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The Vps4p AAA ATPase regulates membrane association of a Vps protein complex required for normal endosome function

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Vps4p is an AAA-type ATPase required for efficient transport of biosynthetic and endocytic cargo from an endosome to the lysosome-like vacuole of *Saccharomyces cerevisiae*. Vps4p mutants that do not bind ATP or are defective in ATP hydrolysis were characterized both *in vivo* and *in vitro*. The nucleotide-free or ADP-bound form of Vps4p existed as a dimer, whereas in the ATP-locked state, Vps4p dimers assembled into a decameric complex. This suggests that ATP hydrolysis drives a cycle of association and dissociation of Vps4p dimers/decamers. Nucleotide binding also regulated the association of Vps4p with an endosomal compartment *in vivo*. This membrane association required the N-terminal coiled-coil motif of Vps4p, but deletion of the coiled-coil domain did not affect ATPase activity or oligomeric assembly of the protein. Membrane association of two previously uncharacterized class E Vps proteins, Vps24p and Vps32p/Snf7p, was also affected by mutations in *VPS4*. Upon inactivation of a temperature-conditional *vps4* mutant, Vps24p and Vps32p/Snf7p rapidly accumulated in a large membrane-bound complex. Immunofluorescence indicated that both proteins function with Vps4p at a common endosomal compartment. Together, the data suggest that the Vps4 ATPase catalyzes the release (uncoating) of an endosomal membrane-associated class E protein complex(es) required for normal morphology and sorting activity of the endosome.

Keywords: ATPase/endosome/transport/vacuole

Introduction

Eukaryotic cells contain a complex vesicle-mediated transport system which selects and delivers proteins and lipids to different subcellular organelles. The endosomal membrane system functions as a central sorting site for both the endocytic and biosynthetic pathways. Endocytosed proteins are first delivered to an early endosome and then are either recycled back to the plasma membrane or delivered to the lysosome/vacuole via a late endosome. Newly synthesized lysosomal/vacuolar proteins also transit through an endosomal intermediate en route from the *trans*-Golgi to the lysosome/vacuole (for review see Gruenberg and Maxfield, 1995).

The diverse sorting and transport functions performed by the endosomal system have been found to require a number of different proteins. *N*-ethylmaleimide-sensitive factor (NSF)/Sec18p (Rodriguez *et al*., 1994; Hicke *et al*., 1997; Robinson *et al*., 1997), Rab proteins (Rybin *et al*., 1996; Ullrich *et al*., 1996; Mukhopadhyay *et al*., 1997) and t-SNAREs (Becherer *et al*., 1996; Bock *et al*., 1997; Burd *et al*., 1997), which are part of the SNARE-mediated membrane docking and fusion machinery (reviewed in Hay and Scheller, 1997; Novick and Zerial, 1997), are essential for endosomal function in yeast and mammalian cells. COPI and ARF are implicated in endosomal traffic in mammalian cells, consistent with vesicle-mediated transport within the endosomal system (Lenhard *et al*., 1992; Whitney *et al*., 1995; Daro *et al*., 1997).

In the yeast *Saccharomyces cerevisiae*, >40 vacuolar protein sorting (*vps*) mutants have been identified (Jones, 1977; Robinson *et al*., 1988; Rothman *et al*., 1989). A subset of 13 *vps* mutants, designated as class E *vps* mutants, accumulate vacuolar, endocytic and *trans*-Golgi markers in an aberrant multilamellar structure, the class E compartment (Raymond *et al*., 1992; Cereghino *et al*., 1995; Rieder *et al*., 1996). Characterization of three class E *VPS* genes, *VPS4*, *VPS27* and *VPS28*, suggested that the class E Vps proteins act at a common step required for efficient transport out of an endosomal compartment, consistent with the class E compartment representing an accumulated endosomal structure (Piper *et al*., 1995; Rieder *et al*., 1996; Babst *et al*., 1997; Finken-Eigen *et al*., 1997; Shirahama *et al*., 1997). *VPS4* and *VPS27* homologs, SKD1 (Perier *et al*., 1994) and Hrs (Komada and Kitamura, 1994), respectively, have been identified in mammalian cells. Hrs has been localized to endosomal membranes (Komada *et al*., 1997), suggesting that the function of the class E Vps proteins is conserved in eukaryotes.

The amino acid sequence of Vps4p identifies it as a member of the AAA (ATPase associated with a variety of cellular activities) family of ATPases. AAA proteins are involved in diverse cellular functions such as membrane fusion (Sec18p/NSF, Cdc48p/p97), protein degradation (YTA10-12, proteosome subunits, FtsH) and chaperone-like activities (YTA10-12, FtsH) (for reviews see Confalonieri and Duguet, 1995; Beyer, 1997; Patel and Latterich, 1998). The defining feature of this family is a conserved ATPase domain of ~220 amino acids present in one or two copies. To date, >200 AAA family members have been identified, and the genome of *S.cerevisiae* alone was found to encode 22 distinct AAA protein candidates.

Vps4p is a soluble protein with a predicted mol. wt of 48 kDa. The protein sequence contains two identifiable motifs: an N-terminal coiled-coil motif and a central AAA domain. *In vitro* tests with purified protein have shown that Vps4p exhibits ATPase activity, and the ATPase activity was found to be essential for Vps4p function

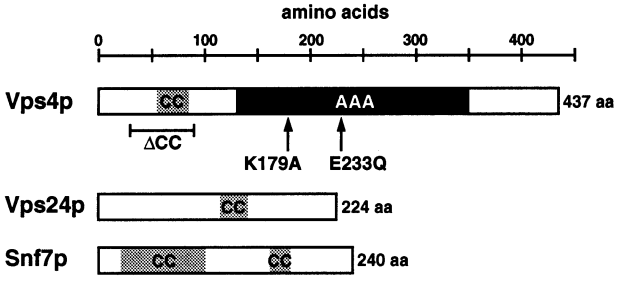

Fig. 1. Schematic protein structure of Vps4p, Vps24p and Snf7p. Putative coiled-coil motifs predicted by COILS (Lupas *et al*., 1991) are marked with gray boxes. The black box indicates the AAA domain of Vps4p. Mutations constructed in Vps4p are marked: ΔCC, deletion of the coiled-coil region; K179A, lysine to alanine exchange at position 179; E233Q, glutamate to alanine exchange at position 233.

in vivo (Babst *et al*., 1997). Upon shift of a temperature-sensitive *vps4* mutant (*vps4*^{ts}) to non-permissive temperature, transport of the newly synthesized vacuolar hydrolase carboxypeptidase Y (CPY) and Ste6p, an endocytosed protein, to the vacuole was blocked. As a result of the impaired transport, the cells accumulated proteins and membranes in the class E compartment, presumably corresponding to an aberrant pre-vacuolar endosome. These data suggested that the function of Vps4p is required for trafficking out of this endosomal intermediate.

We describe here the effect of nucleotide binding and hydrolysis by the AAA domain of Vps4p on its oligomeric state and subcellular localization. In addition, we studied the consequences of the deletion of the N-terminal coiled-coil motif on the biochemical characteristics of Vps4p. Two new class E Vps proteins were identified and we demonstrated that the association of these proteins with an endosomal compartment is affected by Vps4p. Together, the data indicate that the Vps4 ATPase catalyzes the release of a membrane-associated class E Vps protein complex from the pre-vacuolar endosomal compartment.

Results

Vps4p oligomerizes in a nucleotide-dependent manner

Vps4p is a member of the AAA-type ATPase family. Mutations in either a highly conserved lysine (position 179) or glutamate (position 233) in the AAA motif of Vps4p (Vps4p^{K179A} and Vps4p^{E233Q}) previously have been shown to abolish both the ATPase activity and the vacuolar protein sorting function of Vps4p (Figure 1; Babst *et al*., 1997). Studies of analogous mutations in other ATPases predict that Vps4p^{K179A} and Vps4p^{E233Q} should not bind or hydrolyze ATP, respectively (e.g. NSF; Whiteheart *et al*., 1994). To confirm these predictions, we purified wild-type Vps4p, Vps4p^{K179A} and Vps4p^{E233Q} from bacterial lysates and used UV light-induced cross-linking to analyze binding of these proteins to [α-^{32}P]ATP. Reactions were performed in the absence or presence of excess unlabeled AMP or ATP, and products were separated from unbound ATP by SDS–PAGE. As expected, the addition of non-radioactive ATP, but not AMP, successfully displaced [^{32}P]ATP (Figure 2, lanes 2 and 3). Wild-type Vps4p and Vps4p^{E233Q} bound ATP equally well, which indicates that the loss of enzymatic activity of Vps4p^{E233Q} is due to a defect in hydrolysis rather than in binding of ATP (Figure 2, lane 4). In contrast, the mutation in Vps4p^{K179A} completely abolished the binding of the nucleotide (Figure 2, lane 5). Thus, these mutant proteins are useful tools to study the influence of nucleotide binding and hydrolysis on the biochemical characteristics of Vps4p. The mutant protein Vps4p^{E233Q} was particularly useful for the *in vitro* studies, because non-hydrolyzable ATP analogs such as ATP-γS and AMP-PNP did not bind efficiently to Vps4p and therefore were unable to stabilize the ATP-bound state of the wild-type protein (data not shown).

