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Endosomal transport function in yeast requires a novel AAA-type ATPase, Vps4p

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In a late-Golgi compartment of the yeast *Saccharomyces cerevisiae*, vacuolar proteins such as carboxypeptidase Y (CPY) are actively sorted away from the secretory pathway and transported to the vacuole via a pre-vacuolar, endosome-like intermediate. The vacuolar protein sorting (*vps*) mutant *vps4* accumulates vacuolar, endocytic and late-Golgi markers in an aberrant multilamellar pre-vacuolar compartment. The *VPS4* gene has been cloned and found to encode a 48 kDa protein which belongs to the protein family of AAA-type ATPases. The Vps4 protein was purified and shown to exhibit an *N*-ethylmaleimide-sensitive ATPase activity. A single amino acid change within the AAA motif of Vps4p yielded a protein that lacked ATPase activity and did not complement the protein sorting or morphological defects of the *vps4Δ1* mutant. Indeed, when expressed at normal levels in wild-type cells, the mutant *vps4* gene acted as a dominant-negative allele. The phenotypic characterization of a temperature-sensitive *vps4* allele showed that the immediate consequence of loss of Vps4p function is a defect in vacuolar protein delivery. In this mutant, precursor CPY was not secreted but instead accumulated in an intracellular compartment, presumably the pre-vacuolar endosome. Electron microscopy revealed that upon temperature shift, exaggerated stacks of curved cisternal membranes (aberrant endosome) also accumulated in the *vps4^ts* mutant. Based on these and other observations, we propose that Vps4p function is required for efficient transport out of the pre-vacuolar endosome.

Keywords: ATPase/endosome transport/vacuole

Introduction

The structure and function of the subcellular organelles in eukaryotic cells depend on the specific localization and retention of resident proteins. These proteins are transported from their common site of synthesis to the unique compartment in which they function. A well-studied transport pathway in the yeast *Saccharomyces cerevisiae* is responsible for delivery of proteins to the vacuole, the functional equivalent of the lysosome in mammalian cells. The biosynthetic transport pathway for the vacuolar enzyme carboxypeptidase Y (CPY) is well characterized and therefore serves as a general model for vesicle-mediated vacuolar protein sorting (reviewed in Stack *et al.*, 1995). The CPY protein is translocated into the endoplasmic reticulum (ER) and subsequently traverses the Golgi complex. In the trans-Golgi, CPY is sorted away from secretory traffic by binding to the receptor protein Vps10p which carries CPY to a pre-vacuolar endosome-like compartment (Marcusson *et al.*, 1994). From this compartment, Vps10p recycles back to the trans-Golgi for further rounds of sorting, while CPY is transported on to the vacuole (Cereghino *et al.*, 1995; Cooper and Stevens, 1996). A similar pathway is found in higher eukaryotes, where lysosomal proteins carrying a mannose-6-phosphate moiety are recognized in the trans-Golgi network by one of the two mannose-6-phosphate receptors and subsequently transported to the endosome (reviewed in Kornfeld, 1992).

Multiple transport pathways depend on the endosomal membrane system. Endocytosed proteins are first delivered to an early endosome, where they are either recycled back to the plasma membrane or delivered to the lysosome/vacuole via a late/matured endosome. Newly synthesized vacuolar hydrolases also transit through an endosomal intermediate en route from the trans-Golgi to the lysosome/vacuole. The biosynthetic pathway for lysosomal/vacuolar hydrolases thus converges at the endosome with endocytic traffic (Vida *et al.*, 1993; Schimmöller and Riezman, 1993; for a review, see Gruenberg and Maxfield, 1995). Although the general features of the endosomal pathways are well established, the mechanisms which are responsible for organization and function of the compartment are poorly understood.

In *S.cerevisiae*, several selection schemes have been undertaken to identify mutants defective in the delivery of proteins to the vacuole. This has resulted in the identification of >40 complementation groups of vacuolar protein sorting (*vps*) mutants (Jones, 1977; Robinson *et al.*, 1988; Rothman *et al.*, 1989). The *vps* mutants are divided into the classes A–F, based on their vacoular morphology and sorting defects (Banta *et al.*, 1988; Raymond *et al.*, 1992). The 13 class E *vps* mutants accumulate vacuolar, endocytic and late-Golgi markers in an aberrant multilamellar structure, the class E compartment (Raymond *et al.*, 1992; Davis *et al.*, 1993; Cereghino *et al.*, 1995; Vida and Emr, 1995; Rieder *et al.*, 1996). Previous analysis of two class E mutants, *vps27* and *vps28*, indicated that the class E compartment might represent an exaggerated pre-vacuolar endosomal compartment (Piper *et al.*, 1995; Rieder *et al.*, 1996).

We describe here the cloning and characterization of *VPS4*, a class E *VPS* gene which encodes a protein that belongs to the family of AAA-type ATPases. The ATPase activity was found to be required for Vps4p function in vacuolar protein trafficking. The phenotypic characterization of a temperature-sensitive *vps4* allele indicated that

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the immediate consequence of loss of Vps4p function is a block in protein and membrane transport from the pre-vacuolar endosome to the vacuole.

Results

Cloning and sequence analysis of VPS4

The wild-type *VPS4* gene was cloned from a yeast genomic library by complementation of the CPY sorting defect of SEY4-17 (method as described in Burd *et al.*, 1996). The complementing activity was localized to a minimal 1.8 kb *Hin*dIII fragment. DNA sequencing of this fragment revealed that the complementing fragment contains an open reading frame (ORF) of 1314 bp predicted to encode a protein with a mol. wt of 48 168 (accession No. U25842). The haploid yeast strain MBY4 carrying a deletion in the *VPS4* ORF was constructed (*vps4Δ1*; Materials and methods). The phenotype of this deletion strain and the diploid which resulted from crossing MBY4 with SEY4-17 was indistinguishable from that of SEY4-17, indicating that the cloned fragment indeed corresponds to the *VPS4* chromosomal locus. Sequence comparisons with nucleotide databases identified *VPS4* homologs in *Schizosaccharomyces pombe*, mouse and human (Figure 1). Additionally, a 220 amino acid region of the Vps4 protein (Vps4p) was found to be highly homologous to the catalytic domain of AAA-type ATPases. Within this group, the proteins with the strongest overall similarity to Vps4p were Pas8p, a yeast protein involved in peroxisome biogenesis (McCollum *et al.*, 1993), and Yta6p, a possible subunit of the yeast proteosome (Schnall *et al.*, 1994). From the primary amino acid sequence analysis, we postulate a three domain structure for the Vps4 protein: the N-terminal domain, containing a putative ~30 amino acid coiled-coil motif, the central ATPase domain and the highly charged C-terminal domain (Figure 1).

Vps4p functions in vacuolar protein sorting

We analyzed the effect of the *VPS4* deletion (*vps4Δ1*) on the sorting of vacuolar proteins by following the maturation of the vacuolar protein CPY in pulse–chase experiments. Cells were metabolically labeled for 10 min with Trans^{35}S-label and chased for 30 min by the addition of unlabeled cysteine and methionine. The cells were spheroplasted and separated into intracellular and extracellular fractions. CPY was then immunoprecipitated, and the distribution and size of the protein was determined by SDS–PAGE (Rieder *et al.*, 1996). In wild-type cells, the ER-modified form of CPY (p1CPY) is glycosylated further in the Golgi complex, resulting in the p2-form of CPY, and ultimately is transported to the vacuole, where p2CPY is proteolytically cleaved to the mature protein (mCPY). In contrast to wild-type cells, which completely matured CPY during the 30 min chase, the *vps4Δ1* strain secreted ~50% of p2CPY (Figure 2, lanes 7 and 8). To examine morphological changes in the *vps4* mutant, cells were stained with the fluorescent dye FM4-64, a lipophilic dye which is internalized and delivered to the vacuole in an energy- and temperature-dependent manner and thus serves as a marker for bulk membrane endocytosis in yeast (Vida and Emr, 1995). Compared with wild-type, *vps4Δ1* cells showed fewer but enlarged vacuoles and the appearance of a small, brightly stained compartment adjacent to the vacuole (data not shown). This new structure is probably the class E compartment which accumulates proteins destined for the vacuole as previously characterized for class E *vps* mutants (Vida and Emr, 1995; Rieder *et al.*, 1996). This result was confirmed by indirect immunofluorescence of the 60 kDa V-ATPase subunit, which was found, consistent with other class E mutants (*vps28*, Rieder *et al.*, 1996; *vps27*, Piper *et al.*, 1995), in both the class E compartment and the vacuole of *vps4Δ1* cells (data not shown). In summary, the CPY sorting defect and the morphological phenotype of *vps4Δ1* mutants are very similar to those described for the original *vps4* mutants (Banta *et al.*, 1988; Robinson *et al.*, 1988; Raymond *et al.*, 1992).

