Domains within the GARP Subunit Vps54 Confer Separate Functions in Complex Assembly and Early Endosome Recognition

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Tethering complexes contribute to the specificity of membrane fusion by recognizing organelle features on both donor and acceptor membranes. The Golgi-associated retrograde protein (GARP) complex is required for retrograde traffic from both early and late endosomes to the *trans*-Golgi network (TGN), presenting a paradox as to how a single complex can interact specifically with vesicles from multiple upstream compartments. We have found that a subunit of the GARP complex, Vps54, can be separated into N- and C-terminal regions that have different functions. Whereas the N-terminus of Vps54 is important for GARP complex assembly and stability, a conserved C-terminal domain mediates localization to an early endocytic compartment. Mutation of this C-terminal domain has no effect on retrograde transport from late endosomes. However, a specific defect in retrieval of Snc1 from early endosomes is observed when recycling from late endosomes to the Golgi is blocked. These data suggest that separate domains recruit tethering complexes to different upstream compartments to regulate individual trafficking pathways.

INTRODUCTION

Selective transport between organelles requires that transport intermediates be targeted to appropriate downstream compartments. Although the pairing of cognate SNARE proteins on donor and acceptor membranes is critical for membrane fusion, multisubunit tethering complexes contribute to the specificity of vesicle transport *in vivo* by linking transport vesicles to their target membranes in a long-range interaction that may promote SNARE complex formation (reviewed in Whyte and Munro, 2002; Lupashin and Sztul, 2005). Tethering complexes must therefore recognize membrane-associated factors on both the donor and recipient compartment.

Interaction of tethering complexes with activated Rab GTPases is considered important for the initial recruitment of tethers to membranes as well as for subsequent fusion events. Although Rab proteins are important determinants of membrane recognition, a single specificity factor can only be sufficient to explain recruitment to a single compartment. Additional factors implicated in determining organelle identity and membrane recruitment include inositol phospholip-

ids, small GTPases of the Rho, Arf, and Arl families, and vesicle coat components (Munro, 2004). Interactions with such determinants have been demonstrated for both coiled-coil and multisubunit tethering complexes (reviewed in Munro, 2004; Lupashin and Sztul, 2005).

The exocyst, a well-studied tethering complex required for fusion at the cell surface, interacts with active GTPases of the Rab, Rho, and Ral families on secretory vesicles and at the plasma membrane (Guo *et al.*, 1999, 2001; Zhang *et al.*, 2001). Although binding of the exocyst subunit Sec15 to the secretory vesicle Rab protein Sec4 promotes formation of a subcomplex on the vesicle containing six of the eight exocyst subunits, assembly of the full complex occurs only when the vesicle reaches the plasma membrane, where the two remaining subunits are localized by interaction with the Rho-like GTPase Cdc42 (Boyd *et al.*, 2004). However, recent work indicating that Cdc42 restricts the exocyst to sites of polarized growth but does not mediate its association with the plasma membrane *per se* (Roumanie *et al.*, 2005) suggests there is much to be learned about how tethering complexes are recruited to membranes.

Studies in diverse cell types have demonstrated that tethering complexes may first become membrane associated at compartments upstream of the organelle to which they tether vesicles. In mammalian cells, the exocyst components Sec10 and Sec15 have been localized to both the *trans*-Golgi network (TGN) and recycling endosomes (Prigent *et al.*, 2003; Zhang *et al.*, 2004), and in *Drosophila*, the exocyst component Sec5 is found at endocytic coated pits (Sommer *et al.*, 2005). Similarly, the COG complex, which is implicated in tethering vesicles at the *cis*-Golgi that are derived from the ER, later Golgi cisternae, and endosomes, interacts with

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Abbreviations used: CPY, carboxypeptidase Y; GARP, Golgi-associated retrograde protein; TGN, *trans*-Golgi network; VPS, vacuolar protein sorting.

Table 1. Strain descriptions

Strain name	Genotype	Source
BY5563	MAT α <i>his3Δ</i> , <i>leu2Δ</i> , <i>ura3Δ</i> , <i>met15Δ</i> LYS2+ <i>can1Δ::MFA1pr-HIS3</i> , <i>lyp1Δ</i>	Tong and Boone (2005)
BY54 Δ	MATa <i>his3Δ1</i> <i>leu2Δ0</i> <i>met15Δ0</i> <i>ura3Δ0</i> <i>vps54Δ::KanMX4</i>	Open Biosystems
CCY5	MAT α <i>ura3-52</i> <i>leu2-3,112</i> <i>his3-200</i> <i>trp1-901</i> <i>lys2-801</i> <i>suc2-9</i> <i>pho8::ADE2</i> <i>VPS52-myc::HIS5</i> <i>vps54Δ::KanMX4</i>	This study
CCY6	MAT α <i>ura3-52</i> <i>leu2-3,112</i> <i>his3-Δ200</i> <i>trp1-901</i> <i>lys2-801</i> <i>suc2-Δ9</i> <i>pho8Δ::ADE2</i> <i>vps53::TRP1</i> <i>VPS53-myc</i> <i>vps54Δ::KanMX4</i>	This study
CCY7	MAT α <i>ura3-52</i> <i>leu2-3,112</i> <i>his3-200</i> <i>trp1-901</i> <i>lys2-801</i> <i>suc2-9</i> <i>pho8::ADE2</i> <i>VPS51-myc::HIS5</i> <i>vps54Δ::KanMX4</i>	This study
CSY18	MAT α <i>his3Δ</i> , <i>leu2Δ</i> , <i>ura3Δ</i> , <i>met15Δ</i> LYS2+ <i>can1Δ::MFA1pr-HIS3</i> , <i>lyp1Δ</i> <i>VPS52-GFP::URA3</i>	This study
CSY51	MATa <i>his3Δ1</i> <i>leu2Δ0</i> <i>met15Δ0</i> <i>ura3Δ0</i> <i>Vps52-GFP::URA3</i> , <i>vps54Δ::KanMX4</i>	This study
LCY200	MAT α <i>ura3-52</i> <i>leu2-3,112</i> <i>his3-200</i> <i>trp1-901</i> <i>lys2-801</i> <i>suc2-9</i> <i>pho8ΔADE2</i> <i>vps54Δ::TRP1</i>	Conibear and Stevens (2000)
LCY230	MAT α <i>ura3-52</i> <i>leu2-3,112</i> <i>his3-200</i> <i>trp1-901</i> <i>lys2-801</i> <i>suc2-9</i> <i>pho8::ADE2</i> <i>VPS52-myc::HIS5</i>	This study
NQY108	MATa <i>his3Δ1</i> <i>leu2Δ0</i> <i>met15Δ0</i> <i>ura3Δ0</i> <i>vps54Δ::NatMX4</i>	This study
NQY110	MAT α , <i>can1DEL::STE2pr-Sp.Jis5</i> <i>lyp1Δ</i> <i>cyh2</i> , <i>his3Δ1</i> , <i>leu2Δ0</i> , <i>ura3Δ0</i> , <i>met15Δ0</i> , LYS2+, <i>vps54Δ::NatMX4</i>	This study
NQY111	MATa <i>his3Δ1</i> <i>leu2Δ0</i> <i>met15Δ0</i> <i>ura3Δ0</i> <i>Sec7-GFP::URA3</i> , <i>vps54Δ::KanMX4</i>	This study
NQY116	MATa <i>can1Δ::STE2pr-Sp.Jis5</i> <i>lyp1Δ</i> <i>cyh2</i> , <i>his3Δ1</i> , <i>leu2Δ0</i> , <i>ura3Δ0</i> , <i>met15Δ0</i> , LYS2+, <i>vps54Δ::NatMX4</i> ; <i>vps5Δ::KanMX4</i>	This study
NQY113	MAT α <i>ura3-52</i> <i>leu2-3,112</i> <i>his3-200</i> <i>trp1-901</i> <i>lys2-801</i> <i>suc2-9</i> <i>pho8Δ::ADE2</i> <i>vps54Δ::TRP1</i> <i>Ste3DEL365-GFP::HisMX6</i>	This study
Y7096	MAT α , <i>his3Δ1</i> <i>leu2Δ0</i> <i>ura3Δ0</i> <i>met15Δ0</i> LYS2+, <i>can1Δ::STE2pr-Sp.Jis5</i> <i>lyp1Δ</i> <i>cyh2</i>	A. Tong

