Retromer function in endosome-to-Golgi retrograde transport is regulated by the yeast Vps34 PtdIns 3-kinase

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Summary

A direct role for phosphoinositides in vesicular trafficking has been demonstrated by the identification of the yeast *VPS34* gene encoding the phosphatidylinositol 3-kinase responsible for the synthesis of phosphatidylinositol 3-phosphate (PtdIns3*P*). Vps34p binds the protein kinase Vps15p, and it has recently been shown that Vps15p and Vps34p associate with Vps30p and Vps38p to form a multimeric complex, termed complex II. We observed that mutations in the *VPS30* and *VPS38* genes led to a selective sorting and maturation phenotype of the soluble vacuolar protease CPY. Localization studies revealed that the CPY receptor Vps10p and the Golgi-endoprotease Kex2p were mislocalized to vacuolar membranes in strains deficient for either Vps30p or Vps38p, respectively. Interestingly, we measured decreased PtdIns3*P* levels in Δ*vps30* and Δ*vps38* cells and observed redistribution of Vps5p and Vps17p to the cytoplasm in these mutants. Vps5p and Vps17p are subunits of the retromer complex that is required for endosome-to-Golgi retrograde transport. Both proteins contain the Phox homology (PX) domain, a recently identified phosphoinositide-binding motif. We demonstrate that the PX domains of Vps5p and Vps17p specifically bind to PtdIns3*P* in vitro and in vivo. On the basis of these and other observations, we propose that the PtdIns 3-kinase complex II directs the synthesis of a specific endosomal pool of PtdIns3*P*, which is required for recruitment/activation of the retromer complex, thereby ensuring efficient endosome-to-Golgi retrograde transport.

Key words: Vesicular transport, Endosome-to-Golgi retrograde transport, PX domain, Phosphatidylinositol 3-phosphate, *Saccharomyces cerevisiae*

Introduction

Phosphorylated forms of phosphatidylinositol (PtdIns) serve as second messengers in signal transduction, cell proliferation, cytoskeleton organization and in the regulation of membrane trafficking (Fruman et al., 1998; Corvera et al., 1999; Huijbregts et al., 2000; Simonsen et al., 2001). In *Saccharomyces cerevisiae*, evidence for the importance of phosphoinositides in vesicular trafficking was established by the demonstration that the Vps34 phosphatidylinositol 3-phosphate kinase (PtdIns 3-kinase) is required for efficient sorting of proteins from the late-Golgi to the vacuole (Schu et al., 1993). Vps34p is found in a complex with the Vps15 serine/threonine protein kinase, which recruits Vps34p to cellular membranes and activates the PtdIns 3-kinase (Stack et al., 1993; Stack et al., 1995).

Studies from both yeast and mammalian systems have demonstrated that phosphatidylinositol 3-phosphate (PtdIns3*P*) is specifically recognized by a set of proteins containing the FYVE domain. These proteins act as downstream effectors in the regulation of membrane trafficking (Corvera, 2000; Odorizzi et al., 2000; Gillooly et al., 2001). A novel phosphoinositide-binding motif, the Phox homology (PX) domain, has recently been characterized, which also targets proteins to intracellular membranes by interacting with specific phosphoinositides (Sato et al., 2001; Simonsen and Stenmark, 2001; Wishart et al., 2001; Xu et al., 2001; Yu and Lemmon, 2001). The PX domain was first identified in two subunits of the phagocyte NADPH oxidase complex (Ponting, 1996) and contains two conserved basic motifs. More than 100 PX-containing proteins have now been identified by sequence homology. In contrast to mammalian PX domain proteins, which are able to recognize various phosphoinositides, the PX domain proteins of *S. cerevisiae*, which have been characterized by protein-lipid overlay assays, specifically bind PtdIns3*P* (Yu and Lemmon, 2001).

A significant number of the PX domain proteins are localized to membranes or vesicular structures required for vesicular transport between intracellular organelles. In *S. cerevisiae*, vacuolar recruitment of Vam7p, a homologue of the mammalian t-SNARE SNAP-25, has been shown to be modulated via specific interaction between the Vam7p-PX domain and PtdIns3*P* (Cheever et al., 2001).

Vps5p and Vp17p are PX domain proteins that function as subunits of a multimeric complex including Vps26p, Vps29p and Vps35p. This complex, dubbed the retromer, is proposed to form a membrane coat required for cargo retrieval from endosomes to the late-Golgi (Horazdovsky et al., 1997; Kohrer and Emr, 1993; Seaman et al., 1997; Seaman et al., 1998; Nothwehr et al., 1999; Reddy and Seaman, 2001). The cargo molecules in this pathway include the carboxypeptidase Y (CPY) receptor Vps10p (Marcusson et al., 1994; Cereghino et al., 1995; Cooper and Stevens, 1996) and the late-Golgi

Table 1. Strains used in this study

Strain	Genotype	Source
SEY6210	*MATα leu2-3,112 ura3-52 his3-Δ20 trp1-Δ901 lys2-801 suc2-Δ*	Robinson et al., 1988
JCY300	SEY6210; *vps30Δ::HIS3*	Seaman et al., 1997
PBY58	SEY6210; *vps38Δ::HIS3*	This study
PBY51	SEY6210; *vps30Δ::HIS3*, *vps38Δ::HIS3*	This study
EMY18	SEY6210; *vps35Δ::HIS3*	Seaman et al., 1997
BHY152	SEY6210; *vps5Δ::HIS3*	Horazdovsky et al., 1997
KKY11	BHY11; *vps17Δ::HIS3*	Kohrer and Emr, 1993
PBY142	SEY6210; *vps5Δ::HIS3*, *vps17Δ::HIS3*	This study
TVY1	SEY6210; *pep4Δ::LEU2*	Emr Lab Strain Collection
PBY139	SEY6210; *pep4Δ::LEU2*, *vps30Δ::HIS3*	This study
PBY140	SEY6210; *pep4Δ::LEU2*, *vps38Δ::HIS3*	This study
EMY30	SEY6210; *pep4Δ::LEU2*, *vps35Δ::HIS3*	Emr Lab Strain Collection
PHY102	SEY6210; *vps34Δ::TRP1*	Herman and Emr, 1990
vps34^{tsf}	SEY6210; *vps34Δ::TRP1*; *pRS416.vps34^{tsf}*	Stack et al., 1995
PBY34	SEY6210; *VPS10-GFP:TRP1*	This study
PBY35	SEY6210; *KEX2-GFP:TRP1*	This study
PBY70	SEY6210; *VPS17-GFP:TRP1*	This study
PBY82	SEY6210; *KEX2-HA:TRP1*	This study
PBY76	SEY6210; *VPS10-HA:TRP1*	This study
PBY86	SEY6210; *VPS5-GFP:TRP1*	This study
PBY38	SEY6210; *VPS10-GFP:TRP1*, Δ*vps30*	This study
PBY40	SEY6210; *VPS10-GFP:TRP1*, Δ*vps38*	This study
PBY39	SEY6210; *KEX2-GFP:TRP1* Δ*vps30*	This study
PBY30	SEY6210; *KEX2-GFP:TRP1* Δ*vps38*	This study
PBY77	SEY6210; *VPS10-HA:TRP1* Δ*vps38*	This study
PBY124	SEY6210; *Vps10-GFP:TRP1*, *vps34^{tsf}*	This study
PBY123	SEY6210; *Vps17-GFP:TRP1*, *vps34^{tsf}*	This study
PBY88	SEY6210; *Vps5-GFP:TRP1*, Δ*vps34::TRP1*	This study
PBY87	SEY6210; *Vps5-GFP:TRP1*, Δ*vps38::HIS3*	This study
PBY111	SEY6210; *Vps17-GFP:TRP1*, Δ*vps34::TRP1*	This study
PBY112	SEY6210; *Vps17-GFP:TRP1*, Δ*vps38::HIS3*	This study
PBY142	SEY6210; *Vps10-GFP*, Δ*vps5::HIS3*	This study
PBY116	SEY6210; *Vps17-GFP:TRP1*, Δ*fab1::HIS3*	This study
PBY117	SEY6210; *Vps17-GFP*, Δ*mss4::HIS3*; *pYCplacIII.mss4-102*	This study
PBY115	SEY6210; *Vps17-GFP:TRP1*, Δ*stt44::HIS3*; *pRS415.stt4-4*	This study
PBY114	SEY6210; *Vps17-GFP:TRP1*, Δ*pik1::HIS3*; pRS413.pik1-83	This study

endoprotease Kex2p (Wilcox et al., 1992; Voos and Stevens, 1998). Analysis of mutant strains defective for retromer complex function revealed that these cells are unable to recycle Vps10p back to the Golgi. As a consequence, Vps10p is mislocalized to the vacuolar membrane and CPY is secreted in its Golgi-modified p2 precursor form.

