Genetic interactions with the yeast Q-SNARE VT1 reveal novel functions for the R-SNARE YKT6

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Running title
SNARE complexes with Ykt6p and Vti1p
Summary

SNARE proteins are required for fusion of transport vesicles with target membranes. Earlier, we found that the yeast Q-SNARE Vti1p is involved in transport to the cis-Golgi, to the prevacuole/late endosome and to the vacuole. Here we identified a previously uncharacterized gene, VTS1, and the R-SNARE YKT6 both as multicopy and as lowcopy suppressors of the growth and vacuolar transport defect in vti1-2 cells. Ykt6p was known to function in retrograde traffic to the cis-Golgi and homotypic vacuolar fusion. We found that VTI1 and YKT6 also interacted in traffic to the prevacuole and vacuole indicating that these SNARE complexes contain Ykt6p, Vti1p plus Pep12p and Ykt6p, Vti1p, Vam3p plus Vam7p, respectively. As Ykt6p was required for several transport steps R-SNAREs cannot be the sole determinants of specificity. To study the role of the 0 layer in the SNARE motif we introduced the mutations vti1-Q158R and ykt6-R165Q. SNARE complexes to which Ykt6p contributed a fourth glutamine residue in the 0 layer were nonfunctional suggesting an essential function for arginine in the 0 layer of these complexes. vti1-Q158R cells had severe defects in several transport steps indicating that the second arginine in the 0 layer interfered with function.
Introduction

Transport between different organelles is mediated by transport vesicles which bud from the donor compartment (1). Recognition of the correct target requires interactions between specific members of the Rab/YPT family of small GTPases, tether proteins and SNARE proteins (2). These SNARE proteins constitute a large, evolutionary conserved family (3). v-SNAREs are found on transport vesicles, t-SNAREs on target membranes. In most cases SNAREs are attached to the membrane by a C-terminal transmembrane domain or by hydrophobic posttranslational modifications. The SNARE motif, a highly conserved domain of 60 amino acid residues, is found next to the membrane anchor. Four different SNARE motifs form a parallel helical bundle with sixteen layers (numbered from -7 to +8) of interacting amino acid side chains pointing towards the center of the bundle in the neuronal SNARE complex (4). Most layers consist of four hydrophobic amino acid residues. However, in the central 0 layer an arginine (R) from synaptobrevin interacts with three glutamines (Q) from syntaxin 1 and both SNAP-25 helices. These residues are very conserved leading to a reclassification of SNAREs into R- and Q-SNAREs (5). It has been suggested that a parallel four helix bundle with one R- and three Q-SNARE helices is a common feature of SNARE complexes.

The yeast Saccharomyces cerevisiae has proven a powerful model system to study membrane traffic and to test the SNARE hypothesis (6). Twenty-one SNAREs, among them five R-SNAREs, have been identified in yeast (7, 8). All of them have been assigned to one or more traffic steps. However, the exact compositions of the SNARE complexes are not clear for many transport steps. Earlier, we described the Q-SNARE Vti1p which is required for several transport
steps in yeast. Vti1p interacts with the syntaxin related cis-Golgi Q-SNARE Sed5p in a retrograde traffic step to the cis-Golgi (9, 10). The R-SNARE Ykt6p and the Q-SNARE Sft1p have been implicated in this transport step as well (11, 12). Vti1p and the syntaxin-related endosomal Q-SNARE Pep12p are the only SNAREs identified so far in transport from the Golgi to the prevacuolar/late endosomal compartment (9, 13). This transport pathway is used by many vacuolar proteins, for example by carboxypeptidase Y (CPY). These proteins are transported in a second step from the prevacuole to the vacuole (14). A different pathway to the vacuole is used by alkaline phosphatase (ALP) which travels in vesicles from the Golgi to the vacuole without passage through the prevacuole. A third vacuolar pathway is taken by aminopeptidase I (API) and autophagosomes. API is synthesized in the cytosol, packaged into cytosol to vacuole transport (CVT) vesicles enclosed by double membranes in a process similar to autophagocytosis (15). The outer membrane of CVT vesicles and autophagosomes fuses with the vacuole. The same Q-SNAREs Vam3p, Vam7p and Vti1p are required for these three biosynthetic pathways to the vacuole (16 - 20) while an R-SNARE has not yet been identified. The vacuolar R-SNARE Nyv1p has been excluded as the missing R-SNARE because these transport pathways are not affected by deletion of NYV1 and a genetic interaction between the vti1-2 mutant and NYV1 was not observed (20). Vacuoles can also undergo homotypic fusion. The Q-SNAREs Vam3p, Vam7p and Vti1p together with the R-SNAREs Nyv1p and Ykt6p have been implicated in homotypic vacuolar fusion (21 - 23).

We set out to identify proteins which are required together with Vti1p for transport to the vacuole. Genetic interactions have proven a valuable tool for this purpose. vti1-2 is a useful mutant allele for such studies because transport from the Golgi to the prevacuole and all transport steps to the vacuole are blocked
at nonpermissive temperature but transport to the cis-Golgi is not affected. \textit{vti1-2} has the amino acid exchanges S130P in the -8 layer and I151T in the -2 layer of the SNARE motif (24). Two genes were identified as suppressors for \textit{vti1-2}. One was an uncharacterized ORF, the other was the R-SNARE \textit{YKT6} which we identified here as the R-SNARE in transport to the prevacuole and to the vacuole. We investigated the role of the amino acid residues in the 0 layer of Ykt6p and Vti1p. These SNARE complexes are nonfunctional with four glutamine residues and defective with two arginine and two glutamine residues in the 0 layer.
Experimental Procedures

Materials

Reagents were used from the following sources: enzymes for DNA manipulation from New England Biolabs (Beverly, MA), [35S] Methionine from Amersham Pharmacia (Braunschweig, Germany), fixed Staphylococcus aureus cells (Pansorbin) from Calbiochem (San Diego, CA), Zymolyase from Seikagaku (Tokyo, Japan). All other reagents were purchased from Sigma (St. Louis, MO).