COPI coats in both yeast and mammalian cells and is involved in sorting cargo at the ER (Morsomme and Riezman, 2002; Suvorova *et al.*, 2002). Association with upstream compartments before vesicle budding may provide part of the mechanism by which multisubunit tethers become incorporated into specific classes of transport vesicle.

The problem of membrane recognition is compounded by the fact that many tethering complexes are responsible for more than one trafficking pathway and must therefore recognize multiple upstream compartments. Mutations within subunits of the exocyst and COG complexes have been identified that are defective for a subset of pathways controlled by these tethers, supporting the idea that tethers participate in different sets of interactions at each organelle (Mehta *et al.*, 2005; Sommer *et al.*, 2005; Zolov and Lupashin, 2005). For example, mutations in *Vps41*, a subunit of the vacuolar tether HOPS, disrupt Golgi to vacuole transport, whereas vacuolar transport from other compartments remains unaffected (Darsow *et al.*, 2001).

The GARP (Golgi Associated Retrograde Protein) complex, (also referred to as the VFT complex) is a member of the “quatrefoil” family of multisubunit tethering complexes that includes the exocyst and COG complexes (Whyte and Munro, 2002). The GARP complex is responsible for tethering vesicles derived from both early and late endosomes at the TGN (Conboy and Cyert, 2000; Conibear and Stevens, 2000; Siniosoglou and Pelham, 2001; Conibear *et al.*, 2003). Mutation of any one of the four GARP subunits—*Vps51*, *Vps52*, *Vps53*, or *Vps54*—impairs the retrieval of the secretory vesicle v-SNARE *Sncl* from early endosomes, and the recycling of the carboxypeptidase Y (CPY) receptor, *Vps10*, from late endosomes (Conibear *et al.*, 2003; Reggiori *et al.*, 2003). Both of these retrograde pathways also require the Rab protein *Ypt6*, which interacts with *Vps52* when in its GTP-bound form (Siniosoglou and Pelham, 2001, 2002). Deletion of *YPT6* prevents the localization of GARP to the TGN, causing it to

become diffusely localized. However, loss of *Ypt6* does not alter the amount of GARP associated with membranes *in vivo* (Conibear *et al.*, 2003). These observations imply that GARP interacts with vesicles or other small, dispersed organelles by *Ypt6*-independent mechanisms. Because GARP acts in retrograde traffic from two classes of endosomes, distinct components of the complex may determine its interactions with different classes of vesicles.

Here we present evidence that the recognition of upstream compartments is a conserved feature of tethering complexes. We identify a conserved C-terminal domain within the GARP subunit *Vps54* that is specifically required for recycling from early, but not late, endosomes. Furthermore, this C-terminal domain is necessary and sufficient for recruitment to a polarized early endocytic compartment. These results suggest that the GARP tethering complex recognizes distinct features of early and late endosomes and is recruited to upstream compartments before or during the budding of transport vesicles. This may ensure that forming vesicles have the full complement of factors to direct subsequent docking with the correct target organelle.

MATERIALS AND METHODS

Strain and Plasmid Construction

Yeast strains used in this study are described in Table 1. *SpeI*-digested pSEC7-EGFPx3 (Rossanese *et al.*, 2001; gift from B. Glick) was used to integrate a C-terminal GFP tag at the *SEC7* locus in strain BY54 Δ , creating NQY111. NQY108 was made by replacing the KanMX module with NatMX in strain BY54 Δ using *EcoRI*-digested p4339 (Tong *et al.*, 2001). To create a chromosomally encoded *Ste3 Δ 365-GFP* mutant, a GFP tag was inserted immediately after residue 365 of *Ste3* in LCY200 (Conibear and Stevens, 2000) by homologous recombination (Longtine *et al.*, 1998). Strain NQY116 was created by the SGA method (Tong *et al.*, 2001) by mating a *vps5 Δ ::KanMX4* strain with a *vps54 Δ ::NatMX* query strain (NQY110) that contained the URA3-based, *VPS54*-complementing plasmid pLC104. Double mutants lacking pLC104 were selected on 5-fluoroorotic acid (5-FOA). LCY230 was created by transformation with pLC89 linearized with *HpaI* (Conibear and Stevens, 2000).

VPS54 was deleted from LCY230, LCY228, and LCY320 (Conibear and Stevens, 2000; Conibear *et al.*, 2003), creating CCY5, CCY6, and CCY7, respectively, by transformation with a PCR fragment amplified from BY4741 vps54Δ::KanMX4 genomic DNA using oligonucleotides homologous to the 5' and 3' UTR of VPS54. CSY18 was created by transforming BY5563 (Tong *et al.*, 2001) with BglII-linearized pVPS52-EGFPx3 (gift from B. Glick). CSY51 was created by mating CSY18 with BY54Δ and selecting for MATa progeny using the SGA method (Tong *et al.*, 2001).

To express the C-terminus of Vps54 under control of the Gal1 promoter, an EcoRV/Sall fragment from pLC104 (Conibear and Stevens, 2000) was ligated to EcoRV-Sall cut p423-GAL1 (Sikorski and Hieter, 1989), creating pCC5. A HpaI-Sall fragment from pLC104 was ligated to EcoRV-Sall-digested p423-GAL1 to create pCC4. To create high copy LEU2 (pCC6) and URA3 (pLC166)-based plasmids expressing the last 296 residues of Vps54 (as well as a C-terminal 3HA tag) from the ADH promoter, pLC104 (Conibear and Stevens, 2000) was digested with XbaI and Sall and subcloned into XbaI/XhoI-cut pVT102L and pVT102U, respectively (Vernet *et al.*, 1987).

