THE JOURNAL OF BIOLOGICAL CHEMISTRY
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Vol. 274, No. 21, Issue of May 21, pp. 15284–15291, 1999
Printed in U.S.A.

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)

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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 Sec4/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,

* This work was supported in part by National Institutes of Health Grant GM-55301 (to B. F. H.) and March of Dimes Foundation Grant 5-FY97-0119 (to B. F. H.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: GDI, GDP dissociation inhibitor; GDF, GDI dissociation factor; GEF, guanine nucleotide exchange factor; PCR, polymerase chain reaction; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; vps, vacuolar protein sorting; CPY, carboxypeptidase Y.

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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 Vps9p. 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 (*MAT**a** trp1 leu2 his3 LYS2::(lexAop)$_4$-HIS3 URA3::(lexAop)$_8$-lacZ* (30), SEY6210 (*MATa leu2–3, 112 ura3–52 his3-Δ200 trp1-Δ901 lys2–801 suc2-Δ9*(17), CBY1 (SEY6210; *vps9Δ1::HIS3*) (27), GTY1 (BHY10 (25); *vps9Δ2::HIS3 vps21Δ3::NEO*). GTY1 was generated by transforming CBY20 (27) (BHY10; *vps9Δ2::HIS3*) with a *Cla*I/*Pvu*II *vps21Δ3::NEO* fragment of pBHY21–78. *Escherichia coli* strains used were: CW2642 (*araE201 ΔaraFGH::kan^r srl::Tn10 recA59 pro$^-$* [F′ proAB$^+$ *lacI^q*] (31), M15 (*Nal^s Str^s rif^s lac$^-$ ara$^-$ gal$^-$ mtl$^-$ F$^-$ recA$^+$ uvr$^+$*) (QIAGEN, Inc.), XL1Blue (*recA1 endA1 gyrA96 thi-1 hsdR17 supE44*) (Stratagene), and MC1066 (*F$^-$ ΔlacXYZ hsr$^-$ hsm$^+$ spsL galW galK trpC9830 leuB600 pyrF::Tn5*) (32). Bacterial strains were grown in LB medium containing ampicillin (50 μg/ml) 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 Roche Molecular Biochemicals, Life Technologies, Inc., or New England Biolabs. [^{35}S]Pro Mix, [γ-^{32}P]GTP, peroxidase-conjugated anti-rabbit IgG, and peroxidase-conjugated anti-mouse IgG were purchased from Amersham. [^{3}H]GDP was from NEN Life Science Products Inc. Monoclonal anti-HA antibody was obtained from Berkeley Antibody Co. Production of antiserum to Vps21p and carboxypeptidase Y has been described previously (25). Canine His-Rab5 protein was a gift from Marino Zerial (35).