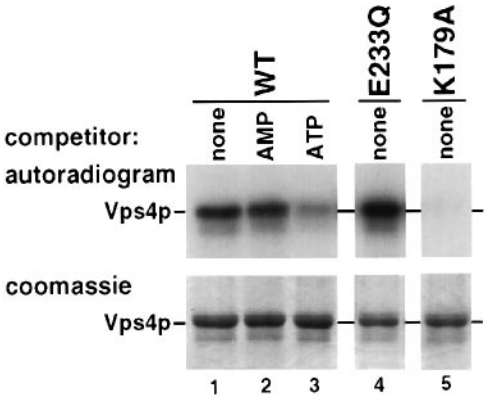

Fig. 2. UV light-induced cross-linking of ATP to Vps4p. Purified wild-type Vps4p (WT) or the mutant proteins Vps4p^{E233Q} (E233Q) and Vps4p^{K179A} (K179A) were cross-linked to [α-^{32}P]ATP in the presence (ATP, AMP) or absence (none) of excess unlabeled nucleotides. The proteins were separated by SDS–PAGE and detected either by exposure to X-ray film (upper panels) or staining with Coomassie (lower panels).

Table I. Molecular weight of wild-type and mutant Vps4p determined by gel filtration analysis

Protein	Nucleotide	Mol. wt (kDa)	Predicted structure
Vps4p	ADP	95	dimer
Vps4p	ATP	95	dimer
Vps4p	no	95	dimer
Vps4p^{E233Q}	ADP	95	dimer
Vps4p^{E233Q}	ATP	440	decamer
Vps4p^{K179A}	ADP	95	dimer
Vps4p$^{\Delta CC/E233Q}$	ADP	85	dimer
Vps4p$^{\Delta CC/E233Q}$	ATP	400	decamer

Several AAA ATPase family members, such as Cdc48p/p97 (Peters *et al*., 1990; Frohlich *et al*., 1995), NSF (Hanson *et al*., 1997) and YTA10-12 (Arlt *et al*., 1996), have been shown to form oligomeric complexes. To examine whether Vps4p oligomerizes, we used gel filtration analysis to determine the molecular weight of purified wild-type and mutant Vps4p in the presence or absence of nucleotides. Under all conditions, wild-type Vps4p eluted at a mol. wt of ~95 kDa relative to standard proteins (Table I), corresponding to the size found for endogenous Vps4p from cell extracts (data not shown). Since the calculated mol. wt of Vps4p is 48 kDa, the 95 kDa form of Vps4p most likely represents a dimer. The same result was obtained in the presence of ADP for both Vps4p^{E233Q} and Vps4p^{K179A} (Table I; Figure 3, P2). In contrast, in the ATP-bound form, the majority of Vps4p^{E233Q} (Vps4p^{E233Q}-ATP) eluted from the gel filtration column with a mol. wt of ~440 kDa (Figure 3, P1). However, the poor resolution

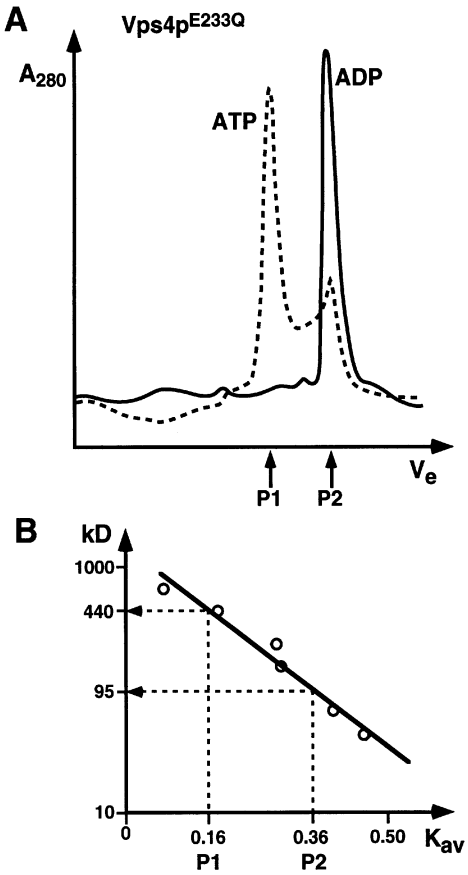

Fig. 3. Gel filtration analysis of Vps4p^{E233Q}. (**A**) Purified Vps4p^{E233Q} was loaded on a Superdex-200HR column and the elution volume (V_e) was determined in the presence of either 1 mM ADP (solid line, P2) or 1 mM ATP (dashed line, P1). (**B**) The molecular weight of the protein in the peak fractions P1 and P2 was calculated using a calibration curve generated by using the following standard proteins: ovalbumin (43 kDa), albumin (67 kDa), aldolase (158 kDa), catalase (232 kDa), ferritin (440 kDa) and thyroglobulin (669 kDa) ($K_{av} = [V_e–V_o]/[V_t–V_o]$; V_o, void volume; V_t, total bed volume).

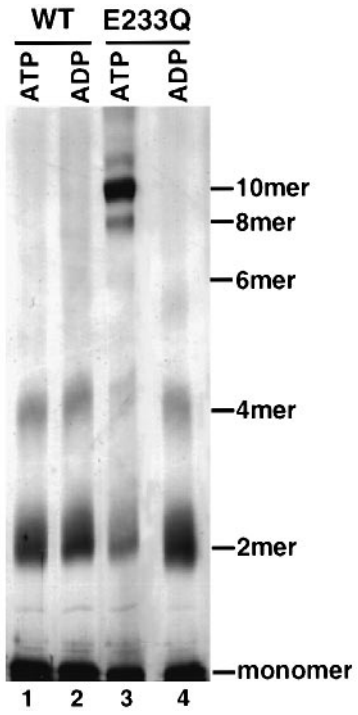

Fig. 4. Chemical cross-linking of Vps4p. Purified wild-type Vps4p (WT) and Vps4p^{E233Q} (E233Q) at a concentration of 1 μM were cross-linked by glutaraldehyde in the presence of 1 mM ATP or ADP. The products were separated by SDS–PAGE using a 3.5–7% acrylamide gradient gel and visualized by silver staining.

in this size range limits our analysis to the prediction that Vps4p^{E233Q}-ATP forms an oligomer of 8–10 subunits.

Oligomerization of Vps4p was also examined by cross-linking purified Vps4p or Vps4p^{E233Q} with glutaraldehyde in the presence of either ADP or ATP. The sizes of the resulting products were determined by SDS–PAGE and silver staining. Consistent with the results of the gel filtration analysis, the majority of cross-linked products of Vps4p-ATP, Vps4p-ADP and Vps4p^{E233Q}-ADP were dimers, whereas the ATP-bound form of Vps4p^{E233Q} was cross-linked into a large oligomer (Figure 4). Comparison with standard proteins showed that the molecular weight of the protein bands produced in these experiments was consistent with Vps4p cross-linking in multiples of two subunits (data not shown). The predominant species of Vps4p^{E233Q}-ATP was the fifth multiple, thus we conclude that the ATP-bound state of Vps4p^{E233Q} most likely forms a stable decamer. Together, data obtained using two independent methods indicate that in the protein concentration range examined (~1–5 μM), Vps4p exists in two different oligomeric forms: an ADP-bound dimer and an ATP-bound decamer.

The fact that we were not able to observe the wild-type protein in the decameric form may be due to disassembly of higher-order oligomers upon ATP hydrolysis. To demonstrate that Vps4 protein, which has a wild-type AAA domain, is able to co-assemble with Vps4p^{E233Q} into the decameric form, we performed a gel filtration analysis on a mixture of Vps4p$^{\Delta CC}$ and Vps4p^{E233Q} in the presence of 1 mM ATP. The coiled-coil deletion protein Vps4p$^{\Delta CC}$ shows the same *in vitro* characteristics as wild-type Vps4p (ATPase activity, oligomerization; see below) but as a result of its lower molecular weight can be easily distinguished from full-length protein by SDS–PAGE. The SDS–PAGE analysis revealed that the decamers formed in this experiment contained ~10% Vps4p$^{\Delta CC}$ (data not shown). This suggests that Vps4p with a functional ATPase domain is able to form higher oligomeric structures, but in order to form a stable decamer only one or two molecules of Vps4p$^{\Delta CC}$ can be incorporated into the Vps4p^{E233Q} complex.