The plasmids described below were created by homologous recombination. Briefly, DNA fragments were cotransformed with linearized vector into yeast, and recombinant plasmids were recovered in *Escherichia coli* using standard methods. To create pCC9, encoding full-length, HA-tagged Vps54, VPS54 sequences were amplified from pLC165 using oligos with homology to pVT102L and VPS54 and cotransformed with XbaI-linearized pCC6. A Vps54 truncation mutant lacking the C-terminal 459 residues was created by integration of a 3xHA::KanMX cassette in pLC104, to create pLC165. Sequences encoding the N-terminal region of Vps54 were introduced into pVT102L by cotransformation of a gel-purified 3-kb Bsu36I/Sall-digested fragment of pLC165 with PstI-cut pCC9 to create pCC10. To introduce a GFP tag at the 3' end of the Vps54 C-terminal fragment in pVT102U, GFP sequences were amplified using oligos homologous to 3' end of the Vps54 open reading frame and cotransformed with XbaI-digested pLC166, creating pCC12. pCC13 was generated by cotransformation of a PCR product encoding residues 597–735 of Vps54 followed by an HA-tag encoded in the reverse oligo, with NcoI-digested pCC5 to yield a construct that expresses residues 593–735 followed by an HA-tag.

To create E689A W691A point mutations in Vps54, a forward primer with mismatches at residues E689 and W691 was used to amplify the C-terminal region of Vps54, using pCC9 as a template. This PCR product was cotransformed with HpaI-cut pCC5 to create pCC19 and with HpaI-cut pLC104 to create pCC14. Similarly, an 85-bp reverse primer with mismatches at residues N805 and S806 was used to amplify an 800-bp fragment of VPS54, which was cotransformed with HpaI-cut pLC104 to create pCC15. The quadruple point mutant of Vps54 (Vps54^{EWNS}) was created by amplifying a 500-bp fragment of Vps54 using both forward and reverse mutagenic primers described above. This PCR product was cotransformed with HpaI-cut pLC104 to create pCC16. Oligonucleotide sequences are available upon request.

Immunoprecipitations and Western Blotting

Ten OD₆₀₀ units of log-phase cells were spheroplasted and stored at –80°C as described previously (Graham *et al.*, 1998). Frozen spheroplasts were lysed in 1 ml NP40 buffer (50 mM Tris, pH 8.0, 50 mM NaCl, 2 mM EDTA, 1.2 M sorbitol, 1% NP40, 1% phenylmethylsulfonyl fluoride). Clarified lysates were adjusted to equivalent protein concentrations as determined by Bradford assay (Bio-Rad, Hercules, CA), incubated with rabbit anti-HA antibodies for 2 h at 4°C, and precipitated with protein G-Sepharose (Amersham Biosciences, Piscataway, NJ). After washing in NP40 buffer, immunoprecipitated proteins were released with Thormer Buffer (8 M Urea, 5% SDS, 50 mM Tris, pH 6.8), and subjected to SDS-PAGE. Coimmunoprecipitating proteins were detected by Western blot using standard blotting procedures with mouse anti-myc 4A6 (Upstate, Charlottesville, VA) or mouse anti-HA (12CA5) antibodies followed by HRP-conjugated secondary goat anti-mouse antibody (Bio-Rad). Detection and densitometry was carried out on a Fluoro-S Multi-Imager (Bio-Rad) using Quantity One software. Stability of GARP subunits was determined from yeast cell lysates prepared from spheroplasts as described above. One OD₆₀₀ unit of each lysate was subjected to SDS-PAGE and Western blotting using monoclonal anti-myc 4AC (Upstate). Secretion of CPY was determined using a colony immunoblot assay (Conibear and Stevens, 2002).

Fluorescence Microscopy

Indirect immunofluorescence microscopy was carried out as described (Conibear and Stevens, 2000, 2002) using the following antibodies: cross-absorbed rabbit anti-HA (gift of T. Stevens), mouse anti-HA (HA.11 Covance Research Products, Berkeley, CA), rabbit anti GFP serum (Molecular Probes, Eugene, OR), and anti-Sec4 monoclonal antibody (mAb) C1.2.3 (gift of P. Novick). Secondary antibodies (Molecular Probes) included goat anti-mouse Alexa Fluor 488 or 594 and goat anti-rabbit Alexa Fluor 488 or 594. To follow proteins expressed under the GAL1 promoter, strains were grown overnight in selective media supplemented with 2% raffinose (for GAL induction experiments) or 2% galactose (for steady state expression) and allowed to double twice in YEP-Galactose before fixation and immunofluorescence. Fluorescence microscopy of GFP-expressing strains was performed by growing strains to log phase in selective media, fixing with 4% paraformaldehyde for

20 min at room temperature and resuspending in 1× PBS. Membranes were stained with the styrl dye FM4–64 (Invitrogen, Carlsbad, CA) by incubating cells on ice for 15 min with 40 μM FM4–64 in YPD. Cells were resuspended in pre-warmed YPD, incubated at 30°C for 5 min, and resuspended in 1% NaN₃, 1% NaF, and 100 mM Tris, pH 8.0. Cells were viewed using a Zeiss Axioplan2 fluorescence microscope (Thornwood, NY), and images were captured with a CoolSnap camera using MetaMorph software (Universal Imaging, West Chester, PA) and adjusted using AutoQuant Deconvolution (Watervliet, NY) and Adobe Photoshop (San Jose, CA).

Electron Microscopy

Conventional and immunoelectron microscopy were performed essentially as previously described (Rieder *et al.*, 1996).

Conventional electron microscopy. Briefly, cells were grown in YPD medium at 26°C to an A600 of 0.5, harvested by centrifugation, and resuspended in fix (3% glutaraldehyde, 0.1 M sodium cacodylate, 5 mM CaCl₂, pH 7.4). Cells were fixed for 1 h at 25°C, washed, and resuspended in 1.2 M sorbitol in phos-citrate buffer (0.1 M K₂HPO₄/0.033 M citric acid). The fixed cells were then treated with glucuronidase and zymolyase for 1 h to remove the cell walls. Cells were embedded in 2% ultralow gelling temperature agarose (Sigma, St. Louis, MO; type IX), stained with osmium/thiocarbohydrazide/osmium, en bloc-stained in Kellenberger's UA (o.n.), and subsequently embedded in low viscosity Spurr resin. Sections were poststained with lead citrate and uranyl acetate. Eighty-nanometer sections were cut on a Leica UCT ultramicrotome (Deerfield, IL) and examined on a Philips EM 410 (Mahwah, NJ) at 80 kV. Images were captured with a Soft Imaging System Megaview III camera (Lakewood, CO). Panels were assembled in Adobe Photoshop with only linear adjustments in brightness and contrast.