Interestingly, phenotypic analysis of *vps30* mutant cells revealed similar phenotypes to those observed in mutants of the retromer complex (Seaman et al., 1997). However, Vps30p could not be identified as part of the retromer complex (Seaman et al., 1998). Recently, Kihara and co-workers demonstrated that Vps30p assembles together with the protein kinase Vps15p, the PtdIns 3-kinase Vps34p and Vps38p to form a complex that is required for maturation of CPY (Kihara et al., 2001). In addition, they characterized a second Vps34p kinase complex that functions in autophagy. These two distinct Vps34p kinase subcomplexes, named complex I and II, differ from each other by only one subunit: Vps38p, is a subunit of complex II, whereas Apg14p is unique for complex I. The *VPS30* gene is allelic to *APG6* (Kametaka et al., 1998) and functions in the autophagy pathway when associated with the complex I-specific subunit Apg14p, or in the CPY pathway together with the unique complex II subunit Vps38p. Since synthesis of PtdIns3*P* in yeast is mediated by the sole PtdIns 3-kinase Vps34p, it suggests that distinct pools of PtdIns3*P* are synthesized for distinct cellular functions.

Based on the observations described above, we investigated whether the PtdIns 3-kinase complex II might influence endosome-to-Golgi retrograde transport. In this study, we show that *vps30* and *vps38* mutant strains are impaired in recycling of cargo molecules from the endosome to the late-Golgi. In addition, PtdIns3*P* levels in *vps30* and *vps38* mutant cells are about threefold lower compared with wild-type cells. We further show that the retromer subunits Vps5p and Vps17p are redistributed to the cytoplasm in *vps30* and *vps38* mutants, indicating a role for PtdIns3*P* in retrograde transport. Taken together, we suggest that complex II, which consists of Vps15p, Vps34p, Vps30p and Vps38p, produces a specific pool of PtdIns3*P* that is needed for recruitment/assembly of the retromer complex, which in turn is required for endosome-to-Golgi retrograde transport.

Materials and Methods

Strains and media

Unless otherwise specified, the *S. cerevisiae* strains used in this study were grown in standard yeast extract-peptone-dextrose (YPD) or synthetic medium (YNB) with the necessary auxotrophic supplements. *S. cerevisiae* strains used in this study are summarized in Table 1. Primers to construct part of the strains listed, are available upon request. Standard LB medium supplemented with 100 μg/ml ampicillin was used to grow *E. coli* strains and to maintain plasmids.

DNA manipulation and plasmids

Restriction enzymes and DNA-modifying enzymes were purchased from Roche (Indianapolis, IN) and Life Technologies Gibco BRL (Gaithersburg, MD). Otherwise standard molecular biology techniques were used (Maniatis et al., 1982). Yeast transformations were performed using the lithium acetate method (Ito et al., 1983) and genomic DNA was isolated as described (Hoffman and Winston, 1987). The *KEX2-HA* gene was cloned by PCR amplification of genomic DNA derived from strain PBY82, with primers containing the 5′ *Spe*I and 3′ *Xho*I sites approximately 500 base pairs upstream and 200 base pairs downstream of the *KEX2-HA* open reading frame. The *KEX2-HA* ORF was then inserted into the *Spe*I-*Xho*I poly-linker site of the pRS416 vector resulting in pPB1. The *Vps5-GFP* gene was cloned by PCR amplification of genomic DNA derived from strain PBY86 with primers containing the 5′ *Sac*II and 3′ *Xho*I sites approximately 500 base pairs upstream and 200 base pairs downstream of the *VPS5-GFP* open reading frame resulting in pPB2. The plasmids pPB3 (*vps5*^{Y322A,R360A}-GFP; pRS416) and pPB4 (*vps5*^{Y322A,R360A}; pRS416) were constructed by PCR-based site-directed mutagenesis. pPB2 was linearized with *BstXI* and was co-transformed with the mutagenized PCR product into strain BHY152 (Δ*vps5*). The recombined plasmid was rescued from the yeast strain and amplified in *E. coli*. The mutation was subsequently confirmed by DNA sequencing. The construction of Vam7-GFP (pTKS35) and GFP-CPS (pGO45) have been described (Odorizzi et al., 1998; Sato et al., 1998).

Pulse-labeling and protein immunoprecipitation

Protein transport assays and immunoprecipitations were carried out as described (Audhya et al., 2000).

Subcellular fractionation and western blot analysis

Subcellular fractionation and immunoblot analyses were performed as previously described (Gaynor et al., 1994; Babst et al., 1998). Monoclonal antibody against the HA or the myc epitope (Boehringer Mannheim Biochemicals) was used at a 1:1500 dilution.

Protein purification and protein-lipid overlay assay

A DNA fragment encoding amino acids 268-406 of Vps5p (which corresponds to the PX domain) was amplified by PCR using primers that added 5′ *Nco*I and 3′ *Xho*I sites. This fragment was inserted into the pGEX-KG vector, and the resulting plasmid was expressed in *E. coli*. The protein was purified with Glutathione Sepharose 4B beads, eluted with 5 mM reduced glutathione, and used immediately after purification. To construct a GST-Vps17PX domain fusion protein, a DNA fragment encoding amino acids 95-238 of Vps17p was amplified by PCR using primers that added 5′ *Xma*I and 3′ *Xho*I sites. The fragment was processed as described above. The construction of a GST-Vam7PX domain fusion protein has been described (Cheever et al., 2001). To construct the GST-Vps5PX domain fusion protein carrying the point mutations Y322A and R360A, DNA from plasmid PB4 encoding amino acids 268-406 of Vps5p was amplified by PCR using primers that added 5′ *Nco*I and 3′ *Xho*I sites. The fragment was then cloned into pGEX-KG vector as described, resulting in pGEX-Vps5PX^{Y322A,R360A}. The GST fusion proteins were then subjected to protein-lipid overlay assays as described (Cheever et al., 2001; Dowler et al., 2002). The lipid-strips were purchased from Echelon.

In vivo analysis of phosphoinositides

Analysis of phosphoinositide levels was carried out as described previously (Audhya et al., 2000; Foti et al., 2001). Briefly, cells were grown in synthetic medium with the appropriate amino acids. Five OD_{600} units of cells from a log-phase culture were harvested, washed and resuspended in inositol-free synthetic medium. Cells were then shifted to the appropriate temperature for 10 minutes, followed by the addition of 50 μCi of *myo*-[2-^{3}H]inositol (Nycomed Amersham), and labeled for 45 minutes. Next, the cells were lysed by mechanical agitation with glass beads in 4.5% perchloric acid to generate extracts. Further processing of extracts is described (Stack et al., 1993). Analysis of ^{3}H-labeled glycerol-phosphoinositols was performed by separation using HPLC (column #4611-1505, Whatman, Clifton, NJ) on a Beckman System Gold HPLC and quantified by liquid scintillation counting by a Packard online radiomatic detector.

FM4-64-labeling of vacuoles and endosomes and fluorescence microscopy

To examine vacuolar structures in vivo, FM4-64 (Molecular Probes, Eugene, OR) labeling was carried out as previously described (Vida and Emr, 1995). The labeling was carried out at a concentration of 16 μM FM4-64 at 30°C for 15 minutes and the cells were chased for a period of 1 hour. To stain endosomal structures, FM4-64 was diluted in YPD to a concentration of 3.2 nM and the labeling procedure was performed as described (Shin et al., 2001). GFP-CPS fluorescent images were collected on a fluorescent microscope equipped with a FITC filter and acquired using a CCD camera (model4995; COHU), an integrator box (model 440A; Colorado Video Inc.) and an LG-3 Frame Grabber. All other fluorescent images were acquired using a Ziess Axiovert S1002TV inverted fluorescent microscope and subsequently processed using a Delta Vision deconvolution system (Applied Precision, Seattle, WA). The software used was Adobe PhotoShop 6.0.

Results

vps30 and *vps38* mutant strains are defective for maturation of the vacuolar protein CPY but not ALP or CPS

In the late-Golgi, vacuolar proteins are sorted away from the secretory pathway and are directed to the vacuole via two distinct transport pathways. One pathway traverses a prevacuolar/late endosome compartment to reach the vacuole. This pathway is referred to as the CPY pathway, as mutants in this pathway are defective for maturation of the vacuolar protease carboxypeptidase Y (CPY) (Bankaitis et al., 1986; Robinson et al., 1988; Rothman et al., 1989). The other route, the alkaline phosphatase (ALP) pathway, bypasses the endosome and was identified by the isolation of mutants harboring maturation defects for ALP (Cowles et al., 1997; Piper et al., 1997).

