Identification of a conserved motif required for Vps35p/Vps26p interaction and assembly of the retromer complex

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The retromer complex is a conserved cytoplasmic coat complex that mediates the endosome-to-Golgi retrieval of vacuole/lysosome hydrolase receptors in yeast and mammals. The recognition of cargo proteins by the retromer is performed by the Vps35p/VPS35 (where Vps is vacuolar protein sorting) component, which together with Vps26p/VPS26 and Vps29p/VPS29, forms the cargo-selective subcomplex. In this report, we have identified a highly-conserved region of Vps35p/VPS35 that is essential for the interaction with Vps26p/VPS26 and for assembly of the retromer complex. Mutation of residues within the conserved region results in Vps35p/VPS35 mutants, which cannot bind to Vps26p/VPS26 and are not efficiently targeted to the endosomal membrane. These data implicate Vps26p/VPS26 in regulating Vps35p/VPS35 membrane association and therefore suggest a role for Vps26p/VPS26 in cargo recognition.

Key words: assembly, endosome, membrane, recruitment, retromer, sorting.

INTRODUCTION

In eukaryotic cells the sorting and transport of membrane proteins in the endocytic and secretory pathways is mediated by cytoplasmic coat proteins that can both recognize cargo through intrinsic sorting motifs and can assemble to deform the membrane into a vesicle or tubule. Different coat proteins function at different sites in the cell and thereby help to generate and maintain the exquisite specificity of protein targeting to the organelles that comprise the endocytic and secretory pathways. A common feature of the various coat proteins is that they are multimeric protein complexes comprising several subunits which assemble into a single functional unit that can perform the tasks of cargo recognition and membrane deformation (reviewed in [1–3]).

A good example of a multimeric coat complex is the retromer complex. The retromer is a conserved cytoplasmic coat that functions in endosome-to-Golgi retrieval and was first characterized in yeast *Saccharomyces cerevisiae* [4,5]. The yeast retromer is comprised of five subunits, each encoded by a *VPS* (vacuole protein sorting) gene. Loss of the retromer function results in a vacuole protein sorting defect and secretion of the vacuolar hydrolase CPY (carboxypepetidase Y), due to the failure to retrieve the CPY sorting receptor, Vps10p [4,6]. Although the yeast retromer is a stable heteropentameric complex, the retromer can be phenotypically and biochemically dissected into two subcomplexes: Vps35p, Vps29p and Vps26p comprise the cargo-selective component of the retromer complex, whereas the sorting nexins Vps5p and Vps17p dimerize to drive vesicle/tubule formation through their BAR (Bin/Aphiphysin/Rvs) domains and intrinsic self-assembly activity (reviewed in [7]). In mammalian cells, VPS35, VPS29 and VPS26 form a complex which can bind cargo such as the CIMPR (cation-independent mannose-6-phosphate receptor) [8]. Snx1 (sorting nexin 1), along with Snx2, 5 and 6 function as the mammalian orthologues of the yeast Vps5p/Vps17p dimer to drive tubulation of endosomal membranes [9,10]. Unlike yeast, the mammalian retromer does not form a stable heteropentameric complex, as the association of the VPS35/29/26 complex with the sorting nexin complex is dynamic and transient [11].

The importance of the retromer in endosomal membrane protein sorting and transport has been revealed recently through a variety of studies that have implicated the retromer in the processing of the amyloid precurser protein [12], transcytosis of the polymeric IgA receptor [13], Wnt gradient formation [14] and trafficking of the Ftr1p protein required for iron homeostasis in yeast [15]. Some progress has been made in understanding how the retromer assembles and the function of the individual subunits. For instance, Vps35p and its mammalian orthologue can bind to cargo proteins such as Vps10p and the CIMPR respectively [6,8]. VPS29 is a phosphoesterase which can dephosphorylate the serine preceding the acidic dileucine sorting motif in the tail of the CIMPR [16]. The structure of VPS26 was recently solved, revealing an unexpected structural similarity to the arrestin family of proteins, hinting that VPS26 may also have a role to play in binding cargo proteins [17]. The association of Vps29p with Vps35p has been shown to depend upon a conserved hydrophobic patch on the surface of Vps29p. Mutation of Val^{109} in yeast Vps29p, or Val^{90} in mammalian VPS29, blocks assembly of Vps29p with Vps35p and prevents VPS29 recruitment to the membrane respectively [18]. A separate hydrophobic patch on Vps29p, centred on Leu^{252}, is essential for the association of the Vps35p/Vps29p/Vps26p subcomplex with the Vps5p/Vps17p members of the yeast retromer complex [18].

Other studies in yeast have shown that Vps26p plays a vital role in mediating the interactions between Vps35p and other retromer components such as Vps5p/Vps17p, and also in regulating the membrane association of Vps35p [19]. As Vps35p/VPS35 must be membrane-associated to recognize cargo proteins such as

Abbreviations used: 3-AT, 3-amino-1,2,4-triazole; CIMPR, cation-independent mannose 6-phosphate receptor; CPY, carboxypeptidase Y; EST, expressed sequence tag; GFP, green fluorescent protein; IMAGE consortium, Integrated Molecular Analysis of Genomes and their Expression consortium [at St. Louis, MO, U.S.A., and at the Human Genome Mapping Project (HGMP), Hinxton Hall, Cambridge, U.K.]; IP, immunoprecipitation; Snx, sorting nexin; TCA, trichloroacetic acid; Vps, vacuolar protein sorting.

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Table 1 Yeast strains used in this study

Strain	Genotype	Source or Reference
SEY 6210	*MATα*, *leu2-3,112 ura3-52*, *his3Δ200*, *trp1-Δ901*, *lys2-801*, *suc2-Δ9*	[24]
MSY2600	SEY 6210 *vps26Δ::LEU2*	[4]
PSY1-29	SEY 6210 *vps29Δ::HIS3*	[21]
EMY18	SEY 6210 *vps35Δ::HIS3*	[21]
MSY10-21	SEY 6210 *VPS10*-6myc	[4]
HF7c	MATα *ura3-52 his3Δ200 trp1-Δ901*, *lys2-801*, *ade2-101 leu2-3*, *112 gal4-542*, *gal80-538*, *LYS2::GAL1-HIS3*, *URA3::*(*GAL4* 17mers)$_3$-*CYC1-LacZ*	Clontech

Vps10p and the CIMPR, the regulatory role that Vps26p plays in Vps35p membrane association has clear implications for the ability of Vps35p to sort cargo proteins. In mammalian cells, binding of VPS26 to VPS35 requires a mobile loop in the C-terminal domain of VPS26 [17]. It is currently unknown, however, how Vps35p associates with Vps26p. In this study, we have identified a critical region of Vps35p that is required for its interaction with Vps26p. This region comprises a highly conserved N-terminal motif, PRLYL, that is predicted to be part of a β-sheet. Mutation of the arginine or adjacent leucine residues results in a *vps35* mutant that cannot bind to Vps26p and therefore fails to retrieve Vps10p from the endosomes to the Golgi. As the PRLYL motif is conserved in mammalian VPS35, we have determined the role this motif has in assembly of the mammalian retromer: it is essential for binding to VPS26 as well as being required for the membrane association of VPS35.