Immunoelectron microscopy. Briefly, exponentially growing cells (30°C) were fixed in suspension for 15 min by adding an equal volume of freshly prepared 8% formaldehyde in 1× phosphate-buffered saline (PBS), pH 7.4. The cells were pelleted, resuspended in fresh fixative (4% formaldehyde, 1× PBS, pH 7.4), and incubated for an additional 18–24 h at 4°C. The cells were washed briefly in PBS and resuspended in 1% low-gelling-temperature agarose. The agarose blocks were trimmed into pieces of 1 mm³, cryoprotected by infiltration with 2.3 M sucrose/20% polyvinyl pyrrolidone, pH 7.4, for 2 h, mounted on cryo-pins, and rapidly frozen in liquid nitrogen. Ultrathin cryosections were cut on a Leica UCT ultramicrotome equipped with an FC-S cryostage and collected onto formvar/carbon-coated nickel grids. The grids were washed through several drops of 2.5% fetal calf serum (FCS), 1× PBS containing 10 mM glycine (pH 7.4), blocked in 10% FCS for 30 min, and incubated overnight in 20 mg/ml monoclonal anti-HA antibody (Covance Research Products, Richmond, CA), and rabbit anti-actin antibody. After washing, the grids were incubated for 2 h in 5-nm Au donkey anti-mouse and 10-nm Au donkey anti-rabbit conjugates (Jackson ImmunoResearch, West Grove, PA). The grids were washed through several drops of PBS followed by several drops of ddH₂O. Grids were then embedded in an aqueous solution containing 3.2% polyvinyl alcohol (10 K)/0.2% methyl cellulose (400 centipoises)/0.1% uranyl acetate. Sections were examined on a Philips 410 transmission electron microscope at 100 kV. Images were captured with a Soft Imaging System Megaview III camera. Panels were assembled in Adobe Photoshop with only linear adjustments in brightness and contrast.

Ste3 Recycling Assay

Yeast strains were grown to log phase in selective media and were resuspended in YPD to a concentration of 0.75 OD₆₀₀/ml and allowed to grow at 30°C for 1 h. 0.75 OD₆₀₀ units of each strain was removed and 5 mM NaN₃ added for the t = 0 time point. A 0.5 volume of media containing a-factor (prepared as described in Chen and Davis, 2000) was added to the remainder, and cells were incubated at 30°C for 45 min to induce the internalization of Ste3Δ365-GFP. 0.75 OD₆₀₀ units of each strain was removed and 5 mM NaN₃ added for the t = 45 min time point. The remaining cells were washed in YPD and allowed to incubate at 30°C for various time points, at which time 5 mM NaN₃ was added. Each sample was washed in 1× PBS prior to visualization by fluorescence microscopy.

Other Techniques

For inhibitor experiments, log phase cultures were treated at 30°C for 2 h with 5 μg/ml alpha factor or 15 μg/ml nocodazole or for 1 h with 200 μM latrunculin B (Sigma) before being processed for immunofluorescence. FACS analysis was performed as described (Haase and Lew, 1997).

RESULTS

The N-Terminal Domain of Vps54 Is Sufficient for Assembly and Stability of the GARP Complex

Vps54 (Luv1/Tcs3/Cgp1), the largest subunit of the GARP tethering complex, contains several domains that are conserved from yeast to humans, including an N-terminal re-

Figure 1. The N-terminus of Vps54 is important for GARP complex assembly and stability. Plasmids encoding HA-tagged full-length Vps54 (FL; pCC9), its N-terminal region (N; pCC10), or C-terminal region (C; pCC6) were expressed in *vps54Δ* strains containing integrated myc-tagged Vps51 (CCY7, lanes 1–4), Vps52 (CCY5, lanes 5–8), or Vps53 (CCY6, lanes 9–12). (A) Whole cell lysates were subjected to immunoprecipitation under nondenaturing conditions using HA antibodies and copurifying HA- and myc-tagged GARP subunits were detected by Western blot. (B) Steady state levels of GARP subunits in the strains described above were determined by anti-myc Western blotting of whole cell lysates. Protein abundance was determined by densitometry and expressed as a percentage of levels in cells expressing full-length Vps54. Note that the N-terminal region of Vps54 largely stabilizes the other GARP subunits.

gion with a predicted coiled-coil motif proposed to act in complex assembly (Whyte and Munro, 2001; Siniosoglou and Pelham, 2002; Whyte and Munro, 2002). To determine if one portion of the protein is sufficient for assembly and stability of the GARP complex, N-terminal (Vps54-N; residues 1–430) and C-terminal (Vps54-C; residues 593–889) fragments were HA-tagged and coexpressed in strains that lacked endogenous Vps54 but contained functional myc-tagged forms of Vps51, Vps52, and Vps53 (Conibear and Stevens, 2000). By coimmunoprecipitation, we determined the N-terminal domain, but not the C-terminal portion of Vps54, was present in a complex that contained each of the other GARP complex subunits (Figure 1A). These results suggest that the N-terminal region of Vps54 is largely sufficient for complex assembly. Because Vps54-N is less stable than the full-length protein, experiments were carried out with domains expressed from high copy plasmids. Nevertheless, copurification of Vps52 and Vps53 with Vps54-N was slightly reduced compared with full-length Vps54 (Figure 1A, lanes 7 and 11), indicating that the C-terminal domain of Vps54 is not required for assembly of the GARP complex but may contribute to its stability.

With the exception of Vps51, loss of one GARP subunit causes the remaining subunits to be rapidly degraded (Conibear and Stevens, 2000). We used this characteristic to determine if the N-terminal domain of Vps54 was sufficient to stabilize the other components of the GARP complex. By Western blot, steady state levels of Vps52 and Vps53 were found to be ~80% of wild-type levels in strains expressing Vps54-N (Figure 1B). Although Vps52 and Vps53 were still present in cells expressing Vps54-C, steady state levels of each protein was reduced to ~30% of wild type, similar to those seen when Vps54 is absent (unpublished data). The ability of the N-terminal region to stabilize other GARP complex subunits is consistent with the idea that the C-

terminal 459 residues of Vps54 are not required for the formation of a stable core complex.

Truncation of Vps54 Causes a Specific Block in Early Endosome Recycling

To evaluate whether a GARP core complex lacking the C-terminal domain of Vps54 is functional for retrograde transport to the TGN, we analyzed the distribution of the marker proteins GFP-Snc1 and CPY. Wild-type localization of these proteins depends on retrograde transport from early or late endosomes, respectively (Cooper and Stevens, 1996; Lewis *et al.*, 2000; reviewed in Conibear and Stevens, 1998; Figure 2A), and each is mislocalized in GARP deletion mutants (Conibear and Stevens, 2000; Conibear *et al.*, 2003). CPY, a vacuolar hydrolase, is secreted from the cell if its receptor, Vps10, fails to recycle from late endosomes back to the Golgi. Overexpression of Vps54-N reduced the amount of secreted CPY in strains lacking endogenous Vps54, establishing that the GARP core complex is partially functional for late endosome recycling (Figure 2B). Consistent with evidence that the Vps54 C-terminal domain is not sufficient for GARP complex assembly, overexpression of Vps54-C did not reduce the amount of CPY secreted from *vps54Δ* mutants.

The v-SNARE Snc1 maintains its steady state plasma membrane localization by continually recycling through early endosomes and is mislocalized to the vacuole in cells lacking a functional GARP complex (Lewis *et al.*, 2000; Conibear *et al.*, 2003). Consistent with the partial rescue of CPY sorting defects, expression of Vps54-N partially restored the cell surface localization of Snc1 in *vps54Δ* mutants. In these cells, GFP-Snc1 was clearly observed at the plasma membrane at sites of polarized growth and was also detected in the vacuole (Figure 2C).