VPS30 and *VPS38* are two previously characterized genes which, when mutated, affect transport of CPY from the Golgi to the vacuole (Luo and Chang, 1997; Seaman et al., 1997; Kihara et al., 2001). Since CPY precursors were often degraded when analyzed by western blot techniques, we monitored processing of CPY by pulse-chase immunoprecipitation. As shown in Fig. 1A, CPY molecules were mostly detected in the Golgi-modified p2 form, which is in agreement with previously published data. In addition, the Δ*vps30*Δ*vps38* double mutant strain exhibited a similar sorting phenotype compared with each single mutant, suggesting that Vps30p and Vps38p act along a common pathway.

To test whether Δ*vps30* and Δ*vps38* mutant cells exhibit defects in the transport of other vacuolar hydrolases that traverse the CPY and ALP pathways, respectively, we examined the delivery of carboxypeptidase S (CPS) and ALP.

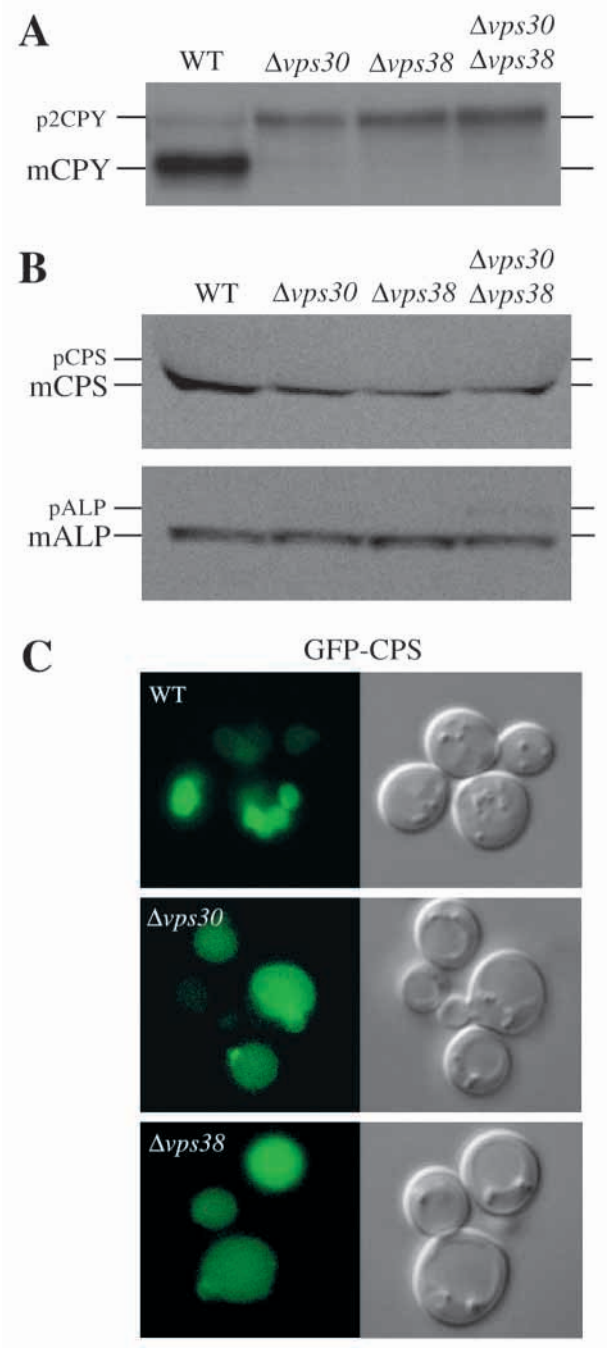

Fig. 1. Vacuolar sorting of ALP and CPS is not affected in Δ*vps30* and Δ*vps38* mutant cells. (A) Wild-type (SEY6210), Δ*vps30* (JCY300), Δ*vps38* (PBY58) and Δ*vps30*Δ*vps38* (PBY51) strains were grown in YNB medium to early log phase and metabolically labeled with [^{35}S] methionine/cysteine at 26°C for 10 minutes and then chased in the presence of excess non-labeled amino acids for 30 minutes. The proteins were immunoprecipitated with antibodies specific to CPY, resolved on SDS-PAGE and visualized by autoradiography. The migration positions of Golgi-modified precursor (p2) and mature (m) CPY are shown. (B) Cells were grown in YPD medium to early log phase. Total cell lysates were subjected to SDS-PAGE, followed by immunoblotting with anti-CPS and anti-ALP antibodies, respectively. CPS samples were treated with endoglycosidase H prior to electrophoresis. Precursor forms of CPS and ALP are indicated (p; 73 kDa and 76 kDa, respectively) and mature forms (m; 69 kDa and 72 kDa, respectively) are shown. (C) Fluorescence and DIC microscopy of cells expressing GFP-CPS (pGO45).

Whole cell lysates from Δ*vps30* and Δ*vps38* mutants were generated, and maturation of CPS and ALP was analyzed using western blot techniques. CPS exists in two glycoforms, which can be separated by SDS-PAGE (data not shown). To exclude co-migration of different CPS-glycoforms, protein lysates were treated with endoglycosidase H, resulting in deglycosylated CPS molecules. In contrast to the CPY maturation defects, we observed normal ALP and CPS processing in both Δ*vps30* and Δ*vps38* cells (Fig. 1B). A minor amount of the unprocessed Golgi-form of ALP (pALP) could be detected in Δ*vps30* Δ*vps38* double-mutant cells.

We also analyzed the processing of CPS and ALP in Δ*vps30* and Δ*vps38* mutants by pulse-chase immunoprecipitation and observed a slight kinetic delay in CPS processing (data not shown) compared with the processing under steady-state conditions (Fig. 1B). ALP processing remained unaffected when analyzed by pulse-chase immunoprecipitation (data not shown). CPS is a biosynthetic cargo molecule that requires a functional multivesicular body (MVB) sorting pathway for its delivery to the vacuolar lumen (Odorizzi et al., 1998). To exclude a defect in the MVB pathway, Δ*vps30* and Δ*vps38* cells were transformed with a plasmid coding for CPS protein fused to green fluorescent protein (GFP). In wild-type cells, GFP-CPS is delivered to the lumen of the vacuole (Fig. 1C). Normal delivery of GFP-CPS to the vacuole was observed in Δ*vps30* and Δ*vps38* mutant cells, indicating a functional MVB pathway (Fig. 1C). Together, these data show a role for Vps30p and Vps38p in vacuolar trafficking of CPY, but not for the vacuolar hydrolases CPS and ALP.

Vp10p and Kex2p are mislocalized to the vacuolar membrane in *vps30* and *vps38* mutant cells

Aberrant sorting of CPY to the vacuole can be caused by either a defect in Golgi-to-vacuole transport or deficient recycling of the CPY receptor Vps10p. The function of Vps10p is to deliver CPY to endosomes, where the receptor releases its cargo before cycling back to the trans-Golgi for further rounds of sorting (Horazdovsky et al., 1997; Nothwehr and Hindes, 1997; Seaman et al., 1997; Seaman et al., 1998; Nothwehr et al., 1999; Reddy and Seaman, 2001).

Since Δ*vps30* and Δ*vps38* mutants showed a vacuolar sorting defect specific for CPY, we wanted to investigate whether Vps10p localization is altered in these mutant strains. To monitor the cellular localization of Vps10p, a chromosomal copy of the *VPS10* locus was tagged with GFP at the 3′-end. The resulting fusion protein appeared to be functional, as no CPY sorting defects were observed (data not shown). In wild-type cells, Vps10-GFP was localized to punctate structures typical of the Golgi and/or endosomes (Fig. 2, upper panel). In Δ*vps38* mutant cells, however, Vps10-GFP was redistributed to the vacuolar membrane in a fashion also observed for Δ*vps30* mutant cells (Fig. 2, middle and lower panel). To visualize vacuolar membranes, cells were incubated with FM4-64, a lipophilic fluorescent dye. FM4-64 incorporates into the plasma membrane, is then transported