Yeast cells harboring *VPS4* on a high-copy number (2 μ) plasmid overexpressed Vps4p at least 10-fold (Figure 3, lane 6). This resulted in a dominant-negative phenotype. Approximately 30% of p2CPY was secreted by these cells (Figure 2, lanes 3 and 4), and staining with FM4-64 dye indicated the presence of a class E compartment (data not shown). Thus the overexpression of *VPS4* resulted in phenotypes similar to the *vps4Δ1* strain. Elevated levels of Vps4p are likely to result in the titration of another protein or proteins required for vacuolar protein sorting.

Sorting phenotype of a temperature-sensitive VPS4 allele

To study the immediate consequences of loss of Vps4p function, we constructed a temperature-sensitive (*ts*) *VPS4* allele. The *VPS4* gene was mutagenized using an error-prone PCR mutagenesis technique (see Materials and methods). The mutagenized DNA was cloned into a centromeric plasmid and transformed into the *vps4Δ1* strain MBY2. A total of 20 000 colonies were screened using a chromosomally integrated CPY–invertase fusion gene that allows *in situ* identification of mutants that secrete CPY–invertase at high temperatures (Paravicini *et al.*, 1992). Several *vps4^{ts}* alleles were generated, and one of them (*vps4ts229*) was selected for further analysis. By exchanging regions of the wild-type gene with the corresponding *vps4^{ts}* gene fragment, we mapped the *ts* mutation(s) to a 309 bp *Nco*I–*Nar*I fragment that codes for the C-terminal part of the ATPase domain. The sequence of this fragment revealed two adenosine to guanosine substitutions resulting in the exchange of two conserved amino acids (Met307→Thr and Leu327→Ser; Figure 1). The *vps4^{ts}* plasmid expressed stable Vps4p at levels similar to wild-type *VPS4* (Figure 3, lane 5).

To assay the vacuolar protein sorting competence of the *vps4^{ts}* mutant, a *vps4Δ1* strain carrying *vps4^{ts}* on a centromeric plasmid (pMB59) was pre-incubated at either 26 or 37°C for 15 min, then subjected to pulse–chase analysis. The distribution and size of the vacuolar proteins CPY, proteinase A (PrA), alkaline phosphatase (ALP) and carboxypeptidase yscS (CPS) were determined by immunoprecipitation and SDS–PAGE (Figure 4). At 26°C, each vacuolar protein tested was matured normally and remained inside the cell, indicating that at low temperature, *vps4^{ts}* cells sort these proteins in a manner indistinguishable from wild-type cells (Figure 4, lanes 2, 3, 6 and 7). In contrast, at the non-permissive temperature (pre-shift of 15 min), newly synthesized CPY accumulated as the Golgi-modified p2CPY precursor, and ~50% was secreted

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S.c.  -MSTGDFLTKGIELVQKAIDLDTATQYEEAYTAYYNGLDYLMLALKYEKN-PKSKDLIRA   58
S.p.  -MSNPDCLSKAISLVKTAIDNDNAEQYPDAYKYYQSALDYFMMALKYEKN-EKSKEIIRS   58
M.m.  MASTNTNLQKAIDLASKAAQEDKAGNYEEALQLYQHAVQYFLHVVKYEAQGDKAKQSIRA   60
H.s.         LQNAIDLVTNATEEDNAKKYEEALRLYQHAVEYFLHAIKYEAHSDKAKESIRA

S.c.  KFTEYLNRAEQLKKHLESEEANAAKKSPSAGSGSNGGNKKISQEEGEDNGGEDNKKLRGA  118
S.p.  KVIEYLDRAEKLKVYLQEKNNQISSKS-RVSNGNVEGSNSPTANEALDS---DAKKLRSA  114
M.m.  KCTEYLDRAEKLKEYLKKKEKKPQKPVKEAQSGPVD-EKGNDSDGEAESDDPEKKKLQNQ  119
H.s.  KCVQYLDRAEKLKDYLRSNENHGKKPVNENQS                EGDNPENKNLQEQ

S.c.  LSSAILSEKPNVKWEDVAGLEGAKEALKEAVILPVKFPHLFKGNRKPTSGILLYGPPGTG  178
S.p.  LTSAILVEKPNVRWDDIAGLENAKEALKETVLLPIKLPQLFSHGRKPWSGILLYGPPGTG  174
M.m.  LQGAIVIERPNVKWSDVAGLEGAKEALKEAVILPIKFPHLFTGKRTPWRGILLFGPPGTG  179
H.s.  LMGAVVMEKSNIRWNDVSGLEGAKEALNEAVILPINFPHLFTGKRTPWRGILLFGPPGTG

S.c.  KSYLAKAVATEAN-STFFSVSSSDLVSKWMGESEKLVKQLFAMARENKPSIIFIDEVDAL  237
S.p.  KSYLAKAVATEAG-STFFSISSSDLVSKWMGESERLVRQLFEMAREQKPSIIFIDEIDSL  233
M.m.  KSYLAKAVATEANNSTFFSISSSDLVSKWLGESEKLVKNLFQLARENKPSIIFIDEIDSL  239
H.s.  KSYLAKAVATEANNSTFFSVSSSDLMSKWLGESEKLVKNLFELARQHKPSIIFIDEVDSL
      A                                                       Q

S.c.  TGTRGEGESEASRRIKTELLVQMNGVGNDSQGVLVLGATNIPWQLDSAIRRRFERRIYIP  297
S.p.  CGSRSEGESESSRRIKTEFLVQMNGVGKDESGVLVLGATNIPWTLDSAIRRRFEKRIYIP  293
M.m.  CGSRSENESEAARRIKTEFLVQMQGVGVDNDGILVLGATNIPWVLDSAIRRRFEKRIYIP  299
H.s.  CGSRNENESEAARRIKTEFLVQMQGVGNNNDGTLVLGATNIPWVLDSAIRRRFERRIYIP

S.c.  LPDLAARTTMFEINVGDTPCVLTKEDYRTLGAMTEGYSGSDIAVVVKDALMQPIRKIQSA  357
S.p.  LPNAHARARMFELNVGKIPSELTSQDFKELAKMTDGYSGSDISIVVRDAIMEPVRRIHTA  353
M.m.  LPEAHARAAMFRLHLGSTQNSLTEADFQELGRKTDGYSGVDISIIVRDALMQPVRKVQSA  359
H.s.  LPEEAARAQMFRLHLGSTPHNLTDANIHELARKTEGYSGADISIIVRDSLMQPVRKVQSA
                T            S

S.c.  THFKDVST------EDDETRKLTPCSPGDDGAIEMSWTDIEADELKEPDLTIKDFLKAIK  411
S.p.  THFKEVYD------NKSNRTLVTPCSPGDPDAFESSWLEVNPEDIMEPKLTVRDFYSAVR  407
M.m.  THFKKVRGPSRADPNCIVNDLLTPCSPGDPGAIEMTWMDVPGDKLLEPVVSMWDMLRSLS  419
H.s.  THFNKVCGPXRTNPSMMIDDLLTPCSPGDPGAMEMXWMDVPGDNLLEPVV

S.c.  STRPTVNEDDLLKQEQFTRDFGQEGN   437
S.p.  KVKPTLNAGDIEKHTQFTKDFGAEG-   432
M.m.  STKPTVNEQDLLKLKKFTEDFGQEG-   444
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Fig. 1. Amino acid sequence alignment of Vps4p homologs from *S.cerevisiae* (S.c.), *Schizosaccharomyces pombe* (S.p.; DDBJ/EMBL/GenBank L33456), *Mus musculus* (M.m.; SKD1, Périer *et al.*, 1994) and *Homo sapiens* (H.s.; DDBJ/EMBL/GenBank H14152, C03377, M85872, F07485). The sequence alignment was done with the assistance of CLUSTAL W (Thompson *et al.*, 1994). Identities between sequences are indicated with black boxes. The solid line marks the AAA domain, the dots indicate the a and d position of a putative coiled-coil motif predicted by COILS (Lupas *et al.*, 1991). Amino acid changes in the mutant proteins Vps4p^{ts}, Vps4p^{K179A} and Vps4p^{E233Q} are indicated beneath the alignment.

over the course of the chase. The strong block in CPY maturation suggested that the Vps4^{ts} protein was completely inactivated at 37°C (Figure 4, lanes 4 and 5). The intracellular pool of p2CPY migrated at a slightly higher molecular mass than observed in wild-type cells, which might result from hyperglycosylation of CPY. PrA, the other soluble vacuolar hydrolase tested, also accumulated inside the cell as a slower migrating, Golgi-modified form; however, no PrA was secreted in the mutant at high temperature. Analysis of the integral membrane proteins ALP and CPS revealed that in *vps4*^{ts} cells at high temperature, maturation of CPS was blocked whereas maturation of ALP was unaffected (Figure 4).

