Curr Genet (1997) 31: 469–480

ORIGINAL PAPER

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The *VPS4* gene is involved in protein transport out of a yeast pre-vacuolar endosome-like compartment

Received: 28 February / 4 April 1997

Abstract Four yeast mutants were isolated in a screen for dominant-negative vacuolar protein-sorting mutants, secreting a carboxypeptidase Y-invertase hybrid protein. In addition to defects in the sorting/transport of soluble vacuolar hydrolases, the mutants accumulated a pre-vacuolar endosome-like compartment. The mutant alleles causing the defects were identified as the members of the *VPS4* gene locus, each harbouring single-point mutations leading to amino-acid exchanges at positions 233 (E233Q), 211 (E211 K), and 178 (G178D). These mutations all reside within a 200 amino-acid-long ATPase module, common to members of the AAA-protein family. The *VPS4* gene product shows homology to the yeast Sec18p (50% similarity and 25% identity), which is involved in several vesicle-mediated protein transport steps and homotypic membrane fusion events. Disruption of the *VPS4* gene leads to a recessive vacuolar protein-sorting phenotype. About 40% of newly synthesized CPY is secreted as the Golgi-modified p2CPY precursor form. Transport of secretory proteins to the plasma membrane is normal as demonstrated by the secretion of invertase and α-factor. The α-factor, however, is secreted as a partially processed precursor, caused by defects in late Golgi function. The *vps4* mutants also exhibit defects in fluid-phase endocytosis, as demonstrated by the accumulation of Lucifer Yellow in a pre-vacuolar endosome-like compartment. Based on the pleiotropic phenotype of the *vps4* mutants and on the sequence homology to NSF/Sec18p, we propose that the *VPS4* gene product is required for efficient transport out of the pre-vacuolar endosome-like compartment.

Key words *Saccharomyces cerevisiae* · *VPS4* gene · AAA-protein family

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Communicated by C. P. Hollenberg

Introduction

Proteins are transported to the lysosome-like vacuole in yeast either via the secretory pathway (Stevens et al. 1982), via the endocytic pathway (Riezman 1985), or via an alternative pathway directly from the cytosol into vacuoles (Harding et al. 1995). Some newly synthesized yeast proteins, like aminopeptidase I and α-mannosidase, are imported into vacuoles directly from the cytosol (Klionsky et al. 1992; Yoshihisa and Anraku 1990), whereas other vacuolar hydrolases like carboxypeptidase Y (CPY) and proteinase A (PrA) traverse the early secretory pathway *en route* to their final destination (Stevens et al. 1982; Klionsky et al. 1988). Proteins entering this pathway are first translocated from their cytoplasmic site of synthesis into the endoplasmic reticulum (ER). After translocation into the ER and the addition of core oligosaccharides, proteins not destined to be retained in the ER are transported to the Golgi complex via transport vesicles (Kaiser and Schekman 1990). In the Golgi complex most proteins undergo further post-translational modifications (addition of outer-chain carbohydrates, elongation of O-linked oligosaccharides) prior to transport out of the late Golgi compartment (Tanner and Lehle 1987). Protein transport to the cell surface appears to occur by a default or bulk-flow mechanism, whereas the transport of vacuolar/lysosomal proteins requires specific sorting signals. Most of the soluble lysosomal proteins of mammalian cells are modified with mannose-6-phosphate residues on N-linked carbohydrate chains, which are recognized by mannose-6-phosphate receptors that mediate sorting and delivery to the lysosome (Figura von and Hasilik 1986; Kornfeld and Mellman 1989). In contrast, the sorting information present in yeast lysosomal/vacuolar proteins is not associated with any specific carbohydrate modification and instead appears to reside within the polypeptide backbone of these proteins (Schwaiger et al. 1982; Johnson et al. 1987; Valls et al. 1987; Klionsky et al. 1988; Klionsky and Emr 1990; Winther et al. 1991). The vacuolar targeting signal of CPY, for example, is minimally defined by four amino-acid res-

idues in the propeptide of CPY (Valls et al. 1990). Similar to lysosomal protein transport in mammals, in yeast a Golgi-resident transmembrane receptor, Vps10p, binds p2CPY in a late Golgi compartment and executes multiple rounds of sorting and transport to a pre-vacuolar endosomal compartment (Marcusson et al. 1994; Cereghino et al. 1995; Cooper and Stevens 1996).

Genetic selections in yeast have identified more than 45 *vps* (*vps* for *v*acuolar *p*rotein *s*orting defective) complementation groups that are involved in the sorting and delivery of vacuolar hydrolases (Jones 1977; Bankaitis et al. 1986; Rothman and Stevens 1986; Robinson et al. 1988; Rothman et al. 1989a, b). All *vps* mutants missort and secrete vacuolar enzyme precursors instead of delivering them to the vacuole. Since protein secretion and post-translational modification seems to be normal in most of the *vps* mutants, these mutants are presumed to be defective either in components of the sorting machinery or in the assembly and maintenance of the acceptor compartment, the vacuole. The *vps* mutants have been grouped into six classes (classes A–F), based on vacuolar morphology, localization of the 60-kDa subunit of the vacuolar H^+-adenosine triphosphatase (V-ATPase), and alkaline phosphatase (Banta et al. 1988; Raymond et al. 1992). The class E *vps* mutants are of special interest, because they are characterized by a novel "class E" compartment. Within this class E compartment, endocytic marker proteins (Ste3p and α-factor), vacuolar hydrolases (e.g. CPY), and Golgi resident proteins (e.g. Vps10p) accumulate (Raymond et al. 1992; Davis et al. 1993; Vida et al. 1993; Piper et al. 1995; Rieder et al. 1996). Therefore, this intermediate pre-vacuolar compartment is thought to be the site where the endocytic protein transport and the Golgi-to-vacuole protein transport converge (Raymond et al. 1992; Davis et al. 1993; Piper et al. 1995; Rieder et al. 1996).

In an attempt to identify factors that are directly involved in vacuolar protein transport, we isolated several dominant-negative yeast mutants that exhibited a vacuolar protein-sorting defect. Analyses of four *vps* mutants, all belonging to one complementation group exhibiting a class E vacuolar morphology, revealed that single point mutations in the P9705.10 ORF caused the vacuolar protein-sorting defect. A more detailed analysis of one of the dominant-negative mutant strains and a null-mutant strain revealed additional defects in endocytosis and in late Golgi function. The P9705.10 ORF is identical with the *VPS4*, *END13*, and *GRD13* genes, which were identified in genetic screens for recessive vacuolar protein-sorting mutants, endocytosis mutants, and Golgi-retention mutants, respectively (Robinson et al. 1988; Munn and Riezman 1994; Nothwehr et al. 1996).

Materials and methods

Strains and media. Table 1 describes all the *Saccharomyces cerevisiae* and *Escherichia coli* strains used in this study. The *E. coli* strain XL1Blue (Stratagene, LA Jolla, Calif.) was grown at 37 °C in standard LB media (Miller 1972) or LB medium supplemented with ampicillin (100 μg/ml). Yeast strains were grown at 30 °C, either in YPD-rich medium (1% yeast extract, 2% peptone, 2% dextrose) or in synthetic complete minimal medium (SD) with the necessary supplements (Sherman 1991). Wickerham's minimal proline medium (WIMP) was used in vivo labelling experiments (Wickerham 1949).