Plasmid Construction—To create the Vps21p, Vps21p-S21N two-hybrid baits, and the Vps9p two-hybrid prey, the coding sequences of these genes were amplified by PCR using pGBY21–5, pBHY21–11 (25), and pPS91 (27), respectively, as templates and oligonucleotides that resulted in PCR products which contained 5′ *Bam*HI and 3′ *Sal*I linkers. These products were ligated into either pVJL11 (36) to create the Vps21p and Vps21p-S21N baits (pGT21–1 and pGT21–2, respectively) or pGADGH (37) to create the Vps9p prey, GT9-1. Expression of these fusion proteins in the L40 strain was confirmed by immunoblotting. To create *CEN* and 2-μm yeast expression plasmids encoding C-terminal HA-tagged Vps9p, a *Sma*I site was created at the 3′ end of the *VPS9* coding sequence eliminating the stop codon using PCR mutagenesis. This amplified product was digested with *Sma*I and *Hin*dIII and inserted into the same sites of pRS416 to create pGT9-3. A *Sma*I-*Eco*RV fragment containing the coding sequence for the HA-epitope from plasmid YEp352-HA was inserted into the *Sma*I site of pGT9-3 to create pGT9HA-1. The *Eco*RV-*Hin*dIII fragment of pGT9HA-1 was subcloned into pRS426 (38) to create the 2-μm plasmid, pGT9HA-2. To create Vps21p and Vps21p-S21N *E. coli* expression constructs, the *VPS21* and *VPS21-S21N* coding sequences were amplified by PCR from pGBY21–2 (25) and pBHY21–11 (25). These products were cloned into the *E. coli* expression vector, pKK223-3 (Pharmacia Biotech) to create the Vps21p and the Vps21p-S21N expression constructs pBHY21–30 and pBHY21–76. pQEVPS9, which contains the *VPS9* gene modified with a NH_2-terminal hexahistidine-coding sequence, was constructed in the same manner as pGT9-1 except pQE31 (QIAGEN, Inc.) was used. The NH_2-terminal sequence of the His$_6$-Vps9p is M-R-G-S-(H)$_6$-T-D-P-Vps9p. A His$_6$-Vps9p yeast expression construct, pEMBL-VPS9 was constructed by cloning the (His)$_6$-*VPS9* gene from pQEVPS9 into pEMBLye30/2 (39). The *E. coli* Ypt7p expression construct was created by amplifying the *YPT7* gene from pBS-YPT7 (40) and cloning the PCR product into pKK223-3 resulting in pKKYPT7. The *vps21::NEO*^{R} construct was generated by first subcloning the *Sma*I/*Spe*I kan MX4 module from pFA6-kanMX4 (41) into pBluescript II (Stratagene) to create pBS-NEO. An *Eco*RV fragment containing the kanMX4 module from pBS-NEO was inserted into the Klenow enzyme-treated *Sal*I/*Bgl*II sites of pBHY21–18 to create plasmid pBHY21–78.

Yeast Two-hybrid Assay—L40 yeast cells (30) were transformed with pGT21-1, pGT21-2, or pVJL11 (36) and either pGT9-1 or pGADGH (37). Transformants were selected and streaked onto SM plates lacking tryptophan, leucine, and histidine. β-Galactosidase filter assays were performed as described previously (30).

Protein Cross-linking—GTY1 (*vps9Δ2::HIS3 vps21Δ3::NEO*) were transformed with low or high copy Vps9HAp expression plasmids (pGT9HA-1 and pGT9HA-2, respectively), and/or with low or high copy Vps21p expression plasmids (pGBY21-5 and pBHY21-28, respectively), or a low copy Vps21p-S21N expression plasmid (pBHY21-11). Strains were grown in appropriate SM to an OD_{600} of 0.8 and spheroplasts were generated, lysed, and treated with the cross-linking agent dithiobis(succinimidylpropionate) as described previously (25). Vps21p and its associated proteins were isolated by immunoprecipitation as described previously (42). The immunoprecipitates were resolved in duplicate by SDS-PAGE and transferred to nitrocellulose membranes. The presence of Vps9HAp and Vps21p was determined by Western analyses (43) using HA monoclonal antibodies or Vps21p antiserum and the ECL chemiluminescent detection system (Amersham, Inc.).

Purification of Vps21p and Ypt7p—Wild-type Vps21p and Vps21p-S21N were purified from *E. coli* CW2642 carrying pBH21-30 or pBH21-76, respectively. Recombinant proteins were induced with 1 mM isopropyl-β-D-thiogalactopyranoside for 2 h at 37 °C. The cells were harvested and washed twice with a buffer containing 50 mM Tris-HCl and 100 mM NaCl. The cell pellets were resuspended in lysis buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM DTT, 100 μg/ml phenylmethylsulfonyl fluoride), and lysed using a Bead-Beater (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-Sephacel column pre-equilibrated with buffer A (50 mM Tris-HCl, pH 7.5, 2 mM $MgSO_4$). 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 Q2 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* CW2642 carrying pKKYPT7. Induction and purification was carried out similarly to the Vps21p procedure with minor modifications. Proteins were eluted from a DEAE-Sephacel column with buffer A containing 200 mM NaCl. The eluate was concentrated, dialyzed, and loaded onto a Q2 column. Proteins were eluted with a linear gradient of 0–300 mM NaCl in buffer A.