Plasmid manipulations were performed in the E. coli strains MC1061 or XL1Blue using standard media.

Yeast strains (Table 1) were grown in rich media (1 % yeast extract, 1 % peptone, 2 % dextrose, YEPD) or standard minimal medium (SD) with appropriate supplements.

Suppressor screen

The growth defect of vti1-2 cells at 37°C was more pronounced in the genetic background of 9Dα cells than SEY6210 cells. To identify multicopy suppressors which allowed vti1-2 cells to grow faster at 37°C we transformed FvMY22 cells with a YEp24 2µ library (25). Plasmids were isolated from colonies which showed improved growth at 37°C and retransformed into FvMY22 cells to confirm suppressor activity. To compare growth rates cells were grown in YEPD to an OD600 between 0,2 and 1,0. After dilution to 0,01 and 0,05 OD600/ml 10 µl were spotted onto YEPD plates and grown at the indicated temperature.

Plasmids and strains
Precise deletions of the \textit{VTS1} ORF were generated by PCR (amplification of 
\textit{HIS3} with oligos annealing to 40 nucleotides of the \textit{VTS1} flanking region) in 
\textit{SEY6210}, \textit{FvMY7} and \textit{FvMY24} resulting in the strains \textit{MDY1}, \textit{MDY4} and \textit{MDY5} 
(\textit{Table 1}), respectively (26). The same method was used to introduce the 
\textit{ykt6\Delta::URA3} mutation into \textit{SEY6211xFvMY6} diploid cells carrying a heterocytote 
\textit{vti1\Delta::HIS3} mutation yielding \textit{BKY6}. A 1,1 kb fragment coding for the \textit{YKT6} ORF 
was PCR-amplified using the oligonucleotides \text{gaggatctcacttcagttggttaattg} and 
ggaattcactgaagaaacaaatcaattct and cloned via BamHI and EcoRI sites into \textit{YEp352} 
(27, pMD1, \textit{table 2}) and \textit{pRS316} (28, pMD21). A 2,3 kb Clal-SpeI fragment encoding 
\textit{VTS1} was isolated from the suppressor plasmid \textit{pBK24} and subcloned into 
pBluescript. The fragment was cut out with KpnI-SpeI and cloned into \textit{YEp352} 
(pMD3) and \textit{pRS316} (pMD9). To introduce a N-terminal triple HA-tag into \textit{VTS1} 
a BamHI site was generated after the start codon by PCR-based site-directed 
mutagenesis (29, oligonucleotides ccaaacatccgtaggaaattg and 
atccatgatttctttgcaattc) and a 126 bp BglII fragment encoding three copies of the 
HA epitope was ligated into the BamHI site (pMD8). \textit{pBK65} was constructed by 
PCR-based site-directed mutagenesis with the oligonucleotides 
cagacgtctcagttggttaattg and cagcaaggtgaaaagttgagataatttg to generate the mutation 
\textit{ykt6-R165Q} and a silent PstI site in pMD1. The inserts of pMD1 and pBK65 were 
subcloned into \textit{YEp351} and \textit{pRS315} to obtain \textit{pBK99} (\textit{YKT6} in \textit{YEp351}), \textit{pBK86} 
(\textit{ykt6-R165Q} in \textit{YEp351}) and \textit{pBK87} (\textit{ykt6-R165Q} in \textit{pRS315}). The mutation Q158R 
and a silent NruI site was introduced into the \textit{VTI1} encoding plasmid \textit{pFvM28} by 
PCR-based site-directed mutagenesis using the oligonucleotides 
cgacattaatcattcattttggtaatttg and cagcaaggtgaaaagttgagataatttg (pBK77). \textit{vti1-Q158R} was 
subcloned into the integration vector \textit{pRS306}, linearized, integrated into \textit{SEY6211}
and the wild-type VTII was looped out on 5-fluorourotic acid plates (30) to construct FvMY38.

Immunoprecipitations of $^{35}$S-labeled proteins

CPY, ALP and API were immunoprecipitated as described earlier (20, 31 - 33). The CPY and ALP antisera were a generous gift from T.H. Stevens. The API antiserum was kindly provided by D. Klionsky. Immunoprecipitates were analyzed by SDS-PAGE and autoradiography. A BAS1000 (Fuji) was used for quantification.