To study the kinetics of onset of the *vps4*^{ts} phenotype, we shifted *vps4*^{ts} cells from permissive to non-permissive temperature for different times prior to pulse–chase ('preshift') and followed the sorting of CPY (Figure 5). When *vps4*^{ts} cells were shifted to 37°C concomitant with the addition of label, CPY maturation was blocked, and p2CPY accumulated inside the cell (lanes 1 and 2). This rapid block in maturation suggests that Vps4p is directly involved in the transport of p2CPY to the vacuole and that the Vps4p^{ts} protein loses its function rapidly after shift to the non-permissive temperature. Significant secretion of p2CPY was observed when *vps4*^{ts} cells were shifted to 37°C 5 min before addition of label, and ~50% of

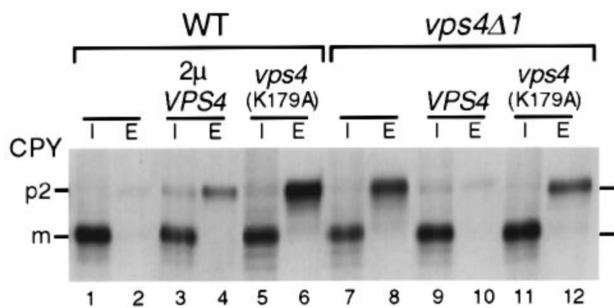

Fig. 2. Sorting of the vacuolar hydrolase CPY analyzed by pulse–chase experiments. Yeast cultures grown at 30°C were labeled with Tran^{35}S-Label for 10 min and harvested 30 min after adding chase. Cells were spheroplasted and separated into intracellular (I) and extracellular (E) fractions. CPY was immunoprecipitated and analyzed by SDS–PAGE. Sorting analysis was performed on wild-type cells SEY6210 (WT) carrying pMB10 (2 μ *VPS4*) or pMB24 (*vps4*^{K179A}) and on *vps4Δ1* strain MBY3 carrying pMB4 (*VPS4*) or pMB24 (*vps4*^{K179A}).

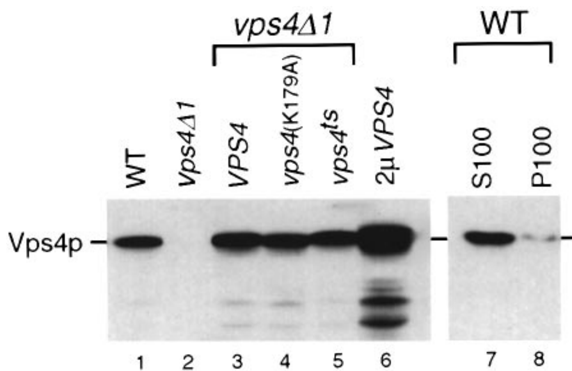

Fig. 3. Immunoblot analysis of yeast extracts using anti-Vps4p antibodies. The left panel shows the immunoblot of cell extracts of SEY6210 (WT), MBY3 (*vps4Δ1*), *VPS4* deletion strain MBY3 carrying pMB4 (*VPS4*) or pMB24 (*vps4*^{K179A}) or pMB59 (*vps4*^{ts}) and SEY6210 carrying pMB10 (2 μ *VPS4*). The right panel shows the fractionation of Vps4p using the wild-type (WT) strain SEY6210 (S100, P100; supernatant and pellet after centrifugation at 100 000 *g*).

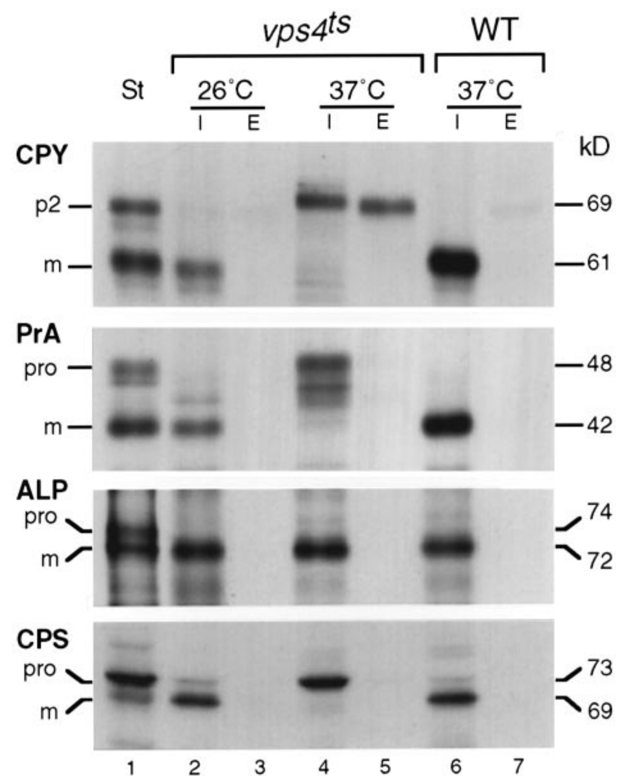

Fig. 4. Vacuolar protein sorting in *vps4*^{ts} cells. Strain MBY3 pMB4 (WT) and MBY3 pMB59 (*vps4*^{ts}) were grown at 26°C and used either directly for pulse–chase experiments at 26°C or pre-incubated for 15 min at 37°C prior to pulse–chase analysis (see Figure 2). Before electrophoresis, CPS samples were treated with endoglycosidase H. An intracellular fraction of MBY3 pMB4 (26°C) analyzed either directly after pulse (ALP) or 5 min after addition of chase (CPY, PrA, CPS) served as standards (St) for the different forms (p2, pro, m) of the vacuolar proteins.

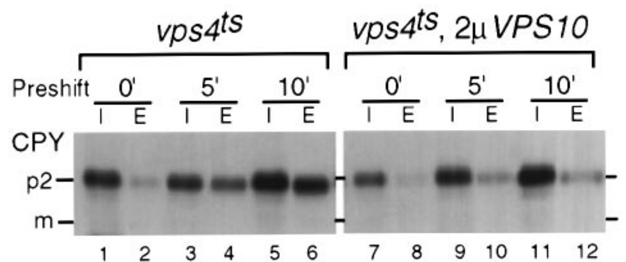

Fig. 5. Kinetic onset of the sorting defect in *vps4*^{ts} and in a *vps4*^{ts} strain overexpressing the sorting receptor Vps10p. Cultures of MBY3 pMB59 (*vps4*^{ts}) or MBY3 pMB59 pEMY10-2 (*vps4*^{ts}, 2 μ *VPS10*) were grown at 26°C and shifted for either 0, 5 or 10 min to 37°C (Preshift) prior to pulse labeling for 5 min and chasing for 30 min at 37°C (see Figure 2).