EXPERIMENTAL

Reagents and media

Most reagents were obtained from Sigma–Aldrich, with the following exceptions: restriction enzymes were purchased from New England Biolabs, Effectene was obtained from Qiagen, Fugene™6 from Roche, and protein-A Sepaharose and ^{125}I-protein-A from Amersham Biosciences. Fluorescently labelled secondary antibodies were obtained from Molecular Probes. Antibodies against mammalian VPS35 and VPS26 are as described in [20]. Yeast strains used are described in Table 1. Standard yeast media was used for culturing the yeast and selecting transformants.

Production of the *vps35* mutant alleles

Generation of the *vps35-27* dominant negative allele (previously named *vps35dn*) has been described previously in [4]. After sequencing to identify the mutations present, the mutations were separated using standard molecular biology techniques by cutting the mutant and wild-type DNA and splicing them together to separate the mutations and generate the *vps35-27a-e* alleles. The Y55H mutation was separated from other mutations using a unique KpnI site. Digestion of wild-type *VPS35* and *vps35-27* with KpnI followed by gel purification of the vector and insert fragments allowed construction of *vps35-27b* and *c*. The Y55H, L99P and F112L mutations were separated from the I247T, S275L and M457T mutations using a unique SpeI site. Digestion of wild-type *VPS35* and *vps35-27* with SpeI followed by gel purification of the vector and insert fragments allowed construction of *vps35-27a* and *d*. The *vps35-27e* allele was generated by digestion of *vps35-27b* with SpeI followed by ligation of the SpeI fragment into wild-type *VPS35*. To produce the *vps35*(L99P), *vps35*(F112L), VPS35(R107A) and VPS35(L108P) mutants, site-directed mutagenesis was performed using the QuickChange kit from Stratagene following the manufacturers' instructions. All mutants and alleles were sequenced to confirm the presence of the required mutation(s).

Yeast metabolic labelling experiments

CPY sorting experiments were performed as described in [19] and are outlined briefly here. To determine how much CPY is secreted, cells grown to a D_{600} of 0.8 were collected by centrifugation and resuspended in 0.5 ml of selective media so that the final concentration was 5 *D* units per ml. The cells were labelled with ^{35}S-methionine for 10 min followed by a 30 min chase. The cells were then transferred to a tube on ice containing 0.5 ml spheroplasting buffer [50 mM Tris/HCl (pH 8.0), 2 M sorbitol, 40 mM sodium azide, 20 mM dithiothreitol and 20 μg/ml α_2-macroglobulin] for 10 min. After addition of zymolyase, the cells were incubated for 15 min at 30 °C to allow spheroplasting to occur. The cells were then centrifuged and the supernatant (extracellular; E) and cell pellet (intracellular; I) fractions were treated with 10 % TCA (trichloroacetic acid) to precipitate the proteins. After centrifugation and washes with acetone, the pellets were dried and resuspended into 100 μl of cracking buffer [6 M urea, 50 mM Tris/HCl (pH 7.5), 1 mM EDTA, 1 % SDS, 1 % β-mercaptoethanol] to solubilize the proteins. The samples were diluted with the addition of 1 ml IP (immunoprecipitation) buffer [50 mM Tris/HCl (pH 7.5), 150 mM NaCl, 0.5 % Tween-20, 0.1 mM EDTA], cleared by centrifugation and transferred to a fresh tube. CPY was recovered from the intracellular and extracellular fractions by IP. In the whole cell CPY sorting assay, 5 *D* units of cells were labelled with ^{35}S methionine for 10 min and chased for 30 min before the whole culture was transferred to ice and precipitated with 10 % TCA. After centrifugation, the pellets were washed with acetone, dried, resuspended into cracking buffer and then lysed by vortexing with glass beads and heating to 70 °C. The samples were diluted with IP buffer, cleared by centrifugation and then transferred to a fresh tube where CPY was recovered by IP.

Cell fractionation experiments to determine the membrane localization/association of Vps10p, Vps35p and Vps29p were performed as described in [4,21]. Native IP experiments to evaluate the assembly of the yeast retromer complex *in vivo* were performed as described in [19,22].

Yeast two-hybrid analysis

VPS35 was cloned into the two-hybrid 'bait' vector pGBT9 by PCR of a full-length mouse VPS35 which introduced a BamHI site at the 5′ end. The PCR product was first cloned using the pCRblunt vector (Invitrogen). VPS35 was then subcloned into pGBT9 by excision from pCRblunt using BamHI followed by ligation into BamHI-cut pGBT9. Mouse VPS26 was obtained as a full-length EST (expressed sequence tag) from the IMAGE consortium {Integrated Molecular Analysis of Genomes and their Expression consortium [at St. Louis, MO, U.S.A., and at the Human Genome Mapping Project (HGMP), Hinxton Hall, Cambridge, U.K.]} and subcloned from pCMVSport using SalI and NsiI to excise VPS26 and ligated into the prey vector pGAD424 which had been cut with SalI and PstI. Mouse VPS29 was obtained as a full-length EST from the IMAGE consortium and subcloned into pGAD424 using EcoRI and NotI to cut at the 5′ and 3′ ends respectively. The NotI site was filled with T4 polymerase and the fragment was ligated into pGBT9 which had been cut with EcoRI and SmaI.