Defects in the early endosome recycling of Chs3 do not completely block its cell surface localization, because of an alternate retrieval pathway from late endosomes (Valdivia *et al.*, 2002). In wild-type cells, Snc1 does not transit late endosomes and consequently its localization is not affected by loss of VPS5, a subunit of the late endosome-specific retromer complex (Seaman *et al.*, 1998; Lewis *et al.*, 2000). However, Snc1 may also be diverted into a late endosome recycling pathway when early endosome retrieval to the TGN is blocked (see Figure 2A). To test this hypothesis, we evaluated the localization of GFP-Snc1 in *vps54Δ vps5Δ* double mutants expressing the N-terminal domain of Vps54 (Figure 2D). Consistent with previous results, Snc1 localization was unaffected by the *vps5* mutation when full-length Vps54 was present. However, the appearance of Snc1 at the plasma membrane became completely dependent on VPS5 function in cells expressing the C-terminally truncated Vps54 allele. In these cells, Snc1 was localized to structures that may correspond to highly fragmented vacuoles. This suggests that GFP-Snc1 maintains its plasma membrane localization by recycling from late endosomes when it cannot follow a retrograde pathway from early endosomes to the TGN.

Overexpression of Vps54-C was not able to rescue the plasma membrane localization of GFP-Snc1, which was largely missorted to the vacuole. We also observed an unusual intracellular structure localized to sites of polarized growth in cells expressing Vps54-C that was unaffected by the presence or absence of VPS5 (Figure 2, C and D, discussed below). Taken together, our results indicate that the conserved C-terminal domain of Vps54 is largely dispensable for GARP complex assembly and for late endosome recycling, but is required for the retrieval of Snc1 from early endosomes.

Figure 2. C-terminally truncated Vps54 is functional in late endosome to TGN transport, but defective in sorting from early endosomes. (A) GARP is required for retrograde traffic from both early and late endosomes. Vacuolar localization of CPY requires retrograde traffic from late endosomes (LE) as represented by the dotted line, whereas plasma membrane localization of Snc1 requires retrograde traffic from early endosomes (EE) as represented by a dashed line. Vps5, a subunit of the retromer, is specifically required for LE-to-TGN transport. (B) Vps54-N partially rescues the CPY sorting defect of *vps54Δ*. Secreted CPY was detected by colony overlay assay from 1/1000 OD₆₀₀ units of *vps54Δ* cells (BY54Δ) transformed with plasmids to express wild-type Vps54 (pCC9), Vps54-N (pCC10), Vps54-C (pCC6), or vector alone (pVT102L). (C) Overexpressing the N-terminal region of Vps54 partially restores localization of Snc1 to the plasma membrane. Snc1-GFP expressed from a CEN plasmid (pCS07) in the strains described in B was visualized by fluorescence microscopy. (D) The ability of Vps54-N to localize Snc1-GFP to the plasma membrane requires late endosome-to-TGN transport. Snc1 was coexpressed with truncated forms of Vps54 in *vps54Δ vps54Δ* double mutants (NQY116) using plasmids described above.

The C-Terminal Domain of Vps54 Localizes to a Polarized Intracellular Compartment

The GARP complex is localized to the yeast TGN at steady state and appears as 3–5 spots by immunofluorescence microscopy (Conibear and Stevens, 2000; Siniosoglou and Pelham, 2001). Consistent with our finding that the GARP core complex is partially functional for retrograde transport to the Golgi, expression of Vps54-N completely restored the TGN localization of GFP-Vps52 in *vps54Δ* cells (Figure 3A). We have previously observed that HA-tagged Vps54 is much more difficult to visualize than Vps52-HA by fluorescence microscopy despite being present in the GARP complex in 1:1 stoichiometry, suggesting the HA epitope is masked when Vps54 is assembled in a complex. Accordingly, both full-length and truncated forms of Vps54 appeared hazy and cytosolic when overexpressed, even though a substantial proportion of each protein was associated with high-speed membranes by subcellular fractionation (unpublished data). Interestingly, the C-terminal domain of Vps54 localized to 1–2 punctate structures with a polarized distribution in both *vps54Δ* and wild-type strain backgrounds (Figure 3B). This localization pattern, which is atypical of the TGN, was present in ~20% of the cell population in *vps54Δ* but was less frequently observed in wild-type backgrounds. Overexpressed full-length Vps54 also localized to a similar structure in ~5% of the cell population.

The localization of Vps54-C varied throughout the cell cycle, roughly corresponding to sites of polarized growth

(Figure 4A). This polarized pattern is reminiscent of the early endosomal marker protein Rcy1 (Galan *et al.*, 2001). As seen for Rcy1, treatment with the actin inhibitor latrunculin B resulted in the mislocalization of Vps54-C-labeled structures, which became more numerous and dispersed (Figure 4B). Treatment of MATa yeast cells with the alpha factor pheromone causes G1 cell cycle arrest and the formation of mating projections referred to as shmoo. Like Rcy1, Vps54-C repolarized to shmoo tips after alpha factor treatment (Figure 4C). After treatment with the tubulin inhibitor nocodazole, which disrupts microtubules and results in G2/M cell cycle arrest, single Vps54-C-labeled structures were seen at the bud neck of large-budded cells. This indicates that the polarized localization of Vps54-C requires an intact actin cytoskeleton, but not microtubules.

Yeast early endosomes are not morphologically well defined. Although some early endosome markers display a polarized localization similar to that seen with Vps54-C, early endosome markers such as Tlg1 localize to a compartment that is virtually indistinguishable from the TGN, whereas other putative early endosome markers are found in smaller, more numerous, puncta (Vida and Emr, 1995). Furthermore, a number of organelles have been reported to display polarized distributions in *Saccharomyces cerevisiae* (Rossanese *et al.*, 2001; Chen *et al.*, 2005). Therefore, a more detailed characterization of the Vps54-C compartment is required to determine its identity.

Figure 3. Differential localization of N- and C-terminal domains of Vps54. (A) Vps54-N, but not Vps54-C, directs the localization of Vps52-GFP to the TGN. The *vps54Δ* strain (CSY51), which contains an integrated copy of Vps52-GFP, was transformed with plasmids for the expression of HA-tagged full-length (pCC9), N-terminal (pCC10) or C-terminal (pCC6) forms of Vps54 and cells were viewed by fluorescence microscopy. (B) Vps54-C localized to a polarized structure. *vps54Δ* cells (BY54Δ) expressing the Vps54 plasmids described in A, as well as wild-type cells (BY4741) expressing Vps54-C (pCC6), were fixed, labeled with a monoclonal anti-HA antibody followed by an FITC-conjugated secondary antibody, and observed by fluorescence microscopy.

Vps54-C Localizes to an Early Endocytic Compartment

The polarized structure that labeled with Vps54-C was strikingly similar to the Snc1-containing compartment previously observed in cells expressing high levels of the Vps54 C-terminal domain (Figure 2, C and D). When Vps54-C was coexpressed with GFP-Snc1 in a strain lacking endogenous Vps54, 100% of the structures that were positive for Vps54-C also contained Snc1 (Figure 5A).