Materials. Pharmacia was the supplier for all DNA restriction and modifying enzymes used in this study. Deoxynucleotides, and 5-bro-

Table 1 Strains used in this study

Strain	Genotype	Source or reference
S. cerevisiae		
SEY6210	*MAT*α *leu2-3,112 ura3-52 his3-*Δ*200 trp1-*Δ*901 lys2-801 suc2-*Δ*9*	(Robinson et al. 1988)
SEY6211	*MAT***a** *leu2-3,112 ura3-52 his3-*Δ*200 trp1-*Δ*901 ade2-101 suc2-*Δ*9*	(Robinson et al. 1988)
SEY6210.5	Diploid of cross SEY6210 and SEY6211	(Herman and Emr 1990)
BHY10	SEY6210; *leu2-3,112*::pBHY11(*CPY-Inv LEU2*)	(Horazdovsky et al. 1994)
BHY11	SEY6211; *leu2-3,112*::pBHY11(*CPY-Inv LEU2*)	(Horazdovsky et al. 1994)
BHY10.5	Diploid of cross BHY10 and BHY11	(Horazdovsky et al. 1994)
D228-7	BHY10.5; *vps4*-d233/*VPS4*	This study
SD228-7	*MAT*α *ura3-52 his3-*Δ*200 trp1-*Δ*901 lys2-801 suc2-*Δ*9* *leu2–3,112*::pBHY11(*CPY-Inv LEU2*) *vps4*-d233	This study
SD228-8	*MAT***a** *ura3-52 his3-*Δ*200 trp1-*Δ*901 lys2–801 suc2-*Δ*9* *leu2–3,112*::pBHY11(*CPY-Inv LEU2*) *vps4*-d178	This study
SD228-9	*MAT*α *ura3-52 his3-*Δ*200 trp1-*Δ*901 lys2-801 suc2-*Δ*9* *leu2–3,112*::pBHY11(*CPY-Inv LEU2*) *vps4*-d211	This study
SD226-A2	*MAT***a** *ura3-52 his3-*Δ*200 trp1-*Δ*901 lys2-801 suc2-*Δ*9* *leu2–3,112*::pBHY11(*CPY-Inv LEU2*) *vps4*-d233	This study
MFY40	SEY6210; *vps4*Δ*1: :LEU2*	This study
MFY41	SEY6211; *vps4*Δ*1: :LEU2*	This study
SEY05179	SEY6210; *vps4*-05179	(Robinson et al. 1988)
SEY1546	SEY6211; *vps4*-1546	(Robinson et al. 1988)
E. coli		
XL1-Blue	*recA1 endA1 gyr A96 thi-1 hsdR17 supE44 relA1 lac* [*F´ proAB lacI*^{q} *Z*Δ*M15 Tn10 (tet*^{r}*)*]	Stratagene, La Jolla, Calif.

mo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) were purchased from Eurogentech. The Trans^{35}S-label was obtained from ICN Radiochemicals. Zymolyase 100T is a product from ICN Biomedicals. CDCFDA and FM4-64 were from Molecular Probes. All other chemicals were purchased from Sigma Chemical. A polyclonal antiserum to CPY was generated by the immunization of rabbits with de-glycosylated CPY essentially as described by Klionsky et al. (1988). Antisera to PrA and α-factor were obtained from Scott Emr (Howard Hughes Medical Institute, San Diego) (Klionsky et al. 1988; Graham et al. 1993). Invertase antiserum was a gift from Ralf Kölling (HHU, Düsseldorf).

Genetic procedures. Crosses, sporulation of diploids, and tetrad analyses were performed by standard genetic techniques (Sherman et al. 1986). Yeast cells were transformed by electroporation with a Bio-Rad Gene Pulser (Becker and Guarente 1991).

Nucleic acid techniques. To construct a genomic library from the haploid yeast strain SD228-7, genomic DNA was isolated from spheroplasts as described by Philippsen et al. (1991) and partially digested with the restriction enzyme *Sau3*A. DNA fragments of 6–15 kb were purified using a Gelase^{TM} kit from Biozym and ligated with the *Bcl*I-digested and de-phosphorylated *E. coli*-yeast shuttle vector pCS19 (Sengstag 1993). Approximately 540 000 independant clones were pooled; 95% of the clones carried an insert with an average size of 8 kb. Double-stranded plasmid DNA templates used for sequencing reactions were isolated with the Qiagen BioRobot 9600 system (QIAGEN, Hilden). The sequence of the 1.8-kb genomic *Hin*dIII DNA fragment (see Fig. 1) harbouring the *VPS4* gene was established by sequencing both strands with specific primers. The sequencing reactions were analyzed with an ABI 373 DNA sequencer from Applied Biosystems, using the dye terminator cycle sequencing kit with Ampli*Taq* DNA Polymerase FS (Perkin Elmer). The sequence of the *VPS4* gene, as well as its predicted protein sequence, were compared to entries of the NBRF, PIR, SwissProt, Genbank and EMBL databases, using the FASTA and TFASTA programs of the University of Wisconsin Genetics Computer Group sequence analysis package (Devereux et al. 1984). We also searched at the NCBI, using the BLAST network service (Altschul et al. 1990; Gish and States 1993). The derived protein sequence of the *VPS4* gene was also searched for known protein sequence motifs with the MacPattern program (Fuchs 1991), the PROSITE dictionary (Bairoch 1991), and the BLOCKS network service (Henikoff and Henikoff 1994). All other molecular-biology techniques were performed as described in Ausubel et al. (1990).

Plasmid constructions. pGK3 is a 5.4-kb genomic clone isolated from a genomic library that was prepared from the haploid yeast strain SD228-7 in the yeast-*E. coli* shuttle vector pCS19 (Sengstag 1993). The plasmid pRSX35 contains a 3502-bp *Sma*I-*Xho*I sub-fragment and the plasmid pRHH18 contains a 1814-bp *Hin*dIII fragment of pGK3 in the yeast-*E-coli* shuttle vector pRS316 (Sikorski and Hieter 1989). The 3.5-kb *Sma*I/*Xho*I-fragment was also subcloned into the pBluescript KSII+ vector (Stratagene) and designated pBSX35. Construction of plasmid pMF4, harbouring a *LEU2* disruption construct of the *VPS4* gene, was accomplished by digesting plasmid pBSX35 with the restriction enzymes *Eco*RV and *Bgl*II, eliminating most of the ORF of the *VPS4* gene. The *Eco*RV/*Bgl*II-cut plasmid pBSX35 was ligated with a 1.6-kb *Bam*HI fragment, isolated from YDp-L (Berben et al. 1991), harbouring the *LEU2* marker gene, followed by a Klenow enzyme treatment and a second ligation reaction. Restriction analyses revealed that the orientation of the *LEU2* marker within plasmid pMF4 is the same as of *VPS4* within pBSX35. The plasmid pRB58 is a YEp24 vector containing a genomic 7.6-kb *Sau*3A fragment in the *Bam*HI site coding for the yeast *SUC2* gene (Carlson and Botstein 1982).

Gap repair. Gap-repair experiments were done according to Orr-Weaver (1983). The plasmid pRSX35 was digested with *Hin*dIII excising the complete *VPS4* gene. The gapped plasmid, harbouring 5′ and 3′ flanking sequences of *VPS4* was used in yeast transformation experiments. Gap-repaired constructs were isolated from yeast transformants by standard procedures (Hoffmann and Winston 1987).

Gene disruption. One-step gene disruption experiments were done according to Rothstein (1983). To disrupt the *VPS4* gene, the haploid wild-type yeast strains SEY6210 and SEY6211 were transformed with a 2.1-kb *Hind*III fragment isolated from pMF4. Leu$^+$ transformants were selected and analyzed by PCR. Using one *leu2*-specific primer PL (5′-GTGATGCTGTCGCCGAAGAAG-3′) and two *VPS4*-specific primers P2R (5′-ACACTCCATGCGTATT-GACC-3′) and P6 (5′-GCATCTATTGGTGGCGAAAC-3′), we could demonstrate correct replacement of *VPS4* by the *LEU2* marker gene in the SEY6210 background (MFY40) and the SEY6211 background (MFY41). Wild-type strains (SEY6210 and SEY6211) exhibit a 700-bp PCR product, amplified by the two *VPS4*-specific primers P6 and P2R, whereas *VPS4*-disrupted strains (MFY40 and MFY41) yield a 590-bp PCR product, generated by the *LEU2*-specific primer PL and the P6 primer, that binds downstream from *VPS4*.