Table 2 Mutations in the *vps35* alleles

Allele	Mutations					
vps35-27	Y55H	L99P	F112L	I247T	S275L	M457T
vps35-27a				I247T	S275L	M457T
vps35-27b		L99P	F112L	I247T	S275L	M457T
vps35-27c	Y55H					
vps35-27d	Y55H	L99P	F112L			
vps35-27e		L99P	F112L			

Native IPs from mammalian cells

Transient transfections were performed using Effectene or Fugene™6 following the manufacturers' instructions. Native IPs of GFP (green fluorescent protein)-tagged VPS35 constructs transiently expressed in HeLa (cervical cancer) cells were performed as described in [18]. VPS26 IPs were performed in an identical fashion using our anti-VPS26 antisera [20]. SDS/PAGE and Western blotting were performed as described in [19].

Immunofluorescence microscopy

Fluorescence microscopy was performed as described in [20].

RESULTS

In previous studies, the generation of a dominant negative *vps35* mutant (*vps35dn*) has been described [4]. This allele of *vps35* is designated *vps35-27* and was produced using random PCR mutagenesis and gapped-plasmid repair. This procedure very often introduces several mutations. Therefore, to identify which mutation (or combination of mutations) is required for the dominant phenotype, the *vps35-27* allele was sequenced and six different mutations were found, all of them in the N-terminal half of the protein (see Table 2). Some of the mutations result in changes to highly conserved amino acids such as the Y55H, L99P and I247T mutations. In Figure 1(A), the sites of the various mutations are shown on an alignment of yeast Vps35p with homologues from plant, chicken and humans. Secondary structure prediction shows that some of the mutations (i.e. Y55H, L99P and I247T) fall in regions that are predicted to form α-helical or β-sheet secondary structure.

To pin-point which mutation(s) confer the dominant-negative phenotype, the *vps35-27* allele was subjected to restriction digest using enzymes that cut at unique sites between the various mutations. The mutant DNA was then ligated into wild-type *VPS35* to generate a new allele carrying one or more of the original six mutations (see Table 2). The resulting alleles were transformed into wild-type yeast and CPY sorting was evaluated by pulse–chase analysis. The dominant-negative phenotype is clearly caused by either the L99P or F112L mutation (or both) (see Figure 1B). Site-directed mutagenesis was therefore used to generate alleles carrying only the L99P mutation or the F112L mutation. Analysis of these alleles conclusively demonstrate that the dominant-negative phenotype of the *vps35-vps27* allele is the result of the L99P mutation, which falls in a highly conserved region predicted to form a β-sheet.

The L99P mutation is able to confer a dominant-negative phenotype. To examine further the importance of this region of Vps35p to its function, the amino acid residues surrounding and adjacent to Leu^{99}, i.e. Pro^{97}, Arg^{98}, Tyr^{100} and Leu^{101}, were mutated individually to alanine. The resulting mutants were ligated into centromeric plasmids for wild-type expression levels and transformed into *vps35*Δ yeast. A whole cell CPY sorting experiment was performed to evaluate the ability of the alanine scanning mutants to complement the CPY sorting defect in *vps35*Δ yeast. In Figure 2(A, upper panel), the CPY sorting experiment shows that the L99P mutant and the R98A mutant are both unable to restore normal CPY sorting in the *vps35*Δ strain. The inability of the L99P and R98A mutants to rescue CPY sorting in the *vps35*Δ strain was not due to a lack of expression of the mutant Vps35 proteins, as all the mutants were expressed and detectable in lysates (Figure 2A, lower panel).

It has previously been demonstrated that Vps35p is required as part of the retromer complex to mediate retrieval of Vps10p from the endosomes to the Golgi [4,5]. Logically, as the L99P mutant is unable to rescue CPY sorting in a *vps35*Δ strain, one would expect that Vps10p should be mislocalized in *vps35*Δ yeast expressing the L99P mutant. In Figure 2(B), the results of a cell fractionation experiment are shown. In wild-type cells, Vps10p is predominantly localized to the P100 fraction, which contains endosomal and Golgi membranes along with small vesicles. However, when *VPS29*, *VPS26* or *VPS35* is deleted, Vps10p becomes mislocalized to the P13 fraction which contains vacuolar membranes [4,21]. Expression of wild-type *VPS35* in the *vps35*Δ strain rescues this phenotype, but expression of the L99P mutant results in Vps10p mislocalization to the vacuolar membrane fraction, identical to that observed in the *vps35*Δ strain with an empty vector (see Figure 2B).

Additionally, when the effect of the L99P mutation upon the localization of Vps29p or the Vps35 protein itself is investigated, it becomes apparent that the L99P mutation confers a phenotype more similar to the effect observed when Vps26p is missing. Loss of Vps35p results in the displacement of Vps29p from the P100 fraction ([4]; and Figure 2B, compare wild-type to *vps35*Δ cells). In yeast expressing the L99P mutant, Vps29p remains associated with the P100 fraction, so the L99P mutant does not behave as the *vps35*Δ strain with respect to Vps29p localization. The L99P mutation does, however, result in a quantifiable shift of the Vps35 protein from the P100 to the S100 fraction. This is very similar to the effect upon Vps35p observed in a *vps26*Δ mutant and hints that perhaps the L99P mutation is causing the loss of interaction between Vps35p and Vps26p. The localization of Vps26p to the P100 fraction was moderately affected by deletion of either *VPS35* or *VPS29* (see Figure 2B, lower panel) and therefore the localization of Vps26p does not lead to any further information regarding whether the L99P mutation in Vps35p affects the interactions with other subunits such as Vps26p. Deletion of both *VPS35* and *VPS29* did not have an additive effect on the P100 localization of Vps26p, which remained associated with the P100 fraction, suggesting that Vps26p can bind to intracellular membranes independently of Vps35p or Vps29p.

The effect of the L99P mutation upon the localization of Vps10p was also investigated in wild-type cells expressing high copy number 2 μ plasmids carrying either wild-type *VPS35* or the *vps35*(L99P) mutant allele. In Figure 2(C), there is little apparent shift of Vps10p from the Golgi (P100) fraction to the vacuolar (P13) fraction and no effect was observed upon the localization of Vps26p. This is consistent with the observation that expression of the mutant *vps35* alleles in a wild-type background results in a more modest CPY sorting defect than was observed in *vps35*Δ cells expressing the *vps35* mutants from low copy centromeric plasmids.