Oligomerization of Vps4p stimulates its ATPase activity

The gel filtration analysis suggested that during the ATPase reaction cycle, Vps4p changes its oligomeric state, implying that the concentration of Vps4p affects its enzymatic activity. To test this, we measured the steady-state ATP turnover of purified Vps4p (measured as ADP/min/Vps4p molecule) as a function of the protein concentration (Figure 5A). These experiments were performed under conditions where substrate was not limiting and no product inhibition was observed. Unlike most enzymes which display constant activity levels over a broad range of protein concentrations, Vps4p ATPase activity was very low at a lower protein concentration (0.1 μM) and strongly increased at a higher protein concentration (1 μM). Gel

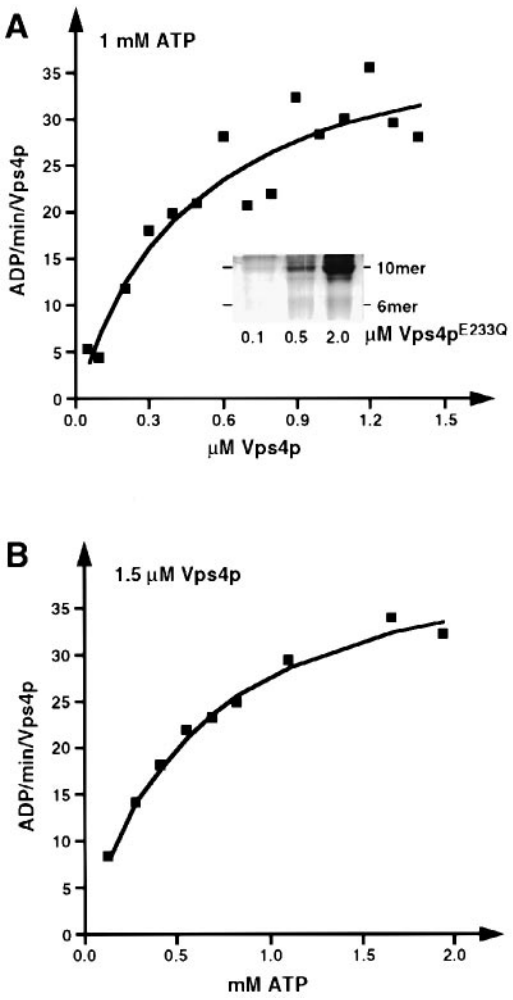

Fig. 5. Analysis of steady-state ATPase activity of Vps4p and concentration-dependent oligomerization of Vps4p^{E233Q}. The ATPase activity of Vps4p was measured as a function of the protein concentration (**A**; in the presence of 1 mM ATP) or as a function of the substrate concentration (**B**; in the presence of 1.5 μM Vps4p). Using SigmaPlot (Jandel Scientific), the data points were fitted to a curve with the function $f = A \times X/(B+X)$. The inset in (A) shows SDS–PAGE analysis of different concentrations of Vps4p^{E233Q} cross-linked by glutaraldehyde, in the presence of 1 mM ATP.

filtration studies showed that Vps4p exists as a dimer even at low protein concentration where ATPase activity was low (0.2 μM; data not shown). These data suggested that Vps4p dimers oligomerized and that the interaction between the dimers dramatically increased the ATPase activity of Vps4p. To support this model further, we cross-linked 2 μg of Vps4p^{E233Q} at different protein concentrations in the presence of 1 mM ATP and analyzed the products by SDS–PAGE (Figure 5A, inset). The results demonstrated that in the range of 0.1–2 μM Vps4p^{E233Q}, the amount of decamers dramatically increased. This is consistent with the idea that oligomerization of Vps4p dimers is responsible for the observed increase in ATPase activity within this protein concentration range.

To determine whether Vps4p oligomers bind ATP in a cooperative manner, we measured the steady-state ATP hydrolysis rate as a function of substrate concentration at high protein concentration (1.5 μM; Figure 5B). Under these conditions, the enzyme kinetics of Vps4p can be described by the Michaelis–Menten equation with a K_m

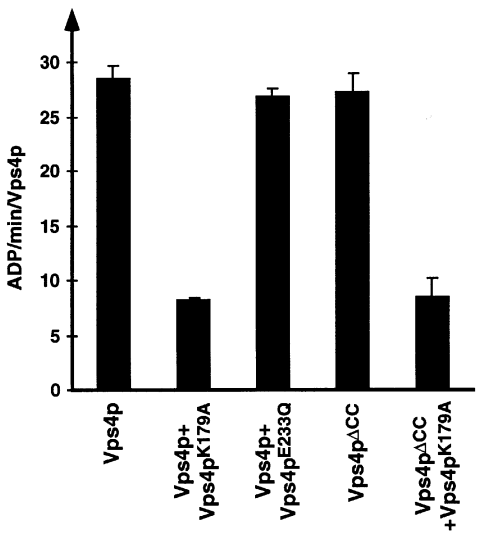

Fig. 6. Effect of the ATPase mutants on the hydrolysis activity of Vps4p and Vps4p$^{\Delta CC}$. The ATPase activity of 1 μM Vps4p or Vps4p$^{\Delta CC}$ was determined in the presence or absence of 5 μM Vps4p^{K179A} or Vps4p^{E233Q}.

of 0.6 μM and a maximal activity of 45 ADP/min/Vps4p. This implies that substrate binding is not cooperative and therefore ATP binding of each ATPase domain within a Vps4p dimer or oligomer occurs independently.

Since oligomerization appears to regulate the ATPase activity of Vps4p, we tested whether the ATPase-deficient Vps4^{K179A} and Vps4^{E233Q} proteins could assemble with wild-type Vps4 protein and influence its activity (Figure 6). The ATPase activity of 1 μM Vps4p was determined either in the presence or absence of either 5 μM Vps4p^{K179A} or Vps4p^{E233Q}. The results showed that a 5-fold excess of Vps4p^{K179A} reduces the activity of Vps4p by a factor of 3.5, whereas the same amount of Vps4p^{E233Q} had no effect. These data indicate that co-oligomerization of wild-type protein with Vps4p^{K179A} suppresses ATPase activity, suggesting that only the interaction between Vps4p dimers in the ATP-bound state induces ATP hydrolysis. Additionally, the data demonstrate that at high concentrations (5 μM), the nucleotide-free or ADP-bound form of Vps4p is able to oligomerize with wild-type Vps4p. In experiments performed at much lower protein concentrations (0.6 μM Vps4p and Vps4p^{K179A}), the addition of Vps4p^{K179A} had no influence on the ATPase activity of the wild-type protein (data not shown). This is consistent with the gel filtration analysis which indicated that Vps4p^{K179A} dimers have a low affinity for each other and presumably also for wild-type Vps4p. The presence of Vps4p^{E233Q} did not affect ATPase activity of the wild-type protein, suggesting either that Vps4p^{E233Q} interacts with wild-type Vps4p and thereby induces ATP hydrolysis and/or that Vps4p^{E233Q} forms stable decameric complexes which do not interact with the wild-type protein.

Together, the biochemical and kinetic analyses of Vps4p suggest that oligomerization of Vps4p-ATP dimers induces hydrolysis of the nucleotide which in turn leads to the dissociation of the oligomer and the formation of ADP-bound dimers. Thus, ATP hydrolysis drives a cycle of association and dissociation of Vps4p oligomers *in vitro*.

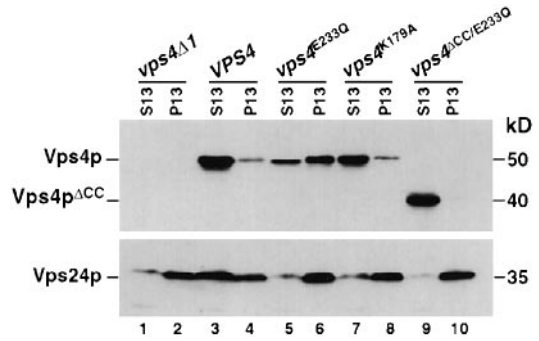

Fig. 7. Fractionation of yeast cells expressing wild-type and mutant Vps4p. Lysates of strains MBY3 (*vps4Δ1*), MBY3 pMB4 (*VPS4*), MBY3 pMB66 ($vps4^{E233Q}$), MBY3 pMB24 ($vps4^{K179A}$) and MBY3 pMB91 ($vps4^{\Delta CC/E233Q}$) grown at 30°C were subjected to centrifugation at 13 000 *g*, and soluble (S13) and pellet (P13) fractions were analyzed by Western blot using Vps4p- and Vps24p-specific antiserum.