Subcellular fractionation

Subcellular fractionation was performed by differential centrifugation as described (34). vts1Δ cells expressing HA-VTS1 from a CEN6 plasmid were spheroplasted, osmotically lysed and centrifuged. Fractions were separated by SDS-PAGE, immunoblotted and detected with HRP-conjugated secondary antibodies via ECL.
Results

Identification of suppressors for vti1-2

The goal of this study was to identify genes which interact with VTI1 in transport from the Golgi to the prevacuolar compartment/late endosome or in transport to the vacuole. These transport steps are blocked in vti1-2 cells at the non-permissive temperature while transport to the Golgi is not affected (9, 20). vti1-2 cells also display a growth defect at 37°C which we utilized for a multicopy (2µ) suppressor screen. As expected, plasmids encoding for VTI1 or the endosomal Q-SNARE PEP12 (20) restored growth of vti1-2 cells at 37°C. Two additional plasmids enhanced growth rates of vti1-2 cells at 37°C (Fig. 1). One plasmid contained an 11 kb fragment of chromosome XI between nucleotides 69,529 and 80,400. The R-SNARE Ykt6p is encoded by this DNA fragment. YKT6 interacts genetically with VTI1 in retrograde traffic to the cis-Golgi (10). Ykt6p is also required for homotypic vacuolar fusion and can be immunoprecipitated with Vti1p (23). A 1,1 kb fragment encoding only YKT6 was subcloned into a 2µ and a centromeric vector to determine whether YKT6 is the suppressor. The growth defect of vti1-2 cells at 37°C was suppressed by overexpression of YKT6 alone from either a centromeric plasmid (1-3 copies) or a 2µ plasmid (10-20 copies per yeast cell, Fig. 1 top). These data indicate that a slight overexpression of YKT6 is sufficient for suppression of the growth defect.

The second suppressing plasmid consisted of chromosome XV nucleotides 1,009,767 to 1,014,198. Two complete reading frames were identified within this fragment: HAP5, a component of a transcription factor and the hypothetical open reading frame YOR359w. A 2,3 kb fragment encoding only YOR359w was subcloned into a 2µ and a centromeric plasmid. Both plasmids improved growth
of \(vti1-2\) cells at \(37^\circ\text{C}\) (Fig. 1 bottom). \(YOR359w\) was also overexpressed in \(vti1-11\) cells which show a severe growth defect at \(37^\circ\text{C}\) in addition to defects in transport to the cis-Golgi, to the prevacuole and to the vacuole (9). The growth defect in \(vti1-11\) cells was not suppressed by overexpression of \(YOR359w\) (data not shown) suggesting that the suppression by \(YOR359w\) is allele specific and not due to a general bypass of \(VTI1\) function. Therefore \(YOR359w\) was named \(VTS1\) (\(vti1-2\) suppressor). \(VTS1\) encodes a predicted protein of 523 amino acid residues without hydrophobic stretches typical for transmembrane domains. Databank searches revealed only two proteins of similar length with an overall sequence homology in the yeast \(Candida albicans\) (28\% amino acid identity, Genbank \# AL033497) and in the fission yeast \(Schizosaccharomyces pombe\) (24\% amino acid identity, Genbank \# CAB89878). These proteins share a SAM (sterile alpha motif) at their C-termini (see discussion).

**Characterization of \(VTS1\)**

To determine what trafficking step is suppressed we examined the effect of overexpression of \(VTS1\) on protein traffic to the vacuole in \(vti1-2\) cells. Carboxypeptidase Y (CPY) is transported from the Golgi first to the prevacuolar compartment and in a second transport step from there to the vacuole (6, 14). \(vti1-2\) cells are blocked in both trafficking steps. \(vti1-2\) cells and \(vti1-2\) cells overexpressing \(VTS1\) were grown at \(24^\circ\text{C}\), shifted to \(32^\circ\text{C}\) for 15 min, pulsed with \(^{35}\text{S}\) cysteine/methionine for 10 min and chased in the presence of unlabeled cysteine/methionine for 30 min. Cells were spheroplasted and CPY was immunoprecipitated. In \(vti1-2\) cells as well as in \(vti1-2\) cells overexpressing \(VTS1\) CPY did not reach the vacuole as indicated by the lack of mCPY within the cells (Fig. 2A, fractions I). Almost all CPY was secreted as the Golgi modified form
p2CPY (E). As CPY transport from the Golgi to the prevacuole is blocked in *vti1-1* cells while traffic to the vacuole is not affected *vti1-1* cells were used to distinguish between these steps (9, 20). Overexpression of *VTS1* in *vti1-1* cells did not suppress the defect in CPY transport from the Golgi to the prevacuole (data not shown). Alkaline phosphatase (ALP) is transported from the Golgi to the vacuole without passing through the prevacuole (6, 14). ALP traffic was investigated in *vti1-2* cells using similar pulse-chase experiments at 36°C (Fig. 2C). In wild-type cells 95% mALP are typical after a 30 min chase (data not shown) while in *vti1-2* cells an average of 47 % mALP (std 4,1) was detected. Overexpression of *VTS1* either from the 2µ library plasmid or from the 2µ *VTS1* plasmid improved ALP traffic to the vacuole considerably as indicated by the rise in vacuolar mALP to an average of 76,8 % (std 3,1) and 70,5 % (std 1,8; n=3), respectively. A slight overexpression of *VTS1* due to the presence of a centromeric plasmid in addition to the genomic copy of *VTS1* suppressed the ALP sorting defect slightly (average of 62 %, std 8). Aminopeptidase I (API) does not enter the secretory pathway but is synthesized in the cytosol and packaged into CVT vesicles surrounded by double membranes in a trafficking pathway related to autophagy (15). API was immunoprecipitated after a 10 min pulse and a 120 min chase at 37°C (Fig. 2B). Overexpression of *VTS1* from either a 2µ or a centromeric plasmid partially suppressed the API sorting defect in *vti1-2* cells (*vti1-2* 25,5 % mAPI, std 9,3; *vti1-2* 2µ *VTS1* 54,8 %, std 8,8; *vti1-2* CEN *VTS1* 52,9 %, std 7,4; n=3). These data indicate that *VTS1* genetically interacts with *VTI1* in traffic to the vacuole but not in traffic from the Golgi to the prevacuole.