p2CPY was secreted from cells after pre-shifts longer than 10 min. This sorting defect is identical to that observed with the *VPS4* deletion strain (Figure 5, lanes 5 and 6). The delay in onset of CPY secretion indicates that this phenotype is a secondary effect of the loss of Vps4p function. In earlier publications, it was speculated that the reason for p2CPY secretion in class E *vps* mutants (includes *vps4*) might be the depletion of the CPY sorting receptor, Vps10p, from the late-Golgi, caused by inefficient recycling of Vps10p from an endosomal compartment back to the Golgi complex (Cereghino *et al.*, 1995; Piper *et al.*, 1995). A prediction of this hypothesis is that overexpression of *VPS10* might increase the concentration of Vps10p in the late-Golgi and thus result in reduced secretion of p2CPY. To test this, we introduced *VPS10* on a multi-copy plasmid into the *vps4*^{ts} strain and followed the onset kinetics of p2CPY secretion after shift to the non-permissive temperature (Figure 5, lanes 7–12). As expected, increased expression of *VPS10* suppressed the secretion phenotype of the *vps4*^{ts} cells but did not suppress the block in p2CPY maturation.

vps4 mutants accumulate precursor CPY in a pre-vacuolar compartment

To address where CPY transport is blocked in the *vps4*^{ts} mutant, we monitored by indirect immunofluorescence microscopy the distribution of CPY in *vps4*^{ts} cells before and after shift to the non-permissive temperature. As expected, the strongest CPY staining at the permissive temperature was localized within the vacuole (Figure 6A and B). However, the vacuolar staining was relatively weak, which might be due to a loss of soluble vacuolar proteins during cell preparation. After 30 min incubation at the non-permissive temperature, the majority of CPY

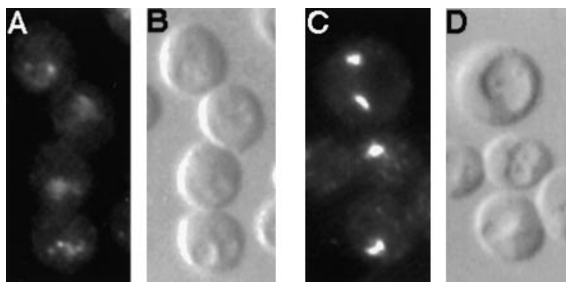

Fig. 6. Cellular distribution of CPY in *vps4^ts^* cells analyzed by indirect immunofluorescence microscopy. MBY3 pMB59 cells (*vps4^ts^*) were grown at 26°C to an optical density (600 nm) of 0.6 and incubated for 30 min either at 26°C (**A** and **B**) or 37°C (**C** and **D**) prior to cell preparation (see Materials and methods). Stained cells were visualized by fluorescence (A and C) or Nomarski (B and D) microscopy.

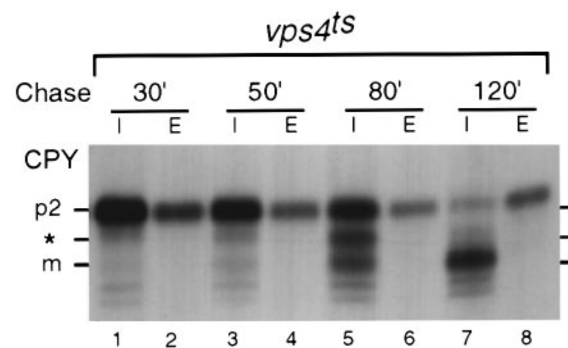

Fig. 7. Maturation of CPY in *vps4^ts^* cells. MBY3 pMB59 cells (*vps4^ts^*) were grown at 26°C and shifted to 37°C 2 min prior to a 10 min pulse labeling. Samples were harvested at the times indicated after addition of chase and further incubated at 37°C. The analysis was performed as described in Figure 2. Different forms of CPY are marked (p2, m, *).

in *vps4^ts^* cells was localized in one or two compartments adjacent to the vacuole (Figure 6C and D). These compartments do not resemble the punctate distribution of the Golgi-resident protein Mnn1p (data not shown), and probably represent class E compartments.

To substantiate further the post-Golgi block conferred by *vps4^ts^*, we monitored in a pulse–chase experiment intracellular and extracellular CPY at late time points after adding chase. This experiment revealed that the amount of CPY inside the cell was constant over 2 h and that during this time, no additional p2CPY was secreted (Figure 7). Studies of mutants in the CPY sorting receptor Vps10p have shown that a block in transport out of the late-Golgi results in the rapid secretion of p2CPY (Marcusson *et al.*, 1994). Our studies thus suggest that in *vps4^ts^* cells at the non-permissive temperature, CPY does not accumulate in the late-Golgi but instead is sorted to a more distal compartment like the pre-vacuolar endosome that accumulates in class E *vps* mutants.

Unlike the *vps4Δ1* strain, which accumulated mCPY inside the cell, *vps4^ts^* cells accumulated exclusively p2CPY at early time points after shift to the non-permissive temperature (compare Figure 2, lane 7 with Figure 4, lane 4). This difference could be explained by the fact that in *vps4Δ1* cells, p2CPY is transported from the Golgi to a class E compartment, which contains active proteases and is competent to mature p2CPY (Raymond *et al.*, 1992; Piper *et al.*, 1995). In contrast, at early time points, *vps4^ts^* cells might not have yet formed a proteolytically active class E compartment, hence no p2CPY is matured. To test this, we followed maturation of CPY at late time points after adding chase (Figure 7). The p2CPY which accumulated inside the cell after shift to the non-permissive temperature was processed within 2 h to the mature form. Maturation occurred via an intermediate form which was not observed in wild-type cells (see *, lane 5 in Figure 7). This suggests that the maturation step did not occur in the vacuole but instead was a consequence of formation of a proteolytically competent class E compartment. In *vps4^ts^* cells which were pre-incubated at the non-permissive temperature for 2 h prior to the pulse–chase experiments, 70% of the newly synthesized, intracellular CPY matured during the 30 min chase (data not shown). This result indicates that after 2 h at non-permissive temperature, newly synthesized CPY was transported to a proteolytically competent class E compartment.

The transport defect in vps4^ts^ cells is reversible

To test if the block in CPY transport observed in *vps4^ts^* cells is reversible, we shifted the cells to 37°C, labeled them for 5 min, and then chased them for 15 min at 37°C. The culture was then shifted back to the permissive temperature, and samples were taken at various time points (Figure 8A). CPY and Vps4p were immunoprecipitated and analyzed by SDS–PAGE (Figure 8B). Most of the p2CPY which accumulated in a pre-vacuolar compartment during incubation at the non-permissive temperature was processed to the mature form within 30 min after shift to the permissive temperature. Compared with the processing observed after a long chase at the non-permissive temperature (Figure 7), CPY maturation observed in this experiment occurred faster and no processing intermediate was observed. This indicated that CPY was transported to the vacuole. The amount of Vps4p^ts^ was constant during the experiment (Figure 8), suggesting that the temperature sensitivity of Vps4p^ts^ was not caused by rapid degradation of the protein at high temperature. Furthermore, we found that the addition of cycloheximide to the culture (100 mg/l) prior to the shift back to 26°C did not influence the maturation kinetics of CPY (data not shown), which argues that *de novo* Vps4p synthesis is not necessary for the observed reversal of the *vps4^ts^* defect. In summary, these data demonstrate that both the loss of Vps4p^ts^ function and the block in CPY transport are reversible.

Morphology of vps4^ts^ cells

Previous characterization of class E *vps* mutants revealed the presence of stacks of curved cisternal membranes (Rieder *et al.*, 1996) known as the class E compartment that is thought to represent an exaggerated pre-vacuolar intermediate compartment (Piper *et al.*, 1995; Rieder *et al.*, 1996). The *vps4^ts^* mutants allowed us to observe the formation of the class E compartment. We characterized by electron microscopy the morphology of *vps4^ts^* cells which were grown at 26°C and shifted to 37°C for various lengths of time. Analysis of >150 sections of cells grown at the permissive temperature revealed the appearance of only a few (<5%) membrane structures similar to class E compartments (Rieder *et al.*, 1996). Several sections of these cells contained aberrant membrane stacks, most of which were adjacent to the vacuole (see arrow in Figure