ATP binding regulates membrane association of Vps4p

Although Vps4p is a predominantly soluble protein, a small fraction appears to be membrane associated (Babst *et al.*, 1997). To determine the relevance of membrane binding on Vps4p function, we examined the role of ATP hydrolysis on the subcellular localization of Vps4p. Cell extracts from yeast expressing wild-type *VPS4* or the mutant genes $vps4^{E233Q}$ and $vps4^{K179A}$ were fractionated by centrifugation at 13 000 *g* into supernatant (S13) and pellet (P13) fractions. Analysis of the samples by Western blot revealed that >90% of both wild-type Vps4p and $Vps4p^{K179A}$ were found in the S13 fraction (Figure 7, lanes 3–4 and 7–8) whereas 60–70% of $Vps4p^{E233Q}$ was localized to the pellet (Figure 7, lanes 5–6). Thus, Vps4p locked in the ATP-bound form appears to associate with membranes or forms a large complex.

$vps4^{ts}$, a temperature-sensitive allele of *VPS4*, was used previously to study the immediate consequences of loss of Vps4p function with respect to sorting of vacuolar hydrolases. Two point mutations in the AAA motif were identified as being responsible for the conditional phenotype of $vps4^{ts}$, suggesting that ATPase activity of $Vps4p^{ts}$ may be impaired at high temperature (Babst *et al.*, 1997). Since ATP hydrolysis appeared to be required for the cytoplasmic localization of Vps4p (see above), we examined $Vps4p^{ts}$ distribution after shift to non-permissive temperature. Cells expressing *VPS4* or $vps4^{ts}$ were either continuously incubated at permissive temperature (26°C) or shifted from permissive to non-permissive temperature (37°C) 20 min prior to lysis. The cell extracts were fractionated by centrifugation at 13 000 *g*, resulting in pellet (P13) and supernatant (S13) fractions. The S13 was separated further by centrifugation at 100 000 *g* into pellet (P100) and supernatant (S100) fractions. Wild-type Vps4p protein and $Vps4p^{ts}$ at the permissive temperature localized primarily to the soluble fraction (Figure 8A). However, after a 20 min incubation at non-permissive temperature (37°C), ~70% of $Vps4p^{ts}$ redistributed to the P13 fraction (Figure 8, lanes 7–8), much like $Vps4p^{E233Q}$ (Figure 7, lanes 5–6). This suggested that at the non-permissive temperature, ATP hydrolysis of $Vps4p^{ts}$ is blocked, resulting in a rapid accumulation of $Vps4p^{ts}$-ATP in a P13 fraction. The small pool of $Vps4p^{ts}$ which was detected

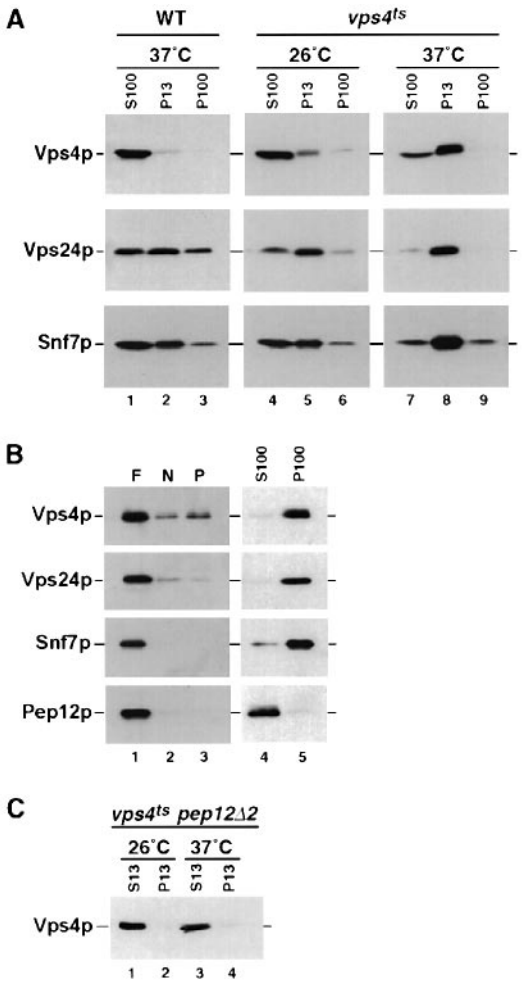

Fig. 8. Effect of loss of Vps4p activity on the subcellular distribution of class E Vps proteins. (**A**) The cells MBY3 pMB4 (WT) and MBY3 pMB59 ($vps4^{ts}$) were grown at 26°C, harvested and spheroplasted either continuously at 26°C or shifted to 37°C for the last 20 min of the spheroplasting reaction. The spheroplasts were lysed and, after a clearing spin, were centrifuged at 13 000 *g* for 10 min, resulting in a pellet (P13) and soluble (S13) fractions. S13 was separated by an additional centrifugation at 100 000 *g* for 45 min (soluble fraction, S100; pellet fraction, P100). The fractions were analyzed by Western blotting. (**B**) The P13 fraction of MBY3 pMB59 ($vps4^{ts}$) cells (spheroplasted at 37°C) was loaded at the bottom of a sucrose density gradient. After 14 h centrifugation at 200 000 *g*, fractions of the floating material (F), the non-floating material (N) and the pellet (P) were collected. Additionally, a parallel P13 fraction was resuspended in buffer containing 1% Triton X-100 and separated by centrifugation at 100 000 *g* for 45 min into a soluble (S100) and a pellet fraction (P100). All fractions were analyzed by Western blotting. (**C**) The strain MBY11 pMB59 ($vps4^{ts}$, *pep12Δ2*) was spheroplasted at 26 or 37°C, fractionated into S13 and P13 and analyzed as described in (A).

in the P13 at 26°C might be due to a partial loss of $Vps4p^{ts}$ function even at low temperature. Unfortunately, $Vps4p^{ts}$ was found to be very unstable when expressed in *Escherichia coli* and, therefore, it was not possible to measure directly the ATPase activity of the temperature-conditional protein.

To determine whether the pelletable pool of Vps4p is membrane associated, the P13 fraction from $vps4^{ts}$ cells at non-permissive temperature was resuspended in buffer and loaded on the bottom of a sucrose step gradient. Following centrifugation, samples were divided into three fractions: the top fraction containing the membrane/organ-

elle-associated floating material (F), the load fraction containing non-floating material (N) and the pellet (P) fraction that corresponds to large non-membrane-associated material. Western blot analysis of these samples showed that the majority of the Vps4p P13 pool as well as a control transmembrane protein (Pep12p) were found in the top fraction of the gradient, indicating that the P13 pool of Vps4p is indeed associated with a membranous compartment (Figure 8B). The P13 fraction was also resuspended in buffer containing 1% Triton X-100 and centrifuged at 100 000 *g* for 45 min. As expected, Pep12p was solubilized by the detergent treatment. However, Vps4p pelleted during this high-speed centrifugation, suggesting that the membrane-associated fraction of Vps4p might be part of a large protein complex (Figure 8B).

Phenotypic analyses of a *VPS4* deletion strain have indicated that Vps4p might act on an endosomal compartment (Babst *et al*., 1997). To test whether the membrane association of Vps4p^{ts} requires a functional endosome, we fractionated *pep12Δ2* cells expressing *vps4^{ts}*. Since Pep12p is a t-SNARE necessary for the transport of proteins and membranes from the late-Golgi to the endosome (Becherer *et al*., 1996; Burd *et al*., 1997), deletion of *PEP12* might result in loss of the endosomal compartment where Vps4p may be located. *pep12Δ2* cells expressing *vps4^{ts}* were either incubated continuously at the permissive temperature (26°C) or shifted from permissive to non-permissive temperature (37°C) 20 min prior to lysis. The cell extracts were separated into S13 and P13 fractions and analyzed by Western blot. Unlike wild-type cells, in the *pep12Δ2* cells, there was no accumulation of Vps4p^{ts} in the pellet fraction at non-permissive temperature (Figure 8C), suggesting that membrane-associated Vps4p is localized to the endosome.