Labelling yeast cells, immunoprecipitation, electrophoresis, and fluorography. Whole yeast cells and spheroplasts were labelled with Trans^{35}S-label as described by Paravicini et al. (1992) with the following modifications. The labelling reactions were performed in WIMP media w/o YE and chased by the addition of 1 vol of WIMP medium containing 10 mM methionine and 5 mM cysteine. For quantitative immunoprecipitations different antisera dilutions were used: anti-CPY 1: 200, anti-PrA 1: 500, anti-α-factor 1:1000, and anti-Invertase 1: 500. After immunoprecipitation three washing steps were performed with the following buffers; (1) 50 mM Tris/HCl pH 7.5, 150 mM NaCl, 0.5% Tween-20, 0.1 mM EDTA; (2) 100 mM Tris/HCl pH 7.5, 200 mM NaCl, 0.5% Tween-20, 2 M urea; (3) 50 mM Tris/HCl pH 7.5, 150 mM NaCl, 0.1 mM EDTA. Prior to electrophoresis, PrA immunoprecipitations were heated in SDS-boiling buffer (125 mM Tris-HCl pH 6.8, 4% SDS, 0.2% bromphenol blue, 20% glycerol, 10% 2-mercapto-ethanol) containing 6 M urea for 10 min at 37 °C. All other immunoprecipitations were heated in SDS-boiling buffer for 5 min and electrophoretically separated in 8.5% SDS polyacrylamide gels as described by Laemmli (1970). After electrophoresis, gels were fixed for 20 min in 10% acetic acid (v/v), 40% methanol (v/v). Fixed gels were washed twice with water (10 min each) and then treated for 20 min with 1 M sodium salycylate, 1% glycerol. Dried-gels were fluorographed at –80 °C using Kodak XAR X-ray films. Quantitative data were obtained with a Fuji BAS 1500 system and analyzed with TINA^{TM} software, version 2.09f.

Microscopy. Yeast cells were stained with the fluorescent dye CDCFDA as described by Roberts et al. (1991). Staining with the lipid-soluble styryl dye FM4-64 was performed as described by Vida and Emr (1995), except that cells were stained for 60 min at 30 °C. LY staining was carried out according to Dulic et al. (1991) with the following modifications: cells were grown to a mid-logarithmic phase in YPUAD (1% yeast extract, 2% peptone, 40 μg/ml uracil, 40 μg/ml adenine, 2% glucose) and staining was performed at 30 °C in 2% glucose for 90 min. Subsequently, the cells were carefully harvested by centrifugation, washed three times and incubated in 2% glucose for 60 min at 30 °C. After immobilizing the cells on Concanavalin A-coated microscope slides photos were taken using a Zeiss Axioplan microscope equipped with a Zeiss MC100 SPOT camera on Kodak TMY 400 films.

Results

Isolation of dominant-negative vacuolar protein-sorting mutants

In an attempt to identify crucial components of the yeast vacuolar protein-sorting apparatus, we isolated dominant-negative mutants with defects in vacuolar protein transport/sorting. The mutant screen we used was originally described by Bankaitis et al. (1986). Bankaitis and cowork-

ers used a plasmid-borne carboxypeptidase Y-invertase (CPY-Inv) fusion construct to select for mutants that mislocalize the CPY-Inv hybrid protein into the media, and thereby allow yeast cells to grow on sucrose as the sole carbon source. The CPY-Inv hybrid protein contains 50 amino-acids of the N-terminal signal sequence of the vacuolar hydrolase carboxypeptidase Y (CPY) that is responsible for correct sorting and transport to the yeast vacuole. We modified this genetic selection procedure in order to select for mutants exhibiting a dominant-negative vps phenotype. Using the diploid yeast strain BHY10.5 (Horazdovsky et al. 1994), harbouring two chromosomal copies of the CPY-Inv fusion construct at the *LEU2* locus, we were able to select several independent spontaneously arising *vps* mutants. All mutants were sporulated and haploid mutant daughter cells were backcrossed with haploid wild-type parent cells to confirm the dominant-negative vps phenotype.

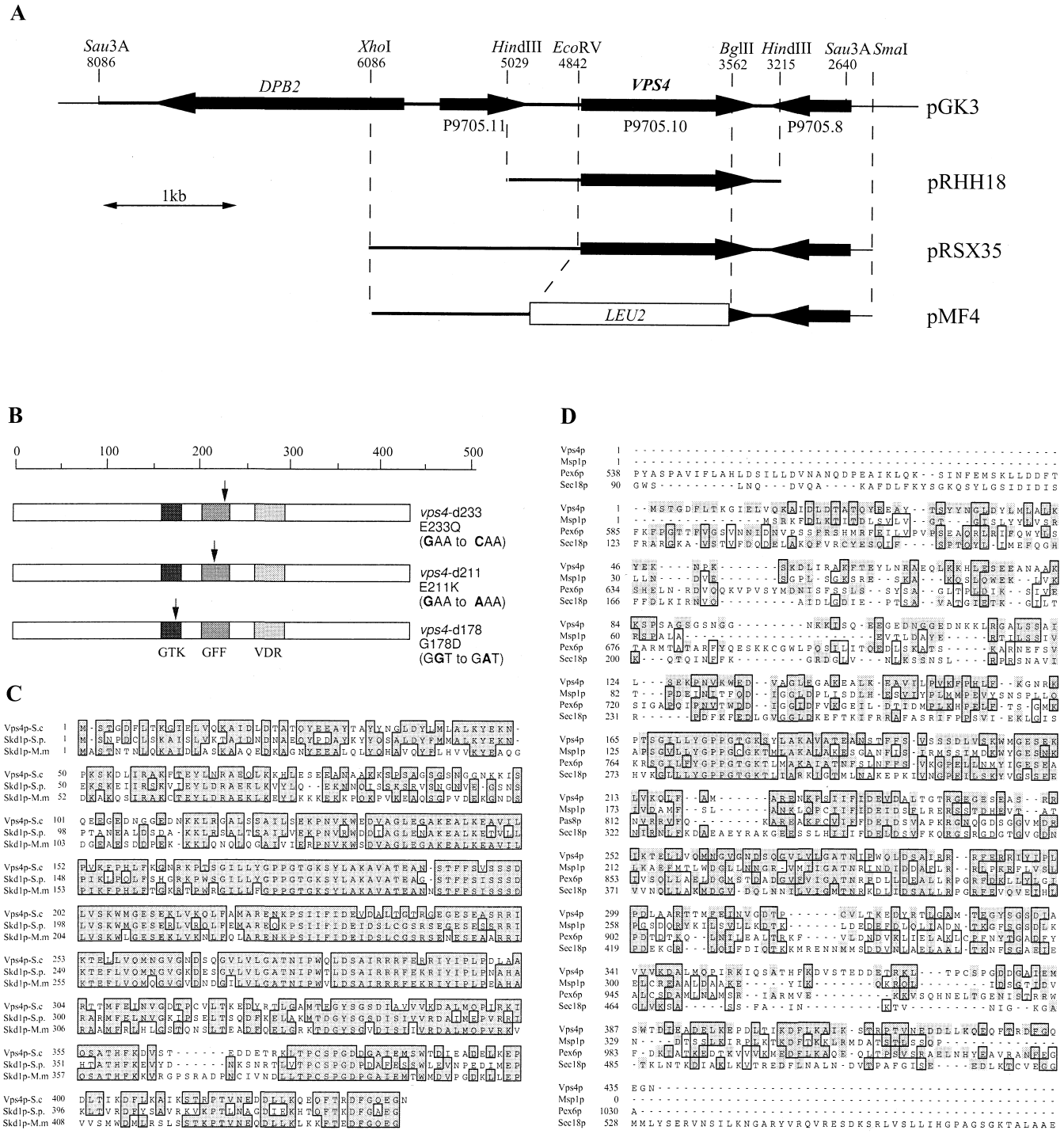
Four of twelve dominant-negative *vps* mutants could be assigned to one complementation group (D228-7, D228-8, D228-9, D226-A2). Details of the mutant screen and complementation analysis will be published elsewhere. All members of this group secreted about 35% of the CPY-Inv fusion protein into the medium as determined by quantitative enzyme activity assays (data not shown). One of the haploid mutant strains (SD228-7) was analyzed in more detail. Staining haploid cells with the vacuole-specific dye CDCFDA or the lipophilic dye FM4-64 revealed, in addition to a central vacuole, several smaller vesicular compartments (see Fig. 4, A2 and B2). These vesicular compartments could not be detected in wild-type cells (see Fig. 4, A1 and B1). These observations are reminiscent of the vacuolar morphology of class E *vps* mutants, which exhibit a novel "class E" compartment (Raymond et al. 1992; Vida and Emr 1995). The class E compartment is a multilamellar structure next to the vacuole (Rieder et al. 1996). It is acidified by an active V-ATPase (Raymond et al. 1992), which we confirmed by staining the class E compartment with the pH-dependent dye CDCFDA (Preston et al. 1989).