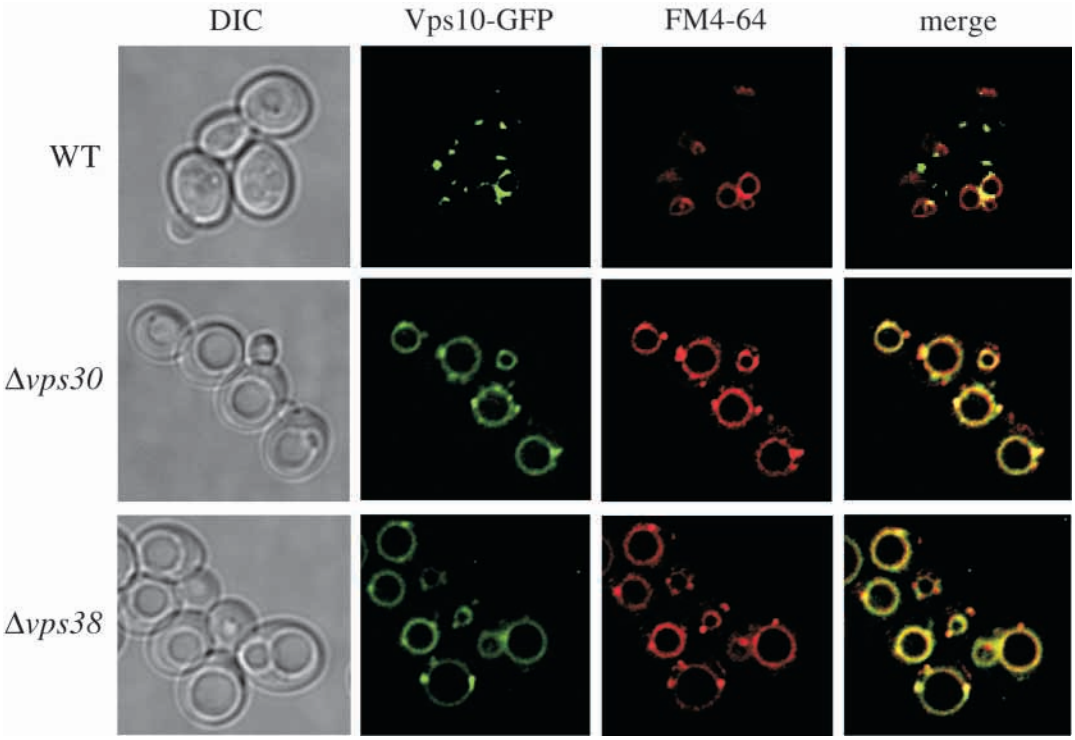

Fig. 2. The CPY sorting receptor Vps10p is mislocalized to the vacuolar membrane in Δ*vps30* and Δ*vps38* mutant strains. Wild-type (PBY34), Δ*vps30* (PBY38) and Δ*vps38* (PBY40) cells expressing Vps10-GFP were labeled in YPD with the fluorescent dye FM4-64 for 10 minutes at room temperature. The dye was washed away, the cells were chased for an additional 60 minutes in YPD and then put on ice. Localization of FM4-64 and Vps10-GFP were compared by fluorescence microscopy.

into cells via the endocytic pathway and ultimately accumulates at vacuolar membranes (Vida and Emr, 1995). In Δ*vps30* and Δ*vps38* strains, co-localization of Vps10-GFP with FM4-64-stained vacuoles was observed, demonstrating that Vps10-GFP is mislocalized to the limiting membrane of the vacuole (Fig. 2).

To confirm the defect of Vps10p localization in Δ*vps30* and Δ*vps38* cells, we investigated the subcellular distribution of Vps10p by cell fractionation. For this purpose, Vps10p was tagged with hemagglutinin (HA) at its C-terminus and introduced into wild-type, Δ*vps30* and Δ*vps38* cells. Total cell lysates were separated by sequential centrifugation into low and high speed membrane pellets (P13, P100, respectively) and the soluble/cytosolic (S100) fraction. The fractions were then analyzed by immunoblotting using anti-HA-specific antibodies. In wild-type cells, Vps10-HA was found almost exclusively in the P100 fraction enriched for Golgi and endosomal markers (Table 2). In Δ*vps38* cells, however, redistribution of Vps10-HA to the P13 pool was observed. This pelletable pool is enriched for proteins residing in the endoplasmic reticulum, plasma membrane and vacuoles (ALP was used as a P13 marker protein; see Table 2). Glucose-6-phosphate-dehydrogenase (G6PDH), a cytosolic protein, served as a marker for the S100 fraction (Table 2). A similar shift of Vps10p from the P100 to the P13 pool was previously reported for Δ*vps30* mutant cells (Seaman et al., 1997). Together, these data show that, in Δ*vps30* and Δ*vps38* mutants, a major fraction of Vps10p is redistributed to the vacuolar membrane leading to the selective CPY missorting phenotype observed in these mutant cells.

Kex2p is an endoprotease responsible for processing of α-factor and M1 killer toxin precursors in the late-Golgi (Gluschankof and Fuller, 1994). Like Vps10p, Kex2p cycles between the Golgi and endosomes (Wilcox et al., 1992; Voos and Stevens, 1998). We fused GFP to the cytosolic tail of Kex2p and the fusion protein allowed processing of pro-α-factor indicating a functional fusion (data not shown). We found that, in Δ*vps30* and Δ*vps38* mutant strains, Kex2-GFP was redistributed to the vacuolar membrane in a very similar manner to that observed for Vps10-GFP in these strains (data not shown). Previous studies revealed that, in mutant cells deficient for endosome-to-Golgi retrograde transport, Kex2p is rapidly degraded in the vacuole (Nothwehr and Hindes, 1997). To investigate the stability of Kex2p in Δ*vps30* and Δ*vps38* strains, a single copy plasmid carrying *KEX2-HA* gene was constructed and transformed into the mutant cells. Kex2-HA protein stability was subsequently assessed by pulse-chase immunoprecipitations using anti-HA specific antibodies. By following the kinetics of Kex2-HA, we observed that Kex2-HA

Table 2. Subcellular distribution of Vps10-HA in Δ*vps38* mutant strains

Strain	Protein	%P13	%P100	%S100
WT	Vps10-HA	10	90	–
Δ*vps38*	Vps10-HA	75	25	–
WT	ALP (vacuole)	90	10	–
WT	G6PDH (cytosol)	–	10	90

ALP, alkaline phosphatase; G6PDH, glucose-6-phosphate-dehydrogenase.

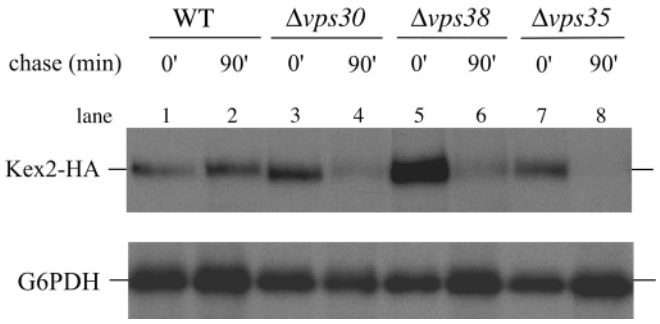

Fig. 3. Vps30 and Vps38 protein functions are required for stability of the Golgi-localized Kex2 protease. Wild-type (SEY6210), Δ*vps30* (JCY300), Δ*vps38* (PBY58) and Δ*vps35* (EMY18) cells carrying plasmid pPB1 (*KEX2-HA*) were grown to early log phase and labeled with [^{35}S] methionine/cysteine at 26°C for 20 minutes followed by the addition of excess non-labeled amino acids (chase). Samples were removed at 0 and 90 minutes after the addition of chase. Kex2-HA (upper panel) was immunoprecipitated from cell lysates using HA-specific antibodies. The same cell lysates were then re-immunoprecipitated with G6PDH-specific antibodies (lower pannel). Kex2-HA and G6PDH were resolved by SDS-PAGE and visualized by autoradiography.