To assess the effect of the L99P mutation on the assembly of the retromer complex in yeast, a native immunoprecipitation experiment was performed. Unfortunately, the Vps35p antiserum does not recognize Vps35p under native conditions, therefore

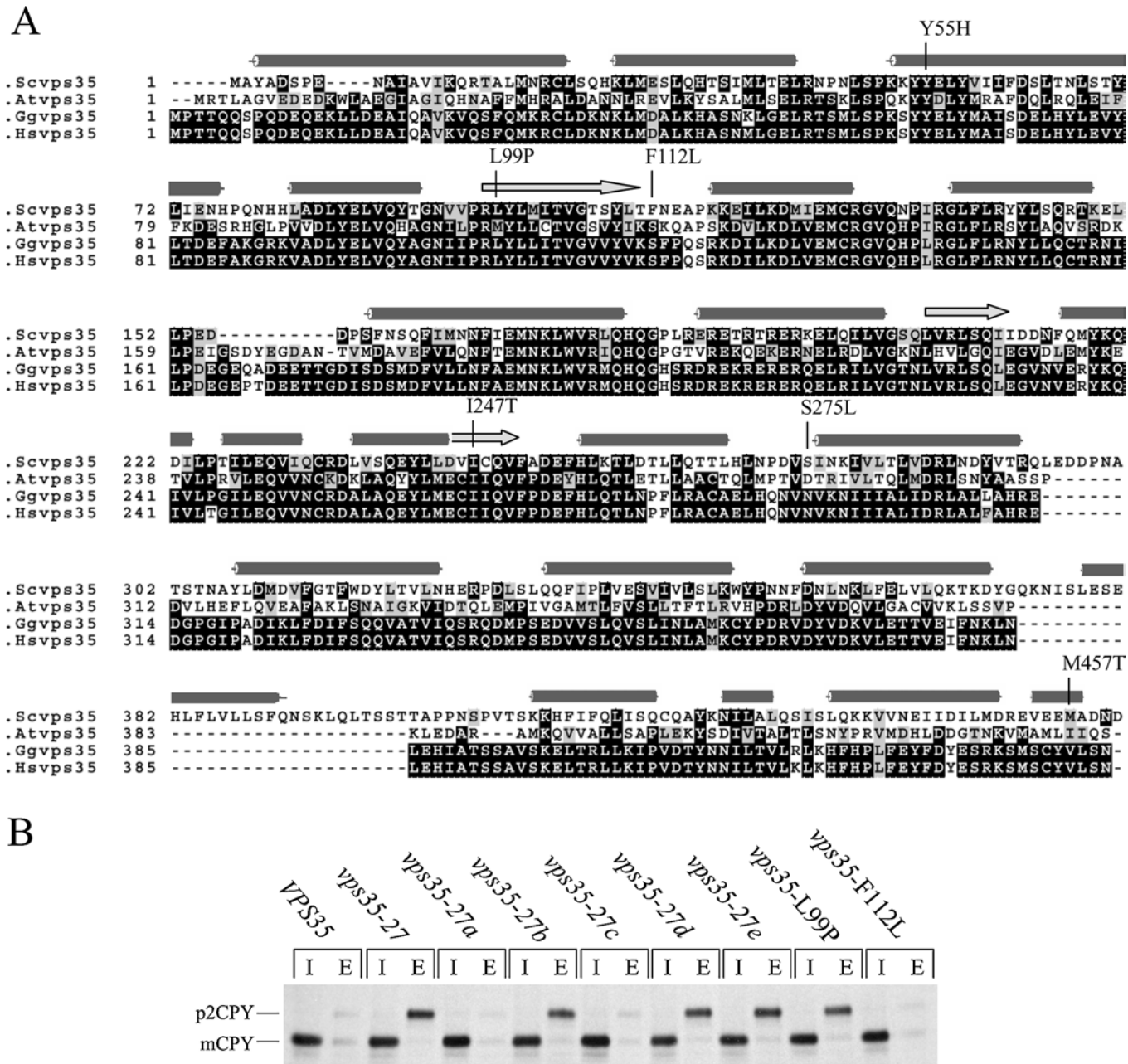

Figure 1 Identification of the mutation that confers the dominant-negative phenotype of the *vps35-27* allele

(**A**) Protein sequence alignment of yeast (*Saccharomyces cerevisiae*, Sc) Vps35p N-terminal half with orthologues from plant (*Arabidopsis thaliana*, At), Chicken (*Gallus gallus*, Gg) and human (*Homo sapiens*, Hs). The position of the various mutations present in the *vps35-27* allele are indicated. Residues highlighted in black indicate the conserved sequence; those highlighted in grey are similar. The yeast Vps35p sequence was analysed by the jpred secondary structure prediction program (http://www.compbio.dundee.ac.uk/~www-jpred/). Regions of α-helical predicted structure are shown as cylinders, arrows depict regions predicted to form β-sheet structure. (**B**) CPY sorting assay of wild-type cells (SEY6210) expressing the *vps35-27* allele and derivatives a-e. Cells were pulse-labelled with ^{35}S-methionine for 10 min and chased for 30 min before the intracellular (I) and extracellular (E) fractions were separated from each other and CPY recovered by immunoprecipitation. Expression of the *vps35-27* allele at high copy number results in secretion of the Golgi-modified (p2) form of CPY. The alleles derived from *vps35-27* that have the L99P mutation secrete p2CPY whilst those without do not. mCPY, mature CPY.

we used the antiserum against Vps5p or Vps26p, both of which have been used previously for native IPs of the retromer [19,22]. When lysate from wild-type cells is incubated with either anti-Vps5p or anti-Vps26p, it is possible to immunoprecipitate the retromer complex as a heteropentamer, each of the subunits being detectable in secondary IPs (see Figure 3A). However, when Vps35p is deleted, it is no longer possible to co-immunoprecipitate all five subunits. In lysates from *vps35*Δ cells, Vps5p remains associated with Vps17p, but no longer assembles with the other members of the complex. Vps26p can only immunoprecipitate itself in lysates from *vps35*Δ cells. This is consistent with previous data which have shown that Vps35p acts as a binding platform necessary for retromer assembly as a heteropentamer [4,19,22]. When lysates from cells expressing the L99P mutant are evaluated, it is observed that the L99P mutation has a similar effect on retromer assembly as is seen in *vps35*Δ cells, i.e. it is