Purification of (His)$_6$-Vps9p—M15 *E. coli* cells carrying pREP4 and pQEVPS9 were grown and induced as described for the Vps21p purification. Cells were harvested, washed, lysed, and (His)$_6$-Vps9p was purified from the crude cell extract using Ni-NTA agarose as described by the manufacturer (Qiagen). (His)$_6$-Vps9p was eluted from the Ni-NTA agarose, 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 0 to 300 mM linear NaCl gradient in 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 $MgSO_4$, 1 mM DTT, 3 mM EDTA, 50 μM [^{3}H]GDP (2.6×10^4 cpm/μl), or [γ-^{32}P]GTP (6.5×10^4 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 $MgSO_4$) 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 Displacement and Exchange Assays—Displacement was monitored by incubating preloaded [^{3}H]GDP or [γ-^{32}P]GTP-Vps21p in 250 μl of 50 mM Tris-HCl, pH 7.5, 1 mM DTT, 1 mM EDTA, 15 μM [^{3}H]GDP (1.5×10^4 cpm/μl), or [γ-^{32}P]GTP (1.3×10^4 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 DTT, 10 mM $MgSO_4$, 4 mM GDP with or without Vps9p and incubated at 30 °C. At each time point, 25 μl of the mixture was removed and diluted in 1 ml of ice-cold buffer C. Protein-bound [^{3}H]GDP or [γ-^{32}P]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 DTT, 10 mM $MgSO_4$, 4 mM GTP, and [γ-^{32}P]GTP (100 μCi/ml). Vps21p-associated [γ-^{32}P]GTP was determined as described above.

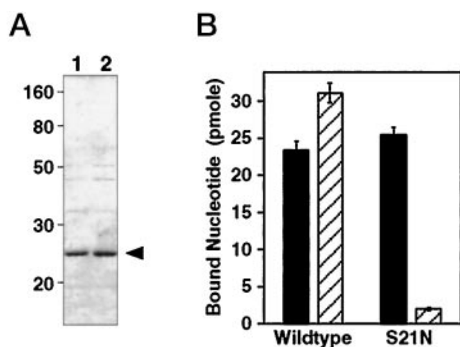

FIG. 1. **Purification and nucleotide binding of wild-type and Vps21p-S21N.** *A*, wild-type and S21N mutant Vps21p were purified as described under "Experimental Procedures." Proteins in the peak fractions from the Q2 column chromatography were subjected to SDS-PAGE (10%) and stained with Coomassie Brilliant Blue. Wild-type Vps21p (*lane 1*) and Vps21p-S21N (*lane 2*) are indicated by the *arrowhead*. *B*, purified wild-type or Vps21p-S21N (54 pmol each) was incubated with 50 μM [^{3}H]GDP (*solid bar*) or 50 μM [γ-^{32}P]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.

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

RESULTS

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 *in vivo* (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. 1*A*), and no other GTP-binding proteins were detected in these fractions by [^{32}P]GTP blot analysis.[2] Purified wild-type and S21N-Vps21p were incubated with [^{3}H]GDP or [^{32}P]GTP in the presence of EDTA. Endogenous nucleotides bound to Vps21p rapidly exchanged with radiolabeled nucleotides under these conditions, reaching maximum binding within 30 min.[2] As shown in Fig. 1*B*, Vps21p-S21N bound substantially more GDP (26 pmol) than GTP (<2 pmol). Whereas, wild-type Vps21p bound slightly more GTP (32 pmol) than GDP (24 pmol). These results demonstrate that Vps21p-S21N, like the Ras N17 protein, preferentially binds GDP.

