Grd19/Snx3p functions as a cargo-specific adapter for retromer-dependent endocytic recycling

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A major function of the endocytic system is the sorting of cargo to various organelles. Endocytic sorting of the yeast reductive iron transporter, which is composed of the Fet3 and Ftr1 proteins, is regulated by available iron. When iron is provided to iron-starved cells, Fet3p–Ftr1p is targeted to the lysosome-like vacuole and degraded. In contrast, when iron is not available, Fet3p–Ftr1p is maintained on the plasma membrane via an endocytic recycling pathway requiring the sorting nexin Grd19/Snx3p, the pentameric retromer complex, and the Ypt6p Golgi Rab GTPase module. A recycling signal in Ftr1p was identified and found to bind directly to Grd19/Snx3p. Retromer and Grd19/Snx3p partially colocalize to tubular endosomes, where they are physically associated. After export from the endosome, Fet3p–Ftr1p transits through the Golgi apparatus for resecretion. Thus, Grd19/Snx3p functions as a cargo-specific adapter for the retromer complex, establishing a precedent for a mechanism by which sorting nexins expand the repertoire of retromer-dependent cargos.
In mammals, retromer is composed of hVps26, hVps29, hVps35 (Haft et al., 2000), and the Bin/Amphiphysin/Rvs (BAR) domain-containing proteins Snx1 and Snx2 (Carlton et al., 2004). Retromer also functions in several trafficking pathways, including transcytosis of the polymeric immunoglobulin receptor (Verges et al., 2004) and retrieval of cargo, such as the cation-independent mannose-6-phosphate receptor from late endosomes back to the Golgi (Arighi et al., 2004; Seaman, 2004). Interestingly, recent studies in Caenorhabditis elegans have also implicated retromer function in establishing long-range Wnt signaling gradients, expanding the role of retromer to include developmental patterning (Coudreuse et al., 2006; Prasad and Clark, 2006). Thus, evidence indicates that retromer participates in endocytic sorting of multiple distinct cargos, but it is not yet known how such diverse cargo is selected by retromer.

The retromer components Vps5p and Vps17p (as well as the mammalian homologues Snx1 and Snx2) are members of the sorting nexin family of proteins, or SNXs, which has been broadly implicated in sorting within the endosomal system (Teasdale et al., 2001; Worby and Dixon, 2002; J. Carlton et al., 2005). This protein family is characterized by the presence of a SNX-PX (phox homology) domain that binds preferentially to phosphatidylinositol-3-phosphate (PtdIns3P), which is a lipid enriched on endosomal membranes (Seet and Hong, 2006). Currently, 28 mammalian and 10 yeast sorting nexin proteins have been identified (Worby and Dixon, 2002; Carlton and Cullen, 2005), and most of the data regarding the functions of these proteins in yeast points to their role in endocytic sorting. Snx4p, Snx41p, and Snx42p, for instance, form a complex that retrieves Snclp from early endosomes (Hettema et al., 2003). Yeast cells that lack Grd19p (the yeast homologue of human Snx3) or cells that lack retromer mislocalize Ste13p and Kex2p to the vacuole (Nothwehr and Hindes, 1997; Voos and Stevens, 1998). In addition, Vps5p, Vps17p, and Mvp1p (another sorting nexin), have been shown to be essential for retrieval of Vps10p from late endosomes (Ekene and Stevens, 1995; Horazdovsky et al., 1997). Thus, there is evidence supporting the notion that these sorting factors cooperate to fulfill their functions in protein retrieval and recycling.

In S. cerevisiae, sorting decisions within the endosomal system have been shown to regulate the abundance, stability, and localization of various nutrient transporters. These sorting decisions are generally based on cues regarding the availability of particular nutrients. The general amino acid permease Gap1p, for instance, is highly expressed at the plasma membrane when cells are grown in media containing a poor nitrogen source, but is rapidly internalized when high concentrations of amino acids are present (Chen and Kaiser, 2002). Similarly, the uracil permease is highly internalized when high concentrations of amino acids are present (Blondel et al., 2004). We have used the yeast high-affinity iron transporter (composed of the proteins Fet3p and Ftr1p) as a model to investigate endocytic sorting events in response to a limiting concentration of extracellular iron. We demonstrate that the plasma membrane localization of Fet3p–Ftr1p in the absence of iron is maintained by endocytic recycling and that the sorting nexin Grd19p mediates this recycling via interactions with both Ftr1p and the retromer complex.