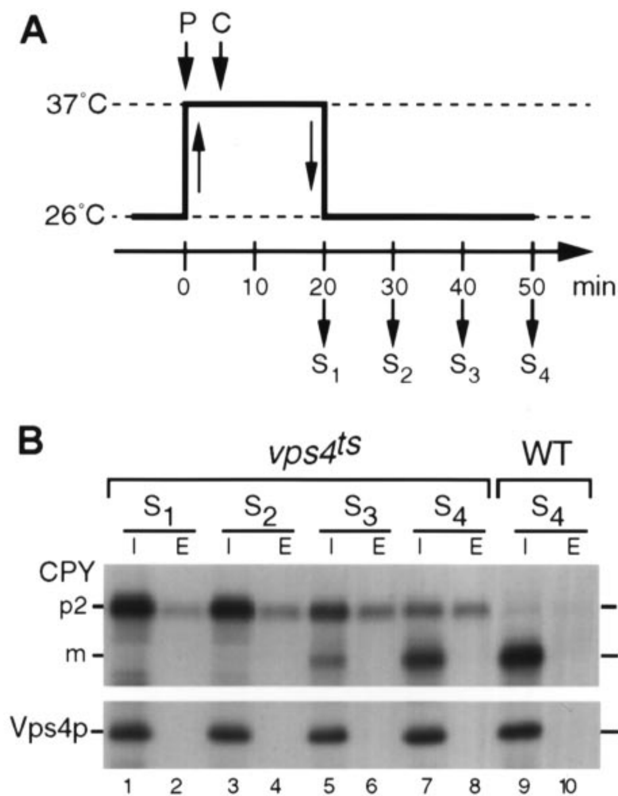

Fig. 8. Reversibility of the sorting phenotype of *vps4^ts^* cells. Cultures of the strains MBY3 pMB59 (*vps4^ts^*) and MBY3 pMB4 (WT) were grown at 26°C and shifted to 37°C; at the same time label was added (P). After 5 min, chase was initiated (C), and the cells were incubated further at 37°C for 15 min. The culture was then shifted back to 26°C, and samples were harvested at the time points indicated (S1–S4; **A**). CPY from the extracellular (E) and CPY and Vps4p from the intracellular (I) fractions were analyzed by immunoprecipitation and SDS–PAGE (**B**).

9A). After 1 h at the non-permissive temperature, the number of cell sections containing stacks of 4–6 curved cisternal membranes increased from <5% to ~30% (*n* = 150; Figure 9B–E). The morphology and frequency of these class E structures is comparable with observations reported in a previous study of a *VPS28* deletion mutant (Rieder *et al.*, 1996).

Vps4p is involved in the endocytic pathway

Several studies have demonstrated that class E mutants exhibit a block in the endocytic pathway (*vps2*, Davis *et al.*, 1993; *vps4*, Munn and Riezman, 1994; *vps27*, Piper *et al.*, 1995; *vps28*, Rieder *et al.*, 1996), which might be a secondary defect due to the accumulation of aberrant endosomal structures. We tested if loss of Vps4p activity directly affects endocytic traffic by comparing Ste6p stability in *vps4^ts^* and wild-type cells. Ste6p, the transporter of the mating pheromone **a**-factor, is rapidly removed from the plasma membrane by endocytosis and subsequently is degraded in the vacuole (Kölling and Hollenberg, 1994). Cells were grown at 26°C and shifted to 37°C 10 min prior to the pulse–chase experiment. The Ste6 protein was immunoprecipitated after different chase times and analyzed by SDS–PAGE (Figure 10). This experiment showed a 2- to 3-fold stabilization of Ste6p in *vps4^ts^* compared with wild-type cells. This suggests that in *vps4^ts^* cells, the transport of Ste6p to the vacuole, where degradation occurs, was impaired. Earlier studies indicated the existence of more than one pathway for the degradation of Ste6p which would explain why in *vps4^ts^* cells Ste6p was only partially stabilized (Kölling and Hollenberg, 1994).

The ATPase activity of Vps4p is critical for sorting of vacuolar proteins

To determine whether the AAA motif of Vps4p is essential for proper vacuolar protein sorting, we used site-directed mutagenesis to change the codons of a highly conserved lysine (position 179) and glutamate (position 233) in the ATPase domain to a codon for alanine or glutamine, respectively ($Vps4p^{K179A}$, $Vps4p^{E233Q}$; Figure 1). Analogous amino acid changes have been shown previously to abolish ATPase activity and function of the NEM-sensitive fusion protein (NSF) (Whiteheart *et al.*, 1994). Western blot analysis showed that $Vps4p^{K179A}$ was expressed in yeast at wild-type levels (Figure 3, lane 4). However, the sorting defect of the *vps4Δ1* strain was not corrected by the mutant $vps4^{K179A}$ gene on a centromeric plasmid (Figure 2, lanes 11 and 12). Additionally, when expressed from a *CEN* plasmid in wild-type cells, $vps4^{K179A}$ acted as a dominant-negative allele causing the same phenotype as described for the deletion strain. These cells missorted ~50% of p2CPY (Figure 2, lanes 5 and 6), and staining with the lipophilic dye FM4-64 revealed the appearance of class E compartments (data not shown). Expression of the other mutant gene $vps4^{E233Q}$ from a *CEN* plasmid either in *vps4Δ1* strain or wild-type cells resulted in the same phenotypes as described for $vps4^{K179A}$ (data not shown). Together, these data clearly demonstrate that the AAA domain is essential for Vps4p function in vacuolar protein sorting.

Biochemical characterization of Vps4p

A polyclonal antiserum raised against a TrpE–Vps4p fusion protein was used to identify the *VPS4* gene product (see Materials and methods). Western blot analysis of wild-type yeast cell extracts identified a protein of M_r ~50 kDa, very near to the predicted molecular weight for Vps4p of 48 kDa. The band was absent in the extracts of the *VPS4* deletion mutant (Figure 3, lanes 1 and 2). Differential centrifugation was then utilized to determine the subcellular localization of the Vps4p. After centrifugation of a yeast cell extract at 100 000 *g*, Vps4p was present mainly in the supernatant (S100), indicating that under these conditions, Vps4p is a soluble protein (Figure 3, lanes 7 and 8).

The Vps4 protein was overexpressed in *Escherichia coli* using a GST fusion vector (see Materials and methods). The resulting GST–Vps4p fusion protein was purified by glutathione affinity chromatography. The fusion protein was proteolytically cleaved with thrombin, resulting in a recombinant Vps4 protein which contains a glycine instead of a methionine at the first position but is otherwise identical to the wild-type protein. Finally, Vps4p was separated from GST and uncleaved fusion protein by ion exchange chromatography (Figure 11; see Materials and methods). The molecular mass of the purified protein was determined by gel filtration analysis (Sephacryl S-200, Pharmacia LKB, Uppsala, Sweden) to be ~60 kDa. There-