Vps21p and Vps9p Physically Interact—*vps21* mutants fall 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 affected 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) and other class D *VPS* gene products. These constructs were used to transform a yeast strain (L40) that contained the *HIS3* and *lacZ* reporter gene constructs under control the *LexA* promoter. As shown in Fig. 2, L40 yeast that

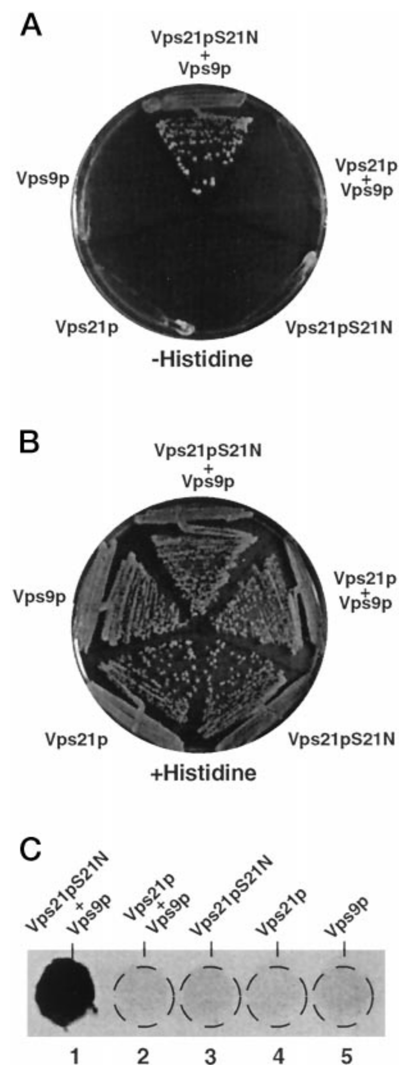

FIG. 2. **Vps21p-S21N interacts with Vps9p in the yeast two-hybrid system.** L40 yeast expressing Gal4AD-Vps9p and LexA-Vps21p-S21N, Gal4AD-Vps9p and LexA-Vps21p wild-type, pGADGH (Gal4AD vector) and LexA-Vps21p-S21N, pGADGH and LexA-Vps21p wild-type, and Gal4AD-Vps9p and pVJL11 (LexA vector) were streaked onto *A*, a YNB plate lacking tryptophan, leucine, and histidine and onto *B*, a YNB plate lacking tryptophan and leucine and incubated at 30 °C for 72 h. These yeast strains were also patched onto 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.

[2] H. Hama and B. F. Horazdovsky, unpublished observations.

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. 2*A*). All strains tested were able to grow on synthetic media that contained histidine (Fig. 2*B*).

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 β-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 β-galactosidase activity (patch 1, Fig. 2*C*). Neither the LexA-Vps21p (wild-type) fusion together with the Gal4AD-Vps9p fusion nor the LexA-Vps21p or Gal4AD-Vps9p fusions alone expressed the β-galactosidase reporter (patches 2–5, Fig. 2*C*). 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 Vps21p-Vps9p two-hybrid results and to examine if the two-hybrid interaction accurately represented an *in vivo* phenomenon. Spheroplasts generated from cells (GTY1; *vps21Δ3, 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. 3*A, 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. 3*A, lane 5*), when Vps9HAp was not expressed (Fig. 3*A, lanes 2* and *3*), or when Vps21p or Vps21p-S21N was not expressed (Fig. 3*A, lane 1*). In addition, approximately 10-fold more Vps9HAp was cross-linked to Vps21p-S21N when Vps9HAp was expressed from a multicopy vector (2 μm) (Fig. 3*A, 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. 3*A, lane 8*) that was not seen in the absence of cross-linking agent (Fig. 3*A, 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. 3*B, lanes 8* and *9*) than strains expressing Vps21p-S21N from a low copy number plasmid (Fig. 3*B, 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