In order to clone the mutant allele that caused mislocalization of the CPY-Inv fusion protein, we established a genomic library of the haploid mutant yeast strain SD228-7 in the yeast-*E. coli* shuttle vector pCS19 (Sengstag 1993). The genomic library was transformed into a haploid (BHY10) and a diploid (BHY10.5) wild-type yeast strain. Approximately 40 000 Ura^+ transformants were assayed by an overlay plate assay (Paravicini et al. 1992) for secreted CPY-Inv activity. Thirty two genomic clones were identified, conferring a dominant-negative vps phenotype in haploid and diploid wild-type yeast cells. DNA restriction analyses of 12 genomic inserts demonstrated that all of them had a 1.8 kb *Hin*dIII fragment in common (Fig. 1 A). DNA sequence analysis of this 1.8-kb fragment revealed a single open reading frame. The same chromosomal region was isolated by gap repair (see Materials and methods) from a haploid wild-type strain (BHY10) and from the three other dominant-negative *vps* mutant strains of the same complementation group (SD228-8, SD228-9, and SD228-A2), and subsequently sequenced. The wild-type sequence of this ORF was identical to the P9705.10 ORF, sequenced by Johnston et al. (Genbank Accession number U25842) in the course of the yeast genome sequencing project.

Fig. 1A–D Genomic organisation and analysis of the *VPS4* gene. **A** restriction map of the genomic clone pGK3 harbouring the dominant *vps4*-d233 allele. *Thin lines* represent vector sequences (pCS19) and *thick lines* represent yeast genomic insert DNA. Possible open reading frames are indicated by *arrows*. The *VPS4* gene was identified in the course of the yeast genome sequencing project as open reading frame P9705.10 on the right arm of chromosome XVI (EMBL database accession number U25842.em_fun). pRHH18 and pRSX35 are both yeast-*E. coli* shuttle vectors (pRS316) harbouring the dominant *vps4*-d233 allele. pMF4 is a pBluescript KS+ vector carrying a *vps4Δ1*: :*LEU2* disruption construct. **B** schematic map of dominant mutations in the *VPS4* gene. *Arrows* indicate the location of three dominant point mutations found in the *VPS4* gene. According to the Blocks Database Version 9.2, three conserved regions (GTK, GFF, and VDR) of the AAA-protein family are indicated by *dark boxes*. **C** sequence comparison of Vps4p and homologous proteins from *S. pombe* (Skd1p-S.p.) and mouse (Skd1p-M.m.). Identical amino-acids are *boxed*. *Gaps* (–) were introduced for optimal alignment, which was created with the Clustal W program (Thompson et al. 1994). Protein sequence data are available from SwissProt under accession numbers P52917 (End13/Vps4 protein), Q09803 (Skd1p from *S. pombe*), and P46467 (Skd1p from mouse). **D** sequence comparison of Vps4p and other AAA protein family members involved in protein transport reactions in *S. cerevisiae*. Identical amino-acids are *boxed* and conserved amino-acids are *shaded*. *Gaps* (–) were indroduced for optimal alignment, which was created with the Clustal W program (Thompson et al. 1994). Protein sequence data are available from SwissProt under accession numbers P52917 (End13/Vps4 protein), P28737 (Msp1p), P33760 (Pex6p), and P18759 (Sec18p)

A detailed database search revealed that recessive mutant alleles of the P9705.10 gene were also identified in screens for recessive vacuolar protein-sorting defective mutants (*vps4*) (Rothman and Stevens 1986; Robinson et al. 1988), for endocytosis mutants (*end13*) (Munn and Riezman 1994), and for Golgi-retention mutants (*grd13*) (Nothwehr et al. 1996). We disrupted the ORF 9705.10 in the two wild-type yeast strains SEY6210 and SEY6211 and analyzed the vps phenotype of the *vps4* null-mutant strains MFY40 and MFY41. Both *vps4* null-mutant strains secreted significant amounts of the CPY-Inv hybrid protein (data not shown), demonstrating a recessive vps phenotype. Subsequent complementation experiments with *vps4* mutant tester strains (SEY4-05179 and SEY4-1546) and *vps4* null-mutant strains (MFY40 and MFY41) clearly demonstrated that the *VPS4* gene and the ORF P9705.10 were identical. Therefore, *VPS4*, *END13*, *GRD13*, and P9705.10 are synonyms for the same gene. Since we have isolated dominant-negative alleles of this gene in a screen for vacuolar protein-sorting mutants, we called the gene *VPS4*.

The *VPS4* gene has the potential to encode a protein with 437 amino-acids and a predicted molecular weight of 48.1 kDa. The protein does not contain an obvious transmembrane domain region or a signal sequence at the N-terminus, and therefore most likely does not enter the secretory protein-transport pathway. Analyses of the primary protein sequence revealed significant homology to mem-



bers of the AAA-protein family (*A*TPases *a*ssociated with a variety of cellular *a*ctivities). Within this family the Skd1p of *Schizosaccharomyces pombe* and *Mus musculus* revealed an overall identity of 62% to Vps4p, indicating that the *SKD1* genes of *S. pombe* and *M. musculus* are homologues of the *S. cerevisiae VPS4* gene (Fig. 1 C). An alignment of Vps4p with related yeast AAA-protein family members (Pex6p, Msp1p, and Sec18p) is shown in Fig. 1D. Pex6p (formerly Pas8p) is required for peroxysome assembly (Distel et al. 1996; Voorn-Brouwer et al. 1993). Msp1p is involved in intramitochondrial protein-sorting (Nakai et al. 1993), and Sec18p participates in several vesicle-mediated protein-transport steps and homotypic membrane fusion events (Eakle et al. 1988; Kaiser and Schekman 1990; Graham and Emr 1991; Rexach and Schekman 1991; Haas and Wickner 1996; Mayer et al. 1996). Although, Sec18p harbours two AAA modules, an optimized alignment between Vps4p which contains one

AAA module and Sec18p shows an overall similarity of 49% and 25% identity.

The AAA module in general is characterized by three conserved motifs (GTK, GFF, and VDR) according to the nomenclature of the BLOCKS-Database (Henikoff and Henikoff 1994) (Fig. 1 B). The N-terminal GTK motif harbours a Walker-type A, also known as a P-loop, motif (Saraste et al. 1990) and the central GFF region represents a Walker-type B motif (Walker et al. 1982). Together with the C-terminal VDR region they are thought to act as an ATP-dependent protein clamp (Confalonieri and Duguet 1995). Interestingly, the point mutations that we found in the dominant *vps4* alleles of SD228-7, SD228-8, SD228-9, and SD226-A2 all reside within the conserved AAA module (Fig. 1 B). The *vps4*-d233 allele (E233Q), found in SD228-7, and the *vps4*-d211 allele (E211K), found in the SD228-9 mutant, reside in the central GFF motif. The *vps4*-d178 allele (G178D), which was isolated twice, from the mutant strains SD228-8 and SD226-A2, affects the P-loop region, which is involved in ATP/GTP binding (Saraste et al. 1990).

