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Characterization of a Novel Yeast SNARE Protein Implicated in Golgi Retrograde Traffic

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The protein trafficking machinery of eukaryotic cells is employed for protein secretion and for the localization of resident proteins of the exocytic and endocytic pathways. Protein transit between organelles is mediated by transport vesicles that bear integral membrane proteins (v-SNAREs) which selectively interact with similar proteins on the target membrane (t-SNAREs), resulting in a docked vesicle. A novel *Saccharomyces cerevisiae* SNARE protein, which has been termed Vti1p, was identified by its sequence similarity to known SNAREs. Vti1p is a predominantly Golgi-localized 25-kDa type II integral membrane protein that is essential for yeast viability. Vti1p can bind Sec17p (yeast SNAP) and enter into a Sec18p (NSF)-sensitive complex with the *cis*-Golgi t-SNARE Sed5p. This Sed5p/Vti1p complex is distinct from the previously described Sed5p/Sec22p anterograde vesicle docking complex. Depletion of Vti1p in vivo causes a defect in the transport of the vacuolar protein carboxypeptidase Y through the Golgi. Temperature-sensitive mutants of Vti1p show a similar carboxypeptidase Y trafficking defect, but the secretion of invertase and gp400/hsp150 is not significantly affected. The temperature-sensitive *vti1* growth defect can be rescued by the overexpression of the v-SNARE, Ykt6p, which physically interacts with Vti1p. We propose that Vti1p, along with Ykt6p and perhaps Sft1p, acts as a retrograde v-SNARE capable of interacting with the *cis*-Golgi t-SNARE Sed5p.

INTRODUCTION

Protein transport and membrane flow in eukaryotic cells is mediated by vesicles that bud from one membrane-bounded compartment and then target to and fuse with the appropriate acceptor compartment (Palade, 1975; Rothman, 1994). Biochemical and genetic analyses in organisms as diverse as yeast, flies, worms, and mammals have produced a partial inventory of the components involved in membrane traffic and an outline of the molecular mechanisms involved.

Vesicle budding is mediated by coat proteins that assemble on nascent buds under the direction of small GTPases (Schekman and Orci, 1996). Cargo proteins are incorporated into budding vesicles either passively or by binding to "cargo receptors" that concentrate the itinerant proteins (Rothman and Wieland, 1996). After vesicle budding, uncoating ensues, and the vesicle docks with the accepter compartment in a process that involves members of three distinct protein families (Pfeffer, 1996). A family of integral membrane proteins, termed soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) (Söllner *et al.*, 1993a), lies at the heart of the docking apparatus: vesicles carry v-SNAREs that specifically interact with cognate t-SNAREs displayed on the tar-

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[1] Abbreviations used: CEN, centromere; CPY, carboxypeptidase Y; ER, endoplasmic reticulum; EST, expressed sequence tag; GST, glutathione *S*-transferase; HA, hemagglutinin; SNARE, soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor; P_{GAL}, *GAL1* promoter; P_{CUP}, *CUP1* promoter.

get compartment. The t-SNAREs are maintained in an inactive state (Pevsner *et al.*, 1994; Lupashin and Waters, 1997) by association with members of the Sec1/Sly1 protein family (Halachmi and Lev, 1996) until they undergo activation through the displacement of the Sec1/Sly1 protein by a member of the Rab-GTPase family (Lupashin and Waters, 1997). The characteristic localization of the SNARE, Rab, and Sec1/Sly1 families to distinct intracellular compartments is thought to delineate the road map for vesicular trafficking and mediate its extraordinary specificity.

The final phase of vesicular transport is initiated after assembly of a v/t-SNARE docking complex when two other components, termed SNAP (Clary *et al.*, 1990) and N-ethylmalemide-sensitive factor (NSF) (Block *et al.*, 1988), which are employed at multiple transport steps (Graham and Emr, 1991; Rothman, 1994), bind to the v/t-SNARE complex (Söllner *et al.*, 1993a,b). NSF is an ATPase (Tagaya *et al.*, 1993; Whiteheart *et al.*, 1994) that harvests the energy of ATP hydrolysis to disassemble the v/t-SNARE complex (Söllner *et al.*, 1993b), thereby initiating the process of membrane fusion. Recently, an additional role for NSF has been uncovered in yeast vacuole-vacuole fusion. In that system, NSF acts to "prime" the vacuoles prior to docking, perhaps by dissociating SNARE complexes and thereby allowing them to engage in subsequent interactions (Mayer *et al.*, 1996; Mayer and Wickner, 1997). After vesicle docking is complete, membrane bilayer fusion ensues, and describing this process represents one of the major challenges ahead.

It is clear that vesicular transport is not a unidirectional process. It has long been appreciated that delivery of newly made secretory proteins or intracellular proteins that reside in the secretory pathway requires vesicular transport from their site of synthesis on the endoplasmic reticulum (ER) to their final destinations (Palade, 1975), a process termed anterograde transport. Transport in the opposite direction, retrograde transport, is also clearly required if cells are to maintain the requisite surface area and volume of their intracellular organelles. Retrograde transport is also necessary for the recovery of components of the transport apparatus, such as SNAREs (Rothman and Warren, 1994) or cargo receptors (Marcusson *et al.*, 1994; Schimmöller *et al.*, 1995; Fiedler *et al.*, 1996), if they are to be utilized more than once. Finally, retrograde transport provides the cell with a mechanism to salvage proteins that have escaped from their resident compartment (Dean and Pelham, 1990; Jackson *et al.*, 1993; Nothwehr *et al.*, 1993; Schutze *et al.*, 1994; Harris and Waters, 1996). To fully appreciate the intracellular vesicular traffic pattern then, both anterograde and retrograde traffic must be understood.

Recently, the realization that SNAREs are central to vesicle docking and that they are arrayed on specific compartments (Bennett, 1995) has prompted efforts to identify and localize the full complement of SNAREs. The recent elucidation of the complete yeast genome sequence should allow the full complement of yeast SNAREs to be identified based on protein sequence homology. Identification of all of the yeast SNAREs, coupled with localization and functional analyses, may provide an ideal model system to develop a high resolution map of the secretory pathway encompassing both anterograde and retrograde traffic.

In this report, we describe a novel yeast SNARE protein, Vti1p, likely to function in retrograde transport to the *cis*-Golgi compartment. This protein has also been examined recently by Fischer von Mollard *et al.* (1997).

MATERIALS AND METHODS

Media, Plasmids, and Strains

YPD, YNB, and sporulation media were prepared as described (Rose *et al.*, 1990). YPGal and YPGlu/Gal media contain 1% yeast extract, 2% Bacto-peptone, 2% galactose, and 0.1% or 1% glucose, respectively. Transformation of yeast was performed as described (Elble, 1992); all other standard genetic techniques were done as indicated (Rose *et al.*, 1990). The *Escherichia coli* strain XL1-Blue (Stratagene, La Jolla, CA), which was used for all molecular genetic manipulations, was grown on standard media (Miller, 1972) and transformed according to Hanahan (1983).

The plasmids used in this study are listed in Table 1 and were constructed as follows. *VTI1* [open reading frame (ORF) YMR197c] was amplified from genomic DNA by pfu of DNA polymerase-(Stratagene) based polymerase chain reaction (PCR) placing a *Sac*II site 535 bp upstream of the initiator methionine codon of *VTI1* and a *Xho*I site 299 bp downstream of the stop codon (5′ primer, 5′-CGCCCGCGGCTTATGTCAACGAGTTTCAAATGCCAAAAC-3′; 3′ primer, 5′-CGCCTCGAGCTAAAAGATAAGGTGAAAAATATGAAAG-3′). The PCR product was digested with *Sac*II and *Xho*I and ligated either into Bluescript II KS− (Stratagene) to generate pPI1 or into pRS426 (Sikorski and Hieter, 1989) to generate pPI2. Plasmid pPI3 was generated by removal of the 750-bp internal *Hin*dIII-*Xba*I fragment of *VTI1* from plasmid pPI1 and replacement with the 2.1-kb *Hin*dIII-*Xba*I fragment from pMR2253 (from M. Rose, Princeton University), which contains a functional *LEU2* gene. To construct pPI6, the plasmid encoding the glutathione *S*-transferase (GST)-Vti1 fusion protein, the *VTI1* ORF was amplified by PCR from genomic DNA, placing *Bam*HI and *Sac*I sites upstream to the sequence encoding the second codon of *VTI1* and a *Xho*I site 299 bp downstream of the stop codon (5′ primer, 5′-GCGAGCTCGGATCCAGTTCCCTATTAATATCATACG-3′; 3′ primer, same as above for pPI1). The *Bam*HI- and *Xho*I-digested PCR product was ligated into pEMBLyex4 (Baldari *et al.*, 1987) digested with the same enzymes. pPI7 and pPI8 were generated by amplification of the *VTI1* ORF by PCR, placing a *Sac*I site upstream of the second codon of *VTI1* and a *Xho*I site 300 bp downstream of the stop codon with the same sets of primers as for pPI6. The PCR products were digested with *Sac*I and *Xho*I and ligated into similarly digested pYATAG200 (from M. Latterich, Salk Institute, La Jolla, CA), which encodes the hemagglutinin (HA)-epitope tag behind the *CUP1* promoter (P_{CUP}), to generate pPI7, or into pYATAG100 (from M. Latterich), to generate pPI8. To construct pPI9, *HA-VTI1* was excised from pPI7 as a 1.4-kb *Pvu*II-*Eco*RI fragment and ligated into *Eco*RI and *Sma*I cleaved pSM578 (from S. Michaelis, Johns Hopkins University School of Medicine, Baltimore, MD). Plasmids pVLhPI1 and pVLmPI2, bearing the cDNA encoding a human and mouse sequences related to Vti1p (hVti1-rp1 and mVti1-rp2) under *GAL1* promoter (P_{GAL}) control, was constructed by ligation of the 600-bp *Eco*RI-*Not*I fragment

Table 1. Plasmids used in this work

Plasmid	Description	Source
pPI1	*VTI1* in Bluescript II KS−	This study
pPI2	*VTI1* in pRS426 (2μ, *URA3*)	This study
pPI3	*VTI1Δ::LEU2* in pRS304 (*YIp*, *TRP1*)	This study
pPI6	P_{GAL}-*GST-VTI1* in pEMBLyex4 (2μ, *URA3*)	This study
pPI7	P_{CUP}-*HA-VTI1* in pYATAG200 (*CEN*, *TRP1*)	This study
pPI8	P_{CUP}-*HA-VTI1* in pYATAG100 (*YIp*, *TRP1*)	This study
pPI9	P_{GAL}-*HA-VTI1* in pSM578 (*CEN*, *TRP1*)	This study
pVLhPI1	*hVti1-rp1* (EcoRI-NotI fragment of EST AA056932) in pYES2 (2μ, *URA3*)	This study
pVLmPI2	*mVti1-rp2* (EcoRI-NotI fragment of EST AA016379) in pYES2 (2μ, *URA3*)	This study
pGST-Sec17	*GST-SEC17* (aa 11–291) in pGEX2T	This study
pSK54	*SLY1-20* in pRS426 (2μ, *URA3*)	This laboratory
pSK60	*YKT6* in pRS426 (2μ, *URA3*)	This laboratory
pSED5	*SED5* in YEP352 (2μ, *URA3*)	H. Pelham
pSFT1	2μ, *SFT1 URA3*	H. Pelham
pJG103	2μ, *SEC22 URA3*	S. Ferro-Novick
pSFN2d	2μ, *BET1 URA3*	S. Ferro-Novick

of expressed sequence tag (EST) AA056932 (for pVLhPI1) or the 800-bp *Eco*RI-*Not*I fragment of EST AA016379 (for pVLmPI2) into a pYES2 (Invitrogen, Carlsbad, CA) vector that had been digested with the same enzymes. To construct the plasmid pGST-Sec17, encoding the GST-Sec17 fusion protein, the *SEC17* ORF was amplified by PCR, placing a *Bam*HI site adjacent to its 12th codon and an *Eco*RI site 3′ to the stop codon (5′ primer, 5′-CGGGATCCGCTGAGAAGAAGGGTGTTCC-3′; 3′ primer, 5′-CGGAATTCTCTAAGTCATCGCTATATG-3′). The PCR product was digested with *Bam*HI and *Eco*RI and ligated into similarly digested pGEX-2T (Pharmacia Biotech, Piscataway, NJ). The first 17 amino acid residues of Sec17p are not essential for function (Griff *et al.*, 1992).