Next we wanted to determine the consequences of a lack of Vts1p. *VTS1* was deleted in wild type, *vti1-1* and *vti1-2* cells. *vts1Δ* cells did not display defects in
traffic of CPY, ALP or API to the vacuole (data not shown). Defects in CPY, ALP and API transport were identical in vti1-2 and vti1-2 vts1Δ and in vti1-1 and vti1-1 vts1Δ cells, respectively, indicating a lack of a synthetic defect in protein transport (data not shown). Neither vts1Δ nor vti1-1 vts1Δ cells displayed a growth defect at 24°C or 37°C (Fig. 3). vti1-2 and vti1-2 vts1Δ cells grew with similar rates at 24°C. At 37°C vti1-2 cells grew slowly while vti1-2 vts1Δ cells did not grow at all. These data indicate that vti1-2 and vts1Δ have a synthetic growth defect at high temperature as an additional genetic interaction. To study the subcellular localization of Vts1p three copies of the influenza HA-tag were introduced at the N-terminus of the VTS1 open reading frame. HA-Vts1p was functional because production of HA-Vts1p in vti1-2 vts1Δ cells restored slow growth at 37°C (data not shown). HA-Vts1p was found exclusively in the supernatant of a 200,000 g centrifugation during subcellular fractionation and was not found on membranes in immunofluorescence experiments (data not shown). These data indicate Vts1p is not associated with membranes under normal conditions. Next we investigated whether Vti1p and HA-Vts1p interact physically. As Vti1p is a membrane protein binding between both proteins could be transient at most or involve only a small amount of Vts1p. HA-Vts1p and Vti1p did not co-immunoprecipitate. Furthermore, they could not be chemically crosslinked (data not shown). In vitro binding assays using recombinant GST-Vti1p and 6-His-Vts1p fusion proteins and vice versa did not reveal a specific interaction between both proteins (data not shown).

In conclusion, we found genetic interactions between VTS1 and vti1-2. This suppression affected transport to the vacuole, was specific for a certain allele of
vti1 and therefore not due to a general effect of VTS1 overexpression. We could not detect biochemical interactions between Vts1p and Vti1p. The mechanism of the genetic interaction between VTI1 and VTS1 remains to be elucidated.

Ykt6p functions in multiple transport steps

After identifying YKT6 as a multicopy suppressor of the growth defect in vti1-2 cells we investigated whether YKT6 overexpression affected different trafficking steps. API transport was followed in vti1-2 cells and vti1-2 cells in which YKT6 was overexpressed from either a centromeric or a 2µ plasmid (Fig. 4A). Overexpression of YKT6 increased the proportion of vacuolar mAPI from 20.9% (std 6.4) to 54.1% (std 0.8) for vti1-2 cells with the 2µ plasmid encoding only YKT6 and to 65.1% (std 0.9) with the 11 kb genomic 2µ plasmid encoding YKT6 (data not shown). A slight overexpression of YKT6 from a centromeric plasmid resulted in intermediate amounts of mAPI. ALP transport was investigated in the same strains (Fig. 4B). Overproduction of Ykt6p from the 2µ plasmid resulted reproducibly in an improved delivery of ALP to the vacuole as indicated by increased amounts of mALP after a 30 min chase period. The amount of mALP detected in vti1-2 cells varied between experiments with an average of 38.6%. In vti1-2 2µ YKT6 cells an average of 57.9% mALP was found, an increase by 21.3 percentage points (std 4.1; n=6). The ALP transport defect was also suppressed by the 11 kb genomic 2µ plasmid encoding YKT6 (increase by 23.1 percentage points, std 4.9, data not shown). A slight overexpression of YKT6 using a centromeric plasmid improved ALP transport to the vacuole somewhat. Next we determined whether this effect is specific for Ykt6p or whether other R-SNAREs can function in this transport step. Snc2p (required for exocytosis and endocytosis, 36, 49) and Sec22p (involved in traffic between ER and Golgi, 43) were
overproduced using 2 µ plasmids. ALP traffic was not suppressed significantly. These data demonstrate that overproduction of Ykt6p suppressed the API and ALP transport defects in \(vti1-2\) cells indicating that Ykt6p has an additional role in biosynthetic transport to the vacuole. We used \(vti1-1\) cells to analyze the role of \(YKT6\) in transport of CPY from the Golgi to the prevacuole which is defective in these cells while transport to the vacuole is not affected (9, 20). Overproduction of Ykt6p in \(vti1-1\) cells resulted in the emergence of vacuolar mCPY (Fig. 4C). The amount of mCPY was increased from 3.4 % (std 0.6) in \(vti1-1\) cells to 20.9 % (std 4.9, \(n=3\)) in \(vti1-1\) cells with a 2µ \(YKT6\) plasmid and to 15.4 % (std 1.2) in \(vti1-1\) cells with a centromeric \(YKT6\) plasmid. By contrast, overproduction of either Snc2p or Sec22p did not suppress the CPY sorting defect. CPY sorting to the vacuole was partially restored by overproduction of Ykt6p in \(vti1-2\) cells at semipermissive temperature (data not shown). These genetic interactions indicate that Ykt6p acts together with Vti1p in traffic from the Golgi to the prevacuole as well. \(vti1-11\) cells are defective in a retrograde traffic step to the cis-Golgi in addition to blocks in traffic from the Golgi to the prevacuole and to the vacuole. Therefore \(vti1-11\) cells accumulate the ER form p1CPY at 36°C (Fig. 4D). Less CPY accumulated within the cell as p1CPY and more p2CPY was secreted into the medium (E) upon overproduction of Ykt6p. These results indicate in accordance with earlier work (10) that \(YKT6\) and \(VTII\) interact genetically in traffic to the cis-Golgi.