The *VPS4* gene is involved in the transport of soluble vacuolar hydrolases

To compare the phenotypic consequences of a complete deletion of the *VPS4* gene with the *vps4*-d233 dominant allele on vacuolar protein-sorting, spheroplasts of wild-type yeast cells (SEY6210), *vps4*-d233 cells (SD228-7), and Δ*vps4* cells (MFY40) were examined for their ability to process and target the hydrolase precursor to the vacuole by pulse-chase experiments (Fig. 2). Spheroplasted cells were metabolically labelled with Trans^{35}S-label for 5 min and chased for 40 min by the addition of unlabelled cystein and methionine. The cells were separated into intracellular (I) and extracellular (E) fractions and CPY was recovered by immunoprecipitation. Within the 40-min chase period wild-type cells completely processed CPY precursor forms (p1CPY, p2CPY) to the mature form of CPY, demonstrated by the presence of mCPY in the I fraction (Fig. 2 A, lane 2). In contrast, the dominant-negative *vps4* mutant strain SD228-7 and the Δ*vps4* strain MFY40 mislocalized about 40% of the Golgi-modified precursor forms of CPY (p2CPY) into the media (Fig. 2 A, lane 6 and 9). Approximately 60% of newly synthesized CPY is found in both *vps4* mutant strains within the cell and is correctly processed (Fig. 2 A, lane 5 and 8). Analyzing a second soluble vacuolar hydrolase, proteinase A (PrA), both *vps4* mutant strains (SD228-7 and MFY40) exhibited a weak vacuolar protein-processing defect. Approximately 5–10% of the PrA precursor forms (proPrA) were not correctly processed within an 40 min chase (data not shown). The processing defects of CPY and PrA observed with yeast spheroplasts were confirmed in pulse-chase experiments with whole yeast cells (Fig. 2 B and C). The wild-type strain SEY6210 and the *vps4* mutant strains SD228-7 and MFY40 were labelled for 5 min with Trans^{35}S-label and chased for 20, 40, and 60 min after the addition of excess unlabelled methionine and cysteine. CPY, PrA, and alkaline phosphatase were recovered by immunoprecipitation. Whereas, in the wild-type strain SEY6210, all the labelled CPY and PrA precursor forms were correctly processed to mCPY

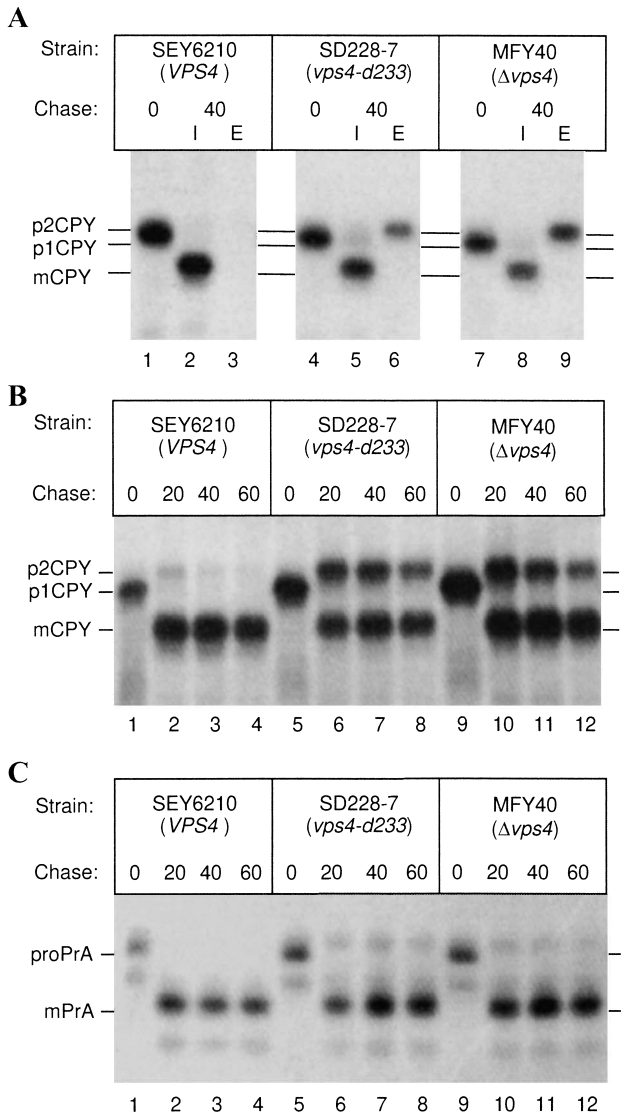


Fig. 2A–C Vacuolar protein-sorting defects analyzed by pulse-chase experiments. Phenotypical consequences of a *vps4* null mutation and the dominant *vps4*-d233 allele on vacuolar protein transport. **A** maturation of CPY in different *vps4* mutant strains. Yeast spheroplasts were labelled with Trans^{35}S-label for 5 min, chased for 40 min, and separated into intracellular (*I*) and extracellular (*E*) fractions. The presence of CPY in each fraction was determined by immunoprecipitation. The migration positions of CPY precursor (p1CPY, P2CPY) and mature forms (mCPY) are indicated by *horizontal lines*. The following strains were used: SEY6210 (wild-type), SD228-7 (*vps4*-d233 allele), MFY40 (Δ*vps4*). **B** and **C** processing kinetics of the vacuolar hydrolases CPY and PrA. Whole yeast cells were labelled with Trans^{35}S-label for 5 min and chased for 0, 20, 40 and 60 min. The presence of CPY (panel **B**) and PrA (panel **C**) in each sample was determined by quantitative immunoprecipitation. The migration positions of CPY precursor forms (p1CPY, p2CPY), the mature form of CPY (mCPY), the PrA precursor form (proPrA), and the mature PrA (mPrA) are indicated by *horizontal lines*. The following strains were used: SEY6210 (wild-type), SD228-7 (*vps4*-d233 allele), MFY40 (Δ*vps4*)