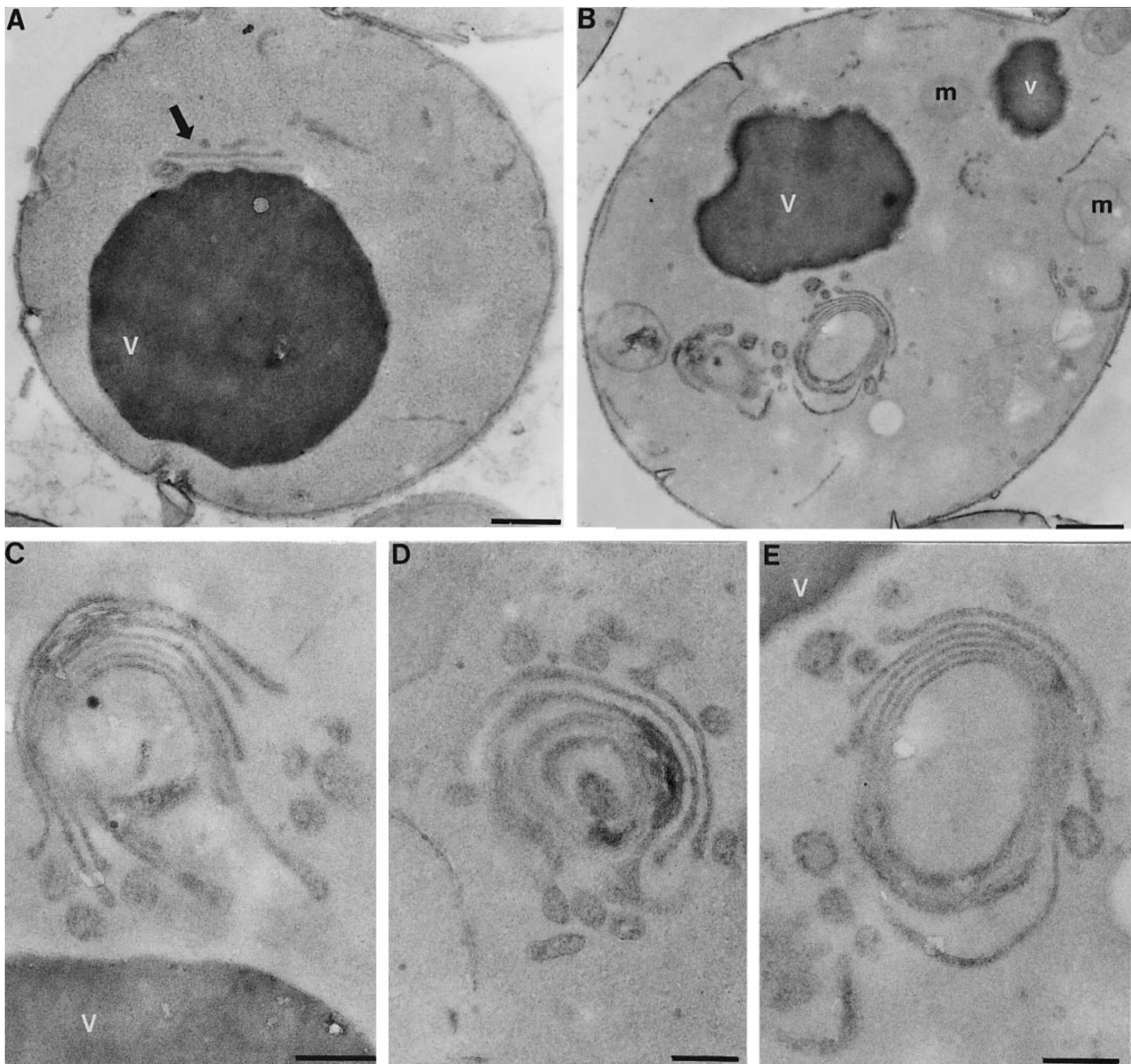

Fig. 9. Electron microscopic analysis of *vps4*^{ts} cells at the permissive and non-permissive temperature. (**A**) Cross-section of an MBY3 pMB59 cell (*vps4*^{ts}) grown at 26°C. The arrow marks aberrant membrane stacks that accumulate in this strain. (**B**) A cell from the same culture after incubation for 1 h at 37°C. (**C**–**E**) Examples of class E compartments formed during 1 h incubation at the non-permissive temperature. (E) The enlarged class E compartment seen in (B). The scale bars in (A) and (B) and (C–E) represent 0.5 and 0.2 µm, respectively (m, mitochondria; v, vacuole).

fore, the recombinant Vps4p is most likely a monomeric protein. The specific ATPase activity of recombinant Vps4p was measured *in vitro* and found to be ~450 nmol/mg/min (Table I). After a 15 min pre-treatment of Vps4p with 1 mM *N*-ethylmaleimide (NEM) at 23°C, a 4-fold inhibition of the ATPase activity was observed. NEM-sensitive ATPase activity has also been described for other members of the AAA family (NSF, Whiteheart *et al.*, 1994; p97, Peters *et al.*, 1990). The mutant protein Vps4p^{K179A}, which carries an amino acid exchange in the AAA motif (see above), was expressed and purified as described for wild-type Vps4p. Using this mutant protein for the *in vitro* activity test, no ATP hydrolysis was detected (Table I).

Discussion

We have cloned and characterized *VPS4*, a gene required for efficient vacuolar protein sorting. The predicted amino acid sequence of Vps4p identifies it as a member of the AAA (ATPases associated with a variety of cellular activities) family. The key feature of this family is a highly conserved ATPase domain of ~220 amino acids present in one or two copies in each protein. This domain is found in a wide variety of proteins with diverse cellular functions, including Cdc48p/p97 required for membrane fusion, several 26S proteosome subunits and FtsH, an *E.coli* proteinase involved in heat shock regulation (for a review about the AAA family, see Confalonieri and

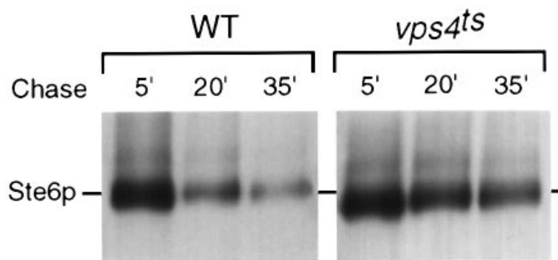

Fig. 10. Stability of Ste6p in wild-type and *vps4^ts^*. Cultures of MBY4 pMB4 pDB192 (WT) and MBY4 pMB59 pDB192 (*vps4^ts^*) were grown at 26°C, shifted to 37°C 10 min prior to the pulse labeling for 15 min. After addition of chase (time 0), the culture was incubated further at 37°C, and samples were harvested at the time points indicated (Chase). The Ste6 protein from these samples was immunoprecipitated and analyzed by SDS–PAGE.

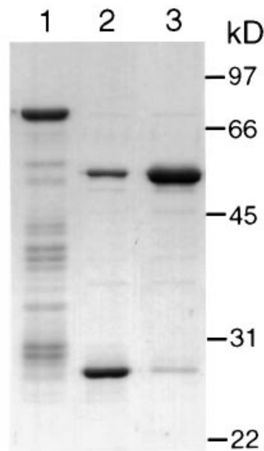

Fig. 11. Purification of recombinant Vps4p using a GST fusion system. GST–Vps4p was overexpressed in *E.coli* and purified by affinity chromatography (1). The fusion protein was cleaved by thrombin (2) and further purified using anion exchange chromatography (3).

Duguet, 1995). The best characterized AAA protein is NSF, the mammalian homolog of yeast Sec18p (Eakle *et al.*, 1988). NSF/Sec18p is required for several transport steps (Graham and Emr, 1991) such as vesicular transport from the ER to the Golgi (Beckers *et al.*, 1989), transport between Golgi stacks (Balch *et al.*, 1984) and the homotypic fusion of endosomes and vacuoles (Diaz *et al.*, 1989; Mayer *et al.*, 1996). Despite the fact that NSF and Vps4p are both AAA family members required for protein trafficking, their structural and biochemical characteristics differ significantly from each other. Vps4p contains one ATPase domain and is a monomeric protein localized to the cytosol, whereas NSF belongs to the AAA subgroup with two ATP-binding domains, forms a trimeric complex and is mostly associated with the membrane (Rodriguez *et al.*, 1994; Whiteheart *et al.*, 1994). In addition, the specific ATPase activity measured for Vps4p is ~30 times higher than the maximal activity described for purified NSF (Whiteheart *et al.*, 1994). Using site-directed mutagenesis to change conserved amino acids in the ATP-binding site (Vps4p^{K179A}, Vps4p^{E233Q}), we demonstrated that ATPase activity is absolutely required for Vps4p function but did not affect the stability of the protein. The analogous point mutation in the two AAA motifs of NSF revealed that ATP hydrolysis by only the N-terminal ATP-binding domain is necessary for NSF-mediated fusion. The second domain was shown to be required for trimer formation and therefore seems to play a structural role in the NSF complex (Whiteheart *et al.*, 1994).

Table I. ATPase activity of wild-type and mutant Vps4p

Protein	Activity (nmol ATP/min/mg)
Vps4p	448 ± 51
Vps4p + NEM	104 ± 28
Vps4p^{K179A}	1 ± 1
No protein	2 ± 3

Normal levels of expression of the mutant genes *vps4*^{K179A} and *vps4*^{E233Q} in wild-type cells or overexpression of wild-type *VPS4* both resulted in a dominant-negative phenotype with sorting defects similar to those observed for the *vps4Δ1* strain. This phenotype might result from depletion of other class E Vps proteins which is caused by the non-functional interaction of these proteins with mutated or overexpressed Vps4p.