The *S. cerevisiae* strains used in this study are listed in Table 2. To construct the GWY150 strain, pPI3 was linearized by *Pvu*II and used to transform the diploid strain GWY30. Leu^+ transformants were purified; the deletion was confirmed by PCR utilizing primers in *LEU2* and flanking the deletion. GWY151 and GWY152 strains were obtained by transformation of GWY150 with pPI2 and pPI6, respectively, followed by sporulation, tetrad dissection, and selection of Leu^+ Ura^+ segregants. GWY153 was obtained by the plasmid shuffle method from GWY151. GWY154 was obtained by transformation of GWY151 with linearized pPI8, followed by a plasmid shuffle. GWY155 was obtained by transformation of GWY150 with pPI9, followed by sporulation, tetrad dissection and selection for Leu^+ Trp^+ segregants. GWY156 was isolated from a cross between RSY271 and GWY152 and GWY157 from a cross between RSY271 and GWY154.

Generation of the vti1 Temperature-conditional Strains

Mutations in *VTI1* were generated by mutagenic PCR (Fromant *et al.*, 1995). The same set of primers used to generate pPI6 were used to PCR amplify a 1.8-kb fragment containing *VTI1* from pPI7 using Taq polymerase (Perkin Elmer-Cetus Corp., Norwalk, CT) under standard conditions except for the presence of 0.5 mM $MnCl_2$. The

Table 2. *S. cerevisiae* strains used in this work

Strain	Genotype	Source
RSY255	*MATα ura3-52 leu2-3,-112*	R. Schekman
RSY271	*MATα sec18-1 ura3-52 his4-612*	R. Schekman
RSY272	*MAT***a** *sec18-1 ura3-52 his4-612*	R. Schekman
GWY30	*MAT***a**/α *ura3-52/ura3-52 leu2Δ1/leu2Δ1 trp1Δ63/trp1Δ63*	This laboratory
GWY147	*MATα ura3-52 his3Δ200 leu2-3,-112 trp1Δ901 sft1Δ::LEU2 psft1-1* (CEN, HIS3)	H. Pelham
GWY150	*MAT***a**/α *ura3-52/ura3-52 leu2Δ1/leu2Δ1 trp1Δ63/trp1Δ63 vti1Δ::LEU2/VTI1*	This study
GWY151	*MAT***a** *ura3-52 leu2Δ1 trp1Δ63 vti1Δ::LEU2 pPI2* (2μ, *URA3*, *VTI1*)	This study
GWY152	*MAT***a** *ura3-52 leu2Δ1 trp1Δ63 vti1Δ::LEU2 pPI6* (2μ, *URA3*, P_{GAL}-*GST-VTI1*	This study
GWY153	*MAT***a** *ura3-52 leu2Δ1 trp1Δ63 vti1Δ::LEU2 pPI7* (*CEN*, *TRP1*, P_{CUP}-*HA-VTI1*)	This study
GWY154	*MAT***a** *ura3-52 leu2Δ1 vti1Δ::LEU2* P_{CUP}-*HA-VTI1*	This study
GWY155	*MATα ura3-52 leu2Δ1 trp1Δ63 vti1Δ::LEU2 pPI9*(*CEN*, *TRP*, P_{GAL}-*HA-VTI1*)	This study
GWY156	*MATα ura3-52 trp1Δ63 vti1Δ::LEU2 sec18-1 pPI6* (2μ, *URA3*, P_{GAL}-*GST-VTI1*)	This study
GWY157	*MATα ura3-52 vti1Δ::LEU2 sec18-1* P_{CUP}-*HA-VTI1*	This study
GWY158	*MAT***a** *ura3-52 leu2Δ1 trp1Δ63 vti1Δ::LEU2 pPIts3* (*CEN*, *TRP1*, P_{CUP}-*HA-vti1-21*)	This study
GWY159	*MAT***a** *ura3-52 leu2Δ1 trp1Δ63 vti1Δ::LEU2 pPIts4* (*CEN*, *TRP1*, P_{CUP}-*HA-vti1-22*)	This study
GWY160	*MAT***a** *ura3-52 leu2Δ1 trp1Δ63 vti1Δ::LEU2 pPIts6* (*CEN*, *TRP1*, P_{CUP}-*HA-vti1-23*)	This study
GWY161	*MAT***a** *ura3-52 leu2Δ1 trp1Δ63 vti1Δ::LEU2 pPIts7* (*CEN*, *TRP1*, P_{CUP}-*HA-vti1-24*)	This study
GWY162	*MAT***a** *ura3-52 leu2Δ1 trp1Δ63 vti1Δ::LEU2 pPIts16* (*CEN*, *TRP1*, P_{CUP}-*HA-vti1-25*)	This study

PCR product was digested with *Sac*I and *Xho*I and ligated into similarly digested pYATAG200. GWY151 [*vti1Δ::LEU2*, pPI2] cells were transformed with the resulting plasmid library, and transformants were selected on SC-Trp plates at 25°C. Trp^+ colonies were replica plated onto plates containing 5-fluoroorotic acid, which selects against *URA3*-containing plasmids (Boeke *et al.*, 1984), to remove the plasmid bearing unmutagenized *VTI1*. 5-Fluoroorotic acid-resistant colonies were replica plated to YPD and incubated at either 25°C or 37.5°C. Temperature-conditional mutants (GWY158 through GWY162) that grew at 25°C but not 37.5°C were isolated.

Subcellular Fractionation

A 100-ml culture of GWY154 expressing HA-Vti1p was grown in YPD to an OD_{600} of 0.6. The cells were collected by centrifugation, washed in distilled water, and resuspended at 30 OD/ml in ice-cold lysis buffer [20 mM HEPES/KOH, pH 7.0, 100 mM KOAc, 2 mM $Mg(OAc)_2$, 1 mM dithiothreitol (DTT), 2 mM EGTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 mM 1,10-phenanthroline, 2 μM pepstatin A, 2 μg/ml aprotinin, 0.5 μg/ml leupeptin]. One-half sample volume of acid-washed glass beads (0.45 mm diameter, Thomas Scientific, Swedesboro, NJ) was added to the cell suspension in a glass tube, which was then vortexed five times for 30 s with 1-min incubations on ice between each burst. The crude lysate was centrifuged at 500 × *g* for 5 min at 4°C in a Sorvall SA600 rotor to remove unlysed cells. The supernatant (Lysate) was diluted to 5 mg protein/ml in lysis buffer and centrifuged at 10,000 × *g* for 15 min at 4°C in the same rotor to generate supernatant (S10) and pellet (P10) fractions. The S10 was then centrifuged at 150,000 × *g* for 60 min at 4°C in a Beckman TLA100.2 rotor to generate high-speed supernatant (S150) and pellet (P150) fractions.

For the membrane extraction experiments (Figure 3B), the lysate was treated on ice for 15 min with lysis buffer or lysis buffer containing either 1% Triton X-100, 0.1 M Na_2CO_3, final pH 11.5, or 1 M NaCl and then separated into S150 and P150 fractions by centrifugation as above.

The sucrose flotation gradient was constructed as follows. Clarified glass bead cell lysate (0.5 ml) from strain GWY154 made in D_2O buffer (20 mM HEPES/KOH, pH 7.0, 100 mM KOAc, 1 mM DTT, 1 mM PMSF, 5 mM 1, 10-phenanthroline, 2 μM pepstatin A, 2 μg/ml aprotinin, 0.5 μg/ml leupeptin in D_2O) was mixed with two volumes of 60% sucrose in D_2O buffer, placed at the bottom of a 12-ml SW41 (Beckman Instruments, Fullerton, CA) tube, and overlaid with 1.5 ml each of 45%, 37%, 33%, 29%, 23%, 17%, and 10% sucrose, respectively, in D_2O buffer. The gradient was centrifuged at 150,000 × *g* for 24 h in a SW41 rotor. Twenty-four 0.45-ml fractions were collected from the top of the tube and analyzed using immunoblotting. Western blots were quantified by analyzing films with a scanning densitometer (Microtek International, Taiwan) and National Institutes of Health Image software.

Separations of the S10 fraction by sucrose density centrifugation was performed as described (Becherer *et al.*, 1996).

Immunofluorescence and Immunoblotting

Indirect immunofluorescence was performed as described by Hardwick and Pelham (1992) with some modifications. Fifty milliliters of log phase GWY154 cells grown in YPD were fixed for 10 min by addition of 1/10th final volume of 37% formaldehyde to the culture. Cells were collected by centrifugation, resuspended in 40 mM KPi (pH 6.5), 0.5 mM $Mg(OAc)_2$, and 3.7% formaldehyde and incubated for 60 min. Cells were washed three times with 40 mM KPi (pH 6.5), once with 40 mM KPi (pH 6.5), and 1.2 M sorbitol and then spheroplasted in the same buffer containing 0.05 mg/ml Zymolyase 100T (Seikagako Kogyo Co., Tokyo, Japan) for 30 min at 30°C. The spheroplasts were washed with 40 mM KPi (pH 6.5) and 1.2 M sorbitol, pipetted onto polylysine-coated microscope slides, and allowed to adhere for 10 min. Slides were plunged into −20°C methanol for 6 min, transferred to −20°C acetone for 1 min, air dried, and incubated in blocking solution [phosphate-buffered saline (PBS), 0.5% bovine serum albumin, 0.5% ovalbumin, 0.2% gelatin, 0.1% Tween 20) for 60 min. Samples were incubated for 2 h at room temperature in a 1:1000 dilution of the anti-Kar2p polyclonal antibodies (from R. Schekman), a 1:200 dilution of the affinity-purified antiSed5p antibodies (Lupashin and Waters, 1997), or a 1:200 dilution of the 12CA5 anti-HA epitope monoclonal antibody (Wilson *et al.*, 1984) in blocking solution. The samples were washed four times with blocking solution, incubated for 1 h at room temperature in a 1:200 dilution of fluorescein isothiocyanate-conjugated goat anti-rabbit or goat anti-mouse immunogloblin (ICN/Cappel, Costa Mesa, CA) in blocking solution, washed again with PBS, and mounted in 90% glycerol and 0.1% *p*-phenylenediamine. Images were obtained using a Bio-Rad MRC600 laser scanning microscope.

Samples for immunoblotting were separated by SDS-PAGE (12% acrylamide, except where noted), electrotransferred to nitrocellulose, and probed with the appropriate primary antibodies according to standard protocols (Harlow and Lane, 1988). Monoclonal antibodies against the HA epitope (12CA5) and Dpm1p (Molecular Probes, Eugene, OR) were used at a dilution of 1:2000. Affinity-purified antibodies against Sed5p, Sec22p, Sly1p, and GST (Lupashin and Waters, 1997) and sera against gp400/hsp150 (Lupashin *et al.*, 1992), Kar2p (from R. Schekman), Pep12p (from E. Jones), Sso1p (from P. Brennwald), and Ykt6p were all used at a dilution of 1:2000. Horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse secondary antibody (Bio-Rad, Hercules, CA) were used at a dilution of 1:3000. Immunoblots were developed with the Renaissance chemiluminescent detection kit (DuPont New England Nuclear, Boston, MA).

Metabolic Labeling and Immunoprecipitation of CPY and Invertase

Strains were grown at room temperature in SC-MET media to an OD_{600} of 0.5. For each strain, 6 OD units of cells were collected by centrifugation, resuspended in 1.0 ml of SC-MET media, and preshifted to 38°C for 30 min. Cells were radiolabeled for 5 min with 100 μCi of Tran^{35}S-label (ICN Radiochemicals, Irvine, CA), followed by a 30-min chase initiated by the addition of 1/25th volume of chase mix (250 mM cysteine, 250 mM methionine). The reactions were stopped by addition of an equal volume of 20 mM sodium azide and placed on ice for 10 min. Cell lysis and immunoprecipitation of CPY were performed as described previously (Sapperstein *et al.*, 1996).