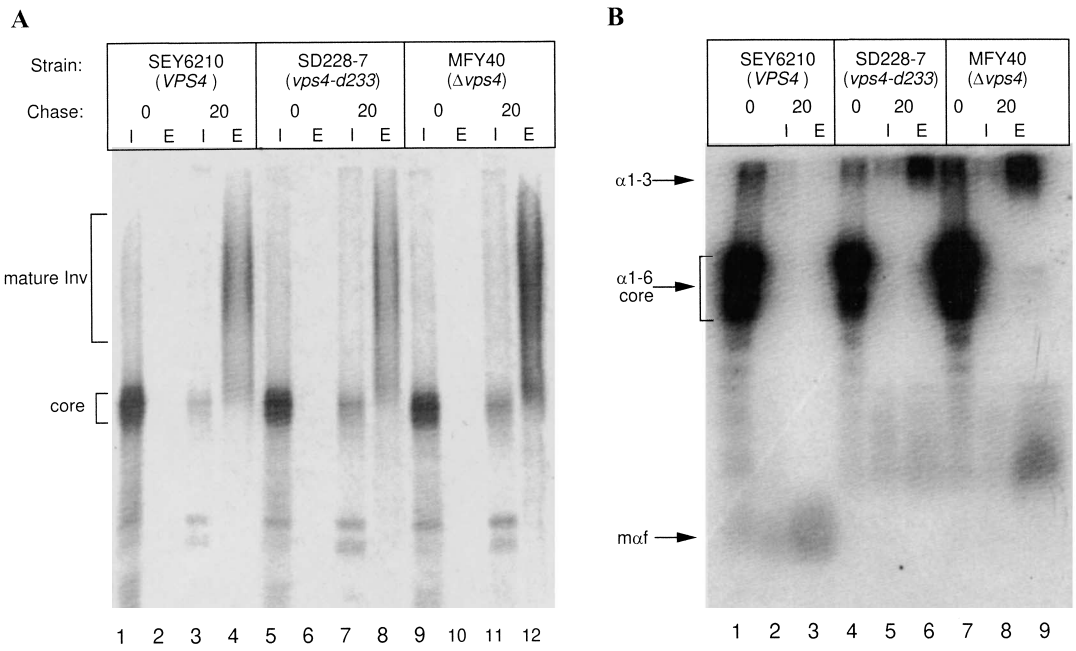


Fig. 3A, B Sorting and processing of secretory proteins in different *vps4* mutant strains analyzed by pulse-chase experiments. **A** secretion of invertase in *vps4* mutant strains.Yeast spheroplasts were labelled with Trans^{35}S-label for 5 min and chased for 20 min. Samples were taken immediately after the pulse and after the 20-min of chase. The samples were separated into intracellular (*I*) and extracellular (*E*) fractions, followed by immunoprecipitation of invertase. The migration position of core-glycosylated and highly glycosylated mature invertase is indicated. The following strains were used: SEY6210 (wild-type), SD228-7 (*vps4*-d233 allele), MFY40 (Δ*vps4*). **B** α-factor processing/secretion in *vps4* mutant strains. Yeast spheroplasts were labelled with Trans^{35}S-label for 5 min and chased for 20 min. Samples were taken immediately after the pulse (0 min) and after the 20-min chase. The 20-min chase-point samples were separated into intracellular (*I*) and extracellular (*E*) fractions. All samples were analyzed by quantitative immunoprecipitation specific for α-factor. The migration position of core glycosylated, alpha 1–6, and alpha 1–3 glycosylated precursor forms of α-factor, and mature α-factor is indicated. The following strains were used: SEY6210 (wild-type), SD228-7 (*vps4*-d233 allele), MFY40 (Δ*vps4*)

and mPrA after a 40-min chase periode (Fig. 2 B, lane 3 and Fig. 2 C, lane 3), both *vps4* mutant strains exhibited the same CPY and PrA processing defects as observed in the spheroplast experiment. The *vps4-d233* strain SD228-7 and the Δ*vps4* strain MFY40 accumulated approximately 40% p2CPY and about 5% proPrA, even after prolonged chase times of 60 min (Fig. 2 B and C, lanes 8 and 12). Transport of the type-II vacuolar membrane protein alkaline phosphatase was completely unaffected in both *vps4* mutant strains (data not shown).

The *VPS4* gene does not affect the transport of secretory proteins but does affect late Golgi function

Secretion of the Golgi-modified p2CPY precursor protein in *vps4* mutant cells (SD228-7 and MFY40) already indicated that the transport of secretory proteins might be unaffected in these *vps4* mutants. In order to test a possible function of Vps4p in secretory protein transport, we performed pulse-chase experiments with wild-type and *vps4* mutant strains, analyzing the transport of the secretory proteins invertase and α-factor. The wild-type strain SEY6210, the dominant *vps4* mutant strain SD228-7, and the *vps4* null-mutant strain MFY40 were transformed with plasmid pRB58 containing the *SUC2 gene* (Carlson and Botstein 1982). Spheroplasted cells were labelled with Trans^{35}S-label for 5 min and chased by the addition of excess unlabelled methionine and cysteine. Samples were taken immediately after the pulse and after a 20-min chase. The samples were separated into intracellular (I) and extracellular (E) fractions and invertase was recovered by immunoprecipitation. As expected, wild-type and both *vps4* mutant strains correctly modified and transported highly glycosylated invertase into the media fraction (Fig. 3 A, lanes 4, 8, 12). α-factor transport in wild-type and *vps4* mutant cells was analyzed in a parallel pulse-chase experiment (Fig. 3 B). In wild-type yeast cells α-factor precursors are processed by the sequential action of the late Golgi enzymes Kex2p, Kex1p, and Ste13p to the mature 2.5-kDa peptide (Fuller et al. 1988; Bryant and Boyd 1993). The wild-type strain SEY6210 secreted correctly processed mature 2.5-kDa α-factor (Fig. 3 B, lane 3). The dominant *vps4* mutant strain SD228-7 and the *vps4* null-mutant strain MFY40, however, secreted highly glycosylated (α 1–3) as well as partially processed precursor forms of α-factor into the media (Fig. 3 B, lanes 6 and 9). In both *vps4* mutant strains, no mature α-factor material could be detected, either in the media (I) or in the pellet (E) fraction. This indicates that correct processing of α-factor precursor, rather than the transport of α-factor, is impaired, suggesting a late Golgi defect in *vps4* mutant strains.