Role for Vps4p in endosome to vacuole transport

VPS4 is one of the 13 class E *VPS* genes required for efficient biosynthetic and endocytic traffic to the vacuole (Raymond *et al.*, 1992). The class E *vps* mutants accumulate vacuolar and endocytic markers in an aberrant pre-vacuolar compartment, the class E compartment. Additionally, ~50% of the vacuolar hydrolase CPY is missorted and secreted by the mutant cells (Robinson *et al.*, 1988; Piper *et al.*, 1995; Vida and Emr, 1995; Rieder *et al.*, 1996; Figure 2). The massive accumulation of pre-vacuolar membranes in class E mutants may cause a variety of secondary defects in protein trafficking. Therefore, we constructed a temperature-sensitive *vps4* allele to address the onset kinetics of the protein sorting and morphology defects that result from inactivation of the Vps4 protein. At the permissive temperature, the *vps4*^{ts} mutant displayed near-wild type morphology and normal vacuolar sorting phenotypes. However, upon shift to non-permissive temperature, the *vps4*^{ts} mutant exhibited a block in protein trafficking to the vacuole, resulting in rapid intracellular accumulation of the soluble hydrolases CPY and PrA and the transmembrane protein CPS in their Golgi-modified precursor forms. The block in transport had an immediate onset and also was reversible when the cells were returned to the permissive temperature. Therefore, the *vps4*^{ts} allele represents a very useful tool to study the immediate consequences of the loss of Vps4p function. Maturation of ALP remained unaffected in *vps4*^{ts} cells at the non-permissive temperature, indicating that the ALP protein is transported to the vacuole via an alternative, Vps4p-independent pathway (see also Burd *et al.*, 1996; Horazdovsky *et al.*, 1996; Rieder *et al.*, 1996). As ALP transport to the vacuole is not affected even in null class E mutants (Raymond *et al.*, 1992), this alternative pathway apparently bypasses the provacuolar endosome utilized for CPY transport.

vps4^{ts} *mutant accumulates pre-vacuolar cisternal membranes*

Mutant cells which either lack the sorting receptor Vps10p or block Vps10p transport out of the late-Golgi (i.e. *vps15*^{ts}

mutants), secrete almost all p2CPY (Herman *et al.*, 1991; Marcusson *et al.*, 1994). The fact that the *vps4*^{ts} strain, even after extended incubation (60 min) at the non-permissive temperature, accumulated p2CPY inside the cell indicates that the block in trafficking is likely to occur in a post-sorting compartment (i.e. endosomal compartment). In addition, indirect immunofluorescence microscopy demonstrated that *vps4*^{ts} cells accumulated p2CPY inside the cell in a pre-vacuolar compartment, most likely the class E compartment, which clearly differs in size and distribution from the Golgi.

In contrast to the immediate block of p2CPY transport to the vacuole, the onset of p2CPY secretion in *vps4*^{ts} cells after shift to the non-permissive temperature was delayed and could be suppressed by overexpression of the sorting receptor Vps10p. These results support a model in which a loss in Vps4p function affects the recycling of Vps10p from the pre-vacuolar endosome back to the Golgi. This defect would lead to depletion of Vps10p from the late-Golgi and thus indirectly result in secretion of p2CPY (see also Cereghino *et al.*, 1995; Piper *et al.*, 1995; Rieder *et al.*, 1996). However, the relatively modest Golgi to endosome sorting defect in *vps4*^{ts} cells indicates that recycling of Vps10p is only partially impaired. Therefore, in comparison with the strong block in forward transport to the vacuole, the recycling defect seems to be a secondary effect of the loss of Vps4p function.

Incubation of *vps4*^{ts} cells at the non-permissive temperature for a long period of time resulted in a phenotype indistinguishable from the *VPS4* deletion strain. After 1 h at the non-permissive temperature, the *vps4*^{ts} cells formed a membrane structure with morphology characteristics of the class E compartment (Rieder *et al.*, 1996). Two hours after inactivation of Vps4p^{ts}, the class E compartment became proteolytically competent, which resulted in maturation of the accumulated p2CPY. Under these conditions, newly synthesized CPY matures rapidly inside the cell. Therefore, while the morphology of the class E compartment suggests that it comprises multiple discrete membrane stacks, the rapid maturation of newly synthesized CPY indicates that the class E compartment is functionally uniform, with respect to soluble hydrolases.

Earlier studies have shown that in class E null mutants (including double class E mutants), a significant pool of the proteins which accumulate in the class E compartment ultimately reach the vacuole (Raymond *et al.*, 1992; Piper *et al.*, 1995; Rieder *et al.*, 1996). Consistent with these data, we found that in *vps4Δ1* cells a substantial amount of the V-ATPase also localized properly to the vacuole. These results, and the fact that class E mutants still have a vacuole, suggest that deletion of a class E *VPS* gene does not lead to a complete block in protein and membrane transport to the vacuole. However, this transport process appears to be very inefficient: results with *vps4*^{ts} cells indicate that this process requires >90 min. Therefore, transport may occur by an alternative mechanism which is different from the normal endosome to vacuole transport system (i.e. autophagy).

Role for Vps4p in endocytosis

Earlier publications demonstrated that endocytic markers accumulate in the class E compartment (Raymond *et al.*, 1992; Davis *et al.*, 1993; Piper *et al.*, 1995; Rieder *et al.*, 1996), which suggests a role for the class E Vps proteins in the endocytic pathway. The endocytosis defect of class E mutants, however, might be caused indirectly by the accumulation of proteins and membranes from the biosynthetic pathway in an endosomal compartment. The yeast Ste6 protein, an ABC-transporter required for secretion of mating pheromone **a**-factor, is removed rapidly from the plasma membrane by endocytosis and subsequently degraded in the vacuole (Kölling and Hollenberg, 1994; Browne *et al.*, 1996). We found that at early time points after shift to the non-permissive temperature, Ste6p was more stable in *vps4*^{ts} cells compared with wild-type cells. This result suggests that Vps4p may function directly in the endocytic pathway to the vacuole. In fact, a screen for mutants defective in endocytosis led to the isolation of *END13*, which was found to be allelic to *VPS4* (Munn and Riezman, 1994). The analysis of α-factor turnover in an *end13* mutant revealed that α-factor is internalized from the cell surface but that delivery to the vacuole and thus degradation was impaired. Therefore, it seems likely that in the *vps4*^{ts} mutant, Ste6p stabilization was caused by a block in transport from a pre-vacuolar compartment to the vacuole. The fact that *vps4* mutants exhibit a block in endocytosis and biosynthetic traffic indicates that Vps4p is acting on the pre-vacuolar endosome, the intermediate compartment common to both pathways (Schimmöller and Riezman, 1993; Vida *et al.*, 1993).

Role of Vps4p as a regulator of class E gene product function

In summary, our data support a model in which the primary role of Vps4p is in endosome function, where Vps4p is required directly for transport of vacuolar and endocytosed proteins from the pre-vacuolar endosome to the vacuole. Proteins destined for the vacuole accumulate in a large stack of cisternal membranes (class E compartment) in *vps4* mutants, which argues that Vps4p function is not required for fusion of transport intermediates with the endosome, but is required for transport out of this pre-vacuolar compartment. The phenotypic similarities among the 13 class E *vps* mutants (including class E double mutants) suggest that the class E Vps proteins act together at the same stage of the transport pathway, possibly as a part of a protein complex. The class E proteins might be involved either directly in the formation of transport intermediates from the endosome or in the maintenance of a dynamic endosomal structure required for transport out of the compartment. In keeping with Confalonieri and Duguet's hypothesis for the function of the AAA proteins (Confalonieri and Duguet, 1995), Vps4p might act as a molecular chaperone or ATP-dependent protein clamp which regulates the assembly or disassembly of a class E protein complex. Given the high degree of sequence conservation between Vps4p, SKD1 (mouse) and homologs in other organisms, it is likely that Vps4p function in protein trafficking is ubiquitous in all eukaryotes.