**Zero layer mutations in \(VTII\) and \(YKT6\)**

SNAREs have conserved arginine (R) or glutamine (Q) residues in the middle of the SNARE motif (5, 35). The crystal structure of the neuronal SNARE complex revealed that three glutamines interacting with one arginine form an
ionic 0 layer in the middle of a parallel four helix bundle (4). Two other structurally characterized SNARE complexes consist also of one R-SNARE and three Q-SNARE helices (36, 37). We wanted to investigate the role of the 0 layer in Ykt6p and Vti1p. The most severely defective vti1-12 allele carries the amino acid exchange Q158R in the 0 layer in addition to an A141S exchange in the -5 layer (24). Site directed mutagenesis was used to create vti1-Q158R (vti1-R) and this construct was integrated into the genome to replace wild type VTI1. CPY did not reach the vacuole but was secreted even at 24°C in the resulting strain. Some p1CPY accumulated in vti1-R cells at 24°C and p1CPY was the predominant intracellular form at 37°C, demonstrating a temperature sensitive block in traffic to the cis-Golgi (data not shown). ALP maturation was almost completely blocked at 30°C (Fig. 5 top). These experiments indicate that SNARE complexes containing a second arginine in the 0 layer contributed by vti1-R protein are defective. Next we examined whether the complementary amino acid exchange in the 0 layer of Ykt6p, R165Q (ykt6-Q) could restore function. Either wild type YKT6 or ykt6-Q were overexpressed in vti1-R cells which also expressed wild type YKT6 from the genomic locus. ALP traffic was followed at 30°C. Overproduction of Ykt6p did not restore ALP transport to the vacuole (Fig. 5 top). By contrast, overexpression of ykt6-Q resulted in a reproducible partial suppression of ALP transport defect indicating that ykt6-Q protein competed with wild type Ykt6p for participation in the vacuolar SNARE complex and that the restoration of a 3Q : 1R ratio in this complex improved function. To determine whether the arginine in the 0 layer of Ykt6p is required for the function of the SNARE complex or if it can be replaced by a glutamine we expressed ykt6-Q either from a centromeric or from a 2µ plasmid in wild type cells. CPY sorting was followed by pulse chase immunoprecipitation (Fig. 5 bottom). Expression of ykt6-Q at levels comparable
to that of wild type YKT6 resulted in a small elevation of CPY secretion. A larger proportion of CPY was secreted upon overexpression of ykt6-Q. The ykt6-Q protein had to be stable and correctly folded to cause these dominant negative effects on CPY transport to the vacuole. We conclude that ykt6-Q protein was incorporated into a SNARE complex which had reduced function or was nonfunctional.

Next we wanted to investigate whether ykt6-Q had residual activity. We designed a yeast strain that allowed us to examine the behavior of ykt6-Q in the absence of wild type Ykt6p either with wild type Vti1p to form a SNARE complex with four glutamines in the 0 layer or with vti1-R protein containing a compensatory amino acid exchange. This would maintain a 3Q : 1R ratio in the 0 layer but the arginine would be provided by Vti1p instead of Ykt6p. A diploid yeast strain was created with the genotype VTI1 / vti1Δ::HIS3  YKT6 / ykt6 Δ::URA3 and transformed with a centromeric plasmid encoding vti1-R and a multicopy plasmid encoding either wild type YKT6 or ykt6-Q. These strains were sporulated and tetrads were dissected (Fig. 6). A maximum of two spores germinated per tetrad in the presence of the ykt6-Q plasmid. The surviving spores were unable to grow on medium without uracil (data not shown) indicating that the plasmid encoding ykt6-Q did not allow for survival of the ykt6 Δ::URA3 cells. A centromeric plasmid encoding ykt6-Q was also unable to support growth of spores with a ykt6 deletion (data not shown). By contrast, all four spores were able to germinate and ykt6 Δ spores survived in the presence of the YKT6 plasmid (Fig. 6, left panel). These results suggest that the replacement of arginine by glutamine in the 0 layer of Ykt6p results in a nonfunctional protein.
and that expression of vti1-R cannot restore growth of ykt6-Q cells (for a summary of phenotypes see table 3).

**Discussion**

**VTS1 as suppressor for vti1-2**

Here we provide the first information about the open reading frame YOR359w. We found allele-specific genetic interactions with the vti1-2 allele in transport to the vacuole and therefore named the gene VTS1 for vti1-2 suppressor. The mechanism of the suppression remains unclear as we could not detect a physical interaction between Vti1p and Vts1p and deletion of VTS1 alone had no detectable phenotype. The lack of phenotype could be explained by an additional protein with a redundant function. However, a gene encoding a protein with significant amino acid homology to Vts1p is lacking in the yeast genome. Proteins with an overall amino acid homology to Vts1p were only identified in the yeast *Candida albicans* and in the fission yeast *Schizosaccharomyces pombe*. The region of highest homology is found close to the C-terminus of these proteins and fits the consensus sequence of a SAM domain (sterile alpha motif) using the SMART program (http://SMART.embl-heidelberg.de, 38). SAM domains have been implicated in low affinity protein-protein interactions and are often found in signalling proteins (39). Other yeast SAM proteins are the Ste11p MAP kinase kinase in the pheromone pathway and Boi1p and Boi2p which bind to Bem1p in bud formation.