For the experiment described in Figure 7B, cells were grown at room temperature in SC-MET media to an OD_{600} of 0.5, collected by centrifugation, resuspended in 1.0 ml of SC-MET media with 0.1% glucose, incubated for 30 min at room temperature, and then for 30 min at 25°C or 38°C as indicated. The cells were labeled as above, chased for 20 min, and spheroplasted for 20 min by addition of 1/3 final volume of 0.3 mg/ml Zymolyase 100T in 2.8 M sorbitol. The spheroplasts were isolated by centrifugation at 3000 × *g* for 5 min and lysed on ice in 1 ml of 0.2 M NaOH and 0.5% β-mercaptoethanol. Proteins from the lysed spheroplasts and the spheroplasting media were precipitated with 10% trichloroacetic acid, resuspended in 2% SDS and 50 mM Tris-Cl (pH 8.0) and diluted in 60 mM Tris-HCl (pH 7.4), 190 mM NaCl, 6 mM EDTA, and 1.25% Triton X-100. Invertase was immunoprecipitated from the lysed spheroplasts (I, for intracellular) and from the spheroplasting media (E, for extracellular). To detect outer chain mannosyl residues, the radiolabeled invertase and CPY were immunoprecipitated, eluted, and reimmunoprecipitated with anti α-1,6-mannose linkage-specific antisera as described (Franzusoff and Schekman, 1989).

For the experiment described in Figure 6, cells were grown at 30°C in 1% glucose/1% galactose containing SC-MET media to an OD_{600} of 0.2, after which they were transferred to SC-MET media containing 5% glucose or 0.1% glucose/2% galactose and allowed to grow for 6 h. Cells were pulse labeled for 5 min and chased in the media containing 5% glucose for the indicated time periods as

described above. Antibodies against invertase, CPY, and α-1,6-mannose-specific linkage were kindly provided by M. Rose (Princeton University) and R. Schekman (University of California, Berkeley, CA).

Suppression Analysis

Strains GWY158 to GWY162 were transformed with the following plasmids: pPI2, pRS426, pJG103, pSFN2d, pSK60, pSK54, p*SED5*, p*SFT1*, pVLhPI1, and pVLmPI2. Purified transformants were grown to stationary phase at 23°C in synthetic media. Suppression was assessed by inoculation of cells from these saturated cultures to YPD or YPGal plates and examination of growth after incubation for 3 d at 37.5°C.

Purification of Recombinant Proteins from E. coli

GST and GST-Sec17p were expressed in XL1-Blue from pGEX-2T and pGEX-Sec17, respectively. Protein expression was induced in 500-ml cultures grown at 37°C to an OD_{600} of 0.5 by the addition of isopropylthiogalactoside to 1.0 mM. After 4 h the cells were harvested (7000 × *g* for 10 min at 4°C in a Sorvall SA600 rotor), resuspended in 25 ml PBS with 0.1 mg/ml lysozyme, incubated on ice for 15 min, and lysed by sonication with three 30-s bursts at half-maximum intensity with a Branson Sonifer 450 (VWR Scientific Products Co., Bridgeport, NJ) separated by 1-min incubations on ice. Triton X-100 was added to 1%, the lysates were rocked at 4°C for 30 min, clarified by centrifugation (12,000 × *g* for 10 min at 4°C in an SA600 rotor), and incubated with 2 ml of glutathione-Sepharose 4B beads (Pharmacia Biotech) with gentle end-over-end mixing for 2 h at 4°C. The beads were washed twice with 25 ml PBS/1% Triton X-100 and loaded into a 2-ml chromatography column. The fusion proteins were eluted with 5 ml of 20 mM reduced glutathione in 50 mM Tris-Cl (pH 8.0) and dialyzed against PBS.

In Vitro GST-Sec17p Binding Reaction

GWY157 spheroplasts were shifted to 37°C for 1 h and lysed in the presence of 1% Triton X-100, as described above for subcellular fractionation. Solubilized protein (0.5 mg) was incubated with purified GST or GST-Sec17p (2 μg each) in buffer B (20 mM HEPES-KOH, 150 mM KOAc, 0.5 mM DTT, 0.05% Tween 20) for 1 h at 4°C and centrifuged at 15,000 × *g* for 15 min to remove aggregated material. The supernatant was incubated for 1 h at 4°C with 40 μl of glutathione-Sepharose 4B beads equilibrated with buffer B. The beads were washed three times with buffer B and bound proteins were eluted with 50 μl of 10 mM reduced glutathione in 50 mM Tris-Cl (pH 7.5), separated by SDS-PAGE (10% acrylamide), and immunoblotted with anti-Kar2p, anti-HA epitope, or anti-Sed5p antibodies.

Analysis of GST-Vti1p Interactions in Whole-Yeast Detergent Extracts

GWY156 cells were grown at room temperature in YPGlu/Gal media to an OD_{600} of 0.5–1.0, collected by centrifugation, resuspended in fresh YPGlu/Gal media, incubated at the permissive (25°C) or restrictive (37°C) temperature for 1 h, and then transferred to ice. Cells were lysed with glass beads in the presence of 1% Triton X-100 as described above. Clarified detergent lysates (~2 mg of protein) were incubated for 1 h at 4°C with 300 μl of glutathione-Sepharose 4B equilibrated with buffer B. The beads were transferred into 1-ml chromatography columns, washed with buffer B, and bound proteins were eluted with 10 mM reduced glutathione in 50 mM Tris-Cl (pH 7.5). Fractions (300 μl each) were collected, proteins were separated by SDS-PAGE followed by immunoblotting with anti-GST, anti-Sed5p, anti-Sso1p, anti-Ykt6p, or anti-Sec22p antibodies.

Immunoprecipitation from Detergent Extracts of Yeast Spheroplasts

GWY157 cells were grown to OD_{600} of 0.5 in YPD media. Cultures (50 ml) were centrifuged for 5 min at 3500 rpm in a Beckman TJ-6 centrifuge, resuspended in 100 mM Tris-SO_4 (pH 9.4)/10 mM DTT at 5 OD/ml, and placed on ice for 10 min. The cells were collected by centrifugation as above, resuspended in the same volume of YPD, 0.8 M sorbitol, and 25 mM HEPES/KOH (pH 7.0). Zymolyase 20T (Seikagak Kogyo Co.) was added to 100 μg/ml, and the cells were incubated at 25°C for 30 min. Spheroplasts were washed in YPD, 0.8 M sorbitol, and 25 mM HEPES/KOH (pH 7.0), resuspended in the same buffer, and incubated for 20 min at 25°C with gentle rocking. The regenerated spheroplasts were collected by centrifugation, resuspended in 20 mM HEPES/KOH (pH 7.0), 200 mM KCl, 1 mM DTT, 1 mM $MgCl_2$, 1 mM PMSF, 0.5 mM 1:10 phenanthroline, 2 μM pepstatin A, 0.5 μg/ml leupeptin, 2 μg/ml aprotinin, and 2% Triton X-100 (Søgaard *et al.*, 1994) at 10 OD/ml, and lysed by repeated pipetting. Debris was removed by centrifugation at 10,000 × *g* for 10 min at 4°C in an SA600 rotor and the cleared extracts were transferred to fresh tubes. Immunoprecipitations were done essentially as described (Sapperstein *et al.*, 1996; Lupashin and Waters, 1997) except that each reaction contained 500 μg of extract protein and 40 μl of a 50% slurry of protein A-Sepharose (Pharmacia Biotech) bearing affinity-purified antibodies to Sed5p, Sec22p, or Sly1p that had been crosslinked to the beads with dimethylpimelimidate as described (Harlow and Lane, 1988).

RESULTS

Identification of Novel Yeast SNARE Proteins

In an effort to identify the full complement of yeast SNARE proteins, we used known SNAREs as query sequences for BLAST (Altschul *et al.*, 1990) and FASTA (Pearson and Lipman, 1988) searches of the complete *S. cerevisiae* genome database (Cherry, Adler, Ball, Dwight, Chervitz, Jia, Juvik, Weng, and Botstein, *Saccharomyces* Genome Database, http://genome-www.stanford.edu/*Saccharomyces*). Predicted proteins were considered SNAREs if 1) they yielded significant BLAST or FASTA scores (p value $< 10^{-2}$); 2) the predicted topology corresponded to a type II integral membrane protein with a very short lumenal domain; and 3) they bore heptad repeats capable of forming a coiled coil in the membrane-proximal region as predicted by the COILS algorithm [with a window size of 21 and the MTDIK matrix (Lupas *et al.*, 1991)]. The Sec9p (SNAP-25 related) group (Weimbs *et al.*, 1997) and Ykt6p (Søgaard *et al.*, 1994; McNew *et al.*, 1997) are exceptions to these criteria because they do not possess transmembrane domains. The results of the searches are presented in Figure 1A as a dendrogram. We found one new member of the Sec9p group of sequences (ORF YMR017w), two new potential members of the Sso1p-Sed5p (t-SNARE) family (ORFs YOL018c and YDR468c), and two new members of the Snc1p/Bet1p (v-SNARE) protein family (ORFs YMR197c and YHL031c).

We have analyzed ORF YMR197c, a new member of the putative v-SNARE family, in detail. Comparison of the YMR197c predicted protein sequence using the GAP and BESTFIT programs from the GCG sequence