To localize Vps4p by indirect immunofluorescence microscopy, we constructed a C-terminal hemagglutinin (HA) epitope fusion for both Vps4p and Vps4p^{E233Q}. Plasmids carrying the genes *VPS4-HA* or *vps4^{E233Q}-HA* were transformed into *vps4Δ1*. Phenotypic analysis showed that *VPS4-HA*, but not *vps4^{E233Q}-HA*, complemented the mutant phenotypes of *vps4Δ1*. Immunofluorescence analysis of these cells showed diffuse staining consistent with a cytoplasmic localization for Vps4p-HA (data not shown). In contrast, fluorescence was localized primarily to a few large structures adjacent to the vacuole in cells expressing *vps4^{E233Q}-HA* (Figure 9A). The same structures were also labeled with antibodies against Vps24p (Figure 9A) and Snf7p (data not shown), consistent with its accumulation in the class E compartment (see below). Together, the subcellular fractionations and immunofluorescence analysis indicate that *in vivo*, Vps4p associates transiently with an endosomal compartment and that this interaction is regulated by the nucleotide-binding state of the AAA domain.

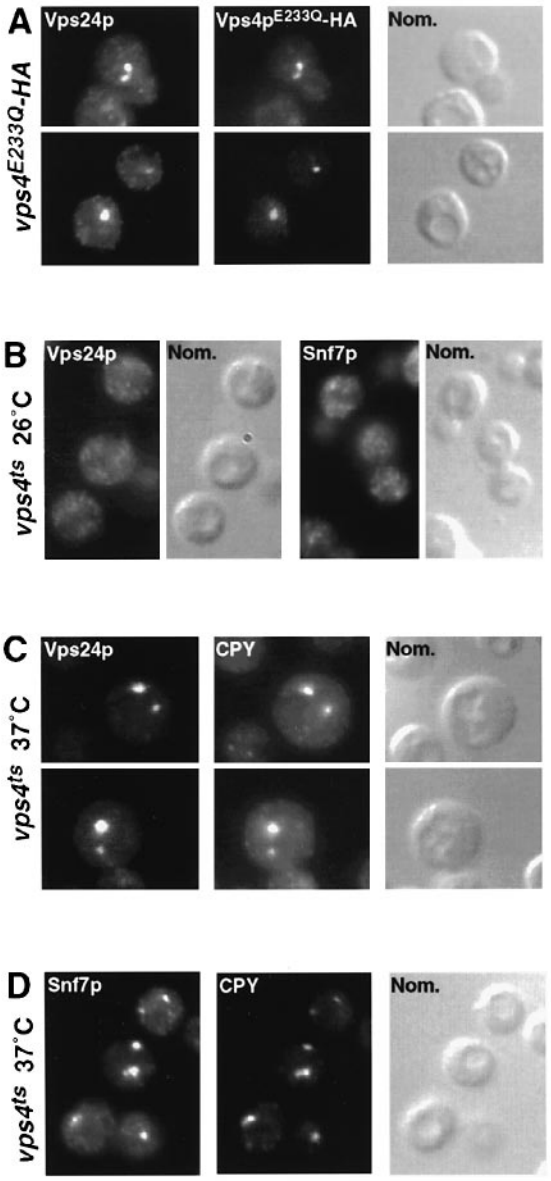

Fig. 9. Subcellular distribution of Vps4p, Vps24p, Snf7p and CPY analyzed by indirect immunofluorescence microscopy. (**A**) The strain MBY3 pMB96 (*vps4^{E233Q}-HA*) was grown at 30°C, spheroplasted and fixed as described in Materials and methods. The immunostaining was performed simultaneously with a monoclonal antibody against the HA epitope and affinity-purified polyclonal antibodies against Vps24p. These antibodies were visualized by Cy2- and Cy3-conjugated tertiary antibodies, respectively. (B–D) The strain MBY3 pMB59 (*vps4^{ts}*) was grown at 26°C and spheroplasted either continuously at 26°C (**B**) or after 15 min shifted to 37°C for the remaining 20 min of the spheroplasting reaction (**C** and **D**). The fixed spheroplasts were co-immunostained with antibodies against either CPY and Vps24p or CPY and Snf7p. The Vps24p- and Snf7p-specific antibodies were visualized by Cy3-conjugated tertiary antibodies whereas the monoclonal CPY antibodies were detected with Cy2 conjugates. The cells were analyzed by fluorescence or Normaski (Nom.) microscopy.

The coiled-coil motif of Vps4p is required for membrane association but not for homotypic oligomerization

Vps4p contains an N-terminal coiled-coil domain which might participate in the oligomerization of the protein (Figure 1). To test this possibility, we deleted 157 bp of *vps4^{E233Q}* which includes the region coding for the coiled-coil motif (*vps4$^{\Delta CC/E233Q}$*). Vps4p$^{\Delta CC/E233Q}$ is predicted to have a mol. wt of 42 kDa, yet gel filtration analysis in the presence of ADP revealed a size of 85 kDa (Table I). This result demonstrates that, as for full-length Vps4p, the ADP-bound form of Vps4p$^{\Delta CC/E233Q}$ forms a dimer. In addition, like Vps4p^{E233Q}, Vps4p$^{\Delta CC/E233Q}$ in the presence of ATP forms a complex in the size range of a decamer. Thus, Vps4p$^{\Delta CC/E233Q}$ shows the same nucleotide-dependent

oligomerization as observed for the full-length protein, indicating that the coiled-coil domain of Vps4p is not required for homotypic oligomerization. Furthermore, the coiled-coil deletion mutant Vps4p$^{\Delta CC}$ exhibited ATPase activity similar to wild-type Vps4p, which also was suppressed by the addition of Vps4p^{K179A} (Figure 6). This indicates that the coiled-coil domain is not necessary for oligomerization-stimulated ATP hydrolysis.

Although deletion of the coiled-coil domain did not affect the *in vitro* characteristics of Vps4p, the mutant gene *vps4*$^{\Delta CC}$ did not complement the *VPS4* deletion strain (data not shown). Fractionation studies of cells expressing *vps4*$^{\Delta CC/E233Q}$ revealed that the coiled-coil deletion mutant did not accumulate in the P13 fraction as did the full-length Vps4p^{E233Q} (Figure 7, lanes 9 and 10). Thus, the coiled-coil domain seems to be necessary for the association of Vps4p-ATP with endosomal membranes.

In vivo distribution of Vps24p and Vps32p/Snf7p depends on Vps4p function

Four class E *vps* mutants, *vps4*, *vps24*, *vps32* and *vps2*, displayed a growth defect at high temperature (data not shown). To address potential interactions among these genes and gene products, two of them, *VPS24* and *VPS32*, were identified and characterized. The *VPS24* gene was cloned from a yeast genomic library (method as described by Burd *et al*., 1996). The gene encodes a 224 amino acid protein with a predicted mol. wt of 26 kDa (DDBJ/EMBL/GenBank accession No. 486052; SGD, YKL041w). Complementation analysis revealed that *VPS32* is allelic to *SNF7*, a gene originally found to be required for the derepression of the gene encoding invertase (Tu *et al*., 1993). The *SNF7* gene encodes a protein of 240 amino acids with a predicted mol. wt of 27 kDa. The primary sequences of Vps24p and Snf7p share several common properties. Both proteins are hydrophilic and contain ~35% charged amino acids. The charges are distributed unequally, resulting in a pI of ~10 for the N-terminal half and a pI of ~4 for the C-terminal half of each protein. In addition, the sequences of both proteins contain coiled-coil motifs (predicted by COILS; Lupas *et al*., 1991; see Figure 1).

Analysis of *VPS24* and *SNF7* deletion strains (*vps24Δ1*, *snf7Δ1*) revealed typical class E *vps* mutant phenotypes (Raymond *et al*., 1992; Piper *et al*., 1995; Rieder *et al*., 1996; Babst *et al*., 1997). In both *vps24Δ1* and *snf7Δ1*, half of the newly synthesized vacuolar hydrolase CPY was missorted and secreted into the periplasm (data not shown). In addition, electron microscopic analysis showed that deletion of *VPS24* leads to the formation of exaggerated stacks of curved cisternal membranes, a structure which is defined as a class E compartment (data not shown).

Antisera raised against Vps24p and Snf7p recognized proteins with apparent mol. wts of ~35 kDa. These proteins were present in extracts from wild-type but not from the corresponding deletion strains (data not shown). The difference between the calculated and apparent molecular weights may be caused by the highly charged nature of both proteins.