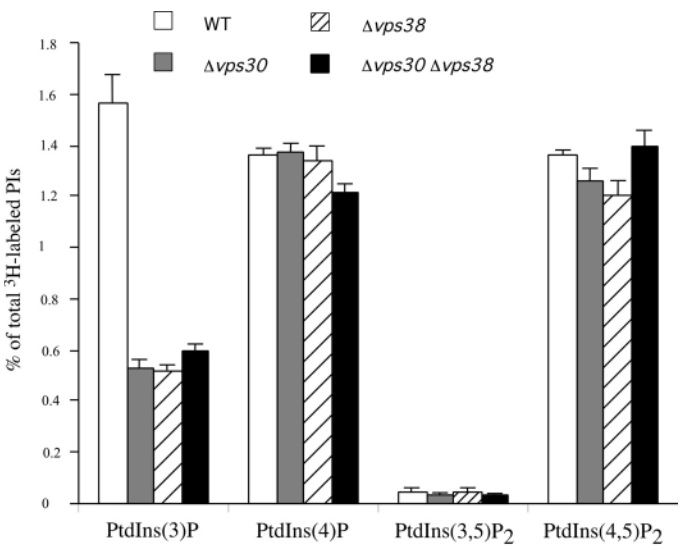

Fig. 4. Δ*vps30* and Δ*vps38* mutant cells exhibit defects in PtdIns3*P* synthesis. Wild-type (SEY6210), Δ*vps30* (JCY300), Δ*vps38* (PBY58), and Δ*vps30*Δ*vps38* (PBY51) cells were grown in YNB to early log phase and labeled with *myo*-[2-^{3}H] inositol at 26°C for 45 minutes. Cellular lipids were recovered, deacylated and separated by HPLC. Levels of deacylated products corresponding to the indicated phosphoinositides are shown. These data represent the means±s.e.m. of at least three independent experiments. PtdIns3*P*, phosphatidylinositol 3-phosphate; PtdIns(4)P, phosphatidylinositol 4-phosphate; PtdIns(3,5)P_2, phosphatidylinositol (3,5)-bisphosphate; PtdIns(4,5)P_2, phosphatidylinositol (4,5)-bisphosphate.

stability was significantly decreased in Δ*vps30* and Δ*vps38* mutant cells (Fig. 3, lanes 4,6), compared with wild-type cells (Fig. 3, lane 2). Kex2-HA degradation was also observed in Δ*vps35* cells deficient in retrograde transport (Fig. 3, lane 8). The cytosolic protein G6PDH remained stable in all mutant cells, indicating that the instability of Kex2p was specific (Fig. 3, lower panel). Further studies revealed that Kex2-HA degradation in Δ*vps30* and Δ*vps38* mutant cells was dependent upon the *PEP4* gene product protease A, a vacuolar proteinase (data not shown). These results demonstrate that Kex2-HA fails to be recycled to the late-Golgi in Δ*vps30* and Δ*vps38* mutants and is instead degraded by vacuolar proteases. In summary, our data strongly suggest that Vps30p and Vps38p are essential for efficient endosome-to-Golgi retrograde transport.

Δ*vps30* and Δ*vps38* cells exhibit defects in PtdIns3*P* synthesis

Previous biochemical studies revealed that the Vps15 protein kinase activity is required for both the stable interaction between Vps15p and Vps34p and the activation of the PtdIns 3-kinase activity of Vps34p (Stack et al., 1993; Stack et al., 1995). Similarly, Vps30p and Vps38p, which form a complex with Vps15p and Vps34p, might regulate PtdIns3*P* synthesis. Thus, we addressed whether Vps30p and Vps38p act as regulators of the Vps15p/Vps34p kinase complex.

To investigate this possibility, we analyzed intracellular PtdIns3*P* levels in Δ*vps30* and Δ*vps38* mutant strains. For this purpose, phosphoinositides were labeled in vivo with [^{3}H]myoinositol, extracted and analyzed using HPLC. We found that in Δ*vps30* and Δ*vps38* cells levels of PtdIns3*P* were significantly decreased (approximately threefold) compared with wild-type cells (Fig. 4). In addition, Δ*vps30*Δ*vps38* double-mutant cells showed no further decrease in PtdIns3*P* compared with each single mutant, again suggesting that Vps30p and Vps38p function together at a common step.

Diminished PtdIns3*P* levels in Δ*vps30* and Δ*vps38* cells specifically affect localization of the retromer subunits Vps5p and Vps17p

Even though Vps30p and Vps38p are not part of the retromer complex, our data clearly show recycling defects of Vps10p and Kex2p in Δ*vps30* and Δ*vps38* mutant cells, mimicking phenotypes observed in retromer mutants. Interestingly, two of the retromer subunits, Vps5p and Vps17p, harbor the PX domain, a PtdIns-binding motif (Sato et al., 2001; Wishart et al., 2001; Yu and Lemmon, 2001). Based on the observation that Δ*vps30* and Δ*vps38* mutant cells showed decreased PtdIns3*P* levels, we wanted to determine whether membrane recruitment of Vps5p and Vps17p may be altered in these mutants.

To monitor the in vivo localization of Vps5p and Vps17p, the proteins were chromosomally tagged at their C-terminus with GFP. Both Vps5- and Vps17-GFP were functional in that CPY transport was not impaired in these strains (data not shown). Using fluorescence microscopy, the fusion proteins were detected as punctate structures (Fig. 5A). To show that the punctate structures corresponded to endosomes, cells harboring the GFP fusion proteins were incubated briefly with FM4-64 dye. The reaction was stopped before the dye was delivered to the vacuolar membrane, thereby allowing visualization of prevacuolar endosomes (Shin et al., 2001). We observed overlapping signals between Vps5/Vps17-GFP and

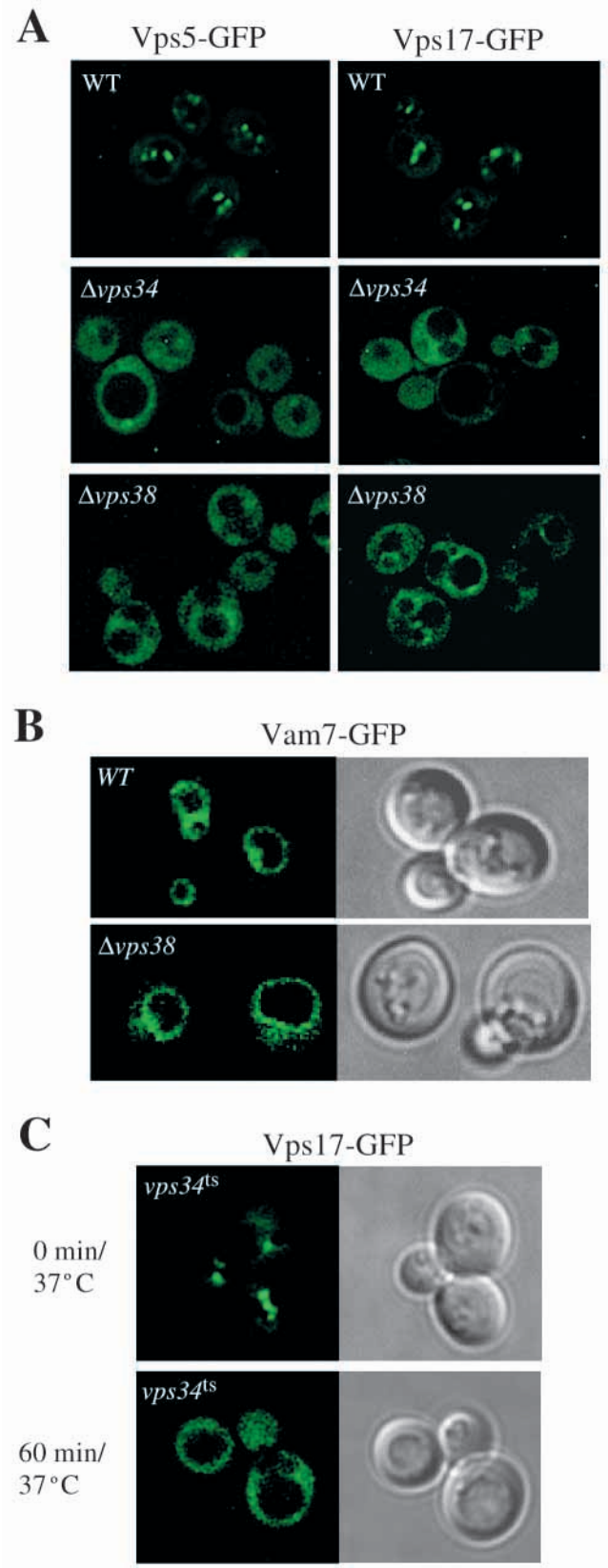

Fig. 5. Recruitment of Vps5p and Vps17p to the endosomal membrane depends on the synthesis of PtdIns3*P* by the Vps34p PtdIns 3-kinase complex II. (A) Localization of Vps5-GFP in wild-type (PBY86), Δ*vps34* (PBY88) and Δ*vps38* (PBY87) cells (left panel) and Vps17-GFP in wild-type (PBY70), Δ*vps34* (PBY111) and Δ*vps38* (PBY112) cells (right panel) by fluorescence microscopy. (B) Fluorescence and DIC microscopy of Vam7-GFP (pTKS35) expressed in wild-type (SEY6210) and Δ*vps38* (PBY58) cells, respectively. (C) Fluorescence and DIC microscopy of *vps34*^{ts} cells expressing Vps17-GFP (PBY123). The cells were incubated at non-permissive temperature (37°C) as indicated.

FM4-64 and concluded that Vps5p and Vps17p are recruited to prevacuolar endosomes (data not shown).