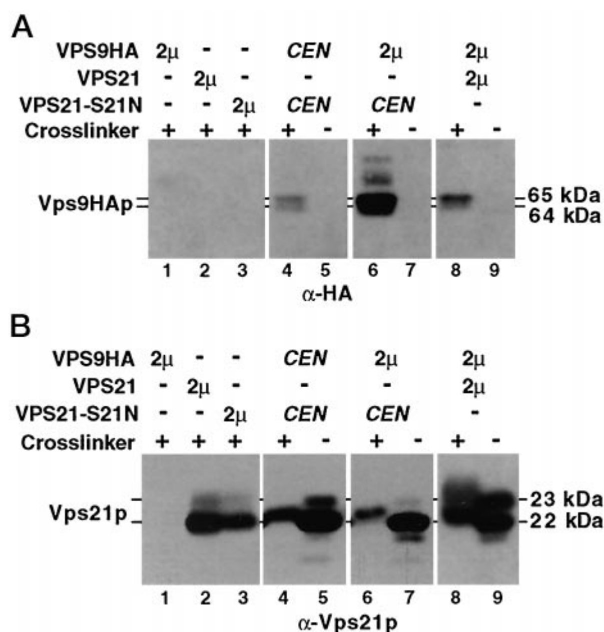

FIG. 3. **Vps21p-S21N is more efficiently cross-linked to Vps9HAp than wild-type Vps21p.** Protein lysates were generated from GTY1 (*vps9Δ2 vps21Δ3*) spheroplasts carrying the following plasmids in the combinations denoted *above panel A* and *panel B*: pGT9HA-1 (*CEN VPS9HA*), pGT9HA-2 (2-μm *VPS9HA*), pBHY21-28 (2-μm *VPS21*), pBHY21-21 (2-μm *VPS21-S21N*), and pBHY21-11 (*CEN VPS21-S21N*). Each lysate was divided into two aliquots. One was treated with the cross-linking agent, dithiobis(succinimidylpropionate) (400 μg/ml), and the other was left untreated. Each aliquot was then subjected to immunoprecipitation with Vps21p antiserum and duplicate samples of the resulting immunoprecipitates were resolved by SDS-PAGE. The resolved proteins were transferred to nitrocellulose membranes and probed with: *A,* HA antibodies to visualize Vps9HAp; and *B*, Vps21p antiserum to visualize the Vps21p levels in each strain. In *panel B*, the film was exposed to the ECL reagent-treated membranes for 3 s (*B, lanes 1–3*), 10 s (*B, lanes 4–7*), and 1 s (*B, lanes 8* and *9*).

Vps21p despite the fact that wild-type Vps21p was expressed from a high copy number vector (compare Fig. 3*A, 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.

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*).

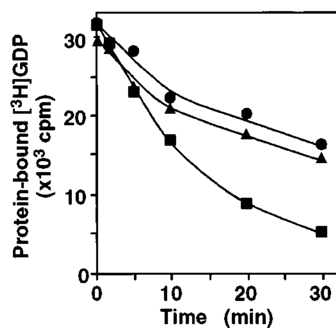

FIG. 4. **Overproduction of Vps9p enhances the guanine nucleotide exchange activity of Vps21p.** Vps21p (88 pmol) was preloaded with 15 μM [^{3}H]GDP and then diluted in 2 mM unlabeled GDP in the presence of lysis buffer (▲), 13,000 × *g* supernatant fractions (approximately 440 μg of protein) from SEY6210 (●) or SEY6210 carrying pPS92 (■) in a 200-μl assay mixture and incubated at 30 °C. At each time point, equal aliquots were removed, mixed with ice-cold buffer, and filtered through nitrocellulose membranes. The membrane filters were washed with buffer, dried, and counted. Radioactive GDP that remained protein bound is presented as a function of time.