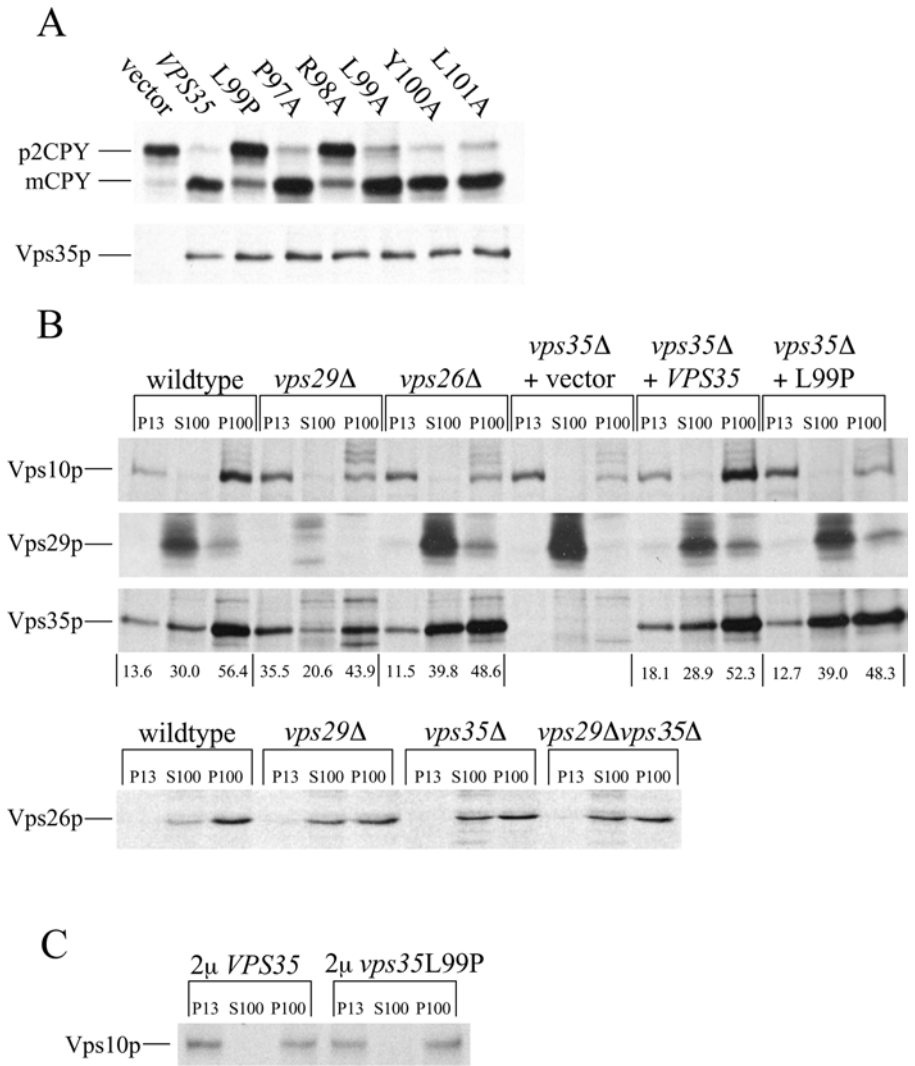

Figure 2 Alanine-scanning mutagenesis of the PRLYL motif and cell fractionation to localize Vps10p, Vps29p and Vps35p

(**A**) CPY sorting of *vps35*Δ cells expressing the PRLYL alanine-scanning mutants. *vps35*Δ cells were transformed with centromeric plasmids (low copy number) to express wild-type *VPS35*, the L99P mutant or the PRLYL motif mutants. Cells were pulse-labelled with ^{35}S-methionine for 10 min and chased for 30 min before the culture was transferred to ice and precipitated with 10% TCA. After lysis, total CPY was recovered by immunoprecipitation. The L99P and R98A mutants both fail to complement the CPY sorting defect in *vps35*Δ yeast. In the lower panel, Vps35p was recovered by immunoprecipitation showing that all the mutants are equally expressed. (**B**) Cells were labelled with ^{35}S-methionine before being fractionated into P13 (vacuolar membrane), S100 (cytosolic) and P100 (Golgi, endosomes and small vesicles) fractions to determine the distribution of Vps10p, Vps29p and Vps35p. Loss of retromer function results in Vps10p being mislocalized to the vacuolar membrane (P13) fraction and a similar phenotype is produced in cells expressing the L99P mutant. When the levels of Vps35p in each fraction is quantified, it is apparent that the behaviour of Vps35(L99P) protein is similar to Vps35p in *vps26*Δ cells. (**C**) Wild-type cells (MSY10-21) expressing either wild-type *VPS35* or the *vps35*(L99P) mutant were fractionated as above and Vps10p and Vps26p were recovered by immunoprecipitation. In a wild-type background there is little discernable shift of Vps10p to the vacuolar (P13) fraction due to the L99P mutation.

impossible to co-immunoprecipitate the retromer complex as a heteropentamer (Figure 3A).

The L99 residue is very highly conserved among orthologues of Vps35p. Therefore, to better understand the role that this region of Vps35p plays in retromer function we next investigated the interactions of mammalian VPS35 using the yeast two-hybrid system. Mouse VPS35 was cloned into the 'bait' vector (pGBT9) of the "Matchmaker" two-hybrid system, and mouse VPS29 and VPS26 were cloned into the 'prey' vector (pGAD424). These constructs were transformed into the two-hybrid reporter strain HF7c and the interaction between VPS35 and VPS26 or VPS29 was determined by growth on plates lacking histidine. No growth was observed when either VPS35 was expressed with empty 'prey' vector or VPS26 or VPS29 was co-transformed with empty 'bait' vector (data not shown) so none of the bait or prey constructs was able to autoactivate.