We subsequently tested whether PtdIns3*P* levels would affect recruitment of Vps5p and Vps17p to the endosomes. Both Vps5-and Vps17-GFP were expressed in Δ*vps34* mutant cells. Neither Vps5-GFP nor Vps17-GFP was associated with endosomal membranes, but were instead distributed in the cytoplasm (Fig. 5A). Importantly, a similar distribution of Vps5- and Vps17-GFP was observed in Δ*vps38* cells (Fig. 5A). This is in agreement with the observation that PtdIns3*P* levels are reduced in these cells.

We also investigated the localization of Vam7-GFP in Δ*vps38* mutant strains. Vam7p, the vacuolar t-SNARE has previously been reported to be targeted to vacuolar membranes in a PtdIns3*P*-dependent manner (Cheever et al., 2001). In wild-type cells, Vam7-GFP localized primarily to the vacuolar membrane (Fig. 5B). Interestingly, we found a very similar distribution of Vam7-GFP localization in Δ*vps38* strains (Fig. 5B). Since Vps38p is the component unique for complex II, and is necessary for retrograde transport, we reasoned that it might specify the site of synthesis of the PtdIns3*P* pool used in this process. Therefore, these observations suggest that the PtdIns3*P* pool synthesized by complex II is specific for retrograde transport.

To confirm that redistribution of Vps5p and Vps17p to the cytosol was primarily due to the loss of PtdIns3*P* synthesis, we expressed Vps17-GFP in a temperature-conditional *vps34*^{tsf} mutant strain (Stack et al., 1995). At the nonpermissive-temperature of 37°C, PtdIns3*P* synthesis is blocked in this strain. At permissive temperature (26°C), we observed endosomal localization of Vps17-GFP (Fig. 5C, upper panel). However, after shifting the cells to the non-permissive temperature of 37°C for 60 minutes, Vps17-GFP predominantly localized to the cytoplasm (Fig. 5C, lower panel). Western blot analysis using anti-GFP antibodies confirmed that during the shift of *vps34*^{tsf} cells to the non-permissive temperature, the Vps17-GFP fusion protein was not degraded, but remained as a stable fusion protein (data not shown). We performed identical experiments using Vps5-GFP expressed in *vps34*^{tsf} mutant strains and found that upon temperature shift to 37°C, Vps5-GFP redistributed to the cytosol as shown for Vps17-GFP (data not shown).

The results described above indicate that PtdIns3*P* is required for recruitment of Vps5p and Vps17p, a prerequisite for efficient endosome-to-Golgi retrograde transport of cargo molecules, such as Vps10p. To test this, Vps10-GFP was expressed in temperature-conditional *vps34*^{tsf} mutant strains. At the permissive temperature (26°C), we observed wild-type localization of Vps10-GFP (Fig. 6). After shifting the cells to the non-permissive temperature of 37°C for 60 minutes, Vps10-GFP was localized to the limiting membrane of the vacuole (Fig. 6), in a manner previously observed in Δ*vps30* and Δ*vps38* cells (Fig. 2). These data are in

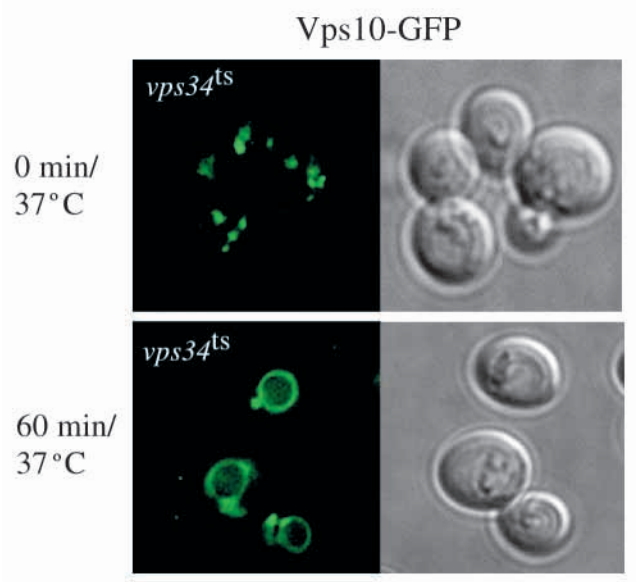

Fig. 6. Proper localization of the CPY receptor Vps10p depends on Vps34p-mediated PtdIns3*P* synthesis. Fluorescence and DIC microscopy of *vps34*^{ts} cells expressing Vps10-GFP (PBY124). The cells were incubated at non-permissive temperature (37°C) as indicated.

agreement with the idea that recruitment of Vps5p and Vps17p to endosomal membranes is triggered by the synthesis of PtdIns3*P* in vivo.

Vps5p and Vps17p bind PtdIns3*P* via their PX domain in vitro and in vivo

Since PtdIns3*P* is required for efficient recruitment of Vps5p and Vps17p to endosomes, we reasoned that these proteins may directly interact with PtdIns3*P* through their PX domain. To test this, the PX domain of Vps5p and Vps17p, respectively, was fused to glutathione S-transferase (GST). The resulting fusion proteins were subsequently purified and used in a protein-lipid overlay assay to determine their capability to bind to phosphoinositides. As shown in Fig. 7A, the GST-PX domain fusion proteins of Vps5p and Vps17p specifically bound PtdIns3*P*. No binding of GST to phospholipids was detected (data not shown).

To compare the PtdIns3*P*-binding specificity of Vps5 and Vps17 PX domain fusion proteins, various concentrations of PtdIns3*P* were spotted onto nitrocellulose membranes. The membranes were then subjected to protein-lipid overlay assays. The GST-Vam7 PX domain fusion protein was used as a positive control for PtdIns3*P*-binding (Cheever et al., 2001). The Vps5 and Vps17 PX domain fusion proteins had approximately a threefold lower affinity for PtdIns3*P* compared with the Vam7 PX domain fusion protein (Fig. 7B). These findings are in agreement with previous studies reporting that the PX domain of Vam7p binds to PtdIns3*P* with higher affinity than the PX domain of Vps5p and Vps17p, respectively (Yu and Lemmon, 2001).

To confirm that PtdIns3*P* is required for endosomal localization in vivo, we expressed Vps17-GFP in PtdIns-kinase mutants defective for the synthesis of other known yeast phosphoinositides and monitored the localization of Vps17-GFP by fluorescence microscopy. In strains deleted for *FAB1*, the PtdIns3*P* 5-kinase (Cooke et al., 1998; Gary et al., 1998), Vps17-GFP was detected on puncate structures (Fig. 7C). Next, we made use of the *stt4*^{tsf} and *pik1*^{tsf} kinase mutants, both defective for PtdIns(4)*P* synthesis (Flanagan et al., 1993; Yoshida et al., 1994; Audhya et al., 2000), and *mss4*^{tsf} strains deficient for PtdIns(4)*P* 5-kinase activity (Desrivières et al., 1998; Homma et al., 1998; Stefan et al., 2002). Upon shift to the non-permissive temperature of 37°C, Vps17-GFP localization did not change in these lipid kinase mutants (Fig. 7C) compared with the permissive temperature (26°C; data not shown). Together with the in vitro lipid binding assays (Fig. 7A,B), the in vivo studies strongly suggest that Vps5p and Vps17p specifically interact with PtdIns3*P*.

Amino acid sequence alignments of multiple PX domains have revealed highly conserved residues and previous studies have shown that point mutations in the conserved residues result in diminished binding to phosphoinositides and redistribution of the corresponding PX domain proteins (Cheever et al., 2001; Xu et al., 2001). To test whether the PX domain of Vps5p was essential for PtdIns3*P* binding and proper protein function, we substituted two highly conserved PX domain residues in the Vps5-GFP fusion protein. Tyr322 and Arg360 were each changed to alanine. Western blot analysis using anti-GFP antibodies showed that the mutant protein was stably expressed (data not shown). Using fluorescence microscopy, we observed that the Vps5-GFP mutant protein did not localize to endosomes, but rather localized to the cytoplasm as observed in Δ*vps38* cells (Fig. 8A). Importantly, we found a strong CPY processing defect for the Vps5^{Y322A,R360A}PX mutant cells, a defect very similar to that of Δ*vps5* mutant strains (Fig. 8B).

Next, we tested the ability of the Vps5^{Y322A,R360A} PX domain to bind PtdIns3*P*. The mutated PX domain of Vps5p was fused to GST and the resulting fusion protein was subjected to the lipid-protein overlay assay as described above. Consistent with the observed loss of function in CPY processing in the Vps5^{Y322A,R360A}PX mutant strain, binding of the mutated Vps5 PX domain fusion protein to PtdIns3*P* was abolished (Fig. 8C, right panel). In summary, a functional PX domain is crucial for the proper recruitment of Vps5p to endosomes, which is a prerequisite for efficient CPY sorting.