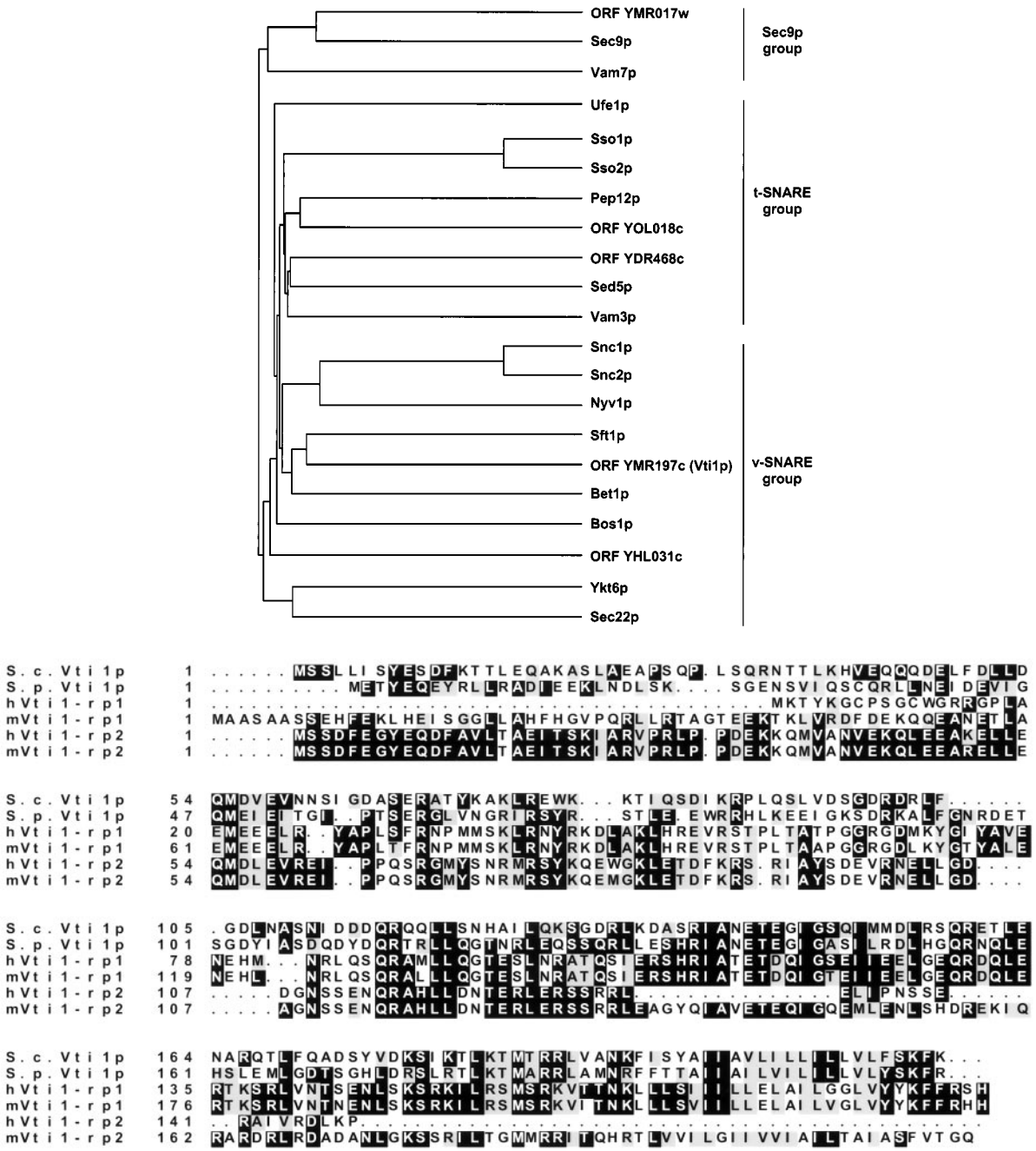


Figure 1. Sequence analysis of yeast SNARE proteins. (A) Nearest-neighbor dendrogram of known and putative yeast SNARE molecules. Common protein names or ORF numbers are indicated. The dendrogram was generated with the PILEUP program of the GCG sequence analysis package. (B) Multiple sequence alignment of *S. cerevisiae* Vti1p (denoted S.c.Vti1p), and Vti1p-related proteins from *S. pombe* (S.p.Vti1), human (hVti1-rp1 and hVti1-rp2), and mouse (mVti1-rp1 and mVti1-rp2). Blackened boxes denote identical residues, and gray shading denotes conserved residues. Optimal alignment was produced with the PILEUP program. S.p.Vti1 is encoded by cDNA clone D89116 and mVti1-rp1, mVti1-rp2, hVti1-rp1, and hVti1-rp2 are encoded by EST AA056932, EST AA016379, EST AA105524, and EST T70362, respectively. In some cases, primary sequence data were obtained from the Washington University Genome Sequencing Center and used to extend the EST sequences to encompass the complete ORFs.

analysis package revealed similarity (32% identity) with the previously described yeast intra-Golgi retrograde v-SNARE Sft1p (Banfield *et al.*, 1995). The next nearest relative of YMR197c, at 23% identity, is the

Table 3. Relative sequence identity of Vti1p-related proteins[a]

	S.p.Vti1p	hVti1-rp1	hVti1-rp2	mVti1-rp1	mVti1-rp2
S.c.Vti1p	38	29	35	27	32
S.p.Vti1p		33	36	30	34
hVti1-rp1			21	87	27
hVti1-rp2				27	90
mVti1-rp1					30

[a] The percentage of amino acid identity between yeast, human, and mouse Vti1p isoforms are displayed. Optimal alignments were produced by the BESTFIT program from the GCG package.

ER-to-Golgi v-SNARE, Bet1p/Sly12p (Newman *et al.*, 1990; Dascher *et al.*, 1991). When we extended our analysis beyond *S. cerevisiae*, we found that the mammalian v-SNARE GOS-28/GS28, which is involved in ER-to-Golgi and/or intra-Golgi traffic (Nagahama *et al.*, 1996; Subramaniam *et al.*, 1996), is also related (21% identity) to YMR197c. We originally termed this new SNARE Igs1p (intra-Golgi SNARE 1 protein), based on it role in intracellular protein traffic. After submission of this work, however, Fischer von Mollard *et al.* (1977) published a report on the same protein. Thus, we have adopted the name they used, Vti1p.

The predicted protein sequence of Vti1p comprises 217-amino acid residues with a predicted molecular mass of 24.7 kDa (Figure 1B). GenBank and dbEST database searches with Vti1p indicated that several proteins are related to Vti1p: a putative protein encoded by *Schizosaccharomyces pombe* cDNA clone D89116, two hypothetical mouse proteins, mVti1-rp1 (mouse Vti1p-related protein 1) and mVti1-rp2, and two hypothetical human proteins, hVti1-rp1 and hVti1-rp2. The aligned amino acid sequences of the putative Vti1p-related proteins are shown in Figure 1B, and their pairwise sequence identities are shown in Table 3. The human and mouse orthologous pairs of proteins (i.e., the hVti1-rp1/mVti1-rp1 pair and the hVti1-rp2/mVti1-rp2 pair) are very similar (87 and 90% identity, respectively). The human paralogues are more distantly related to each other and to their yeast orthologues. The existence of two distinct mammalian homologues is interesting since a similar situation has been demonstrated for the yeast v-SNARE Sec22p (Hay *et al.*, 1996, 1997; Paek *et al.*, 1997). These observations may reflect the difference between a more streamlined yeast secretory pathway and the more highly elaborated mammalian protein transport machinery. It is also possible that the yeast protein is involved in more than one transport step, as recently proposed (Fischer von Mollard *et al.*, 1997), whereas in mammalian eukaryotic cells these distinct functions could be performed by related, yet distinct proteins.

VTI1 Encodes an Essential Yeast Golgi Membrane Protein

To determine whether *VTI1* is an essential gene, the viability of haploid *igs*Δ disruptants was examined. The *VTI1* ORF was replaced by the sequence of *LEU2* in a diploid strain, the presence of both the wild-type and disrupted *VTI1* alleles in the heterozygous diploid was confirmed by PCR, the strain was sporulated, and tetrads were dissected. All 24 tetrads examined displayed 2^+:2^- segregation for viability at 25°C (Figure 2). The same 2^+:2^- segregation pattern was evident when the spores were incubated at 30°C (our unpublished results). Microscopic examination of the inviable segregants revealed microcolonies of ~20 cells, indicating that the *vti1*Δ spores can germinate. All viable spores were Leu^-, and therefore contained the wild-type *VTI1* allele. These data indicate that *VTI1* is essential for vegetative growth. Transformation of the *vti1*Δ/VTI1 heterozygous diploid strain with a low-copy number centromere- (CEN) based plasmid-bearing VTI1 prior to sporulation and dissection complemented the lethality of the *vti1*Δ haploid segregants (our unpublished results). In addition, the vti1Δ strain could be rescued by expression of HA epitope-tagged Vti1p (HA-Vti1p) or a GST-Vti1p fusion protein (GST-Vti1p) expressed from CEN-based plasmids (our unpublished results), indicating that these fusion pro-

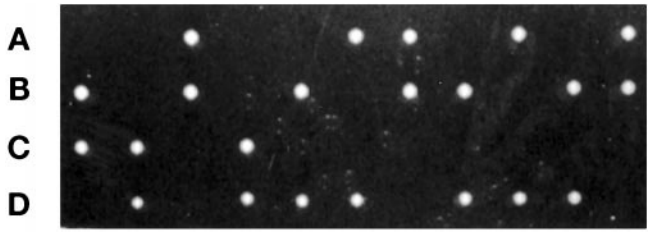


Figure 2. *VTI1* is an essential gene. The *VTI1/vti1Δ::LEU2* heterozygous deletion strain was sporulated, tetrads were dissected, and the segregants incubated at 25°C for 4 d on YPD plates. All viable segregants were *Leu*$^-$. A–D indicate the segregants within each tetrad.

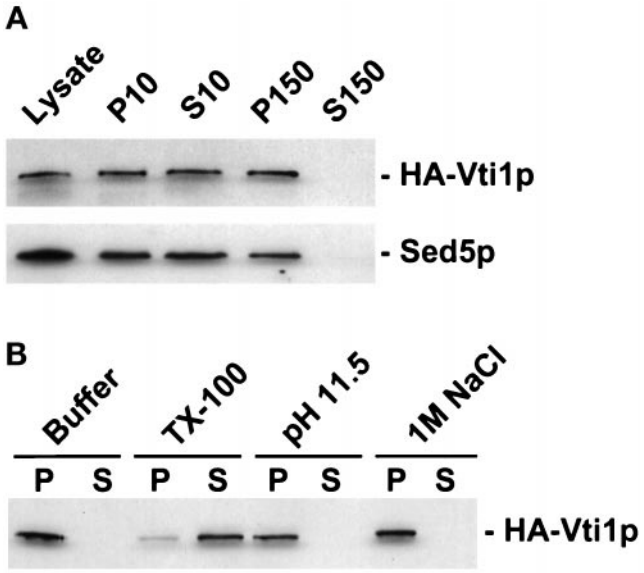


Figure 3. Vti1p is membrane associated. (A) The GWY154 strain (*HA-VTI1*) was grown to logarithmic phase, lysed with glass beads, and centrifuged at 500 × *g* for 3 min. The supernatant (Lysate) was centrifuged at 10,000 × *g* for 15 min, yielding the P10 pellet and S10 supernatant fractions, and the S10 was centrifuged again at 150,000 × *g* for 60 min to generate the P150 pellet and S150 supernatant fractions. Aliquots (derived from 0.2 OD_{600} units of cells) of each fraction were separated by SDS-PAGE and immunoblotted with the anti-HA epitope monoclonal antibody or anti-Sed5p affinity-purified antisera. (B) Aliquots of lysate were extracted with buffer, 1% Triton X-100, 0.1 M Na_2CO_3, or 1 M NaCl. After centrifugation at 150,000 × *g*, HA-Vti1p was detected in pellet (P) and supernatant (S) fractions via immunoblotting.

teins, which were used in experiments described below, are functional.

We used the *vti1*Δ strain carrying genomic HA-Vti1p to initiate a biochemical analysis of Vti1p. Immunoblot analysis with an anti-HA epitope monoclonal antibody (Wilson *et al.*, 1984) specifically recognized one protein with an apparent molecular mass of ~28 kDa, which was absent in the wild-type parent strain (our unpublished results). The intracellular localization of HA-Vti1p was examined by subcellular fractionation via differential centrifugation (Figure 3A). HA-Vti1p-expressing cells were lysed, unbroken cells were removed, and the lysate was centrifuged at 10,000 × *g*, yielding pellet (P10) and supernatant (S10) fractions. The S10 was further centrifuged at 150,000 × *g* to generate high-speed pellet (P150) and supernatant (S150) fractions. The proteins in each fraction were resolved by SDS-PAGE and immunoblotted to localize HA-Vti1p and, as a control, the *cis*-Golgi t-SNARE Sed5p. HA-Vti1p behaves as an integral or peripheral membrane protein, partially sedimenting at 10,000 × *g* and completely sedimenting at 150,000 × *g* (Figure 3A).

To test the prediction that Vti1p is an integral membrane protein, we extracted cell lysates under several conditions that perturb membrane proteins and examined the sedimentation behavior of Vti1p (Figure 3B). Neither 1 M NaCl nor 0.1 M Na_2CO_3 (pH 11.5) treatments, which are expected to release peripheral membrane proteins (Fujiki *et al.*, 1982), extracted Vti1p from membranes, whereas 1% Triton X-100, which solubilizes membranes, did solubilize Vti1p, suggesting that Vti1p is an integral membrane protein. Furthermore, fusion of myc epitope-tagged yeast vacuolar alkaline phosphatase to the carboxyl-terminus of Vti1p produced a protein of ~90 kDa that was glycosylated, as shown by interaction with concanavalin A-Sepharose beads (our unpublished results), suggesting that the carboxyl-terminus of Vti1p is lumenally oriented.

To further examine the intracellular location of HA-Vti1p, a cell lysate was subjected to isopycnic flotation through a sucrose/D_20 density gradient, the gradient was fractionated, and aliquots were subjected to immunoblotting (Figure 4A). Markers of the endoplasmic reticulum (Dpm1p), *cis*-Golgi (Sed5p), and prevacuolar (Pep12p) compartments were used as internal controls. HA-Vti1p was present in two peaks whose distribution was most like that of the *cis*-Golgi marker Sed5p. The more buoyant HA-Vti1p peak (fraction 13) also coincided with the prevacuolar marker Pep12p (Becherer *et al.*, 1996).