To investigate the relationship between Vps4p, Vps24p and Snf7p, subcellular fractionations were performed. In wild-type and *vps4*^{ts} cells at permissive temperature, both proteins were equally distributed between the P13 and S100 fractions, with a minor pool in the P100 fraction (Figure 8A, lanes 1–6). Sucrose density gradient analysis indicated that the P100 fraction of Vps24p and Snf7p is not membrane associated (data not shown). After a 20 min incubation of *vps4*^{ts} cells at non-permissive temperature, both Vps24p and Snf7p were found almost exclusively in the P13 fraction (Figure 8A, lanes 7–9). Like Vps4p^{ts}, the P13-localized fraction of Vps24p and Snf7p was membrane associated and was not solubilized by the detergent Triton X-100 (Figure 8B). Together, the subcellular fractionation of the *vps4*^{ts} cells demonstrated that Vps24p and Snf7p behave much like Vps4p^{ts}. Furthermore, Vps24p and Snf7p accumulated in P13 not only in *vps4*^{ts} cells at non-permissive temperature but in all *VPS4* mutant backgrounds tested (Figure 7; Snf7p, data not shown). This suggests that Vps4p function in general is necessary for the dissociation of these proteins from a membrane. Fractionation of all 13 class E *vps* mutant strains revealed that Vps24p accumulated on a membrane only in *VPS4* mutant cells (data not shown). In addition, the class E Vps protein Vps28p did not show the shift to the P13 fraction of *VPS4* mutant cells as found for Vps24p and Snf7p (data not shown). These data indicate that the observed accumulation of Vps24p and Snf7p in the P13 fraction is a specific phenotype associated with the loss of Vps4p function.

To define the compartment where Vps24p and Snf7p accumulated, we monitored the distribution of Vps24p and Snf7p by indirect immunofluorescence microscopy in *vps4*^{ts} cells at both permissive and non-permissive temperatures. At permissive temperature, Vps24p and Snf7p were localized to several small dots suggestive of small membranous organelles (Figure 9B). However, following 20 min of incubation at non-permissive temperature, the majority of Vps24p and Snf7p localized to one to three large spots adjacent to the vacuole, reminiscent of the appearance and localization of class E compartments. Because class E compartments have been shown previously to accumulate CPY (Raymond *et al*., 1992; Babst *et al*., 1997), we performed double labeling experiments and found that both Vps24p and Snf7p co-localize with CPY, indicating that these proteins associate with a class E compartment in a *VPS4* mutant background (Figure 9C and D).

Discussion

Vps4p is an AAA-type ATPase necessary for efficient protein transport from an endosomal compartment to the yeast vacuole/lysosome. The accumulation of proteins and membranes in a large aberrant structure in a *VPS4* deletion strain is most consistent with a role for Vps4p in formation of transport intermediates exiting the endosome (Babst *et al*., 1997; Finken-Eigen *et al*., 1997). In this study, we demonstrate that nucleotide binding and hydrolysis by the AAA domain direct changes in the oligomeric structure of Vps4p and in the subcellular distribution of Vps4p as well as two other class E gene products, Vps24p and Vps32p/Snf7p. Based on these data, we postulate that the Vps4 ATPase catalyzes disassembly of an endosome-bound class E Vps protein complex and thereby functions

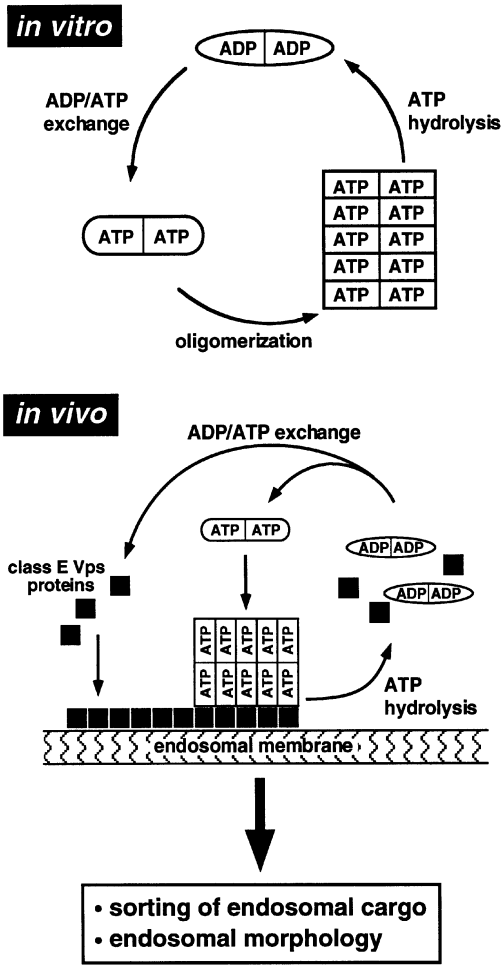

Fig. 10. Model for the ATP-driven cycle of Vps4p *in vitro* and *in vivo*. Ellipses and rectangles represent Vps4p dimers in different nucleotide-binding states and oligomeric structures. Black squares represent class E Vps proteins such as Vps24p and Snf7p.

to regulate the dynamic structure and transport activity of the pre-vacuolar endosome.

Oligomeric structure and ATPase activity of Vps4p are coupled

Previous gel filtration analysis in the presence of high salt revealed that recombinant Vps4p behaves like a monomeric protein (Babst *et al*., 1997). However, using a more physiological buffer system, our present study revealed a dynamic, nucleotide-dependent oligomeric structure of Vps4p. In either the nucleotide-free or ADP-bound state, purified Vps4p forms stable dimers. Additionally, gel filtration analysis of yeast cell extracts revealed that endogenous Vps4p is also primarily dimeric. Therefore, the dimer appears to represent the basal state of native Vps4p and the predominant form at steady state. Analysis of the oligomeric state of a mutant protein, Vps4p^{E233Q}, which is impaired in ATP hydrolysis, indicated that in the ATP-locked state, Vps4p dimers oligomerize into a stable complex composed of approximately five dimers (decamer; Figures 3 and 4). Wild-type Vps4p was not observed to form this decamer, consistent with rapid hydrolysis of the bound ATP (Babst *et al*., 1997), resulting in disassembly of the oligomer.

We observed a strong increase in decamer formation of Vps4p^{E233Q}-ATP in the protein concentration range from 0.1 to 2 μM. In the same concentration range, the ATPase activity of wild-type Vps4p dramatically increased, suggesting that oligomerization of Vps4p dimers activates the ATPase. Furthermore, the K_m for ATP of Vps4p at low concentrations was reduced (data not shown), indicating that hydrolysis, and not nucleotide exchange, is the rate-limiting factor responsible for the low ATPase activity of dimeric Vps4p. The rate of ATP hydrolysis was found to be dependent not only on the protein concentration but also on the nucleotide-binding state of the interacting Vps4 protein. The addition of ADP-locked mutant Vps4p did not induce, but instead inhibited the rate of ATP hydrolysis by wild-type Vps4p, indicating that only interaction between ATP-bound Vps4p dimers results in increased hydrolysis (Figure 6).

Together, the *in vitro* data support a model in which the ATPase activity of Vps4p drives a cycle of assembly and disassembly of Vps4p dimers/oligomers (Figure 10). Two characteristics of Vps4p are responsible for this cycling between the dimeric and oligomeric forms: the nucleotide-dependent affinity of the Vps4p dimers for each other and regulation of ATP hydrolysis by the oligomeric state. The fact that we did not observe wild-type Vps4p in the decameric form indicates that ATP hydrolysis and the resulting dissociation of the protein complex occur much faster than nucleotide exchange and/or oligomerization. Therefore, Vps4p remains in the ATP- or ADP-bound dimeric form for most of its cycle time. Furthermore, the study of the enzyme kinetics demonstrated that ATP binding by Vps4p is not cooperative, consistent with our model which predicts that Vps4p dimers bind ATP and that this drives oligomerization. However, we cannot exclude a model in which ATP hydrolysis occurs before the complete assembly of the decamer because we are only able to observe Vps4p decamers *in vitro* when ATP hydrolysis is impaired.

Homotypic oligomerization has been described for several members of the AAA family and in at least two cases it was demonstrated that oligomerization requires the presence of nucleotides (NSF: Nagiec *et al*., 1995; Hanson *et al*., 1997; YTA10-12: Arlt *et al*., 1996). However, Vps4p is the first example of an AAA-type ATPase for which the oligomeric state changes as a result of ATP hydrolysis. Notably, in this respect, Vps4p resembles dynamin, a GTPase which is involved in the scission of clathrin-coated vesicles (Takei *et al*., 1995). *In vitro* studies showed that self-assembly of dynamin induces its GTPase activity and that the oligomeric structure of dynamin is affected by the nucleotide-binding state (Warnock *et al*., 1996; Muhlberg *et al*., 1997). Therefore, it has been suggested that dynamin has a nuclease-driven cycle of assembly and disassembly, much like we propose for Vps4p.