**Ykt6p participates in three SNARE complexes**

It has been reported that Ykt6p is involved in retrograde traffic to the cis-Golgi (11) as well as in homotypic vacuolar fusion in yeast (23). Here we have
shown by lowcopy as well as by highcopy suppression that \textit{YKT6} is also required for biosynthetic transport of ALP and API to the vacuole and for transport of CPY from the Golgi to the prevacuole. The observed suppression by a slight overexpression of \textit{YKT6} minimizes the possibility that Ykt6p replaced an endogenous SNARE in these complexes. Furthermore, this suppression was specific for Ykt6p as it was not observed upon overexpression of any of the other yeast R-SNAREs, which might have been able to occupy the same position as Ykt6p in a SNARE complex (Sec22p, Snc2p and, as previously shown, Nyv1p (20)). A requirement for Ykt6p in biosynthetic transport to the vacuole is supported by a recent study with the temperature-sensitive strain \textit{ykt6-1} (40). \textit{ykt6-1} cells secrete CPY into the medium at all temperatures as shown by western blotting suggesting a block in a post Golgi traffic step. However, it remained unclear whether transport to the prevacuole or to the vacuole was affected. Recombinant Ykt6p bound to all SNAREs implicated in ER to Golgi and intra Golgi traffic (Bet1p, Bos1p, Sec22p, Sed5p, Sft1p, Gos1p, Vti1p) as well as to Pep12p (40). However, demonstration of binary \textit{in vitro} interactions with Ykt6p are not sufficient to identify functionally relevant SNARE complexes because mammalian SNAREs are known to form promiscuous complexes \textit{in vitro} which not necessarily reflect their \textit{in vivo} function (41, 42).

Our functional data indicate that the R-SNARE Ykt6p together with the Q-SNARE Vti1p form three different SNARE complexes with the syntaxin-related Q-SNAREs Sed5p, Pep12p and Vam3p localized to the Golgi apparatus, the prevacuole and the vacuole, respectively (Fig. 7). Therefore Ykt6p or Ykt6p and Vti1p on the transport vesicle are not sufficient to ensure specificity in membrane traffic. Specific recognition of the target membrane could result from interactions of Ykt6p with an additional specific SNARE protein on the vesicles. Two
different R-SNAREs have been shown to function both in anterograde transport and in their respective recycling pathway in two distinct SNARE complexes. The R-SNARE Sec22p forms a complex with Bet1p, Bos1p and Sed5p in traffic from the ER to the Golgi (43 - 46) and with the ER syntaxin Ufe1p in Golgi to ER traffic (47). The redundant R-SNAREs Snc1/2p are required for exocytosis together with Sec9p and Sso1/2p (36, 48) and for endocytosis with Tlg1p and Tlg2p (49).

Increasing evidence suggests that two different molecular interactions contribute to specificity in membrane traffic. The first selective recognition of vesicle and target membrane takes place during tethering. In this reaction specific rab/ypt proteins interact with tethering factors on opposite membranes (2). Docking then provides a signal for SNARE complex formation, the second specific pairing event.

Earlier studies have shown that transport of ALP from the Golgi and of API from CVT vesicles/autophagosomes to the vacuole requires the Q-SNAREs Vam3p, Vam7p and Vti1p (16 - 20). Our data indicate that the R-SNARE Ykt6p is the fourth member of this SNARE complex. Most likely, the same SNARE complex is required for CPY transport from the prevacuole to the vacuole because defects in VAM3 or VAM7 block CPY transport to the vacuole. We were unable to study this directly in vti1 mutant cells as Vti1p and Ykt6p are also used in the preceding Golgi to prevacuolar transport step. In vitro formation of the SNARE complex required for yeast biosynthetic traffic to the vacuole has been demonstrated using recombinant soluble fragments of Vam3p, Vam7p, Vti1p and Ykt6p (50). This SNARE complex would be similar in structure to the neuronal SNARE complex consisting of synaptobrevin, syntaxin 1 and SNAP-25 (4) and to the recently identified mammalian late endosomal SNARE complex with endobrevin, syntaxin 7, syntaxin 8 and vti1b (37). Therefore we provide further
evidence that SNARE complexes have a common structure of one R- and three Q-SNARE helices. The amino acid sequence of the SNARE motif in vti1b is related to the N-terminal helix of SNAP-25 and that of syntaxin 8 is similar to the C-terminal helix of SNAP-25. Yeast Vti1p shares the highest amino acid similarities with the mammalian homologs vti1a and vti1b making it likely that they occupy the same position in SNARE complexes. In addition, yeast Vti1p and the N-terminal helix of SNAP-25 share a glycine in the highly conserved -3 layer while an alanine is found in this position in the C-terminal helix of SNAP-25 as well as in Vam7p. These three SNARE complexes have different membrane anchors. Synaptobrevin and syntaxin 1 have transmembrane domains while SNAP-25 is palmitoylated. Endobrevin, syntaxin 7, syntaxin 8 and vti1b all have C-terminal transmembrane domains. Only Vam3p and Vti1p are attached by transmembrane domains in the SNARE complex described here. Ykt6p has a C-terminal farnesylation consensus sequence while Vam7p is partially soluble (51) and has a single cysteine residue as a potential palmitoylation site.