Wild-type VPS35 can interact with both VPS26 and VPS29. Using the inhibitor of the *HIS3* reporter gene, 3-AT (3-amino-1,2,4-triazole), it is possible to gauge the strength of the interaction between VPS35 and VPS26 or VPS29. Strong interactions will be resistant to inhibition with 3-AT while weak interactions will result in poor expression of the *HIS3* gene and yeast growth will be inhibited by 3-AT. The interaction of VPS35 with VPS26 is robust and can allow growth in the presence of 2 mM 3-AT. The interaction between VPS35 and VPS29 is weaker and is susceptible to inhibition in the presence of 2 mM 3-AT. The VPS35 mutants R107A and L108P (equivalent to R98A and L99P in yeast) are compromised in their ability to interact with VPS26 and virtually no growth is seen on plates containing 2 mM 3-AT. The VPS35(L108P) mutant behaves as wild-type VPS35 with respect to its interaction with VPS29, the VPS35(R107A) mutant, however, appears to be able to interact with VPS35 even

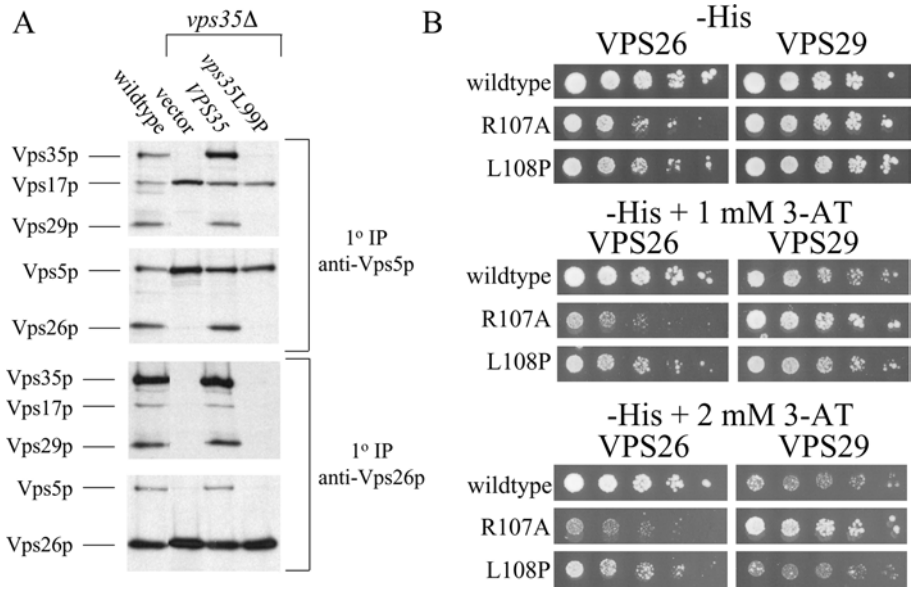

Figure 3 The L99P/L108P mutation in yeast Vps35p or mammalian VPS35 abolishes the interaction with Vps26p/VPS26

(**A**) Yeast cells were labelled with ^{35}S-methionine before lysis. An IP of the retromer complex was done under native conditions using antisera against either Vps5p or Vps26p. After the primary IP, the samples were re-solubilized and the IP of individual retromer proteins was done under non-native conditions. The Vps35p(L99P) mutant does not assemble into the retromer complex. (**B**) Yeast two-hybrid analysis of the interaction between mammalian VPS35, VPS26 and VPS29. VPS35 wild-type, R107A and L108P mutants in the two-hybrid 'bait' vector were transformed into the two-hybrid reporter strain HF7c along with either VPS26 or VPS29 in the 'prey' vector. Transformants were grown in liquid culture before being spotted onto YNB (yeast nitrogen base) plates lacking histidine (but with either 0, 1 or 2 mM 3-AT added) in a serial dilution. Growth on the −His plates is indicative of an interaction between bait and prey, the strength of the interaction can be gauged by the ability of the yeast to grow in the presence of 3-AT. The R107A and L108P mutations in VPS35 abolishes the interaction with VPS26.

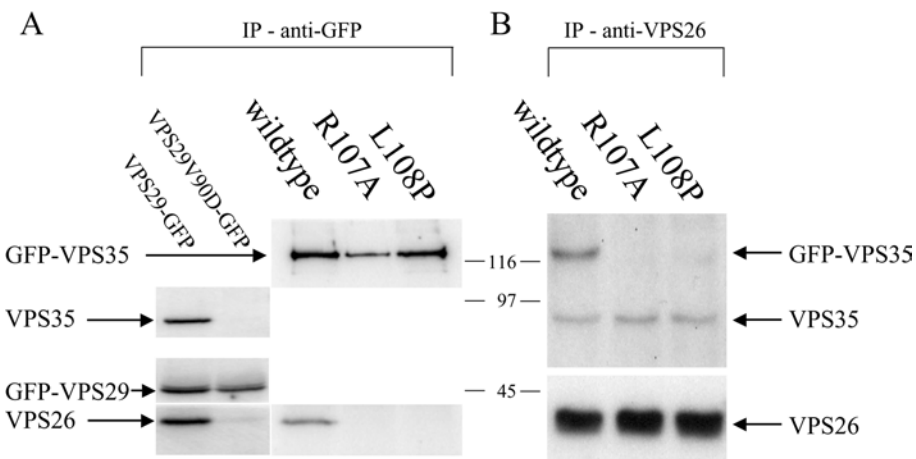

Figure 4 The R107A and L108P mutations abolish the interaction with mammalian VPS35 *in vivo*

(**A**) HeLa cells were transiently transfected with either GFP-tagged wild-type VPS35, R107A or L108P mutants. Additionally, cells stably expressing either VPS29–GFP or VPS29(V90D)–GFP constructs were used as positive and negative controls. After lysis, the lysates were incubated with anti-GFP antisera. Wild-type GFP–VPS35 binds VPS26 but the R107A and L108P mutants do not. The VPS29–GFP protein was able to co-immunoprecipitate with VPS35 and VPS26 but the V90D mutant does not. (**B**) Similarly, when anti-VPS26 antisera is used to immunoprecipitate the lysates, wild-type GFP–VPS35 can co-immunoprecipitate with VPS26 but the R107A and L108P mutants both fail to interact with VPS26.

in the presence of 2 mM 3-AT, suggesting that the VPS35(R107A) mutant has a stronger interaction with VPS29 than wild-type VPS35.