The trafficking pathway followed by GFP-Snc1 has been well described: GFP-Snc1 is internalized from the plasma membrane and first delivered to early endosomes before

being recycled to the TGN to be incorporated into newly forming secretory vesicles (Lewis *et al.*, 2000). To determine if the expression of Vps54-C slows the passage of Snc1 through an organelle that is part of its normal recycling itinerary, we examined the colocalization of the Vps54-C structure with established markers of the TGN, secretory vesicles, and endocytic compartments. The late Golgi marker Sec7-GFP localized to numerous punctate structures that showed no overlap with Vps54-C (Figure 5B). The secretory vesicle Rab protein, Sec4, has been shown to have a polarized localization pattern due to the accumulation of

Figure 4. The C-terminal domain of Vps54 localizes to sites of polarized growth throughout the cell cycle. (A–C) HA-tagged Vps54-C (pCC6) was visualized by immunofluorescence microscopy in fixed *vps54Δ* cells (BY54Δ). (A) Representative images of unbudded cells or cells with small, medium, or large buds show polarization of Vps54-C through the cell cycle. (B) Localization of Vps54-C to sites of polarized growth was abolished by treatment with the actin inhibitor latrunculin B. (C) Treating cells with the mating pheromone alpha-factor induced G1 arrest and the relocation of Vps54-C to shmoo tips. Treatment with the microtubule inhibitor nocodazole induced G2/M arrest but did not disrupt the cortical localization of Vps54-C. Cell cycle arrest was confirmed by FACS analysis of DNA content (shown on the right). Peaks corresponding to 1n, 2n, and 4n DNA content are indicated.

Figure 5. Vps54 C-terminus colocalizes with early endocytic markers but not markers of late Golgi or secretory vesicles. (A) Snc1-GFP localizes to a punctate polarized structure that colocalizes with the HA-tagged C-terminus of Vps54. (B) Vps54-C does not colocalize with the late-Golgi marker Sec7-GFP in NQY111 cells. (C) Vps54-C and Sec4 colocalize at the bud tip but not in the mother cell. Treatment with latrunculin B disperses polarized staining patterns and abolishes the colocalization of Vps54-C with Sec4 (D) but not that of Snc1-GFP and Vps54-C (E). (F) Vps54-C colocalizes with the styryl dye FM4-64 after a short chase. *vps54Δ* cells (BY54Δ) expressing GFP-tagged Vps54-C (pCC12) were allowed to take up FM4-64 for 5 min at 30°C after labeling the plasma membrane for 15 min on ice and viewed by fluorescence microscopy of unfixed cells. (G) Vps54-C partially but consistently colocalizes with Rcy1-GFP (arrows). *vps54Δ* cells expressing GFP-tagged Rcy1 from the GAL1 promoter (pJMG95) and coexpressing HA-tagged Vps54-C from a plasmid (pCC6) were visualized by double label indirect immunofluorescence microscopy after 8 h of galactose induction. In A–C, E, and G, epitope tags were visualized by double label indirect immunofluorescence microscopy using anti-HA and anti-GFP antibodies. In C and D, the localization of Sec4 was visualized using a mAb directed against Sec4.

secretory vesicles at sites of cell growth (TerBush and Novick, 1995). Indeed, Sec4 was present at bud tips in newly budding cells, where it partially colocalized with Vps54-C (Figure 5C). Vps54-C was often found in two puncta, one near the bud neck of the mother and the other near the tip of the new bud. In these cells, Sec4 colocalized only with Vps54-C present in the new bud.

Vps54-C may be localized to two intracellular compartments, both of which contain Snc1, but only one of which corresponds to secretory vesicles. It is also possible that Vps54-C, Snc1, and Sec4 are associated with different compartments that are independently targeted to sites of polarized growth. To test this hypothesis, we took advantage of the observation that the polarized localization of these organelles depends on the actin cytoskeleton (Ayscough *et al.*, 1997). After a 60-min treatment with Lat B, Sec4-containing secretory vesicles dispersed throughout the cell in finely punctate pattern (Figure 5D). In contrast, Vps54-C and Snc1 colocalized in structures that were larger, less numerous and clearly distinct from secretory vesicles (Figure 5E).

To visualize early endosomal compartments, GFP-tagged Vps54-C was expressed in the *vps54Δ* strain and cells were incubated with the lipid dye FM4-64 for 5 min at 30°C (Figure 5F). FM4-64 clearly labeled Vps54-C compartments after this brief incubation, in addition to numerous small, dispersed organelles similar to previously described early endosomes (Vida and Emr, 1995). Full-length Vps54 has previously been found to colocalize with FM4-64 (Conboy and Cyert, 2000). We also performed indirect immunofluorescence microscopy on *vps54Δ* strains coexpressing Rcy1-GFP under the control of a galactose-inducible promoter (Galan *et al.*, 2001) together with HA-tagged Vps54-C. Rcy1-GFP and Vps54-C-HA were rarely visible in the same cells. However, when both signals were present, the proteins consistently colocalized (Figure 5G). The overlap was not always precise, consistent with reports that Rcy1 is found in part on Sec7-containing Golgi compartments (Chen *et al.*, 2005). Taken together, these data suggest that Vps54-C localizes to an early endocytic compartment that is accessible to Snc1, FM4-64, and a proportion of Rcy1.

Figure 6. Morphology of Vps54-C-expressing cells by electron microscopy. (A–C) Clusters of irregular 100–200-nm organelles in the bud and in the mother cell near the mother-bud junction seen in representative cells from a *vps54Δ* strain (LCY200) expressing Vps54-C (pCC6) but not vector alone are indicated with an asterisk. (D) Enlarged view of structures that are boxed in C. (E) Immunoelectron microscopy of the same strain double-labeled with anti-HA antibodies conjugated to 5-nm gold particles, and anti-actin particles conjugated to 10-nm gold particles. HA staining is found on clustered membrane profiles (closed arrowheads) and on the limiting membrane of vesicular profiles (arrows). Scale bars, 0.20 μ m.

By conventional electron microscopy, clusters of 100–200-nm irregularly shaped membrane compartments were observed in or near the small buds of cells expressing Vps54-C that were not found in control strains (Figure 6). These compartments, which have luminal content of varying densities and appear to contain internal membranes, were frequently

found in two distinct groupings within the bud and near the bud neck of the mother cell, reminiscent of the pattern seen by fluorescence microscopy. Double indirect immunoelectron microscopy performed on cryo-sections indicated that Vps54-C was associated with clusters of membranes near the bud neck that were often close to, but not coincident with, patches of actin. Vps54-C appeared to be associated with the limiting membrane of vesicular compartments (Figure 6E, arrows) but was more frequently found along disordered membrane profiles that may correspond to tubular elements (arrowheads). These observations suggest that Vps54-C localizes to aberrant compartments that may correspond to enlarged, clustered early endosomes.

Point Mutations within the C-terminal Region of Vps54 Prevent Localization to the Polarized Early Endosome and Block Early Endosome Recycling

The C-terminal region of Vps54 contains two conserved stretches of amino acids that may form independent subdomains (Figure 7A). When expressed individually, neither region was sufficient for endosome localization (Figure 7B). However, alanine substitution of residues E689 and W691, which lie within the first conserved region, completely abolished localization of Vps54-C to the polarized structure. These residues are invariant in species ranging from yeast to man, suggesting that the C-terminus of Vps54 engages in conserved interactions that are important for recruitment to early endosomes.