Analysis of yeast cell extracts revealed that the cellular concentration of Vps4p is estimated at 0.2–0.3 μM and that Vps4p is predominantly dimeric, suggesting that the *in vivo* ATPase activity of the majority of Vps4p is very low. This is consistent with a model in which the oligomerization of Vps4p dimers *in vivo* is induced by interaction with its membrane-associated substrate/receptor (e.g. Vps24p, Snf7p). This would ensure that ATP

hydrolysis occurs only after a complex of Vps4p and substrate/receptor is formed. In contrast, the high Vps4p concentration used in the *in vitro* ATPase activity tests induced homotypic oligomerization and, therefore, hydrolysis occurs without co-factors such as a substrate/receptor. It is likely that this putative substrate/receptor serves to promote a local high concentration of Vps4p. We propose that, similar to the *in vitro* system, an ATP-driven cycle of association/dissociation also exists *in vivo*, playing an essential role in the function of Vps4p (Figure 10).

Membrane association of Vps4p is regulated by the nucleotide-binding state and requires the coiled-coil motif

Subcellular fractionation demonstrated that the mutant protein Vps4p^{E233Q} accumulates in cells on a large membranous compartment, whereas the wild-type protein was found to be primarily soluble. Thus, in the ATP-locked state, Vps4p binds membranes, and nucleotide hydrolysis apparently releases Vps4p from membranes to the cytosol. The temperature-conditional mutant protein, Vps4p^{ts}, contains two amino acid exchanges in the AAA motif which may impair ATP hydrolysis at restrictive temperature and, therefore, lock Vps4p^{ts} in the ATP-bound, membrane-associated state (Figures 7 and 8).

Immunofluorescence microscopy localized Vps4p^{E233Q} to the class E compartment, a large multilamellar structure formed in all class E *vps* mutant backgrounds (Figure 9). The class E compartment accumulates proteins and membranes from both the biosynthetic and endocytic pathways and most likely represents an aberrant endosomal structure. These results suggest that Vps4p normally associates with an endosomal intermediate. Localization to the endosome was supported further by the observation that Vps4p no longer associates with a membrane in mutant cells in which the endosomal system is perturbed (e.g. *pep12Δ2*).

The protein sequence of Vps4p shows no obvious structural or hydrophobic elements which would account for its transient membrane association. However, deletion of the N-terminal coiled-coil motif of Vps4p (Vps4p$^{\Delta CC}$), a structure implicated in protein–protein interactions, abrogated membrane association. Because the mutant protein Vps4p$^{\Delta CC}$ has near wild-type ATPase activity as well as nucleotide-dependent oligomerization comparable with wild-type Vps4p, loss of membrane association of Vps4p$^{\Delta CC}$ is not caused by a dramatic change in the oligomeric structure of the protein; rather, it is a specific phenotype associated with the coiled-coil domain. Vps4p may bind to an endosomal compartment through the coiled-coil domain interacting with a membrane-associated protein complex, possibly directly with the Vps24 and Snf7 proteins which also contain coiled-coil domains (see below). Furthermore, after detergent extraction of intracellular membranes, Vps4p remained in a pelletable fraction, arguing that Vps4p interacts with a large membrane-bound protein complex rather than with a single receptor protein.

Vps4p regulates membrane association of other class E Vps proteins

The 13 class E *vps* mutants display very similar sorting and morphological phenotypes (Raymond *et al*., 1992; Piper *et al*., 1995; Rieder *et al*., 1996; Babst *et al*., 1997; Finken-Eigen *et al*., 1997), consistent with the idea that they function together as a complex or at a common step in the endocytic pathway. We have identified two new class E *VPS* genes, *VPS24* and *SNF7*/*VPS32* which encode small hydrophilic proteins. Similarly to Vps4p, the membrane-bound pools of Vps24p and Snf7p were found in a large detergent-insoluble structure, most likely a membrane-associated protein complex. In the *vps4Δ1* strain or in cells expressing a non-functional allele of *VPS4*, the cytoplasmic pool of both proteins shifts to a membranous compartment. In addition, immunofluorescence experiments revealed that upon inactivation of Vps4p^{ts}, the Vps24 and Snf7 proteins rapidly redistributed from small punctate cytoplasmic compartments to the larger class E compartment (Figure 9). Together, the data indicate that in wild-type cells, Vps24p and Snf7p transiently associate with an endosome and that the dissociation from this compartment requires the function of Vps4p. Analysis of the other 10 class E mutant strains has shown that accumulation of Vps24p on the endosomal membrane is a specific phenotype of *VPS4* mutant strains. Based on this result and the fact that both Vps24p and Snf7p have putative coiled-coil domains, we speculate that Vps4p may interact via the coiled-coil domains directly with the membrane-associated pools of these proteins, and thus Vps24p and Snf7p may function as a receptor(s) for cytosolic Vps4p.

Structural and functional similarities among the AAA family members

A well-characterized AAA family member is NSF/Sec18p, a protein required for SNARE-mediated membrane fusion in eukaryotic cells (Beckers *et al*., 1989; Graham and Emr, 1991; reviewed in Sudhof, 1995). NSF is composed of an N-terminal domain which binds to SNARE complexes followed by two AAA domains, D1 and D2 (Nagiec *et al*., 1995; Hanson *et al*., 1997). The domains of NSF seem to share functional similarities with the corresponding domains in Vps4p. The N-terminal domains of both proteins are thought to interact with their target proteins, and in both cases the interaction might be regulated by the nucleotide-binding state of the adjacent AAA domain (D1 in the case of NSF). Additionally, nucleotide binding by the AAA domains of each protein is required for the interactions between the AAA domains within the oligomeric complex. The C-terminal D2 domain of NSF is necessary for trimer formation and, therefore, seems to be a stable structural element of the NSF oligomer. Similarly, the C-terminus of Vps4p might be involved in dimerization of the protein. Therefore, although they are quite different in their primary sequences, the AAA family members might share a common modular architecture: an N-terminal target-interacting domain which is regulated by the adjacent AAA ATPase domain followed by a structural element involved in oligomerization.

Analysis of AAA family members and their close relatives has also revealed similarities in their functions. The common theme of AAA protein function seems to be a protein unfolding or disassembly activity as has been ascribed to molecular chaperones. The loss of Vps4p function leads to the accumulation of at least two other class E Vps proteins, Vps24p and Snf7p, on an endosomal

compartment. This suggests that Vps4p activity is necessary for the disassembly of a class E Vps protein complex.

Model for Vps4p as a dissociation factor of an endosomal coat

Our current model for *in vivo* Vps4p function predicts an ATP-driven cycle similar to that found *in vitro* (Figure 10). Vps4p dimers in the ATP-bound form are recruited from the cytoplasm to an endosome, leading to Vps4p decamer association with a class E Vps protein complex via coiled-coil interactions. The oligomerization of Vps4p dimers triggers the hydrolysis of ATP, resulting in a conformational change in the coiled-coil domain which in turn disassembles the class E Vps protein complex. In addition, ATP hydrolysis destabilizes the decameric Vps4p complex and results in the formation of soluble ADP-bound dimers. ADP/ATP exchange closes the functional cycle of Vps4p. The soluble class E Vps proteins can then bind again to endosomal membranes to perform their function.

In this model, Vps4p acts as a dissociation factor for a class E Vps protein complex bound to the cytoplasmic face of endosomes. This complex could define or stabilize structural/morphological properties of the endosome (e.g. tubules) required for proper sorting of anterograde and retrograde traffic within the intermediate endosomal compartment. Sorting could be performed either by direct interaction of class E Vps proteins with a specific set of transmembrane proteins or by selectively excluding a set of transmembrane proteins from diffusing into certain subdomains of the endosomal network. Together, class E Vps protein complexes might act on endosomal membranes as a coat in which the cycle of assembly and disassembly is regulated by the Vps4 ATPase. The presence of a mammalian homolog of Vps4p (mouse SKD1) suggests that the function of the endosomal system in mammalian cells may also require a class E Vps-like protein complex(es) regulated by an AAA-type ATPase.

Materials and methods

Materials

Monoclonal antibodies specific for CPY or the HA epitope were purchased from Molecular Probes (Eugene, OR) and Boehringer Mannheim (Germany), respectively.