To better resolve the *cis*-Golgi from the prevacuolar compartment, we separated a 10,000 × *g* supernatant of lysed spheroplasts by sedimentation through a shallow sucrose gradient (Figure 4B). Under these conditions the Sed5p and Pep12p peaks were separated. HA-Vti1p could be found in both the Sed5p and the Pep12p regions of the gradient. Recently, Fischer von Mollard *et al.* (1997) have shown colocalization of Vti1p with both prevacuolar and TGN markers.

We also carried out an immunofluorescence study in *S. cerevisiae* cells expressing HA-Vti1p as the sole source of Vti1 protein. We observed a small number of punctate structures (Figure 5, Vti1p) that did not coincide with the plasma membrane or vacoule nor with nuclear envelope or ER, which were revealed by anti-Kar2p staining (Figure 5, Kar2p). The staining observed for Vti1p resembled that of Sed5p (Figure 5, Sed5p): numerous punctate structures were found throughout the cytoplasm.

Our observations, along with those of Fischer von Mollard *et al.* (1997), suggest that Vti1p is a type II integral membrane protein of the Golgi apparatus and the prevacuolar compartment.

Vti1p Is Required for Intra-Golgi Protein Transport

To investigate the function of Vti1p, we tested the effect of its depletion on cell growth and protein trans-

port. *HA-VTI1* was placed behind the galactose-inducible/glucose-repressible *GAL1* promoter (P_{GAL}) on a low-copy number CEN plasmid. *vti1*Δ cells bearing the P_{GAL}-*HA-VTI1* construct were grown in medium containing galactose, where they grew as rapidly as wild-type cells in the same media (our unpublished results), indicating that the HA-Vti1p was functional. However, when HA-Vti1p expression was repressed by transferring the cells to glucose-containing medium, the strain gradually ceased to grow (Figure 6A). To examine the role of Vti1p in protein transit through the secretory pathway, we analyzed the kinetics of maturation of vacuolar CPY 6 h after shift to high-glucose media, a time point when the level of HA-Vti1p was significantly reduced (Figure 6B), but growth was not inhibited (Figure 6A). CPY is a useful probe of secretory pathway function, because it is sequentially modified as it transits the secretory pathway: upon translocation into the ER, it receives N-linked oligosaccharides resulting in the p1 form, subsequent transport to the *medial* Golgi, and finally, to the vacuole, generates the p2 and mature (m) forms, respectively. Cells grown in the galactose-containing medium efficiently transported CPY to the vacuole; most of the CPY was matured within 10 min of synthesis, and no p1 or p2 forms remained after 40 min (Figure 6C, Galactose). In contrast, cells grown in glucose-containing medium, where the amount of HA-Vti1p was greatly reduced (Figure 6B), matured only about half of the CPY within 10 min of synthesis, and some p1 and p2 forms were still found after 40 min (Figure 6C, Glucose). These data suggest that movement of the ER/*cis*-Golgi p1 form of CPY to the compartments that generate the p2 (*medial*/*trans*-Golgi) and m forms (vacoule) is compromised by depletion of Vti1p activity. The persistence of both the p1 and p2 forms of CPY when the level of Vti1p is low suggests that transport is defective at multiple steps within the Golgi. This result, however, does not by itself allow us to determine whether Vti1p acts in anterograde or retrograde protein transport, since intracellular transport of the CPY is very sensitive to defects in either of these processes (Stevens *et al.*, 1982; Banfield *et al.*, 1995; Gaynor and Emr, 1997).

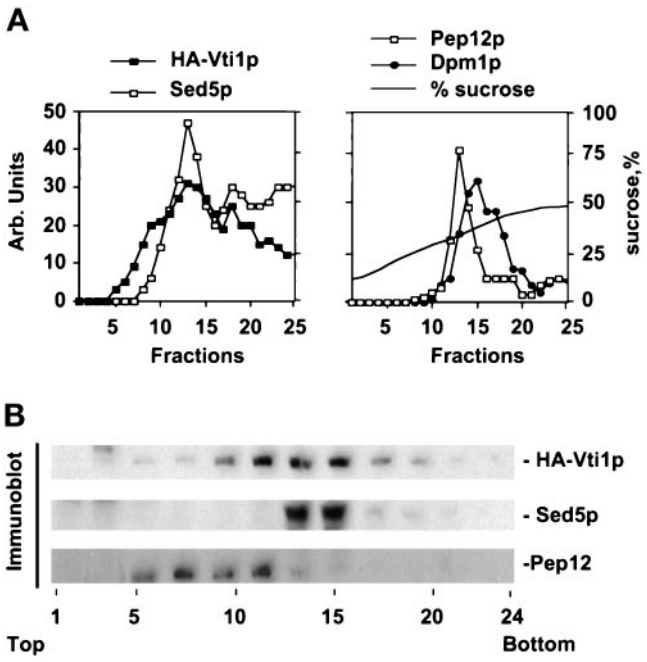


Figure 4. Vti1p partially colocalizes with the Golgi t-SNARE Sed5p and prevacuolar t-SNARE Pep12p on sucrose gradients. (A) A clarified glass bead cell lysate (500 × *g* supernatant) from GWY154 was mixed with two volumes of 60% sucrose/D_2O, placed at the bottom of a 10–45% sucrose step gradient, and subjected to centrifugation at 150,000 × *g* for 24 h. Fractions were collected and subjected to SDS-PAGE, and the locations of HA-Vti1p, the Golgi marker Sed5p, prevacuolar marker Pep12p, and ER marker Dpm1p were determined by immunoblotting. Sucrose density was measured with a refractometer. (B) Immunoblots of fractions from sucrose density centrifugation of the S10 fraction on a shallow sucrose gradient.

Golgi Function Is Disrupted in vti1 Temperature-Sensitive Mutants

To further analyze the role of Vti1p in intracellular protein transport, we prepared temperature-sensitive (ts) alleles of *HA-VTI1* by PCR-mediated mutagenesis (Fromant *et al.*, 1995) and studied their secretion phenotypes at the restrictive temperature. We obtained five ts mutants of *VTI1*, all of which ceased growth at 37.5°C but differed from each other by their growth rates at lower temperatures (e.g., *vti1–23* grows as well as wild type at 30°C, but very slowly at 20°C; *vti1–25* grows poorly at 30°C). All five ts mutants harbor approximately equivalent amount of HA-Vti1p after prolonged incubation at semipermissive conditions, suggesting that stability of the mutant proteins was not compromised (Figure 7D, lowest panel). We did note, however, that the electrophoretic mobility of some of the mutant forms of HA-Vti1p was slightly aberrant .

To test the effect of the ts mutations on the intracellular transport of CPY, we used *vti1*Δ cells bearing either *VTI1* or one of the *vti1* ts alleles on CEN plasmids. The cells were shifted to the restrictive temperature for 30 min, pulse labeled for 5 min, chased for 30 min, and CPY was immunoprecipitated and analyzed by SDS-PAGE and autoradiography (Figure 7A). After 5 min of pulse label, most of the CPY in the wild-type cells and the *vti1–21*, *vti1–22*, and *vti1–23* mutants was present as the ER/*cis*-Golgi p1 form with a small amount of the *medial*/*trans*-p2 form detectable; in *vti1–24* and *vti1–25*, the p1 form was found exclusively. After 30 min of chase, neither p1 nor p2 forms of CPY were detectable in the wild-type cells, since all

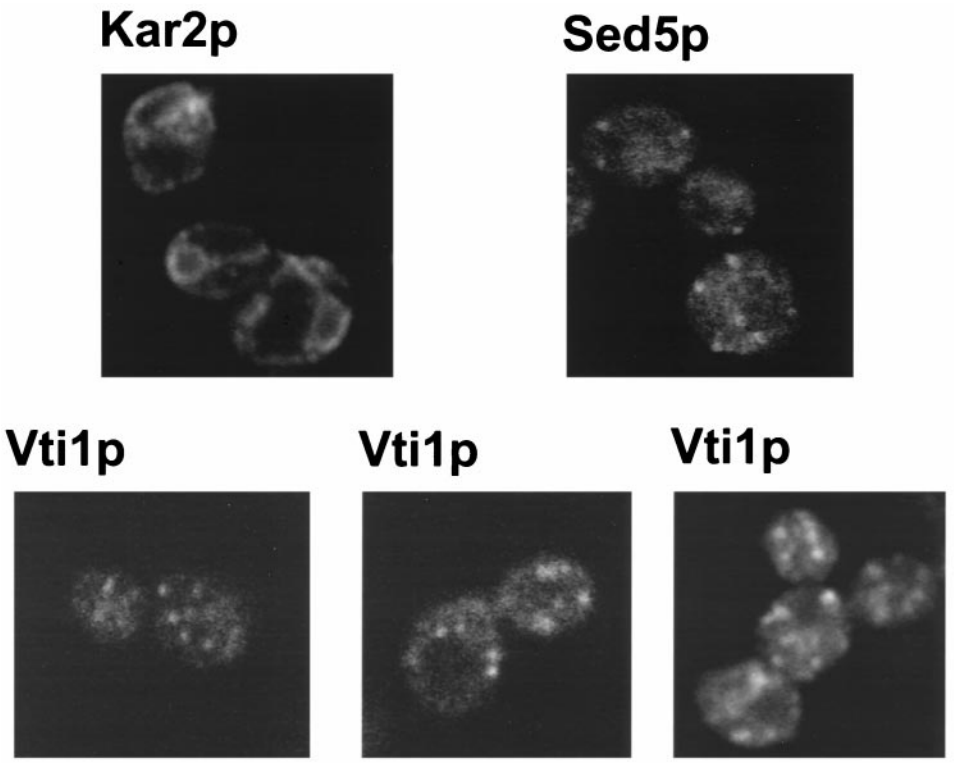


Figure 5. Vti1p is localized to punctate structures throughout the cytoplasm. The indicated proteins were detected in strain GWY154 by indirect immunofluorescence.

of the CPY had been processed to the mature (m) form. In the *vti1–21*, *vti1–22*, and *vti1–23* strains, CPY was detected after the chase predominantly in the *medial/trans*-Golgi p2 form with a small amount of p1 form present. In contrast, in the *vti1–24* and *vti1–25* strains the ER/*cis*-Golgi p1 form of CPY was predominant, with a small amount of p2 form present (Figure 7A). Some of the p1 and most of the p2 form of CPY that accumulated in *vti1–24* mutants was modified with Golgi-specific α-1,6-mannose glycosylation (Figure 7B), suggesting that delivery of CPY from the ER to the Golgi was not significantly compromised. All *vti1* mutants were able to deliver CPY to the vacuole at the permissive temperature (our unpublished results).

The ability of the *vti1* ts strains preincubated at the restrictive temperature to accumulate the p1 and p2 Golgi forms of CPY, in conjunction with their inability to generate the vacuolar mature (m) form suggested that transport of CPY through the Golgi toward the vacuole is compromised. In addition, the reduced intensity of the p2 form after the chase relative to the intensity of the p1 form before the chase suggested that some CPY was either degraded intracellularly or secreted from the cells. We found that all tested *vti1* ts mutants secreted some p2 CPY at the semirestrictive temperature (Figure 7D). We have observed similar missorting of CPY in mutants of previously described intra-Golgi v-SNAREs Sft1p (Banfield *et al.*, 1995) and Ykt6p (Søgaard *et al.*, 1994; McNew *et al.*, 1997) when they were incubated at semirestrictive conditions (our unpublished results).