Point mutations that abolish the localization of Vps54-C might also be expected to disrupt the early endosome trafficking function of full-length Vps54. However, we did not observe an appreciable change in GFP-Snc1 plasma membrane localization when the corresponding mutant, Vps54^{EW}, was expressed at endogenous levels in either *vps54Δ* or *vps54Δ vps5Δ* strains (Figure 8A). Because redundant interactions contribute to the membrane association of other tethering factors, two highly conserved residues in the second subdomain of Vps54-C, N805 and S806, were also changed to alanine. The resulting quadruple mutant form of Vps54

Figure 7. Conserved residues in the C-terminus of Vps54 are required for localization to the polarized, early endocytic structure. (A) Alignment of Vps54 from *S. cerevisiae* with homologues in *Schizosaccharomyces pombe* (O14093), *Drosophila melanogaster* (Q9VLC0), *Caenorhabditis elegans* (Q22639), and human (NP_057600) showing evolutionarily conserved regions in the C-terminal domain. Gray shading shows conservation across 50% of sequences, whereas black shading indicates more highly conserved or invariant residues. Arrows indicate residues that were mutated to create Vps54^{EW} or Vps54^{EWNS}. Sequence alignment was generated with T-coffee (Notredame *et al.*, 2000) (B) *vps54Δ* strains (BY54Δ) expressing either Vps54-C (pCC5), truncated forms that each contain one of the conserved regions in the C-terminus (Vps54-C⁵⁹³⁻⁷³⁵, pCC13; Vps54-C⁷²⁴⁻⁸⁸⁹, pCC4), or Vps54-C harboring point mutations (Vps54-C^{EW}, pCC19) from a galactose-inducible promoter were grown to log phase in galactose, fixed, and processed for immunofluorescence microscopy.

Figure 8. Point mutations in the C-terminal Vps54 domain abolish sorting from early endosomes without affecting late endosome to TGN transport. (A) The early endosome-sorting defect of Vps54^{EWNS} is apparent in cells deficient in late endosome recycling. Vps54 (pLC104) and the indicated mutant forms (Vps54^{EW}, pCC14; Vps54^{NS}, pCC15; Vps54^{EWNS}, pCC16; Vps54-N, pCC10) were coexpressed with Snc1-GFP (pCS07) in *vps54Δ vps5Δ* (NQY116) double mutant strains or in a *vps54Δ* single mutant (BY54Δ). Snc1-GFP was visualized by fluorescence microscopy of fixed cells. Neither the E689A W691A nor N805A S806A mutations alone were sufficient to destroy the early endosome sorting function of Vps54, but plasma membrane localization of Snc1 was abolished in *vps54Δ* strains expressing the Vps54 quadruple mutant. (B) The Vps54^{EWNS} mutant remains functional for late endosome-to-TGN transport. CPY secretion was determined by a colony overlay assay for *vps54Δ* strains (BY54Δ) expressing wild-type and mutant forms of Vps54 as described in A.

(Vps54^{EWNS}), when expressed from its endogenous promoter, was present at the same level as wild-type Vps54 (unpublished data). Furthermore, this mutant fully complemented the CPY and Snc1 sorting defects of a *vps54Δ* strain (Figure 8B). Strikingly, the Vps54 quadruple mutant caused the localization of Snc1 to become completely dependent on retrograde trafficking from late endosomes. In *vps54Δ vps5Δ* cells expressing Vps54^{EWNS}, Snc1 was no longer observed at the cell surface and instead was mis-sorted to fragmented vacuolar compartments (Figure 8A), a result that is similar to that seen for Vps54-N. However, Vps54-N was only partially functional for late endosome retrieval even when expressed at high levels, whereas Vps54^{EWNS} was stable and fully functional for late endosome retrieval when present at endogenous levels. These data demonstrate that Vps54 contains a conserved determinant that is specifically required for retrograde trafficking from the early endosome.

GARP Is Not Required for Endocytic Recycling of the α -Factor Receptor Ste3

Ste3, the receptor for the mating pheromone α -factor, undergoes both constitutive and ligand-induced endocytosis through separable mechanisms (Chen and Davis, 2000). Truncating the C-terminal 105 residues of Ste3 blocks constitutive endocytosis but not ligand-stimulated uptake (Chen and Davis, 2000). In the presence of ligand, Ste3 Δ 365

enters early/recycling endosomes that may appear as single polarized structures in the mating projections of highly polarized shmoo. It was recently shown that a truncation mutant of the exocyst component Sec5 is defective for endocytic recycling of the pheromone receptor Ste3 but remains competent for Snc1 recycling and for exocytosis (Sommer *et al.*, 2005). This suggests that the exocyst has an additional role at an early endosome trafficking step that does not affect Snc1.

To determine if GARP is required for the recycling of Ste3 Δ 365 from the yeast early/recycling endosome, we expressed either full-length Vps54 or its N-terminal domain in a *vps54Δ* strain in which endogenous Ste3 was truncated by integrating a GFP tag (Figure 9). As previously described, Ste3 Δ 365GFP was present on both the plasma membrane and the vacuole in the absence of pheromone and was redistributed to intracellular structures after α -factor treatment to stimulate endocytosis. Removal of α -factor induced the rapid redistribution of Ste3 Δ 365GFP to the plasma membrane. However, the ability of Ste3 Δ 365GFP to recycle back to the cell surface was unaffected by expression of Vps54-N or -C terminal domains, or even by complete loss of Vps54 (Figure 9; unpublished data). The finding that endocytic recycling of Ste3 does not require GARP-dependent retrograde transport to the TGN raises the possibility that Snc1 and Ste3

Figure 9. Vps54 is not required for recycling of Ste3 Δ 365-GFP. (A) *vps54* Δ Ste3 Δ 365-GFP (NQY113) cells expressing full-length Vps54 (pCC9), empty vector, or Vps54-C (pCC6) were visualized by fluorescence microscopy before treatment with a-factor ($t = 0$), immediately after a 45-min incubation with a-factor ($t = 45$ min, a-factor) and 30 min after removing a-factor from the media ($t = 30$ -min wash). Ste3 Δ 365-GFP is recycled from endosomes back to the plasma membrane in both wild-type (Vps54) and *vps54* Δ cells after the 30-min wash at 30°C. Expression of Vps54-C does not impair Ste3 Δ 365-GFP recycling or affect its intracellular localization.

may transit different classes of early endosomes during their transport.

DISCUSSION

GARP is a multisubunit tethering complex that directs the Golgi targeting of retrograde vesicles derived from both early and late endosomes. We find that mutations within a conserved C-terminal domain of the GARP subunit Vps54 specifically abolish retrograde trafficking from early endosomes to the Golgi, without affecting transport on the late endosome-to-Golgi recycling pathway. Furthermore, this C-terminal domain of Vps54 is necessary and sufficient for association with an organelle that has the characteristics of an early endosome. These results suggest that separate domains within the GARP tethering complex dictate recruitment to upstream compartments (Figure 10). The incorporation of tethering factors into budding transport vesicles

may ensure that newly formed vesicles contain targeting information to specify their ultimate destination.