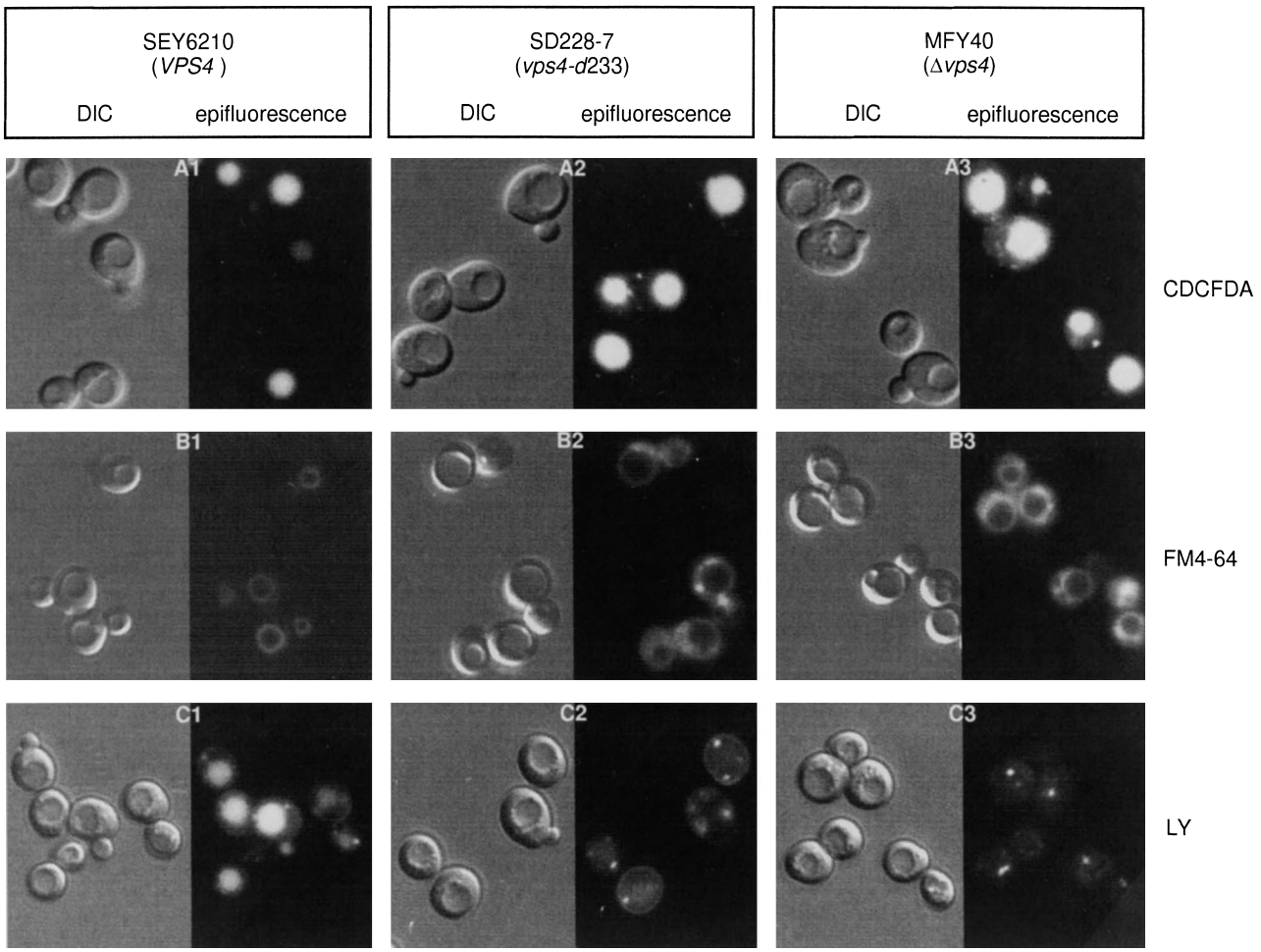


Fig. 4 Vacuolar morphology of wild-type and *vps4* mutant cells. All strains, SEY6210 (wild-type), SD228-7 (*vps4*-d233) and MFY40 (Δ*vps4*), were grown at 30 °C to a final OD_{600} = 1 and stained at 30 °C. Stained cells were viewed by Normarski optics (*DIC*) and epifluorescence (CDCFDA). CDCFDA staining: cells were grown in YPD and stained with the vacuole-specific dye CDCFDA for 20 min. FM4-64 staining: cells were grown in YPD and stained with the lipid-soluble dye FM4-64 for 60 min. LY staining: cells were grown in YPUAD and stained with the fluid-phase endocytosis marker LY in 2% glucose for 90 min. Subsequently, the cells were washed and incubated for an additional 60 min in 2% glucose

The *VPS4* gene is involved in endocytic transport to the yeast vacuole

Recessive *vps4* strains were classified by Raymond et al. (1992) as class E *vps* mutants accumulating a pre-vacuolar/endosomal compartment. As stated earlier, these pre-vacuolar/endosomal compartments found in *vps4* mutant cells can be stained with the vacuole-specific dye CDCFDA or the lipid-soluble dye FM4-64 (Fig. 4). After staining wild-type cells (SEY6210) with FM4-64 a typical ring-shaped vacuolar structure was observed (Fig. 4, B1). The *vps4* mutant strains SD228-7 and MFY40, however, exhibited a pronounced staining of small peri-vacuolar compartments when stained with FM4-64 (Fig. 4, B2 and B3). The vacuolar membrane was only weakly stained by FM4-64. Since FM4-64 is a marker for bulk membrane endocytosis (Vida and Emr 1995), the faint vacuolar FM4-64 staining suggested a defect in endocytosis. In addition, other class E *vps* mutants (*ren1*/*vps2*, *vps27*, and *vps28*) also exhibit defects in endocytosis (Davis et al. 1993; Piper et al. 1995; Rieder et al. 1996). Therefore, we asked whether the dominant-negative *vps4*-d233 allele or the Δ*vps4* allele also cause defects in endocytosis. When we incubated wild-type cells (SEY6210) with LY, a marker for fluid-phase endocytosis (Riezman 1985), we found an accumulation of LY in the vacuoles (Fig. 4, C1). No vacuolar staining with LY was observed in the *vps4* mutant strains SD228-7 and MFY40. Instead, LY accumulated in the small peri-vacuolar class E compartments (Fig. 4, C2 and C3). The complete lack of vacuolar LY staining in *vps4* mutant strains indicated a block in fluid-phase endocytosis, presumably between the pre-vacuolar/endosomal class E compartment and the vacuole. This is consistent with earlier findings by Munn and Riezman (1994), demonstrating that a recessive *vps4*/*end13* allele (*end13*-1) is not defective in α-factor uptake, but rather in the degradation of internalized α-factor in the vacuole.

Discussion

In a screen for dominant-negative vacuolar protein-sorting (*vps*) mutants we isolated four dominant alleles of the yeast *VPS4* gene. We cloned the four alleles and also the wild-type gene. We constructed a Δ*vps4* mutant strain and analyzed the phenotypic consequences of different *vps4* alleles. A previously isolated recessive *vps4* mutant strain was classified by its vacuolar morphology as a class E *vps* mutant (Raymond et al. 1992). Class E *vps* mutants in general are characterized by a novel vesicular structure next to the vacuole. In this compartment vacuolar, endocytic, and late Golgi markers accumulate (Raymond et al. 1992; Rieder et al. 1996; Cereghino et al. 1995; Piper et al. 1995; Davis et al. 1993). The class E compartment represents a normal functional Golgi-to-vacuole intermediate and not an aberrant dead-end compartment. This was shown by the analysis of a temperature-sensitive allele of another class E *vps* mutant, *vps27*. Upon reactivation of Vps27p function, endocytosed Ste3 receptor, trapped in the class E compartment, could be transported to the vacuole (Piper et al. 1995). The class E compartment, is therefore thought to represent an exaggerated pre-vacuolar endosomal compartment, where both biosynthetic and endocytic traffic converge (Raymond et al. 1992; Davis et al. 1993; Wilsbach and Payne 1993; Vida and Emr 1995; Piper et al. 1995; Rieder et al. 1996). Based on these observations, it is conceivable that a defect in protein transport out of the pre-vacuolar/endsosomal class E compartment would lead to defects in biosynthetic and endocytic protein-transport reactions. Our detailed analyses of a dominant *vps4* mutant (SD228-7) and a Δ*vps4* strain (MFY40) substantiate this view. We were able to demonstrate that both mutants exhibit a defect in fluid-phase endocytosis, as shown by a defect in the uptake of LY. The *vps4* mutant strains SD228-7 (*vps4*-d233) and MFY40 (Δ*vps4*) accumulated LY in the class E compartment, whereas in wild-type cells LY was transported to the vacuole. This defect in a late step in fluid-phase endocytosis is consistent with the observation of endocytosis defects in other class E *vps* mutants. For example, in addition to fluid-phase endocytosis defects, *vps27*, *vps28*, and *ren1*/*vps2* mutant cells exhibit defects in receptor-mediated endocytosis, as shown by the accumulation of endocytosed a-factor receptor (Ste13p) in the endosomal class E compartment (Davis et al. 1993; Piper et al. 1995; Rieder et al. 1996). Consistent with the Ste13p accumulation in a pre-vacuolar/endosomal class E compartment is the observation that a yeast *end13* mutant, harbouring a recessive allele of the *VPS4* gene, accumulated endocytosed α-factor in the class E compartment (Munn and Riezman 1994). This indicates that *vps4* mutants, similiar to other class E mutants, exhibit a block in a late step in fluid-phase and receptor-mediated endocytosis. With the lipophilic styryl dye FM4-64, which is a marker for bulk membrane endocytosis, we could stain the vacuolar membrane weakly and the pre-vacuolar class E compartment intensely in the dominant *vps4* mutant strain SD228-7 and the Δ*vps4* strain MFY40. Similar observations were made with the class E *vps* mutants *vps27* and Δ*vps28* (Vida and Emr 1995; Rieder et al. 1996). The predominant staining of the class E compartment with the lipophilic dye FM4-64 could be due to its multilammellar structure, as shown in Δ*vps28* mutant strains (Rieder et al. 1996).