It has been suggested that a pentameric SNARE complex with Vam7p, Vam3p, Vti1p and two R-SNAREs, Ykt6p and Nyv1p is required for homotypic fusion of vacuoles in an in vitro assay (23). Antibodies against these five SNAREs block in vitro vacuolar fusion. Vacuoles isolated from strains with deleted or mutant VAM3, VAM7, VTI1 or NYV1 are defective in this assay (21 - 23) while data for mutant YKT6 are not available. We have shown earlier that Nyv1p is not required for biosynthetic traffic to the vacuole (20). A quaternary SNARE complex with recombinant Vam3p, Vam7p, Vti1p and Nyv1p was sufficient to drive fusion of liposomes in vitro (50). Nyv1p and Ykt6p were competing for the same binding site in this assay as well as in SNARE complex formation in vitro. This indicates that two different quaternary SNARE
complexes exist on the yeast vacuole, each with the same three Q-SNAREs but with a different R-SNARE, either Nyv1p or Ykt6p. The observed co-immunoprecipitation of Nyv1p and Ykt6p (23) could be explained by association of the two different quaternary SNARE complexes.

Only two Q-SNAREs (Vti1p and Pep12p) and one R-SNARE (Ykt6p) have been identified for transport from the Golgi to the prevacuole. An additional Q-SNARE is probably involved in this step which should have sequence homology to the C-terminal helix of SNAP-25. Tlg1p and Vam7p are candidates but have been identified as part of different SNARE complexes already. Tlg1p, the syntaxin-related Tlg2p and the R-SNARE Snc1p are required for endocytosis and may form a four helix bundle with Vti1p (49, 52, 53).

Implication for role of the 0 layer in the SNARE complex

We generated mutations in the 0 layer of \textit{VTI1} and \textit{YKT6} to study its role in vesicular traffic. Yeast cells expressing only \textit{vti1}-Q158R were viable but displayed severe defects in transport to the vacuole even at 24°C. These data indicate that SNARE complexes with two R-SNAREs are defective in vacuolar transport. 0 layer mutations were recently studied in the exocytic SNARE complex in yeast (54, 55). Sec9p contributes two Q-helices, Sso1p or Sso2p one Q-helix and Snc1p or Snc2p one R-helix to this SNARE complex. Introduction of a second arginine into the 0 layer resulted in severe defects for \textit{sso2}-Q228R, \textit{sso1}-Q224R and \textit{sec9}-Q622R and lethality for \textit{sec9}-Q468R similar to the defects we observed in \textit{vti1}-Q158R cells. Secretion was normal in exocytic SNARE complexes with four glutamines in the 0 layer generated by mutating the arginine residue in the 0 layer of \textit{SNC1} or \textit{SNC2} to glutamine (54, 55). By contrast, we found that cells expressing only \textit{ykt6}-R165Q were not viable. \textit{ykt6}-Q had a dominant negative
effect on CPY transport from the Golgi to the vacuole if expressed in wild type cells. These data suggest that the \textit{ykt6}\textit{}\textunderscore Q\textit{ protein was able to bind into a SNARE complex and rendered it non-functional. Therefore the arginine in the 0 layer of Ykt6p is required for traffic from the Golgi to the vacuole as well as for its essential function in Golgi traffic. As transport to the vacuole is not required for survival under optimal growth conditions a block in vacuolar traffic would not result in lethality. Restoration of a 3Q:1R ratio in \textit{snc}\textit{}\textunderscore Q\textit{ cells resulted in a functional exocytic SNARE complex. In efforts to form 3Q:1R SNARE complexes in cells expressing \textit{vti1}\textit{}\textunderscore R\textit{ we overexpressed \textit{ykt6}\textit{\textunderscore Q\textit{ in the presence of wild type Ykt6p. This resulted in a slight suppression of the ALP-sorting defect. These data indicate that SNARE complexes with \textit{ykt6}\textit{\textunderscore Q\textit{ and \textit{vti1}\textit{\textunderscore R\textit{ have partial function in traffic to the vacuole. However, it is unclear to what degree wild-type Ykt6p versus \textit{ykt6}\textit{\textunderscore Q\textit{ protein are present in SNARE complexes. Cells expressing \textit{vti1}\textit{\textunderscore R\textit{ did not survive in the presence of \textit{ykt6}\textit{\textunderscore Q\textit{ without Ykt6p. These data indicate either that Ykt6p is involved in an essential SNARE complex without Vti1p or that the essential SNARE complex does not function if the arginine is contributed by Vti1p and the glutamine by Ykt6p. Therefore our data demonstrate a more critical role for the arginine in the 0 layer of Ykt6p than for the arginine in the 0 layer of Snc1/2p.
References


Acknowledgments

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Abbreviations

The abbreviations used are: SNARE, soluble NSF attachment protein receptor; v-SNARE, vesicle associated SNARE; t-SNARE, target membrane-associated SNARE; Q-SNARE, SNARE with glutamine in the 0 layer; R-SNARE, SNARE with arginine in the 0 layer; CPY, carboxypeptidase Y; ALP, alkaline phosphatase; API, aminopeptidase I
Figure Legends

Figure 1
Overexpression of VTS1 or YKT6 partially suppressed the growth defect of vti1-2 cells at 37°C. Dilutions of wild-type (WT), vti1-2, and vti1-2 cells overexpressing YKT6 either from a 11 kb library plasmid or as the only open reading frame on a 2µ or a CEN6 plasmid (top panel) or VTS1 from a 4,4 kb library plasmid, as the only open reading frame on a 2µ or a CEN6 plasmid (bottom panel) were incubated at 24°C or at 37°C on plates with rich medium.