To further test the effect of the ts mutations on protein secretion, we analyzed the transport of the yeast periplasmic enzyme invertase. Invertase synthesis was derepressed for 30 min on low-glucose media prior to the 30-min shift to the *vti1* ts restrictive temperature, the cells were pulse labeled for 5 min, chased for 20 min, spheroplasted, and then separated into intracellular (I) and extracellular (E) fractions from which invertase was immunoprecipitated. We found that invertase was efficiently secreted from the *vti1–22* and *vti1–24* mutants at both the permissive and restrictive temperatures (Figure 7C) and only a small fraction of protein was retained intracellularly (about 10% or 35% of the total invertase at the restrictive temperature, respectively). Invertase secreted by both mutants at the restrictive temperature migrated faster in SDS-PAGE than the hyperglycosylated forms secreted from wild-type cells or from mutant cells at 25°C (Figure 7C), suggesting that it was underglycosylated. To observe post-ER glycosyl modifications in the *vti1–22* and *vti1–24* strains, we reimmunoprecipitated the invertase with an antisera against the α-1,6-mannose linkages, which are added in the *cis*- and *medial*-Golgi (Franzusoff and Schekman, 1989). Both the hyperglycosylated forms secreted at 25°C and, to a lesser degree, the intermediate form produced at 38°C were modified with α-1,6–mannose (Figure 7C). These

results suggest that invertase export, unlike CPY traffic, was not seriously affected in the *vti1* ts mutants.

To examine secretion in the *vti1* ts mutants further, we harvested the cells, transferred them to fresh media, shifted the mutant strains to a semipermissive temperature (35°C) for 5 h, and analyzed the cells (I) and the extracellular medium (E) for several protein markers (Figure 7D). Similar to invertase, we found that secretion of gp400/hsp150, an *O*-glycosylated protein that is primarily localized extracellularly (Lupashin *et al.*, 1992; Russo *et al.*, 1992), occurred efficiently in all of the *vti1* ts strains. In wild-type cells, gp400/hsp150 is secreted as a heteroligomer (7C and Lupashin *et al.*, 1992) as a result of partial proteolytic cleavage by the Kex2p protease (Russo *et al.*, 1992). We noted that gp400/hsp150 secreted by the *vti1* ts strains migrated on SDS-PAGE as a single band, which may indicate defects in the Kex2p processing, glycosylation, or both. The lack of Kar2p in the media (Figure 7D) also suggests that HDEL-dependent retrieval of the ER luminal proteins (Dean and Pelham, 1990) from the *cis*-Golgi to the ER was not seriously affected by the *vti1* mutations. These results indicate that a partial loss of Vti1p function compromises traffic of CPY without seriously affecting the transit of invertase or gp400/hsp150 to the cell surface.

Vti1p Is a Putative Intra-Golgi Retrograde v-SNARE

SNAREs, SNAP receptors by definition, are known to physically interact with SNAP molecules, which facilitate subsequent binding of NSF (Clary *et al.*, 1990; Whiteheart *et al.*, 1992; Söllner *et al.*, 1993a,b). Yeast SNAP is encoded by the *SEC17* gene (Griff *et al.*, 1992) and NSF is encoded by *SEC18* (Eakle *et al.*, 1988; Wilson *et al.*, 1989). To examine the possibility that Vti1p interacts with Sec17p, we incubated purified bacterially expressed GST or GST-Sec17p fusion protein with a cell lysate from a yeast strain expressing HA-Vti1p. GST and GST-Sec17p, as well as any associated proteins, were harvested by glutathione-Sepharose affinity. Bound proteins were eluted, separated by SDS-PAGE, transferred to nitrocellulose, and stained with Ponceau S to ensure that equivalent amounts of GST and GST-Sec17p were recovered from the binding reaction (our unpublished results). The immunoblots (Figure 8) were developed with anti-HA epitope antibodies to detect HA-Vti1p, as well as with anti-Sed5p (as a positive control) and anti-Kar2p (as a negative control) antibodies. Neither HA-Vti1p, Sed5p, nor Kar2p bound detectably to GST. However, both HA-Vti1p and Sed5p were found to be associated with GST-Sec17, whereas Kar2p was not. These data strongly indicate that Vti1p is a bona fide SNARE.

Since Sed5p and Vti1p are both localized to the Golgi (at least partially), we tested whether they can interact with each other in a *SEC18*-sensitive manner. Lysates were made from a *sec18* strain expressing HA-Vti1p after incubation at the permissive and restrictive temperatures, Sed5p was immunoprecipitated, and precipitate was probed for the presence of

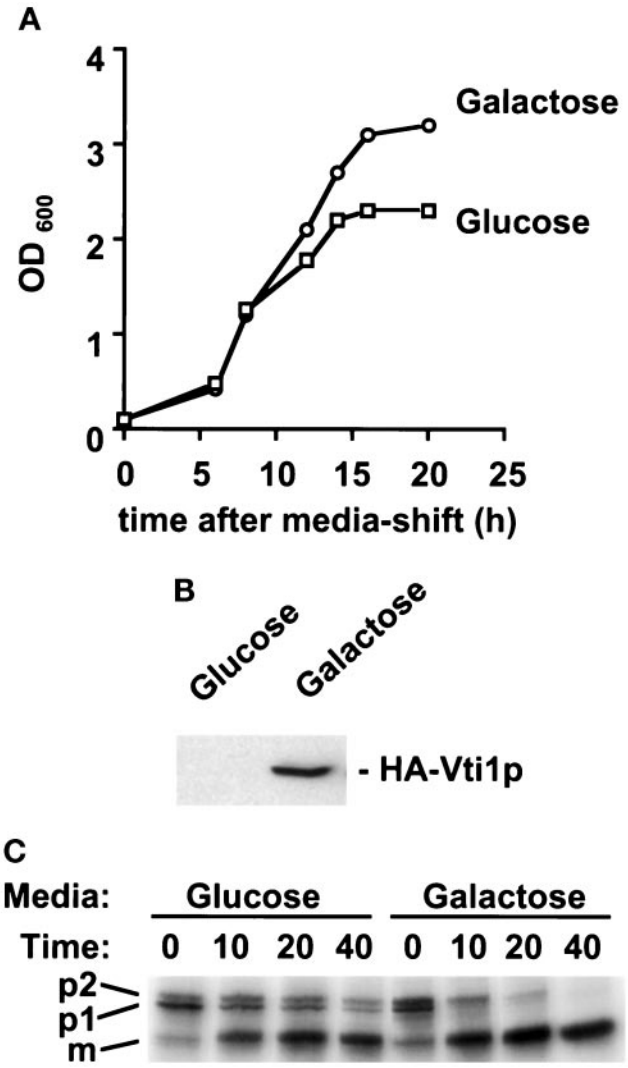


Figure 6. Depletion of HA-Vti1p results in the accumulation of the Golgi forms of CPY. (A) Growth curve of the GWY155 (*vti1*Δ strain carrying P_{GAL}-HA-VTI1) grown in galactose-containing media (Galactose) and glucose-containing media (Glucose). (B) Immunoblot detection of HA-Vti1p in whole-cell extracts after 6 h of growth on each medium shown in A. Note that upon long exposure of the immunoblot, HA-Vti1p is detectable in glucose-grown cells. (C) Autoradiograph of pulse-chase analysis of CPY processing after 6 h of growth on each media shown in A. Cells were pulse labeled for 5 min and chased in the glucose-containing media for times indicated. CPY was isolated from cell extracts by immunoprecipitation and resolved by SDS-PAGE (8% acrylamide). The different forms of CPY are indicated on the left. p1 indicates the ER and *cis*-Golgi form, p2 denotes the outer-chain glycosylated *medial/trans*-Golgi form, and m marks the mature, vacuolar form.

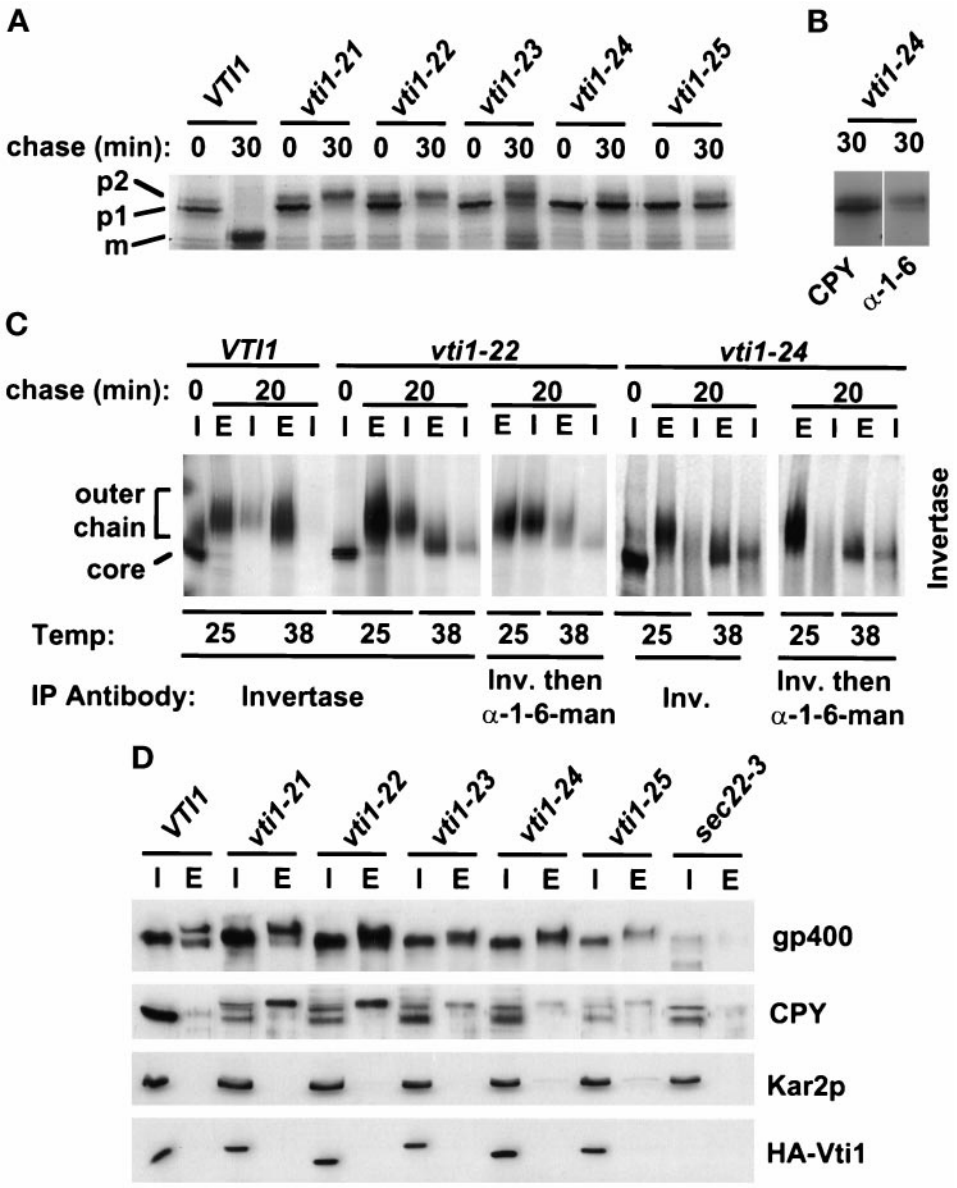


Figure 7. *vti1* ts mutants are defective in the Golgi sorting and glycosylation, but secretion is not severely compromised. (A) *vti1* ts mutants block CPY transport at different stages. Autoradiograph of CPY immunoprecipitations from an *igs*Δ strain bearing *HA-VTI1* (*GWY153*) or one of five HA-Vti1 ts mutant alleles (*GWY158-GWY162*). Cells were shifted to the restrictive temperature of 38°C for 30 min, pulse labeled for 5 min, and chased for 30 min at the same temperature. (B) The immunoprecipitated CPY was eluted and reprecipitated with anti-CPY and anti-α-1,6-mannose-specific antiserum and analyzed by SDS-PAGE and autoradiography. (C) Invertase is secreted in the underglycosylated form. Autoradiograph of invertase immunoprecipitations from an *igs*Δ strain bearing the *HA-VTI1* (*GWY153*), *HA-vti1–22* ts (*GWY159*), or *HA-vti1–24* ts mutant alleles (*GWY161*). Cells were preincubated in low-glucose media for 30 min, shifted to the restrictive temperature of 38°C for 30 min, pulse labeled for 5 min, and chased for 20 min at the same temperature. Cells were converted to spheroplasts, and invertase was immunoprecipitated from lysed spheroplasts (I) and spheroplasting media (E). The immunoprecipitated invertase was eluted and reprecipitated with anti-invertase and anti-α-1,6-mannose-specific antiserum and analyzed by SDS-PAGE and autoradiography (D) *vti1-ts* cells secrete gp400/hsp150 and CPY but not ER-resident protein Kar2p at a semipermissive temperature. Mutant cells were grown at 35°C for 5 h in fresh YPD medium. The extracellular medium (E) and total cell lysates (I) were subjected to SDS-PAGE (10% acrylamide) followed by immunoblotting with anti-gp400/hsp150, anti-CPY, anti-Kar2p, and anti-HA epitope antibodies. We determined that the small amount of Kar2p detectable in the culture media of *vti1–24* and *vti1–25* cell was due to the cell lysis (our unpublished results).