As expected, disruption of the *VPS4* gene also resulted in a defect in the transport of soluble vacuolar hydrolases. Approximately 40% of the Golgi-modified p2CPY precursor and 5–10% of proteinase A precursor material is secreted in mutant cells. This moderate mislocalization defect of vacuolar hydrolases was also found in other class E mutants (*vps27* and *vps28*) (Piper et al. 1995; Rieder et al. 1996). The weak proteinase A processing defect could be explained by the observation that proteinase A most likely utilizes at least two mechanisms for vacuolar protein-sorting (Westphal et al. 1996). However, the amount of correctly processed mCPY found in pulse-chase experiments with *vps4* mutant cells is astonishing if one assumes a block in protein transport from the class E compartment to the vacuole. Analyses of other class E *vps* mutants suggested that the observed moderate CPY sorting defect is the consequence of a lack of CPY receptor activity (Vps10p) in late Golgi function, when the sorting of soluble vacuolar hydrolases takes place (Piper et al. 1995; Rieder et al. 1996). The CPY receptor, a late Golgi transmembrane protein, accumulates in the pre-vacuolar class E compartment in Δ*vps27* and *vps28* mutants and is rapidly degraded (Cereghino et al. 1995; Piper et al. 1995). Over-expression of Vps10p in the class E mutants *vps23*, *vps27*, and *vps28* suppressed the CPY sorting defect (Piper et al. 1995; Rieder et al. 1996). Since over-expression of the Vps10p in the dominant *vps4* mutant strain SD228-7 also partially suppressed the CPY sorting defect (data not shown), we suggest that, due to rapid degradation of Vps10p in *vps4* mutant cells, a lack of CPY receptor activity in the late Golgi complex leads to the mislocalization of Golgi-modified p2CPY to the cell surface. About 60% of p2CPY remains inside the cells and is correctly processed. Although it is difficult to prove, we believe that, due to a block in protein transport out of the class E compartment, the activation of CPY occurs within the class E compartment and not in the vacuole. Several lines of evidence suggest that the class E compartment represents a proteolytical active pre-vacuolar compartment. First, we and others have shown that the class E compartment is acidified due to its content of active V-ATPase, and therefore can be stained with the pH-dependent fluorescent dyes CDCFDA and quinacrine (Preston et al. 1989; Raymond et al. 1992). Second, the late Golgi membrane proteins Kex2p and Vps10p are degraded in the class E compartment of *vps28* cells in a proteinase A-dependent manner (Cereghino et al. 1995). Similar observations were made in Δ*vps27* mutant cells (Piper et al. 1995).

In addition to the mislocalization of soluble vacuolar hydrolases, the *vps4* mutant strains SD228-7 and MFY40 exhibited a severe defect in α-factor processing. Golgi-modified high-glycosylated and partially processed α-factor precursor is secreted in *vps4* mutant cells, whereas in wild-type cells α-factor is sequentially processed by late

Golgi-resident proteins (Kex2p, Kex1p, and Ste13p) (Fuller et al. 1988; Bryant and Boyd 1993) and secreted as mature α-factor. A simple explanation for the observed α-factor processing-defect in *vps4* mutant cells is a late Golgi defect, leading to reduced α-factor processing activities. As stated above, defects in late Golgi functions have been demonstated in several class E *vps* mutants. In the *vps4* mutant strains SD228-7 and MFY40 we found a reduced steady state level of the α-factor processing enzyme Kex2p (data not shown) and, in the class E *vps28* null-mutant strain, Kex2p has also been shown to be de-stabilized (Cereghino et al. 1995). The observed late Golgi defect of class E *vps* mutants obviously generates a pleiotropic phenotype. Similar to the vacuolar protein-sorting defect observed in class E *vps* mutants, where a reduced level of the CPY receptor (Vps10p) in the late Golgi compartment caused mislocalization of p2CPY, the reduced α-factor processing activity (Kex2p) in the late Golgi function caused secretion of partially processed α-factor precursors. But how can the Vps4p affect late Golgi function and a late step in endocytosis? We favour a model where Vps4p is necessary for the transport of proteins out of the pre-vacuolar/endosomal class E compartment, thereby affecting endocytosis, vacuolar protein transport, and retrograde protein transport from the class E compartment to the late Golgi system. The yeast Vps4p shows a 62% identity to the *M. musculus* Skd1 protein, indicating that *VPS4* could be the yeast homologue of *SKD1*. Vps4p is also similiar (31% identity) to the yeast Cdc48p that participates in the fusion of ER membranes (Latterich et al. 1995). Interestingly, the mouse *SKD1* gene was identified in a screen for multicopy supressors of the growth deficiency of potassium transport mutants in *S. cerevisiae* (Périer et al. 1994) together with the mouse *SKD2* gene, which is the mouse homologue of NSF/Sec18p (Périer et al. 1994). NSF/Sec18p is also a member of the AAA-family and shows 50% similiarity (25% identity) to Vps4p. For the yeast Sec18 protein it could be demonstrated that it participates in several vesicle-mediated protein transport reactions in the secretory pathway. It was shown that Sec18p is required sequentially for protein transport from the ER to the Golgi complex, through multiple Golgi compartments, and from the Golgi complex to the cell surface. For late-Golgi-to-vacuole transport, however, Sec18p is not necessary (Graham and Emr 1991). In vitro studies of vacuolar membrane fusion events demonstrated further that Sec18p is necessary for the initial priming step in homotypic vacuolar membrane fusion. Without Sec18p membrane fusion is blocked (Haas and Wickner 1996; Mayer et al. 1996). According to the SNAP/SNARE hypothesis of Rothman and co-workers, NSF/Sec18p is an essential component of the intracellular membrane fusion apparatus (Söllner et al. 1993; Rothman 1994). By analogy to Sec18p function, Vps4p could be necessary for protein targeting/transport out of the class E compartment, which would lead to the observed pleiotropic phenotype of the *vps4* mutants. Whether this block in protein transport from the class E compartment is due to a defect in an early priming step or a membrane fusion step is not yet clear.

Vps4p contains a single AAA module with a Walker-type A, also known as the P-loop, and a Walker-type B motif (Walker et al. 1982; Saraste et al. 1990). They are indicated as GTK and GFF motifs according to the BLOCKS database (Henikoff and Henikoff 1994) and are supposed to form putative ATP binding (GTK) and hydrolyzing (GFF) sites (Walker et al. 1982; Saraste et al. 1990). Our findings that single amino-acid exchanges at conserved residues in the AAA module of Vps4p (E233Q, E211 K, and G178D) caused severe protein transport defects, demonstrated the importance of the GTK and GFF motifs within the AAA module. Analysis of a mutant NSF allele, harbouring a mutation (E329Q) that is equivalent to the dominant mutation found in the *vps4*-d233 allele (E233Q), showed that this conserved amino-acid in the GFF motif is essential for the ATPase activity and normal function of NSF in membrane fusion (Whiteheart et al. 1994). Whiteheart et al. (1994) also found that the E329Q exchange in NSF caused a dominant-negative phenotype. NSF, which acts as a trimer, is inactivated if the trimer is assembled by two wild-type and one mutant NSF subunits. The functional importance of the conserved G178 residue in the P-loop region (GTK motif) of Vps4p is outlined by a point mutation in the P-loop motif of the RecA protein of *Escherichia coli*. A G71D exchange in the RecAp P-loop motif, which is equivalent to the Vps4p G178D exchange, leads to a loss of RecAp function (Logan and Knight 1993). The observed pleiotropic consequences of mutant Vps4 proteins demonstrated the importance and central role of the Vps4p in anteriograde and retrograde protein transport pathways out of a pre-vacuolar compartment where vacuolar protein transport and endocytotic pathways converge. However, detailed studies with purified wild-type and mutant Vps4 protein are necessary to unravel the biochemical and molecular function of the Vps4 protein.

Acknowledgements We thank Scott D. Emr for supplying *vps4* tester strains and antisera specific for PrA and a-factor. The invertase-specific antiserum was a generous gift from Ralf Kölling. The CPY-specific antiserum was generated by Otto Bodor. We would also like to thank Sybilla Müller for excellent technical assistence and John Williams for carefully reading the manuscript. This work was supported by a DFG grant to K. K.

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