Discussion

In this study, we have characterized the biological role of the yeast PtdIns 3-kinase complex II, consisting of Vps15p, Vps34p, Vps30p and Vps38p. Based on our findings, we conclude that complex II plays an important role in endosome-to-Golgi retrograde transport. First, vacuolar sorting of CPS and ALP were essentially unaffected in Δ*vps30* and Δ*vps38* strains, respectively, while CPY maturation was severely defective in these strains. Second, we observed mislocalization of the CPY receptor Vps10p as well as the Golgi protease Kex2p to the vacuolar membrane in Δ*vps30* and Δ*vps38* cells. These phenotypes are commonly seen in mutant cells deficient in endosome-to-Golgi transport. The most interesting finding, however, was that Vps5p and Vps17p were mislocalized from endosomal membranes to the cytosol in Δ*vps30* and Δ*vps38* cells. Vps5p and Vps17p are subunits of the retromer complex, which is required for endosome-to-Golgi transport. In addition,

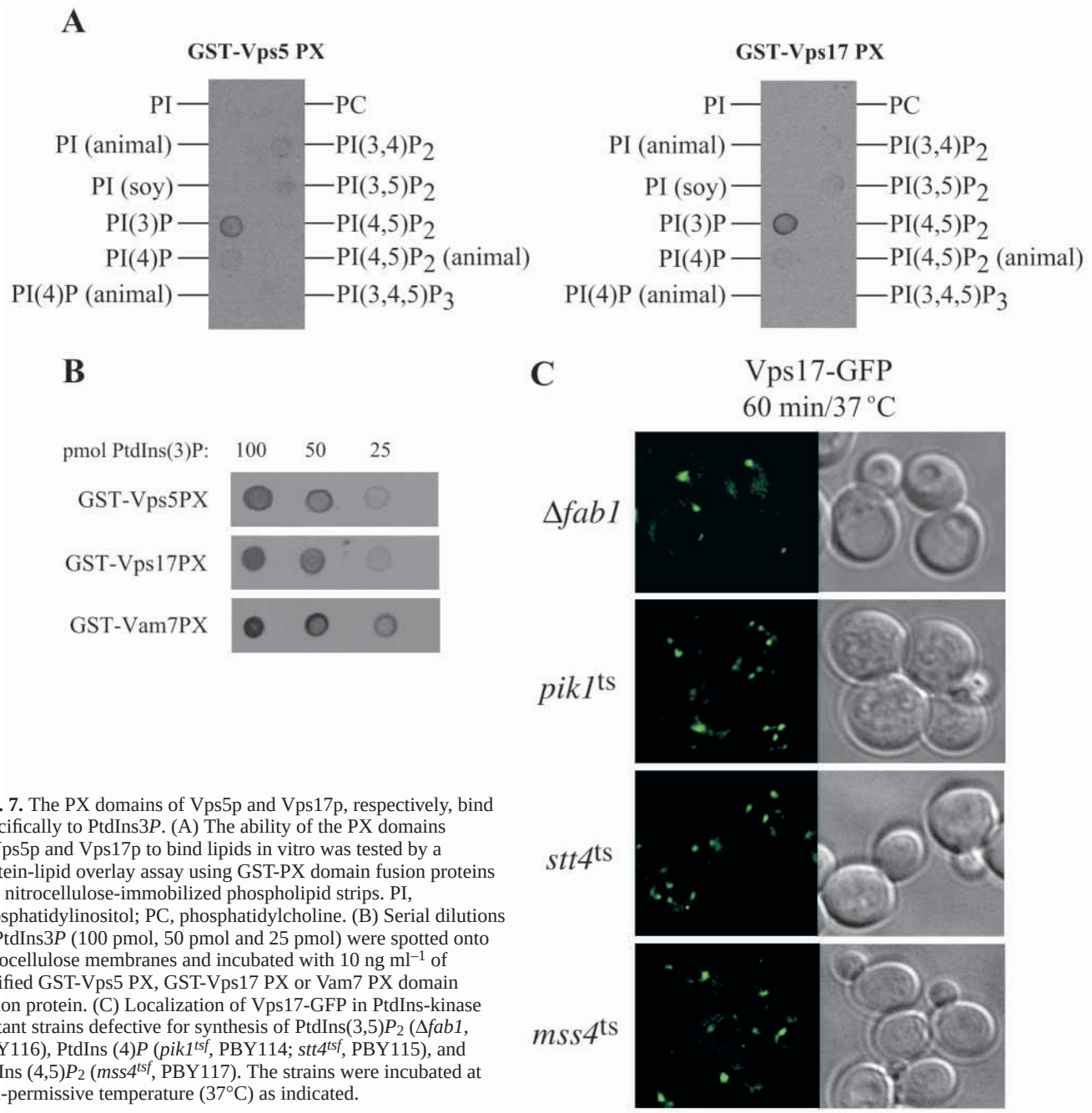

Fig. 7. The PX domains of Vps5p and Vps17p, respectively, bind specifically to PtdIns3*P*. (A) The ability of the PX domains ofVps5p and Vps17p to bind lipids in vitro was tested by a protein-lipid overlay assay using GST-PX domain fusion proteins and nitrocellulose-immobilized phospholipid strips. PI, phosphatidylinositol; PC, phosphatidylcholine. (B) Serial dilutions of PtdIns3*P* (100 pmol, 50 pmol and 25 pmol) were spotted onto nitrocellulose membranes and incubated with 10 ng ml^{-1} of purified GST-Vps5 PX, GST-Vps17 PX or Vam7 PX domain fusion protein. (C) Localization of Vps17-GFP in PtdIns-kinase mutant strains defective for synthesis of PtdIns(3,5)P_2 (Δ*fab1*, PBY116), PtdIns (4)*P* (*pik1*^{tsf}, PBY114; *stt4*^{tsf}, PBY115), and PtdIns (4,5)P_2 (*mss4*^{tsf}, PBY117). The strains were incubated at non-permissive temperature (37°C) as indicated.

both proteins contain PX domains that specifically bind PtdIns3*P*. Importantly, analysis of total phosphoinositides in Δ*vps30* and Δ*vps38* mutants revealed significantly decreased levels of PtdIns3*P*. In support of these findings, Kihara and co-workers observed a partial shift of Vps34p to the cytosolic pool in Δ*vps30* and Δ*vps38* mutant cells (Kihara et al., 2001).

Previous studies demonstrated that the yeast t-SNARE Vam7p is localized to vacuolar membranes in a manner that is dependent on its PX domain and on the synthesis of PtdIns3*P* (Cheever et al., 2001). Importantly, in Δ*vps38* mutant cells, Vam7-GFP still localized to vacuolar membranes. In addition, Vam7p function is maintained in Δ*vps38* mutants as transport of ALP and CPS to the vacuole, which is dependent on Vam7p function, is not blocked. We therefore propose that complex II is responsible for the synthesis of a specific pool of PtdIns3*P* that is required for recruitment of the retromer complex to endosomes, which directs endosome-to-Golgi retrograde transport.

How can the cell generate spatially distinct pools of PtdIns3*P*? In *S. cerevisiae*, Vps34p is the sole PtdIns3*P* kinase, nevertheless there are multiple downstream effectors of PtdIns3*P* that act in different pathways within the cell. These effectors include proteins containing a FYVE domain (5 gene products in yeast) or a PX domain (15 gene products in yeast) (Odorizzi et al., 2000; Sato et al., 2001). The synthesis of different PtdIns3*P* pools may be achieved by recruiting the Vps15p-Vps34p kinase complex to specific subcellular compartments via the formation of distinct protein complexes.