We next examined the ability of the mammalian VPS35 mutants to interact with VPS26 *in vivo* using native IPs. Cells were transfected with GFP-tagged VPS35 constructs, lysed, and the lysates treated with antibodies against either GFP or VPS26. Additional controls are provided in the form of GFP-tagged VPS29 constructs. Wild-type VPS29–GFP can assemble with VPS35 and VPS26 but the VPS29(V90D)–GFP mutant cannot [18]. These constructs therefore act as positive and negative controls respectively. In Figure 4(A), wild-type GFP–VPS35 is able to co-immunoprecipitate VPS26, but the R107A and L108P mutants fail to interact with VPS26, although the R107A mutant was relatively poorly expressed compared with wild-type GFP–VPS35 or the L108P mutant. Similarly, when antibodies against VPS26 are used to immunoprecipitate the lysates, anti-VPS26 can immunoprecipitate wild-type GFP–VPS35 (along with

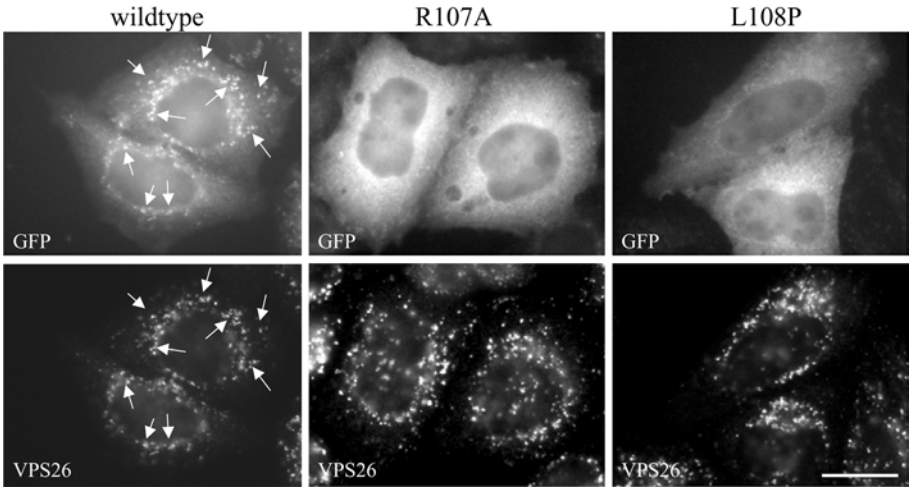

Figure 5 The R107A and L108P mutations in VPS35 prevent recruitment to the endosomal membrane

HeLa cells were transiently transfected with GFP-tagged VPS35 constructs. After fixation, the cells were labelled with antibodies against VPS26. Wild-type VPS35 can be recruited to the membrane to co-localize with VPS26 but the R107A and L108P mutants are only visible in the cytoplasm. Scale bar = 20 μm.

endogenous VPS35) but the R107A and L108P mutants were not detected in these immunoprecipitates (Figure 4B).

The interaction between Vps35p and Vps26p has been shown to be important for the membrane association of Vps35p ([19]; this study, Figure 2B). To determine if the localization of VPS35 to endosomal membranes requires interaction with VPS26, we have investigated the localization of GFP-tagged VPS35 constructs by fluorescence microscopy. In Figure 5(A), wild-type GFP–VPS35 can target to membranes and colocalize with VPS26, but the R107A and L108P mutants are detected only in the cytoplasm. The mutation of the R107 or L108 residues in mammalian VPS35 therefore blocks recruitment of VPS35 to the endosomal membrane.

DISCUSSION

In this study we have identified a conserved region of Vps35p and its mammalian orthologue, VPS35, that is required for the interaction with Vps26p/VPS26. The mutation that confers the dominant-negative phenotype observed in cells expressing the *vps35-27* allele was determined to be a leucine to proline substitution at residue 99, which is in the middle of the conserved motif PRL/MYL. It is worth noting that, of all the Vps35p orthologues present in the database, only the *Arabidopsis* orthologue has the methionine residue instead of the first leucine. All of the other Vps35p orthologues conform to the PRLYL consensus. The *vps35-27* allele was first described in [4] but the site of the mutation(s) that conferred the phenotype was not yet known. The *vps35-27* allele was found to be suppressed by overexpression of *VPS29* [4]. In this study, we have found that the L99P mutation results in a Vps35 protein that can no longer bind to Vps26p. Therefore, the cause of the dominant effect of the *vps35-27* allele is thought to be the titration of Vps29p into inactive complexes, hence overexpression of *VPS29* is able to restore CPY sorting in cells expressing the *vps35-27* allele.

When the L99P *vps35* mutant is expressed at wild-type levels in a *vps35*Δ background, the CPY sorting phenotype is identical to a null mutant. Alanine scanning mutagenesis of the PRLYL motif revealed that the arginine is also critical for Vps35p function, as mutation to alanine also results in a null phenotype. Interestingly, the other alanine scanning mutants were all able to complement the CPY sorting defect in the *vps35*Δ strain, indicating that some residues of the PRLYL motif are more important than others and that the L99P mutation, which confers a null phenotype, may be due to local instability in that region of the Vps35p due to the introduction of a proline instead of leucine. All of the *vps35* alleles were readily detectable, so none of the mutations resulted in a protein that was intrinsically unstable and rapidly degraded.

Loss of retromer function has been shown to result in the mislocalization of the vacuolar hydrolase receptor, Vps10p, to the vacuole [4,5,21]. This is also true for the Vps35(L99P) mutant, which provides the first clue as to the underlying defect in the Vps35(L99P) mutant. When the localization of Vps29p in cells expressing the L99P mutant was examined, it was found to be similar to those cells expressing wild-type Vps35p and not to the *vps35*Δ cells in which Vps29p becomes cytosolic. When the distribution of Vps35p was investigated, it was found that the Vps35(L99P) protein was present in the cytosolic (S100) fraction to a similar degree to that observed in a *vps26*Δ strain. This hints at a defect in the Vps35p–Vps26p interaction.

The loss of the interaction between Vps35p and Vps26p, which was suggested by the data from Figure 2(B), was confirmed by native IP from yeast lysates and by two-hybrid analysis of the interaction between mammalian VPS35 and VPS26. In yeast, the L99P mutant did not assemble into the retromer complex in the IP with antibodies against either Vps5p or Vps26p. Our antiserum against Vps35p does not recognize the protein under native conditions, so it was not possible to immunoprecipitate Vps35p directly to assess the interactions of the L99P mutant. It has been previously demonstrated that Vps26p facilitates the proper assembly of Vps35p/Vps29p with Vps5p/Vps17p [19]. Therefore, the inability of Vps5p to co-immunoprecipitate Vps35p/Vps29p is consistent with a loss of interaction between Vps35p and Vps26p due to the L99P mutation. Similarly, the inability of Vps26p to co-immunoprecipitate any of the other retromer subunits from cells expressing the L99P mutant is in line with the hypothesis that the L99P mutant cannot bind to Vps26p.