HA-Vti1p (Figure 9A, Sed5 IP). We found that HA-Vti1p associated with the *cis*-Golgi t-SNARE Sed5p only at the *sec18* restrictive temperature, suggesting that Vti1p is a v-SNARE that participates in vesicular

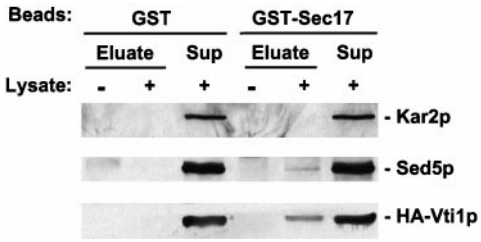


Figure 8. Vti1p physically interacts with Sec17p. Spheroplasts of GWY154 cells (*HA-VTI1*) were lysed in the presence of Triton X-100. Solubilized proteins (0.5 mg) were incubated with purified bacterially expressed GST or GST-Sec17p (2 μg each) in buffer B (20 mM HEPES-KOH, 150 mM KOAc, 0.5 mM DTT, 0.05% Tween 20) for 1 h at 4°C and centrifuged at 15,000 $\times$ *g* for 15 min. The supernatant was incubated for 1 h at 4°C with 40 μl of glutathione-Sepharose 4B equilibrated with buffer B. Beads were washed three times with buffer B, and bound proteins were eluted with 50 μl of 10 mM reduced glutathione in 50 mM Tris-Cl (pH 7.5), separated by SDS-PAGE (10% acrylamide) followed by immunoblotting with anti-HA, anti-Sed5p, or anti-Kar2p antibodies. Beads that had not been incubated with yeast lysate were included as a primary antibody specificity control.

transport to the *cis*-Golgi. Since Sed5p can interact with the ER-to-Golgi v-SNARE Sec22p and form a separate complex with its negative regulator Sly1p (Søgaard *et al.*, 1994; Lupashin and Waters, 1997), we tested the interaction of Vti1p with each of these proteins. Although the immunoprecipitations of Sec22p and Sly1p were complete (Figure 9A, Supernatants), no interaction of Vti1p with either of these proteins was detected (Figure 9A, Sec22 and Sly1 IP). These data suggest that the t-SNARE Sed5p can enter into at least two distinct v/t-SNARE complexes: one containing Sec22p and another containing Vti1p.

In an effort to confirm this data, we attempted to immunoprecipitate HA-Vti1p with the aim of identifying interacting factors. Unfortunately, the recovery of HA-Vti1p was incomplete (Figure 9A, anti-HA IP and Supernatant). We therefore examined copurification of several factors with GST-Vti1p (Figure 9B) in a *sec18* strain. The GST-Vti1p-expressing *sec18* cells were grown to midlogarithmic phase, spheroplasted, and incubated for 1 h at the *sec18* permissive or restrictive temperature. After incubation, lysates were prepared and GST-Vti1p was recovered on glutathione-Sepharose beads. Eluates from the beads were analyzed for their Sed5p, Sso1p, Ykt6p, or Sec22p content (Figure 9B). Ykt6p was discovered as a component of the *cis*-Golgi v/t-SNARE complex (Søgaard *et al.*, 1994), and is thought to be a v-SNARE acting in ER-to-Golgi transport (McNew *et al.*, 1997). Sso1p is a plasma membrane t-SNARE (Aalto *et al.*, 1993). Interestingly, we found that the inactivation of Sec18p resulted in accumulation of Vti1p/Sed5p and Vti1p/Ykt6p complexes. No Vti1p/Sec22p or Vti1p/Sso1p complexes accumulated at the *sec18* restrictive temperature.

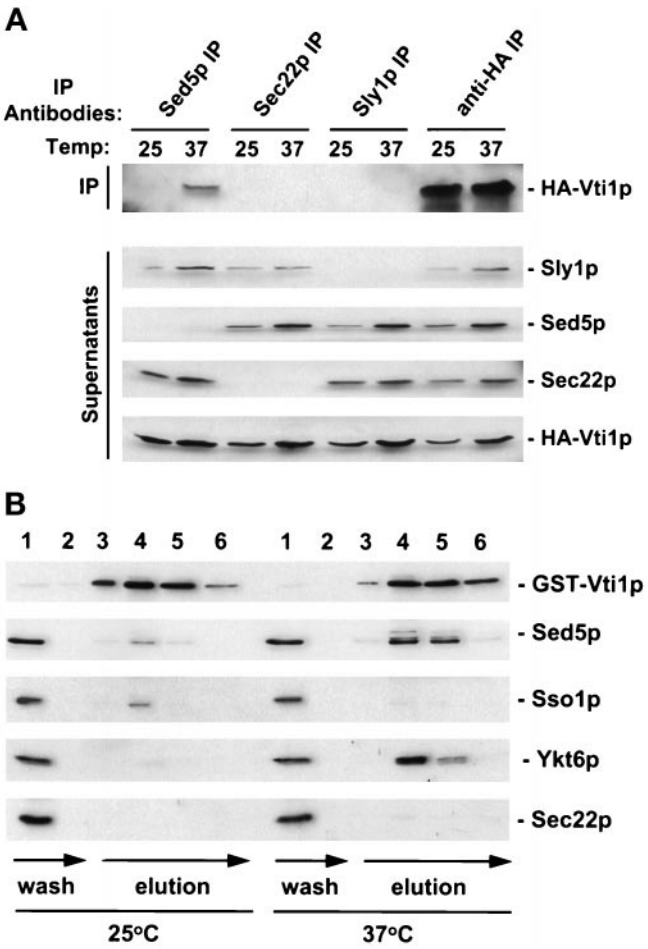


Figure 9. *Vti1p* interacts with Sed5p and Ykt6p in a *SEC18*-sensitive manner. (A) Interaction of Vti1p and Sed5p is specific. Spheroplasts of GWY157 cells (*sec18–1* with chromosomal *HA-VTI1*) were incubated at the permissive (25°C) or restrictive (37°C) temperature for 1 h, lysed in the presence of Triton X-100, and proteins immunoprecipitated (IP) with affinity-purified antibody to Sed5p, Sec22p, Sly1p, or the anti-HA monoclonal antibody (12CA5). The IPs (upper panel) and one-fifth of the corresponding supernatants (lower panel) were immunoblotted to detect HA-Vti1p, Sed5p, Sec22p, and Sly1p. (B) GST-Vti1p interactions. GWY156 cells (*sec18–1* with *GST-VTI1*) were incubated at the permissive (25°C) or restrictive (37°C) temperature for 1 h, chilled on ice, and lysed with glass beads in the presence of 1% Triton X-100. Clarified detergent lysates (~2 mg of protein) were incubated for 1 h at 4°C with 300 μl of glutathione-Sepharose 4B equilibrated with buffer B (see MATERIALS AND METHODS). Beads were transferred into 1-ml chromatography columns, washed with buffer B (wash), and bound proteins were eluted (elution) with 10 mM reduced glutathione in 50 mM Tris-Cl (pH 7.5). Fractions (300 μl each) were collected and proteins were separated by SDS-PAGE followed by immunoblotting with anti-GST, anti-Sed5p, anti-Sso1, anti-Ykt6p, or anti-Sec22p antibodies.

Genetic Interactions of VTI1

The protein–protein interaction studies and the analyses of the effects of Vti1p depletion or mutation on protein trafficking suggested that Vti1p is required for intra-Golgi protein traffic. To gain more

insight into the role of Vti1p, we undertook an analysis of the genetic interactions of *VTI1* with other genes involved in ER-to-Golgi and intra-Golgi traffic. High levels of Vti1p were expressed from a 2-μm plasmid in each of the following ts strains, but suppression of the growth defect at the restrictive temperatures was not evident: *sec22–3*, *bos1–1*, *bet1–1* (ER-to-Golgi v-SNAREs), *uso1–1* (ER-to-Golgi transport factor), *sly1^{ts}* (t-SNARE-associated regulatory protein), *sec19–1* (GDP dissociation inhibitor for Ypt1p and Sec4p), *sec7–1* (ER-to-Golgi and intra-Golgi docking factor), *ypt1–3* (ER-to-Golgi Rab-like GTPase), *ypt6^{ts}* (intra-Golgi Rab-like GTPase), *sec4–8* (Golgi-to-plasma membrane Rab-like GTPase), *sec21–1* and *sec21–2* (γ-COP), *sec27–1* (β'-COP), and *ret1–1* (α-COP), *sec35–1*, and *sec34–2* (novel ER-to-Golgi transport factors). In contrast, high levels of Vti1p expression could partially suppress the *sft1–1* temperature-sensitive growth defect (Figure 10A).

We also tested whether any of several known genes could act as multicopy suppressors of the growth defects of the *vti1* ts alleles. As shown in Figure 10B, the temperature sensitivity of all tested *vti1* ts strains could be completely or partially suppressed by overexpression of the SNARE *YKT6* from a 2-μm plasmid. In addition, the *vti1–23* and *vti1–24* temperature-sensitive growth defect was partially suppressed by a multicopy plasmid carrying genes encoding the putative intra-Golgi retrograde t-SNARE *SFT1* (Banfield *et al.*, 1995), the ER-to-Golgi t-SNARE *SED5* (Hardwick and Pelham, 1992), or a dominant mutation of *SLY1*, termed *SLY1–20* (Ossig *et al.*, 1991). *SEC22* and *BET1* (Newman *et al.*, 1990; Ossig *et al.*, 1991), v-SNAREs involved in ER-to-Golgi transport, were also tested for the ability to suppress *vti1* ts mutants, but these genes did not improve the growth of *vti1* ts strains at the restrictive temperature.

Finally, to test whether any of the mammalian sequences that encode Vti1p homologues (Figure 1B) were capable of complementing a Vti1p defect, we tested the ability of the human homologue, hVti1-rp1, and the mouse homologue mVti1-rp2 to suppress the growth defects of the *vti1* ts alleles. Expression of hVti1-rp1 under control of P_{GAL} completely suppressed the growth defect of the *vti1–25* (Figure 10C) and partially suppressed the growth defect of other *vti1* ts alleles (our unpublished results). In contrast, suppression by the mouse homologue, mVti1-rp2, was very weak.