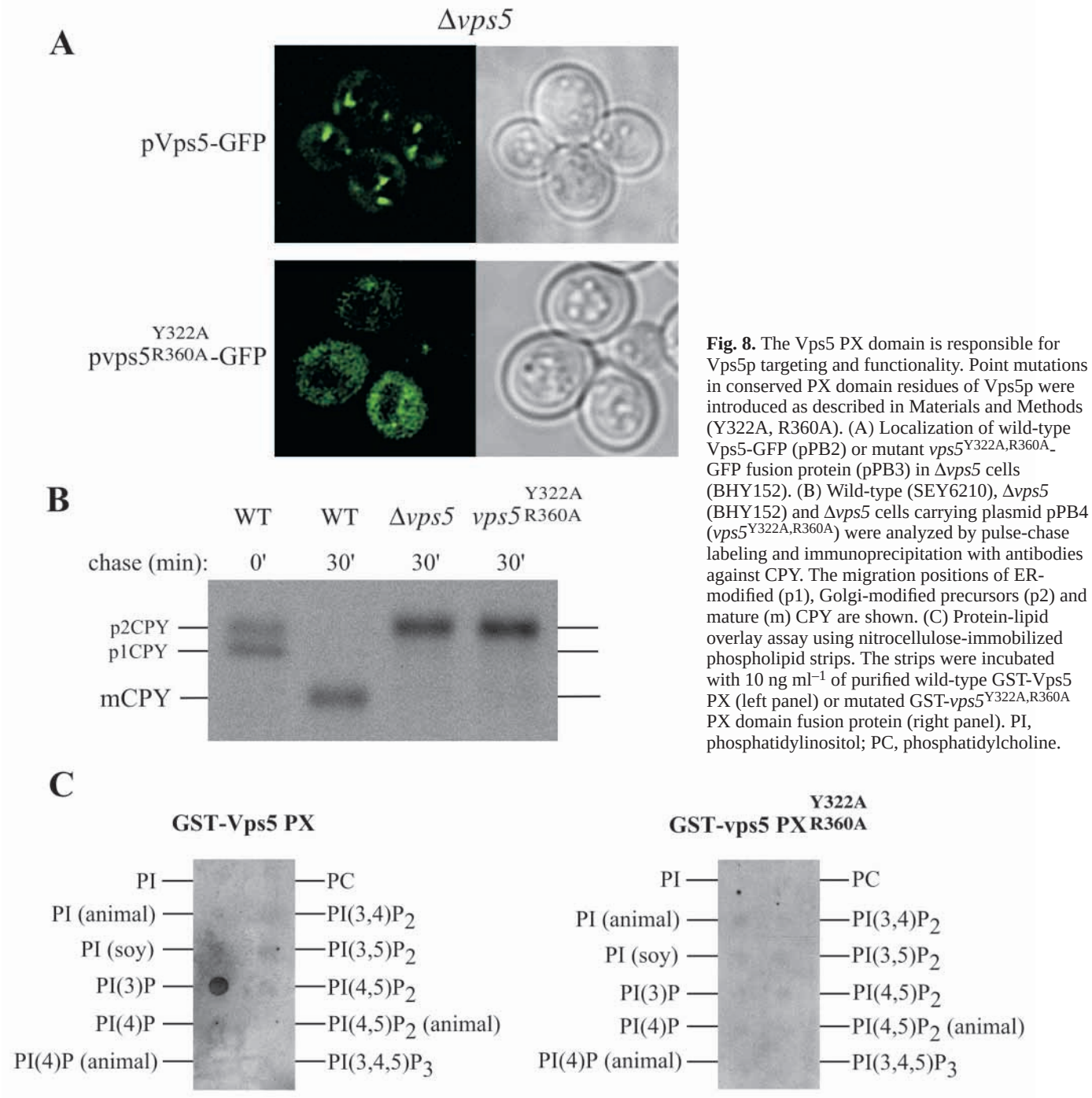

Fig. 8. The Vps5 PX domain is responsible for Vps5p targeting and functionality. Point mutations in conserved PX domain residues of Vps5p were introduced as described in Materials and Methods (Y322A, R360A). (A) Localization of wild-type Vps5-GFP (pPB2) or mutant *vps5*^{Y322A,R360A}-GFP fusion protein (pPB3) in Δ*vps5* cells (BHY152). (B) Wild-type (SEY6210), Δ*vps5* (BHY152) and Δ*vps5* cells carrying plasmid pPB4 (*vps5*^{Y322A,R360A}) were analyzed by pulse-chase labeling and immunoprecipitation with antibodies against CPY. The migration positions of ER-modified (p1), Golgi-modified precursors (p2) and mature (m) CPY are shown. (C) Protein-lipid overlay assay using nitrocellulose-immobilized phospholipid strips. The strips were incubated with 10 ng ml^{-1} of purified wild-type GST-Vps5 PX (left panel) or mutated GST-*vps5*^{Y322A,R360A} PX domain fusion protein (right panel). PI, phosphatidylinositol; PC, phosphatidylcholine.

Indeed, two Vps34p kinase subcomplexes, complex I and II, have been discovered, each of them functioning in different cellular processes (Kihara et al., 2001). Vps30p and Vps38p assemble together with the protein kinase Vps15p and the lipid kinase Vps34p to form complex II, which is required for CPY maturation. In contrast, complex I has been reported to function in autophagy and differs from complex II only by one subunit; Vps38p is replaced by Apg14p. Vps38p and Apg14p may individually regulate localization and/or activity of the corresponding Vps34p kinase complex, thereby generating distinct PtdIns3*P* pools, either for autophagy or for CPY sorting. Since the Vps15p-Vps34p kinase complex is also required for Golgi-to-vacuole anterograde transport, it seems likely that additional complexes exist in the cell. This idea is supported by the fact that PtdIns3*P* is still detected in Δ*vps30*Δ*vps38* double mutant strains. In Δ*vps15* mutants cellular PtdIns3*P* levels are almost completely absent (Stack et al., 1993) and no PtdIns3*P* can be detected in Δ*vps34* cells (Schu et al., 1993). The Vps15p-Vps34p kinase complex may cycle between membranes and the cytosol, forming distinct subcomplexes, when interacting partners are available at the target membrane.

Our data are consistent with a model in which complex II and the retromer communicate with each other. Our

preliminary findings indicate that Vps30p and Vps38p are able to associate in the cytosol prior to their recruitment to the membrane (P.B., unpublished). One could imagine that an increase of cargo molecules in the endosome might trigger recruitment of Vps30p-Vps38p to endosomal membranes, followed by binding to Vps15p-Vps34p to form the final complex II. The synthesis of a specific PtdIns3*P* pool by complex II that is localized to a subdomain of the endosome could then trigger assembly of the retromer and ultimately vesicle formation for retrograde transport.

Data obtained from yeast and mammalian cells show that PtdIns3*P* synthesis occurs mainly at endosomal membranes. In vivo localization studies using a PtdIns3*P*-specific GFP-FYVE domain fusion protein revealed that PtdIns3*P* is enriched in prevacuolar compartments (Burd and Emr, 1998). Vacuolar membrane labeling was also observed, albeit to a lesser extent. These results were confirmed by studies using electron microscopy techniques to localize PtdIns3*P* (Gillooly et al., 2000). In agreement with these findings, many proteins containing PtdIns3*P*-binding modules, are recruited to endosomal membranes (Odorizzi et al., 2000; Teasdale et al., 2001; Stenmark et al., 2002).

The prevacuolar endosome represents a complex sorting compartment where biosynthetic and endocytic cargoes merge and are then sorted to the vacuole. In addition, other proteins such as the CPY receptor Vps10p are recycled back to the late-Golgi from this compartment. PtdIns3*P* appears to be a key regulator in each of the endosomal sorting events. However, PtdIns3*P* alone can hardly be sufficient to provide specificity in these different pathways. Apparently, it is the combination of PtdIns3*P*-binding domains acting together with protein-protein interaction domains that ensures the specific location and function of each effector protein. Previous studies revealed that the yeast t-SNARE Vam7p contains both the PX domain and an α-helical coiled-coil domain, which are required to stabilize the interaction of Vam7p with the vacuolar membrane by binding PtdIns3*P* and to the vacuolar t-SNARE Vam3p, respectively (Sato et al., 1998). Within the retromer complex, Vps35p provides the cargo-selective protein-protein interaction by directly binding to Vps10p (Nothwehr et al., 1999; Nothwehr et al., 2000). It is possible that, together with the PtdIns3*P*-dependent recruitment of Vps5p and Vps17p, specific recruitment and/or assembly of the retromer complex at the prevacuolar endosome is assured. Thus, spatially restricted synthesis of PtdIns3*P* could concentrate PtdIns3*P* effectors at the membrane, thereby allowing the effector proteins to interact with specific membrane-bound molecules via other protein-protein interaction domains.

Recently, SNX1 and SNX2, homologues of Vps5p, were found to associate with human orthologues of Vps26p, Vps29p and Vps35p (Haft et al., 1998). The structural similarities to the yeast retromer strongly suggest that the mammalian complex performs a related function in protein trafficking. In summary, the present data suggest that the restricted localization of distinct Vps15p-Vps34p complexes results in PtdIns3*P* synthesis at specific organelles, such that membrane microdomains enriched in PtdIns3*P* are generated. Further studies, including in vitro reconstitution assays will be required to elucidate the mechanisms involved in regulating the dynamic organization of these lipid microdomains and to understand, how these domains recruit and activate the appropriate set of effector molecules.

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