Assembly of the GARP Complex

Members of the quatrefoil family of tethering complexes, which includes COG, the exocyst, and GARP, are related by conserved sequences in their N-termini that include predicted amphipathic helices thought to be important for complex formation (Whyte and Munro, 2002). The predicted amphipathic helix within the N-terminal portion of Vps54 was previously shown to be necessary, but not sufficient, for GARP assembly (Siniossoglou and Pelham, 2002). Here, we find that a larger N-terminal region consisting of 430 amino acids that includes this helical motif is sufficient to direct the formation of a GARP core complex. In contrast, a C-terminal domain containing the last 296 aa of Vps54 is largely dispensable for complex assembly, but directs the recruitment of Vps54 to early endosomes. Computer modeling based on Ginzu domain predictions (Robetta; Chivian *et al.*, 2003; Kim *et al.*, 2004) indicate that the N- and C-terminal domains may be separated by a long central portion with significant homology to myosin heavy chain (unpublished results). This arrangement may position the C-terminal domain at some distance from the central core region where it would be accessible to membrane recruitment factors.

Recruitment of GARP to Upstream Compartments

At steady state, many yeast tethering complexes are localized to the compartment at which they function in docking and fusion (TerBush and Novick, 1995; Conibear and Stevens, 2000; Whyte and Munro, 2001). Such observations led to early models that tethering complexes act as a “molecular fly paper” at the target membrane to trap specific classes of transport vesicles (Pfeffer, 1996). Our data support an alternate model (Figure 10), that tethering complexes associate with transport vesicles at the time of their formation and may even contribute to cargo sorting. The localization of the C-terminal domain of Vps54 to an early endosomal compartment suggests that GARP itself may become incorporated into forming transport vesicles as they bud from the donor compartment. Furthermore, Snc1 does not appear to accumulate in transport vesicles in a *Vps54*^{EWNS} mutant but is instead missorted to late endosomes, suggesting that loss of functional GARP complex impairs either the sorting of Snc1 into retrograde vesicles at the early endosome or formation of the vesicles themselves. However, missorting at the early endosome may be a secondary defect resulting from mislocalization of critical sorting factors in GARP mutants.

There is evidence that the processes of tether recruitment and vesicle formation are tightly coupled for other tethering

Figure 10. Model of Vps54 function in retrograde transport from early endosomes. (A) In wild-type cells, distinct domains within the GARP complex target the complex to retrograde vesicles budding from early and late endosomal membranes. GARP subsequently directs the tethering of these vesicles to the TGN through interactions with Rab and SNARE proteins (not illustrated). (B) Loss of the Vps54-C domain (dark gray appendage) specifically blocks GARP complex recruitment to early endosomes, resulting in the rerouting of Snc1 to late endosomes (light gray arrow), where it is retrieved via the retrograde pathway used by Vps10 (gray arrow). According to this model, a separate, as-yet-unidentified domain (light gray appendage) recruits GARP to late endosomes.

factors. Vps41, a subunit of the HOPS tethering complex involved in vacuole fusion, associates with AP-3-coated vesicles destined to fuse with the vacuole through an interaction between an N-terminal region of Vps41 and the AP-3 subunit Apl5 (Darsow *et al.*, 2001). Mutations that block the interaction of Vps41 with the AP-3 adaptor complex also block the formation of the AP-3-coated transport intermediate. Other tethers have also been shown to interact with vesicle coat proteins. The polarized cell-specific adaptor coat protein AP-1B may play a role in the recruitment of at least two subunits of the exocyst complex to transferrin receptor positive endosomes (Folsch *et al.*, 2003).

What recruits Vps54-C to the polarized early endosome? By analogy with other tethering complexes, Vps54 may interact with components of a vesicle coat that is required specifically for the retrograde sorting of Snc1. However, coat proteins specific for early endosome retrieval have not been characterized. The candidate coat protein Snx4 is likely involved in retrieval of Snc1 at both early and late endosomes, because Snc1 is predominantly missorted to the vacuole in its absence (Hettema *et al.*, 2003). Furthermore, Snx4 and its interacting partners Snx41 and Snx42 are found at the late endosome/MVB where they colocalize with Snf7 (Huh *et al.*, 2003). Gene deletions of other candidates such as Ypt6 and Arl1 (Siniosoglou and Pelham, 2001; Panic *et al.*, 2003), which are known GARP interactors, and Arl3, which is required for localization of Arl1, do not prevent association of Vps54-C with the polarized endocytic compartment (unpublished results). Therefore, further work will be required to identify the binding partner of the Vps54-C domain.

Although mutation of only one of the two conserved regions within the Vps54 C-terminal domain was sufficient to prevent the localization of the Vps54-C domain to early endosomes, mutation of both regions was required to block early endosome retrieval *in vivo*. Both may contribute to a single functional interface that retains only partial function in *Vps54^{EW}* mutants. Alternatively, Vps54 may have two binding motifs that interact redundantly with determinants at the early endosome membrane. In fact, the ER tethering factor Dsl1 interacts with two different subunits of the COPI coat through independent binding sites, and knockout of both motifs is required for complete loss of COPI binding *in vitro* and of loss of function *in vivo* (Andag *et al.*, 2001).

Early Endosome Recycling Pathways

Irregular 100–200-nm organelles were observed by electron microscopy only in *Vps54-C*-expressing cells, suggesting that the overexpression of this domain induces the expansion of a normally dynamic early endosomal compartment. However, GFP-tagged forms of other proteins involved in early endosome recycling, including Gyp2 and Rcy1, are observed in similar structures when expressed at endogenous levels (Galan *et al.*, 2001; Lafourcade *et al.*, 2003), implying that a morphologically similar early endosome also exists in wild-type cells.

A truncation mutant of the exocyst component Sec5 was previously shown to be impaired in recycling the α -factor receptor, Ste3, from early endosomes but was not defective for Snc1 sorting (Sommer *et al.*, 2005). We observe the opposite phenotype for a truncated *vps54* mutant: a defect in the recycling of Snc1, but not Ste3. Snc1 may recycle from the early endosome to the Golgi, a pathway that requires GARP, whereas Ste3 may be sorted directly to the plasma membrane. Alternatively, the plasma membrane recycling of Snc1 and Ste3 could involve different endocytic compartments that are not well characterized in yeast but which may be analogous to the sorting and recycling endosomes of

mammalian cells. Recently, human homologues of GARP complex subunits were shown to form a complex that localizes to the TGN and to a perinuclear region described as the recycling endosome (Liewen *et al.*, 2005). Because the C-terminal region of Vps54 is evolutionarily conserved, the interactions that direct the recruitment of GARP to early endosomes are likely to be present in higher cells.

Because late endosomes are thought to mature from early endosomes (for review see Maxfield and McGraw, 2004), it was conceivable that GARP might interact with a targeting determinant common to both. The discovery of a domain with a specific role in retrograde transport from early endosomes indicates that the GARP complex is likely to recognize distinct determinants on each of the two classes of endosomes and implies that another domain mediates retrograde transport from late endosomes. Further work will be directed toward identifying additional functional domains in the remaining GARP subunits. How tethering complexes are able to recognize and distinguish multiple membranes is important to our understanding of how organelle identity is determined. Our observations support a model for tethering complex function whereby distinct regions of the complex recognize features of each membrane with which it interacts.

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