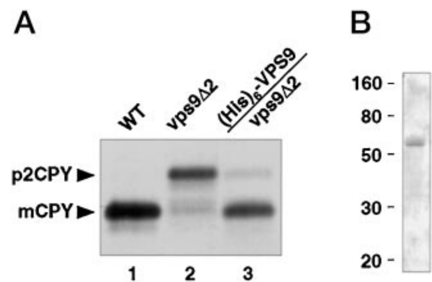

FIG. 5. **Characterization of recombinant (His)$_6$Vps9p.** *A*, SEY6210 (wild-type, *lane 1*), CBY20 (*vps9Δ2*, *lane 2*), and CBY20 carrying pEMBLVPS9 (*vps9Δ2* + (His)$_6$Vps9p, *lane 3*) were labeled with [^{35}S]methionine and cysteine for 10 min at 30 °C. Unlabeled methionine and cysteine were added and the cells were incubated for another 30 min. Cells were lysed and subjected to immunoprecipitation with CPY antiserum. Immunoprecipitated CPY was resolved by SDS-PAGE and visualized by fluorography. *B*, (His)$_6$-Vps9p was purified as described under "Experimental Procedures." The peak fraction from Q2 column chromatography was subjected to SDS-PAGE (10%) and stained with Coomassie Brilliant Blue. The 58-kDa (His)$_6$-Vps9p is indicated by an *arrowhead*.

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. 5*A*, wild-type, *vps9Δ2*, and *vps9Δ2* cells expressing (His)$_6$-Vps9p were pulse-labeled with [^{35}S]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. 5*A, lane 1*). In the *vps9*Δ2 cells CPY delivery to the vacuole was blocked and Golgi-modified p2CPY accumulated (Fig. 5*A, lane 2*). When recombinant (His)$_6$-Vps9p was expressed in the *vps9*Δ2 cells, CPY was processed to its mature form (mCPY) (Fig. 5*A, 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. 5*B*). When purified Vps9p was added to the displacement assay, release of pre-bound [^{3}H]GDP was stimulated in a dose-dependent manner (Fig. 6*A*). In addition, Vps9p acted catalytically. At a molar ratio of 1:4 (Vps9p:Vps21p), Vps9p was able to significantly stimulate nucleotide displacement (Fig. 6*A*, ●). Stimulation of Vps21p-dependent GTP association was also observed (Fig. 6*B*). This result clearly demonstrates that Vps9p stimulates

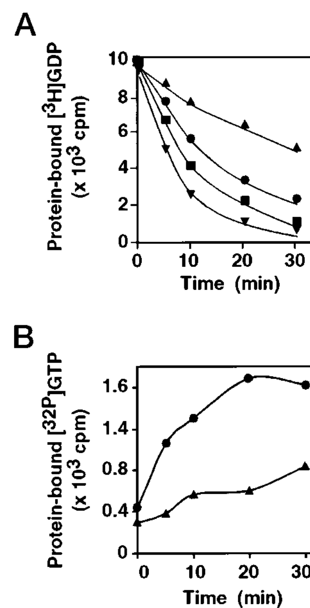

FIG. 6. **Vps9p stimulated both GDP displacement and GDP/GTP exchange.** *A*, Vps21p (100 pmol) was preloaded with 15 μM [^{3}H]GDP and diluted in 2 mM unlabeled GDP in the absence (▲) or presence of 25 (●), 50 (■), or 100 (▼) pmol of purified (His)$_6$-Vps9p in a 200-μl assay mixture. Protein-bound [^{3}H]GDP was determined as described in the legend to Fig. 4. *B,* nucleotide exchange activity was monitored by incubating Vps21p (200 pmol) in the presence (●) or absence (▲) of Vps9p (200 pmol) in 200 μl of 50 mM Tris-HCl (pH 7.5), 1 mM DTT, 10 mM $MgSO_4$, 4 mM GTP, and [γ-^{32}P]GTP (100 μCi/ml). Vps21p-associated [γ-^{32}P]GTP was determined as described in the legend to Fig. 4.