DISCUSSION

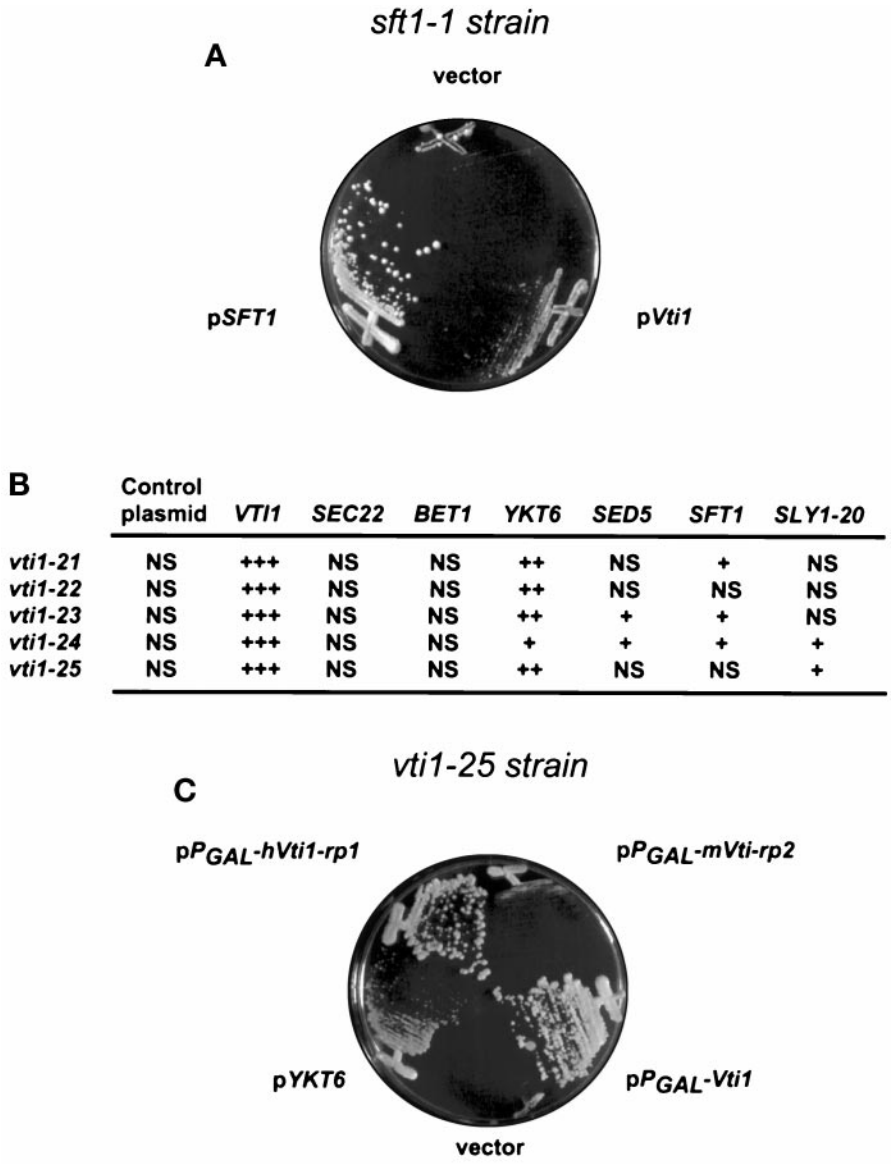
In this study, we used protein homology searches of the complete yeast genome database to identify five ORFs that could encode novel SNARE-like proteins. We have performed a functional analysis of one of these, named Vti1p. This 24.7-kDa type II transmembrane protein is most closely related to the putative intra-Golgi retrograde v-SNARE Sft1p (Banfield *et al.*, 1995) and is essential for vegetative growth of yeast cells. Subcellular fractionation and immunofluorescence suggested that Vti1p is localized to the Golgi apparatus and perhaps to the prevacuolar compartment as well (see also Fischer Von Mollard *et al.*, 1997). We have confirmed that Vti1p is a SNARE by showing that it physically interacts with yeast SNAP (Sec17p).

Reduction of the cellular level of Vti1p, expressed under the control of the glucose-repressible *GAL1* promoter, resulted in a defect in the intra-Golgi transport of CPY prior to cessation of cell growth. Both the p1 (ER/*cis*-Golgi) and p2 (*medial*/*trans*-Golgi) forms of CPY accumulated intracellularly upon loss of Vti1p, suggesting that this SNARE is required, either directly or indirectly, for CPY transport through multiple compartments of the Golgi.

To further define the role of this Golgi SNARE, we generated a set of temperature-conditional *vti1* mutants and studied their secretion phenotypes. Pulse-chase analysis of CPY transport in these strains indicated that the mutants accumulate the p1 and/or p2 forms of CPY at the restrictive temperature. In addition, we found that some p2 CPY was secreted in the *vti1* ts mutants. In a similar pulse-chase experiment, we found that the secretion of invertase was not significantly compromised, but that the secreted invertase lacked the extensive outer-chain glycosylation which is normally added in the Golgi. Another extracellular protein, termed gp400/hsp150, was also secreted by the *vti1* ts strains.

This combination of secretion phenotypes is unusual. For example, the accumulation of p1 and p2 suggests an intra-Golgi block, whereas secretion of p2 CPY indicates that anterograde transport to the cell surface can still occur and that vacuolar protein sorting may be defective. The continued secretion of invertase and gp400/hsp150 in the *vti1* ts mutants also indicates that anterograde transport is operational, but the finding that the secreted invertase is underglycosylated suggests a defect in Golgi glycosylation.

In an effort to understand how a loss of Vti1p function could lead to this spectrum of secretion phenotypes, we identified the SNAREs with which Vti1p interacts. Since Vti1p is localized, at least partially, to the Golgi apparatus and it is most homologous to Sft1p (Banfield *et al.*, 1995) and Bet1p (Newman *et al.*, 1990), two v-SNAREs known to interact with the *cis*-Golgi t-SNARE Sed5p (Søgaard *et al.*, 1994; McNew *et al.*, 1997), we tested whether Vti1p could interact with Sed5p. Indeed, Vti1p physically interacts with Sed5p in a complex that accumulates in the absence of NSF (Sec18p) activity. This suggests that Vti1p is a v-SNARE that can target vesicles to the *cis*-Golgi. Inter-



	Control plasmid	*VTI1*	*SEC22*	*BET1*	*YKT6*	*SED5*	*SFT1*	*SLY1-20*
vti1-21	NS	+++	NS	NS	++	NS	+	NS
vti1-22	NS	+++	NS	NS	++	NS	NS	NS
vti1-23	NS	+++	NS	NS	++	+	+	NS
vti1-24	NS	+++	NS	NS	+	+	+	+
vti1-25	NS	+++	NS	NS	++	NS	NS	+

Figure 10. Multicopy suppression of *sft1–1* and *vti1 ts* growth defect. (A) Vti1p overexpression suppresses the growth defect of the *sft1–1*. Growth at 35°C on YPD plates of the *sft1–1* strain carrying multicopy plasmids bearing *VTI1*, *SFT1*, or no insert as indicated. (B) Growth at (37.5°C) on YPD plates of the indicated mutants carrying 2μ multicopy plasmids bearing the indicated genes. +++, wild-type growth; ++, good growth; +, poor growth; NS, no suppression. (C) Human Vti1p homologue (hVti1-rp1) expression suppresses the growth defect of the *vti1–25* mutant allele. Growth at 37°C on YPGal plates of the *vti1–25* strain carrying multicopy P_{GAL} plasmids bearing *VTI1*, hVti1-rp1, mVti1-rp2, or no insert as indicated. *YKT6* was not under P_{GAL} control.

estingly, the Vti1p/Sed5p complex is distinct from the well-characterized Sec22p/Sed5p complex (Søgaard *et al.*, 1994) that targets ER-derived vesicles to the *cis*-Golgi. It has previously been proposed, on the basis of genetic evidence, that Sed5p can act as the *cis*-Golgi docking site for both anterograde ER-to-Golgi vesicles as well as retrograde intra-Golgi vesicles (Banfield *et al.*, 1995). This concept has recently been supported by the observation that the mammalian homologue of Sed5p, syntaxin 5, can enter into at least two unique v/t-SNARE complexes (Hay *et al.*, 1997). Our protein–protein interaction data demonstrate that a similar situation exists in yeast for Sed5p and also suggests that Vti1p is a retrograde v-SNARE.

Support for the idea that Vti1p is a retrograde *cis*-Golgi-directed was obtained by analysis of the multicopy suppression profile of the *vti1* ts mutants. First, we found that overexpression of Sed5p, or Sly1–20p, which physically interacts with Sed5p and facilitates its function (Dascher *et al.*, 1991, Søgaard *et al.*, 1994,

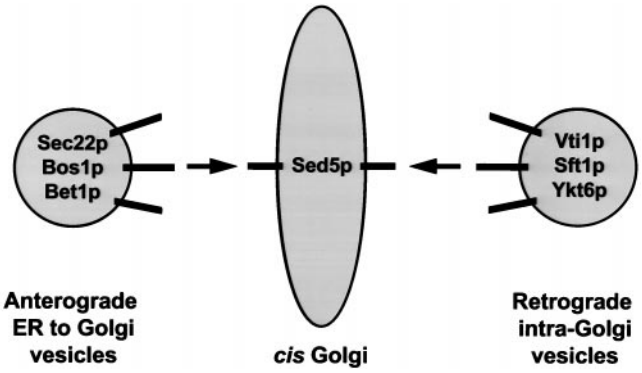


Figure 11. Model for Vti1p function.

Grabowski and Gallwitz, 1997), could suppress *vti1* mutants in an allele specific manner. This finding is consistent with the physical interaction of Vti1p with Sed5p. In contrast, overexpression of the ER-to-Golgi v-SNARE Sec22 did not suppress any of the *vti1* ts mutants, reminiscent of its lack of physical interaction with Vti1p. Interestingly, overexpression of Sft1p, which is a putative retrograde intra-Golgi v-SNARE (Banfield *et al.*, 1995), could weakly suppress several *vti1* ts alleles; likewise overexpression of Vti1p suppressed *sft1–1*. Finally, high levels of Ykt6p, an isoprenylated v-SNARE involved in early Golgi traffic (McNew *et al.*, 1997), efficiently rescued all of the *vti1* ts mutants.

In agreement with this genetic suppression data, we found that Vti1p physically interacts with Ykt6p. To summarize then, Vti1p can physically interact with the *cis*-Golgi t-SNARE Sed5p, and the v-SNARE Ykt6p, but not with the anterograde ER-to-Golgi v-SNARE Sec22p. *VTI1* also genetically interacts with the putative intra-Golgi v-SNARE *SFT1*.

These protein–protein interaction and genetic analyses suggest a model for the role of Vti1p in vesicular traffic (Figure 11). We propose that Sed5p marks the *cis*-Golgi docking site for ER-to-Golgi vesicles bearing the anterograde v-SNAREs Sec22p, Bet1p, and Bos1p as well for retrograde intra-Golgi vesicles that display the v-SNAREs Vti1p, Sft1p, and Ykt6p.

This model provides the framework for interpretation of the secretion phenotypes exhibited by the *vti1* mutants. First, we suggest that anterograde transport of invertase and gp400/hsp150 is largely unaffected by loss of Vti1p activity because the primary function of this SNARE is in retrograde transport. The inefficient anterograde transport of CPY could be a secondary manifestation of the retrograde transport defect if CPY requires recycling of components that facilitate its forward movement, e.g., Vps10p (Marcusson *et al.*, 1994). This model was also proposed recently by Gaynor and Emr (1997) who found similar transport phenotypes when studying the role of Sec21p, the γ-subunit (γ-COP) of yeast coatomer (Hosobuchi *et al.*, 1992), in retrograde transport from the Golgi to the ER. Whereas mutants in γ-COP exhibited a CPY-specific ER-to-Golgi transport defect, mutants in Vti1p allowed CPY transit from the ER-to-Golgi, but compromised further forward transport. Similar effects on CPY and invertase transport were demonstrated previously in the *sft1* mutant (Banfield *et al.*, 1995). Finally, we suggest that the inefficient glycosylation of invertase seen in the *vti1* mutants is due to mislocalization of the Golgi glycosyltransferases such as the α-1,6-mannosyltransferase, Och1p, which must be recycled to maintain its *cis*-Golgi localization (Harris and Waters, 1996).

The recent work of Fischer von Mollard *et al.* (1997) provides another model that is not mutually exclusive with ours. In their work they showed that Vti1p interacts with both Sed5p and with Pep12p, the t-SNAREs that marks the prevacuolar compartment (Becherer *et al.* 1996). This suggests that Vti1p may be involved in docking of late Golgi-derived vesicles with the prevacuolar compartment (Fischer von Mollard *et al.*, 1997) as well as retrograde transport to the *cis*-Golgi.

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