Strains and media

Saccharomyces 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) or synthetic medium supplemented with essential amino acids as required for maintenance of plasmids (YNB) (Sherman *et al*., 1979). Luria–Bertani (LB) medium was used for growth of *E.coli* cells (Miller, 1972). For selection of plasmids, 100 μg/ml ampicillin was added to the media.

The *VPS24* deletion strain BWY102 was constructed by transforming SEY6210 wild-type strain with a plasmid containing *VPS24* with the *HIS3* gene inserted into the *Nde*I–*Nco*I sites (*vps24Δ1*). The mutant MBY11 was constructed by transforming the strain CBY16 with a DNA fragment containing the *TRP1* gene flanked by ~200 bp of *VPS4* upstream and downstream DNA (*vps4Δ1*). Yeast cells were selected for the presence of the *TRP1* or *HIS3* gene and the deletions were 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 as described (Ito *et al*., 1983). The plasmids used in this study are listed in Table II. The plasmid pMB31 was partially digested with *Acc*I, filled in with T4 DNA polymerase (Boehringer Mannheim, Germany) and subsequently digested with *Eco*47III. The plasmid finally was religated, resulting in pMB40. pMB63 was constructed by replacing the 0.4 kb *Kpn*I–*Eco*47III fragment from pMB31 with the corresponding fragment of pMB49 (Babst *et al*., 1997). The plasmid pMB66 is a derivative of pMB49. A DNA fragment coding for three HA epitopes was inserted into the blunted *Spe*I site of the plasmids pMB4 and pMB66, resulting in pMB76 and pMB96, respectively. The 1.5 kb *Sal*I–*Xmn*I fragment of pMB4 was replaced by the corresponding fragment of pMB40, resulting in the plasmid pMB50. The plasmids pMB90 and pMB91 were constructed by replacing the 0.3 kb *Bst*EII–*Nco*I

Table II. Strains and plasmids used in this study

Strain/plasmid	Genotype/description	Reference/source
S.cerevisiae		
SEY6210	*MATα leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9*	Robinson *et al*. (1988)
BWY102	*SEY6210; vps24Δ1* (*VPS24::HIS3*)	this study
MBY3	*SEY6210; vps4Δ1* (*VPS4::TRP1*)	Babst *et al*. (1997)
MBY11	*SEY6210; pep12Δ2* (*PEP12::LEU2*), *vps4Δ1* (*VPS4::TRP1*)	this study
E.coli		
XL1-blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB lacIqZΔM15* Tn*10*(tet^r)]	Stratagene Cloning Systems, CA
Plasmids		
pRS413	*HIS3* Ap^R *CEN*	Christianson *et al*. (1992)
pGEX-2T	Ap^R	Pharmacia LKB, Uppsala, Sweden
pJT4	*URA3* Ap^R (pRS316) *SNF7*	Tu *et al*. (1993)
pMB4	*HIS3* Ap^R (pRS413) *VPS4*	Babst *et al*. (1997)
pMB24	*HIS3* Ap^R (pRS413) *vps4*^{K179A}	Babst *et al*. (1997)
pMB31	Ap^R (pGEX-2T) *GST–VPS4*	Babst *et al*. (1997)
pMB40	Ap^R (pGEX-2T) *GST–vps4*$^{\Delta CC}$	this study
pMB42	Ap^R (pGEX-2T) *GST–vps4*^{K179A}	Babst *et al*. (1997)
pMB59	*HIS3* ApR (pRS413) *vps4ts229*	Babst *et al*. (1997)
pMB63	Ap^R (pGEX-2T) *GST–vps4*^{E233Q}	this study
pMB66	*HIS3* Ap^R (pRS413) *vps4*^{E233Q}	this study
pMB76	*HIS3* Ap^R (pRS413) *VPS4-HA*	this study
pMB90	Ap^R (pGEX-2T) *GST–vps4*$^{\Delta CC/E233Q}$	this study
pMB91	*HIS3* Ap^R (pRS413) *vps4*$^{\Delta CC/E233Q}$	this study
pMB96	*HIS3* Ap^R (pRS413) *vps4*^{E233Q}*-HA*	this study

fragment from pMB40 and pMB50, respectively, with the corresponding fragment of pMB66.

Biochemical assays

With the use of the GST fusion plasmids pMB31 (*GST–VPS4*), pMB40 (*GST–vps4*$^{\Delta CC}$), pMB42 (*GST–vps4*^{K179A}), pMB63 (*GST–vps4*^{E233Q}) and pMB90 (*GST–vps4*$^{\Delta CC/E233Q}$), wild-type and mutant Vps4 proteins were overexpressed in *E.coli* and purified as described (Babst *et al*., 1997). For *in vitro* ATPase activity assays, 0.1–2 μM Vps4p was incubated in 20 μl of reaction buffer [buffer A (0.1 M potassium acetate, 20 mM HEPES pH 7.4, 5 mM magnesium acetate), 0.1–2 mM ATP, 1 μCi of [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]. The data were interpreted using SigmaPlot (Jandel Scientific). For gel filtration analysis, ~50 μg of protein was loaded on a Superdex-200HR column (Pharmacia, Uppsala, Sweden) and separated in the presence of buffer A (± 1 mM nucleotide). UV cross-linking experiments were performed as previously described (Sarkar *et al*., 1985). Briefly, 20 μl samples containing 2 μg of purified protein in reaction buffer [buffer A, 15 μCi [α-^{32}P]ATP (3000 Ci/mmol), ± 0.1 mM nucleotide] were illuminated by a UV lamp (254 nm, UVGL-58, UVP Inc., CA) at 2–3 cm distance for 15 min at 0°C. The protein was precipitated by adding 10% trichloroacetic acid (TCA), washed with acetone and analyzed by SDS–PAGE. Chemical cross-linking of purified Vps4p was performed at a protein concentration of 1 μM (5 μg in 100 μl) in buffer A (± 1 mM nucleotide) with 0.02% glutaraldehyde. After 5 min incubation at room temperature, the reaction was terminated by the addition of an equal volume of 1 M glycine. The products were TCA-precipitated, washed with acetone and analyzed by SDS–PAGE.

Subcellular fractionation and Western blot analyses were performed as previously described (Babst *et al*., 1997). For temperature shift experiments, cells were spheroplasted for the first 15 min at the permissive temperature and then shifted to the non-permissive temperature for the remaining 20 min of the spheroplasting reaction. For sucrose density gradients, the 13 000 *g* pellet fraction from a subcellular fractionation was resuspended in 1 ml of lysis buffer (0.2 M sorbitol, 50 mM potassium acetate, 20 mM HEPES pH 6.8, 2 mM EDTA) supplemented with 60% sucrose and loaded beneath 2 ml of 55% and 2 ml of 35% sucrose steps. After a 14 h spin at 200 000 *g*, three samples were collected: the top 3 ml (F), the remaining 2 ml (N) and the sediment (P). The proteins contained in the F and N fractions were TCA-precipitated and washed with acetone.

Preparation of antiserum against Vps24p and Snf7p

The 560 bp *Nco*I–*Xba*I fragment of *VPS24* was fused to the *trpE* gene of the *E.coli* expression vector pATH3 (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). The 490 bp *Xba*I–*Acc*I fragment of pJT4, containing part of *SNF7*, was inserted into the GST fusion vector pGEX-2T (Pharmacia LKB, Uppsala, Sweden). The fusion protein was purified by affinity chromatography and used to immunize New Zealand White rabbits. Both fusion proteins were coupled to Sepharose and the resulting resins were used for affinity purification of the corresponding antiserum (Harlow and Lane, 1988).

Fluorescence microscopy

Cells of a 12 ml culture were harvested at an OD_{600} = 0.8, resuspended in 3 ml of spheroplasting medium [YNB medium, 1 M sorbitol, 40 mM phosphate buffer pH 6.5, 30 μg/ml zymolyase (Seikagaku Co., Tokyo, Japan)] and incubated for 35 min. In the case of temperature shift experiments, the final 20 min of the spheroplasting reaction were performed at the non-permissive temperature. Formaldehyde was added to a concentration of 4% and the samples were incubated further for 1 h at 30°C. The fixed cells were washed with SHA (1 M sorbitol, 0.1 M HEPES pH 7.5, 5 mM NaN_3), incubated for 10 min in SHA with 1% lauryl-dimethylamineoxide and washed twice with SHA. These cells were then incubated for 2 h with the primary antibody, 1 h with the secondary antibody and finally stained for 1 h with Cy2- or Cy3-conjugated tertiary antibody (Jackson ImmunoResearch Laboratories, PA).

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