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 GEP) (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 [^{3}H]GDP or

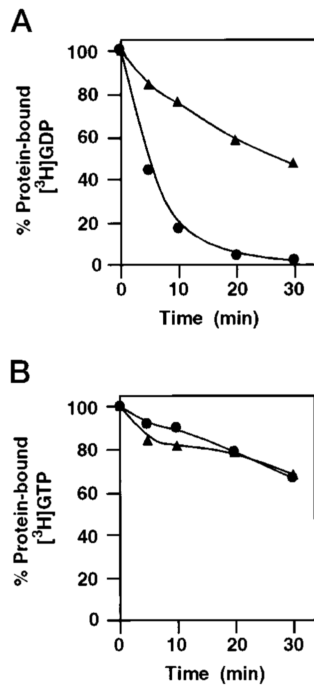

FIG. 7. **Vps9p does not stimulate GTP/GDP exchange.** *A*, Vps21p (177 pmol) was preloaded with 15 μM [^{3}H]GDP or *B*, 15 μM [γ-^{32}P]GTP and diluted in 2 mM unlabeled GDP in the absence (▲) or presence (●) of (His)$_6$-Vps9p (88 pmol). Protein-bound [^{3}H]GDP and [γ-^{32}P]GTP were determined as described in the legend to Fig. 4.

[γ-^{32}P]GTP in the presence or absence of Vps9p. As shown in Fig. 7*A*, pre-bound [^{3}H]GDP release was stimulated by adding purified Vps9p. However, the same amount of Vps9p did not stimulate the release of [γ-^{32}P]GTP from Vps21p (Fig. 7*B*). This result demonstrates that Vps9p only stimulates GDP release but not GTP release from Vps21p and that Vps9p does not stimulate the GTPase activity of Vps21p.

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 [^{3}H]GDP and their intrinsic guanine nucleotide exchange activities were determined using the [^{3}H]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. 8*C*). However, the presence of Vps9p stimulated the intrinsic nucleotide exchange rates of Vps21p (Fig. 8*A*) and mammalian Rab5 (Fig. 8*B*). These results demonstrate that Vps9p recognizes sequence variations among different Rab proteins.

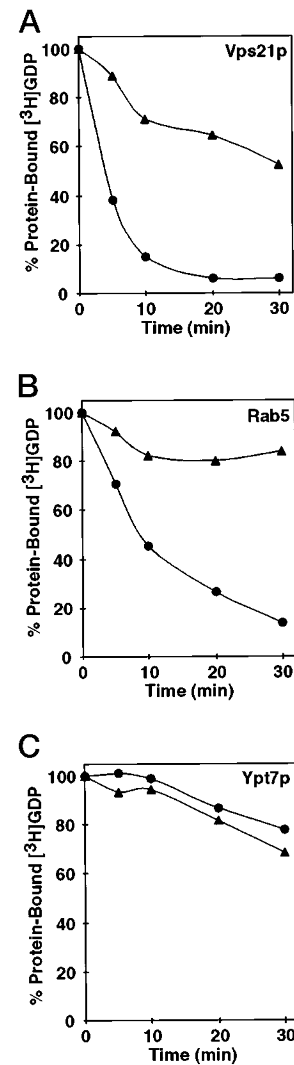

FIG. 8. **Nucleotide exchange activity of Vps9p is specific for Vps21p and Rab5.** *A*, purified Vps21p (490 pmol); *B*, Rab5 (490 pmol); *C*, Ypt7p (490 pmol) were preloaded with 15 μM [^{3}H]GDP and diluted in 2 mM unlabeled GDP in the absence (▲) or presence (●) of (His)$_6$-Vps9p (180 pmol). Protein-bound [^{3}H]GDP was determined as described in the legend to Fig. 4.

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. Protein cross-linking confirmed this interaction. 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*Δ mutant strains (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 Ypt7p, mutations in the *YPT7* gene manifest several phenotypes distinct from *vps21* or *vps9* mutants. Finally, *vps9* mutants show no obvious defects in other protein localization pathways. Based on these data, we conclude that the major role of Vps9p in vacuolar protein transport is to stimulate the guanine nucleotide exchange of Vps21p.

