Beach, D. (1993) *Genes Dev.* 7, 2378–2391
38. Sikorski, R. S., and Hieter, P. (1989) *Genes Dev.* 22, 19–27
39. Horazdovsky, B. F., Delahodde, A., and Jacq, C. (1986) *Cell* 46, 837–844
40. Wichmann, H., Heneg, L., and Gallwitz, D. (1992) *Cell* 71, 1131–1142
41. Wach, A., Brachar, A., Poehmann, R., and Philippsen, P. (1994) *Yeast* 10, 1793–1808
42. Horazdovsky, B. F., Davies, B. A., McLaughlin, S. A., Yoon, S.-H., Seaman, M. N. J., and Emr, S. D. (1997) *Mol. Cell. Biol.* 8, 1529–1541
43. Dunn, S. (1986) *Annu. Rev. Biochem.* 55, 144–153
44. Feig, I. A., and Cooper, G. M. (1988) *Mol. Cell. Biol.* 8, 3235–3242
45. Horazdovsky, B. F., Cowles, C. R., Mustol, P., Holmes, M., and Emr, S. D. (1996) *J. Biol. Chem.* 271, 33657–33651
46. Stack, J. H., Herman, P. K., Schu, P. V., and Emr, S. D. (1993) *EMBO J.* 12, 2253–2254
47. Romero, G., Chau, V., and Biltonen, R. L. (1985) *J. Biol. Chem.* 260, 6167–6174
48. Lai, C. C., Boguski, M., Broek, D., and Powers, S. (1993) *Mol. Cell. Biol.* 13, 4894–904
Vps9p Is a Guanine Nucleotide Exchange Factor

1345–1352

49. Burton, J. L., Burns, M. E., Gatti, E., Augustine, G. J., and De Camilli, P. (1994) EMBO J. 13, 5547–5558
50. Moya, M., Roberts, D., and Novick, P. (1993) Nature 361, 460–463
51. Rosa, J. L., Casaroli-Marano, R. P., Buckler, A. J., Vilaro, S., and Barbacid, M. (1996) EMBO J. 15, 4262–4273
52. Horiuchi, H., Lippe, R., McBride, H. M., Rubino, M., Woodman, P., Stenmark, H., Rybin, V., Wilm, M., Ashman, K., Mann, M., and Zerial, M. (1997) Cell 90, 1149–1159
53. Sprang, S. R., and Coleman, D. E. (1998) Cell 95, 155–158
54. Sprang, R. S. (1997) Annu. Rev. Biochem. 66, 639–678
55. Collins, R. N., Brennwald, P., Garrett, M., Lauring, A., and Novick, P. (1997) J. Biol. Chem. 272, 18281–18289
56. Sasaki, T., Kikuchi, A., Araki, S., Hata, Y., Isomura, M., Kuroda, S., and Takai, Y. (1990) J. Biol. Chem. 265, 2333–2337
57. Ullrich, O., Stenmark, H., Alexandrov, K., Huber, L. A., Kaibuchi, K., Sasaki, T., Takai, Y., and Zerial, M. (1993) J. Biol. Chem. 268, 18143–18150
58. Ullrich, O., Horiuchi, H., Bucci, C., and Zerial, M. (1994) Nature 368, 157–160
59. Soldati, T., Shapiro, A. D., Svejstrup, A. B., and Pfeffer, S. R. (1994) Nature 369, 76–78