The data from the two-hybrid analysis of the interactions between VPS35 and VPS26 or VPS29 strongly agrees with the data obtained from the studies in yeast. Wild-type VPS35 and VPS26 interact strongly and the strength of the interaction is

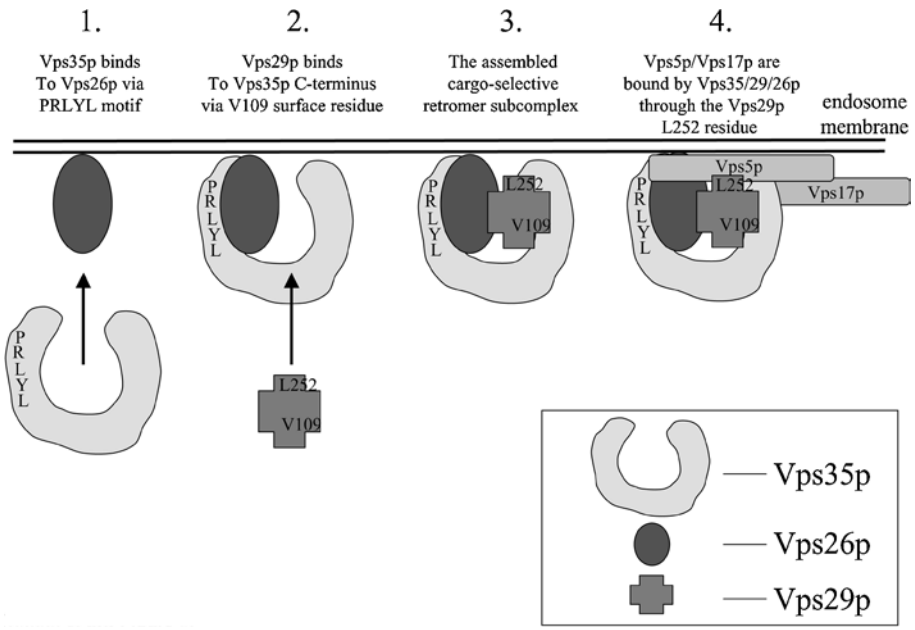

Figure 6 A schematic model depicting key features of the assembly of the retromer complex

Vps35p/VPS35 binds to Vps26p/VPS26 via the PRLYL motif, the arginine and adjacent leucine residues being critical for this binding. Vps29p/VPS29 binds at the C-terminus of Vps35p/VPS35 through a conserved valine (Val^{109} or Val^{90}) on Vps29p/VPS29 [18,23]. Vps29p is necessary for the assembly of the pentameric retromer complex containing Vps5p and Vps17p, and the Leu^{252} residue of Vps29p is essential for this interaction. The assembly of retromer could occur on the endosomal membrane, or the cargo-selective subcomplex (Vps35/29/26p) could assemble in the cytoplasm prior to the recruitment onto the membrane.

resistant to inhibition with 2 mM 3-AT. The L108P and R107A VPS35 mutants were only able to interact weakly with VPS26 and growth of the yeast containing these combinations of bait and prey was inhibited by 2 mM 3-AT. Curiously, the interaction between VPS35 and VPS29 was actually increased due to the R107A mutation. This observation hints that perhaps VPS29 can interact with a region of VPS35 close to where VPS26 interacts.

Native IP experiments with GFP-tagged VPS35 constructs transiently transfected into mammalian cells confirmed that the R107A and L108P mutants do not interact with VPS26 *in vivo*, although the R107A mutant did not efficiently transfect, resulting in lower expression of this mutant than was observed for the L108P mutant. The reason for the relatively poor expression of the R107A mutant is currently unknown but was observed on repeated occasions. One possibility is that the ability of the VPS35(R107A) mutant to interact with VPS29 (see Figure 3B) resulted in this construct being dominant-negative, and therefore toxic, to cells expressing it. Loss of the interaction between VPS35 and VPS26 results in VPS35 being unable to localize to endosomal membranes. Wild-type GFP–VPS35 colocalized with VPS26, whereas the R107A and L108P mutants were present only in the cytoplasm.

In summary, we have identified a critical conserved region of Vps35p/VPS35 that is necessary for the interaction with VPS26. Loss of interaction with Vps26p results in a null phenotype and mislocalization of Vps10p to the vacuole. Mutation of Arg^{107} or Leu^{108} in mammalian VPS35 abolishes the interaction with mammalian VPS26 and results in VPS35 being unable to target to the membrane. We have previously shown that a conserved valine residue on Vps29p/VPS29 is essential for the interaction with Vps35p/VPS35. Mutation of Val^{109} or Val^{90} in Vps29p or VPS29 respectively results in an inability to bind Vps35p/VPS35 and an inability to target to endosomal membranes [18]. With this data in mind we can propose a model of recruitment of the retromer to the membrane that is controlled by Vps26p/VPS26. Through unknown mechanisms (that may require Snx1 and Snx2 in mammalian cells [11]), Vps26p/VPS26 is recruited to endosomal membranes. Vps35p/VPS35 follows and binds Vps26p/VPS26 through the latter's mobile loop [17] and the PRLYL motif in Vps35p/VPS35. Vps29p/VPS29 binds last and this is mediated through the C-terminus of Vps35p/VPS35 [23] and the Val^{109}/Val^{90} residues in Vps29p/VPS29 [18]. It is also possible that Vps35p can interact with Vps29p independently of Vps26p, which has been demonstrated through the use of crosslinking in *vps26*Δ cells [4]. Vps29p is required for the subsequent assembly of the cargo-selective Vps35/29/26p subcomplex with Vps5/17p to create the pentameric retromer complex. In yeast, this involves the Leu^{252} residue of Vps29p which is necessary for this interaction [18]. In this model, the assembly of the retromer on the endosomal membrane is stepwise and ordered and facilitates the retromer in its function of membrane protein sorting for endosome-to-Golgi retrieval.

An alternative model is also possible in which the cargo-selective subcomplex of Vps35/29/26p assembles as a unit prior being recruited onto the membrane. The Vps35p/29/26p subcomplex may then interact with the membrane via multiple weak interactions occurring through the Vps35p and Vps26p subunits, both of which appear able to associate with the membrane (at least partially) independently of the other subunits. Hence, loss of the interaction between Vps35p and Vps26p (due to the L99P mutation) does not (in yeast) result in a complete loss of membrane association. The interactions that govern assembly of the retromer complex are summarized in Figure 6.

Note added in proof (received 19 September 2007)

During the publication of the present paper, an independent study by Steve Nothwehr, Peter Arvan and co-workers has identified the PYLRL motif as critical for retromer assembly [25].

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