Several guanine nucleotide exchange factors for Rab proteins have been described. These include Rab3 GEP (13) and yeast Sec2p (10) which have been shown to be specific guanine nucleotide exchange factors for Rab3A, C, D, and Sec4p, respec-

tively. Surprisingly, Vps9p does not share significant sequence similarity to Rab3 GEP or Sec2p, indicating that this group of proteins is quite diverse. However, a mammalian Vps9p sequence homologue, Rabex5, has been recently been identified as a nucleotide exchange factor for Rab5 (52). Since Rab5 is a sequence homologue of Vps21p, it is likely that Vps9p and Rabex5 may represent a novel class of nucleotide exchange factors that modulate the activity of Vps21p/Rab5.

Stimulating the release of bound nucleotide appears to be the primary action of guanine nucleotide exchange factors. Based on structural studies, it has been postulated that guanine nucleotide exchange factors may interfere with the ability of GTPases to bind Mg^{2+} and therefore reduce their affinity for guanine nucleotides (reviewed in Ref. 53). Upon nucleotide release, GTPases and nucleotide exchange factors form relatively stable complexes that are devoid of guanine nucleotide (21, 47–49). The formation of this complex has been demonstrated *in vitro* by depleting guanine nucleotide or by introducing mutations in GTPases that reduce the affinity for guanine nucleotide (21, 47–49). The strong association seen between Vps9p and Vps21p-S21N relative to wild-type Vps21p suggests that Vps9p act in a similar manner. Serine 21 of Vps21p is located in the highly conserved nucleotide-binding pocket shared by most GTPases. Where structural information is known, the hydroxyl group of this conserved serine (or threonine) has been shown to participate in coordinating both the γ-phosphate of GTP and Mg^{2+} (54). Therefore, loss of the hydroxyl group in the serine 21 to asparagine Vps21p mutant is anticipated to result in a protein with low affinity for GTP, which could stabilize the GDP-bound Vps21p-exchange factor complex and/or the nucleotide-free Vps21p-exchange factor complex. An interesting distinction of Vps21p/Vps9p association is that Vps9p does not seem to associate with GTP-bound Vps21p. Association between many GTPases and their nucleotide exchange factors occurs irrespective of the bound guanine nucleotide. This subsequently results in GTP as well as GDP release, at least *in vitro* (48, 49, 55). In our assays, nucleotide exchange of GTP-bound Vps21p was not stimulated by Vps9p, indicating that Vps9p has very low, if any, affinity for GTP-bound Vps21p. The strong preference of Vps9p for GDP-bound Vps21p would drive the equilibrium in only one direction generating the GTP-bound, activated form of Vps21p *in vivo*.

GDP-bound Rab proteins are extracted from the target membrane by Rab GDP dissociation inhibitor, or GDI, and exist as Rab/GDI heterodimers in the cytoplasm (56, 57). Upon arrival to a specific membrane compartment, Rab GDI is thought to be displaced by the action of a GDF, which allows Rab proteins to relocate to the membrane (9, 58, 59). Subsequently, a nucleotide exchange factor would activate the Rab protein by stimulating GDP release followed by GTP binding. Interestingly, all detectable Vps9p is found in a soluble cell fraction (27). The cytosolic localization of Vps9p indicates that recruitment of Vps21p to the membrane is independent of, and most likely precedes, the action of Vps9p, which is consistent with the temporal model of Rab recruitment/activation (58, 59). Since Vps9p appears to be able to reach any membrane compartment, there must be a mechanism that prevents Vps9p from activating Vps21p at the target membrane, where most of the GDP-bound Vps21p is localized (25). It is likely that there is a specific GDF for Vps21p and that the localization of the GDF determines where Vps21p is to be activated by Vps9p. In this process, the GDF may present Vps21p to Vps9p at the correct donor membrane. It is conceivable that mutations that inactivate the Vps21p GDF would result in similar phenotypes associated with *vps9* and *vps21* mutants as well as other class D *vps* mutants (19). We are currently examining the role of proteins affected in other class D *vps* mutants in the process of membrane recruitment and activation of Vps21p.

Acknowledgments—We thank members of the Horazdovsky laboratory for helpful discussions during the course of this study. We also thank Michael White for yeast two-hybrid system reagents and suggestions and Marino Zerial for providing us with purified Rab5.

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