Materials and methods

Materials

Tran^{35}S-label was purchased from ICN Radiochemicals (Irvine, CA). Antisera against CPY, PrA, ALP and CPS have been characterized

Table II. Strains and plasmids used in this study

Strain or plasmid	Genotype or description	Reference or source
S.cerevisiae		
SEY6210	*MATα leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9*	Robinson *et al.* (1988)
SEY6210.1	SEY6210; *MAT***a**	P.Scott, unpublished
BHY10	SEY6210; *leu2-3,112*::pBHY11 (CPY-Inv *LEU2*)	Rothman and Stevens (1986)
SEY4-17	SEY6210; *vps4-17*	Robinson *et al.* (1988)
MBY2	BHY10; *VPS4::TRP1*	this study
MBY3	SEY6210; *VPS*4::TRP1	this study
MBY4	SEY6210.1; *VPS4::TRP1*	this study
E.coli		
XL1-blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB lacI*^{q}*ZΔM15* Tn*10*(tet^r)]	Stratagene Cloning Systems, CA
Plasmids		
pRS413	HIS3 Ap^R *CEN*	Christianson *et al.* (1992)
pRS425	*LEU2* Ap^R 2 μ	Christianson *et al.* (1992)
pGEX-2T	Ap^R	Pharmacia LKB, Uppsala, Sweden
pMB4	HIS3 ApR (pRS413) *VPS4*	this study
pMB10	*LEU2* Ap^R (pRS425) *VPS4*	this study
pMB24	*HIS3* Ap^R (pRS413) *vps4*^{K179A}	this study
pMB31	Ap^R (pGEX-2T) *GST–VPS4*	this study
pMB42	Ap^R (pGEX-2T) *GST–VPS4*^{K179A}	this study
pMB49	*HIS3* Ap^R (pRS413) *vps4*^{E233Q}	this study
pMB59	*HIS3* Ap^R (pRS413) *vps4ts229*	this study
pEMY10-2	*URA3* Ap^R (pRS426) *VPS10*	Marcusson *et al.* (1994)
pDB192	*URA3* Ap^R (pSEY8) *STE6*	Browne *et al.* (1996)

previously (Robinson *et al.*, 1988; Klionsky and Emr, 1989; C.Cowles and S.D.Emr, unpublished). Antiserum against Ste6p was a generous gift of David Bedwell.

Strains and media

The *S.cerevisiae* and *E.coli* strains used in this work are listed in Table II. Yeast strains were grown in standard yeast extract–peptone–dextrose (YPD) (Sherman *et al.*, 1979) or synthetic medium supplemented with essential amino acids as required for maintenance of plasmids. Luria–Bertani (LB) medium was used for growth of *E.coli* cells (Miller, 1972). For selection of plasmids, 75 μg/ml ampicillin was added to the media.

The *VPS4* deletion strains MBY2, MBY3 and MBY4 were constructed by transforming the wild-type strains BHY10, SEY6210 and SEY6210.1, respectively, with a DNA fragment containing the *TRP1* gene flanked by ~200 bp of *VPS4* upstream and downstream DNA. Yeast cells were selected for the presence of the *TRP1* gene, and the deletion of *VPS4* was confirmed by PCR analysis of the chromosomal DNA.

DNA manipulations

Recombinant DNA work was performed using standard protocols (Sambrook *et al.*, 1989). Transformation of *S.cerevisiae* was done by the lithium acetate method of Ito *et al.* (1983). The plasmids used in this study are listed in Table II. The 1.8 kb *Hin*dIII fragment containing the *VPS4* gene was cloned either into the *Hin*dIII site of pRS425 or into the *Eco*RV site of pRS413 to obtain pMB10 and pMB4, respectively. Site-directed mutagenesis was performed on pMB4 using the Transformer system from Clontech Laboratories (Palo Alto, CA) and the mutagenic oligonucleotides VPS4K179A (5′GGACCACCA-GGTACCGGTGCATCATATTTG3′) and VPS4E233Q (5′TATTTTTA-TAGATCAAGTGGATGCG3′). The mutations in the resulting plasmids pMB24 and pMB49 were confirmed by sequencing. These plasmids express the mutant Vps4p with the amino acid exchange of lysine at position 179 to alanine, or glutamate at position 233 to glutamine, respectively.

The *VPS4* gene was cloned into the *Bam*HI site of the GST expression vector pGEX-2T using a PCR-based linker DNA fragment. This construct was confirmed by sequencing. The resulting plasmids pMB31 (wild-type *VPS4*) and pMB42 (*vps4*^{K179A}) express in *E.coli* the fusion protein with the amino acid sequence GST–‘LeuValProArgGlySerThrGlyGlu-PheLeu′–Vps4p (thrombin cleaves after Arg).

A temperature-sensitive allele was constructed by PCR-based mutagenesis (Muhlrad *et al.*, 1992). A *VPS4*-containing DNA fragment was amplified under dATP limiting conditions (0.02 mM) using primers complementary to the upstream and downstream region of the gene. The resulting mutagenized DNA fragments were co-transformed with a gapped plasmid (pMB4 derivative *Spe*I–*Eco*RV digested) into the yeast strain MBY2 and grown on plates selecting for the plasmid. A colorimetric invertase plate assay (Paravicini *et al.*, 1992) was used to identify colonies that were Vps$^+$ at 26°C and Vps$^-$ at 38°C. Putative *vps4^{ts}* colonies were retested, and plasmid linkage of the temperature-dependent sorting phenotype was confirmed. The *vps4^{ts}* allele *vps4ts229* of the plasmid pMB59 was chosen for further phenotypic analysis.

Preparation of antiserum against Vps4p

The 940 bp *Eco*47III–*Bgl*II fragment of *VPS4* was fused to the *trpE* gene of the *E.coli* expression vector pATH1 (Dieckmann and Tzagoloff, 1985). The fusion protein was purified by SDS–PAGE and used to immunize New Zealand White rabbits as previously described (Horazdovsky and Emr, 1993).

Western blot analysis

Yeast strains were grown at 30°C in 5 ml of YPD or minimal medium to an optical density at 600 nm of 1.0 and harvested by centrifugation. For whole cell extracts, yeast cells were resuspended in 0.2 ml of loading solution (2% SDS, 0.1 M Tris–HCl, pH 6.8, 10% glycerol, 0.1 mg/ml bromophenol blue, 30 mM 2-mercaptoethanol) and lysed by glass beading and boiling. For fractionation studies, the cells were spheroplasted and lysed as previously described (Gaynor *et al.*, 1994). After a clearing spin (500 *g* for 5 min), the lysate was centrifuged for 45 min at 100 000 *g* to generate pellet (P100) and supernatant (S100). The proteins of both fractions were precipitated by adding trichloroacetic acid to a final concentration of 10%. The pellets were washed twice with acetone, resuspended in 0.2 ml of loading solution, and boiled for 5 min. The proteins of 10–20 μl of sample were separated by SDS–PAGE and transferred to a nitrocellulose membrane (Hybond-C Extra, Amersham, Arlington Heights, IL). This filters were incubated with anti-Vps4p antiserum (5000-fold dilution) and developed using a chemiluminescence detection system (ECL, Amersham).

Fluorescence microscopy

Cells for indirect immunofluorescence microscopy were prepared as described by Rieder *et al.* (1996). Fixed cells were stained using a monoclonal antibody specific for CPY (Molecular Probes, Inc., Eugene, OR) or affinity-purified rabbit anti-Mnn1p antibody (Graham *et al.*, 1994).

Electron microscopy analysis

The strain MBY3 pMB59 was grown at 26 or 37°C in YPD to an optical density at 600 nm of 0.5. The cells were harvested and prepared for electron microscopy as previously described, except that the time of cell wall digestion was reduced to 1 h (Rieder *et al.*, 1996).

Purification and ATPase activity test of Vps4p and Vps4p^{K179A}

The *E.coli* strain XL1-blue pMB31 or XL1-blue pMB42 was grown at 37°C in selective medium to an optical density at 600 nm of 1.0. Protein expression was induced by adding isopropyl-β-D-thiogalactoside to 0.2 mM, and the culture was incubated further at 30°C for 2 h. The GST–Vps4p fusion protein was affinity purified using glutathione-coupled Sepharose as described by Pharmacia (Uppsala, Sweden) and subsequently incubated with thrombin (Sigma Chemical Co, St Louis, MO; 15 U per mg of GST–Vps4p) for 3 h at 23°C. The resulting Vps4p was separated from GST and uncleaved fusion protein using ion exchange chromatography (MonoQ, Pharmacia). The protein was stored in buffer A (20 HEPES, pH 7.5, 0.15 M NaCl) with 10% glycerol. The protein concentration was determined spectroscopically by the method of Gill and von Hippel (1989).

To test the *in vitro* ATPase activity, ~1.5 μg of purified Vps4p was incubated in 20 μl of reaction buffer [buffer A, 5 mM $MgCl_2$, 1 mM ATP, 1 μCi [α-^{32}P]ATP (3000 Ci/mmol)] at 30°C. Samples were taken at different time points and analyzed by thin layer chromatography [PEI-Cellulose F (Merck, Darmstadt, Germany), 0.75 M KH_2PO_4, pH 3.5].

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