Figure 2
Overexpression of VTS1 partially suppressed the API and ALP but not the CPY sorting defects in vti1-2 cells. vti1-2 cells with the indicated plasmids were grown at 24°C and preincubated for 15 min at the restrictive temperatures. (A) CPY traffic was followed by pulse-chase labeling at 32°C and immunoprecipitation from cellular extracts (I) and the medium (E). (B) API was immunoprecipitated from cells pulsed for 10 min and chased for 2 h at 37°C. (C) ALP was immunoprecipitated from cell extracts after a chase period of 0 min, 10 min or 30 min at 36°C, respectively. Immunoprecipitates were analyzed by SDS-PAGE and autoradiography.

Figure 3
Deletion of VTS1 did not result in a growth defect in wild type or vti1-1 cells but resulted in a synthetic growth defect in vti1-2 cells at 37°C. VTS1 was deleted in wild type, vti1-1 and vti1-2 cells. Dilutions of cells from the resulting vts1Δ strains and their parent strains were incubated at 24°C or at 37°C on plates with
rich medium. While *vti1-2* cells grew slowly at 37°C, *vti1-2 vts1Δ* cells were unable to grow at this temperature.

**Figure 4**
Overproduction of Ykt6p in *vti1-ts* cells partially restored transport of API, ALP and CPY to the vacuole. Overexpression of the R-SNAREs *SNC2* or *SEC22* did not significantly improve vacuolar transport of ALP or CPY. (A, B) *vti1-2* and *vti1-2* cells overexpressing *YKT6, SNC2* or *SEC22* from a *CEN6* or a 2µ plasmid were grown at 24°C. API and ALP were immunoprecipitated after a chase period of 2h (API) or 30 min (ALP) at 37°C and 36°C, respectively. (C, D) CPY was immunoprecipitated from cellular extracts (I) and the medium (E) after pulse-chase labeling at 36°C. Overproduction of Ykt6p partially restored transport of CPY to the vacuole in *vti1-1* cells and reduced the ER-accumulation of p1CPY in *vti1-11* cells.

**Figure 5**
Overexpression of *ykt6-Q* improved transport of ALP to the vacuole in *vti1-R* cells but had a dominant negative effect on CPY sorting in wild type cells. Overproduction of Ykt6p did not have an effect. Wild type *YKT6* or *ykt6*-R165Q (0 layer mutation) were overexpressed using 2µ plasmids in cells expressing only *vti1-Q158R* (0 layer mutation). ALP traffic was analyzed using pulse-chase immunoprecipitations. *ykt6-Q* was expressed from a centromeric plasmid or overexpressed from a 2µ plasmid in wild type cells. Wild type *YKT6* was overexpressed as a control. CPY was immunoprecipitated from cellular extracts...
(I) and medium (E) after pulse-chase labeling. Cells were grown and pulse-chase labeled at 30°.

Figure 6
Expression of \( ykt6\text{-}R165Q \) did not allow for survival of spores in the absence of \( YKT6 \) in cells expressing either \( VTI1 \) or \( vti1\text{-}Q158R \). Diploid yeast cells with the genotype \( VTI1/vti1\Delta::HIS3 \ YKT6/ykt6\Delta::URA3 \) were transformed with a centromeric plasmid encoding \( vti1\text{-}R \) and with a \( 2\mu \) plasmid encoding either \( YKT6 \) or \( ykt6\text{-}Q \). Cells were sporulated and tetrads dissected. Whereas four spores germinated and \( ykt6\Delta::URA3 \) spores survived upon expression of wild type \( YKT6 \) only two spores germinated and no viable \( \text{Ura}^+ \) spores were found in the presence of the \( ykt6\text{-}Q \) plasmid.

Figure 7
Model for Ykt6p and Vti1p function in membrane traffic. Ykt6p and Vti1p are part of three distinct SNARE complexes: together with Sft1p and Sed5p in retrograde traffic to the cis Golgi, with Pep12p in traffic from the Golgi to the prevacuole, and with Vam7p and Vam3p in traffic to the vacuole. Five SNAREs (Ykt6p, Vti1p, Vam7p, Vam3p and Nyv1p) have been implicated in homotypic vacuolar fusion, but may be present in two different SNARE complexes. Vti1p has also been coimmunoprecipitated with the early endosomal SNAREs Tlg1p and Tlg2p.
### Table 1. Yeast strains used in this study

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Table 2. Plasmids used in this study

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Table 3. Phenotypes of cells producing Ykt6p and Vti1p with arginine or glutamine residues in the 0 layer

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<td>R + Q</td>
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**Fig. 1** Dilcher et al.
Fig. 2 Dilcher et al.
Fig. 3  Dilcher et al.
Fig. 4 Dilcher et al.
Fig. 5 Dilcher et al.
Fig. 6 Dilcher et al.
Fig. 7 Dilcher et al.
Genetic interactions with the yeast Q-SNARE VTI1 reveal novel functions for the R-SNARE YKT6
Meik Dilcher, Beate Köhler and Gabriele Fischer von Mollard

J. Biol. Chem. published online July 9, 2001

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