Results

Retromer and Grd19/Snx3p are required to maintain Ftr1p-GFP at the plasma membrane via endocytic recycling

The high-affinity reductive iron transporter in S. cerevisiae is composed of two proteins, Fet3p and Ftr1p (Askwith et al., 1994; Stearman et al., 1996), whose biosynthesis and localization are regulated by the concentration of extracellular iron. When iron is limiting in the growth medium, the protein complex is highly expressed and is localized to the plasma membrane. However, when the extracellular concentration of iron is high, transcription ceases and the protein complex undergoes regulated endocytosis and delivery to the lysosome-like vacuole, where it is degraded (Felice et al., 2005). When cells are grown in media containing intermediate concentrations of iron (10–100 μM), transcription of FET3 and FTR1 is barely detectable, yet the abundance of the Fet3 and Ftr1 proteins remains high and the proteins are localized to the plasma membrane (Felice et al., 2005). As the abundance of several other nutrient transporters is regulated by protein sorting within the endosomal–lysosomal system (Blondel et al., 2004; Rubio-Texeira and Kaiser, 2006), we hypothesized that the localization and stability of Fet3p and Ftr1p might be at least in part, to recycling of endocytosed Fet3p–Ftr1p. According to this hypothesis, Fet3p–Ftr1p are internalized constitutively and are recycled back to the plasma membrane under low-iron conditions; if they were not recycled, they would be delivered to the vacuole and degraded. To test our hypothesis, a GFP tag was integrated upstream of the stop codon of the FTR1 locus in a collection of yeast deletion mutants with known defects in protein trafficking within the endocytic system (Table S1, available at http://www.jcb.org/cgi/content/full/jcb.200609161/DC1), and this collection was visually screened to identify mutants in which Ftr1p-GFP was partially localized to the vacuole when cells were grown in iron-deficient medium. This screen identified nine genes required to restrict Ftr1p-GFP localization to the plasma membrane under these conditions (Fig. 1 A). Five of these genes, VPS5, VPS17, VPS26, VPS29, and VPS35, encode subunits of retromer, which is a protein complex that has been implicated in the trafficking of cargo proteins from endosomes to the Golgi (Seaman et al., 1997, 1998). Another gene, GRD19/SNX3, encodes a member of the sorting nexin family of proteins, and it has also been implicated in trafficking from endosomes to the Golgi (Voos and Stevens, 1998). The other three genes, YPT6, RIC1, and RGP1, encode components of the Ypt6 Golgi Rab GTPase module that functions at the Golgi in endosome-to-Golgi trafficking pathways (Fig. S1 A; Siniossoglou et al., 2000; Bensen et al., 2001). In all of these mutants, Fet3p-GFP was also mislocalized to the vacuole (Fig. S1 B). We have focused here on investigating the roles of Grd19p and retromer in endocytic recycling.

In principle, Grd19p and retromer could be functioning in one of two ways to localize Ftr1p to the plasma membrane under iron-limiting conditions. They could act in the biosynthetic pathway to deliver Fet3p–Ftr1p to the plasma membrane, or they could function after Fet3p–Ftr1p has been delivered to the
plasma membrane to maintain them there. These models can be distinguished experimentally because in the latter model endocytosis would be required for delivery of Ftr1p-GFP to the vacuole in the mutants. To test this, double mutants were constructed in which the endocytosis-defective end4-1 allele was combined with a retromer gene deletion, vps17Δ, and Ftr1p-GFP localization was determined. Cells were grown in iron-deficient medium, and whole-cell extracts were prepared and immunoblotted with an anti-GFP antibody and an antibody to Pgk1p (3-phosphoglycerate kinase) which served as the loading control. AU, arbitrary units. The amount of Ftr1p-GFP detected from the native FTR1 locus were grown in iron-deficient medium and analyzed by fluorescence microscopy. (B) Quantitative immunoblot analysis of steady-state levels of Ftr1p-GFP in wild-type (WT) cells and the indicated deletion mutants. Cells were grown in iron-deficient medium, and whole-cell extracts were prepared and immunoblotted with an anti-GFP antibody and an antibody to Pgp1p (3-phosphoglycerate kinase) which was used as a loading control. ECF was used to detect the antibodies, and the ECF signals were quantified using ImageQuant software and normalized to the loading control. AU, arbitrary units. The amount of Ftr1p-GFP detected from the wild-type strain was set to 1.0, and the means and SDs for each of the indicated strains were calculated from three independent experiments.

The C-terminal cytoplasmic tail of Ftr1p is required for endocytic recycling

Because endocytic recycling signals are not well characterized, we sought to design a systematic strategy to identify those features of Fet3p and Ftr1p that are responsible for directing endocytic recycling. We designed a novel assay based on the trafficking itinerary of the vacuolar protein sorting receptor, Vps10p, which cycles between endosomes and the Golgi to mediate the sorting of cargo proteins to the vacuole (Marcussen et al., 1994; Cooper and Stevens, 1996). After delivery of Vps10p–cargo complexes to the late endosome, retrieval of Vps10p back to the Golgi requires retromer, which recognizes a retrieval signal in the C-terminal cytoplasmic tail of Vps10p (Nothwehr et al., 1999). Removal of the cytoplasmic tail results in vacuolar delivery and degradation of Vps10p, and, as a result, cargos normally destined for the vacuole, such as carboxypeptidase Y (CPY) are instead secreted (Cereghino et al., 1995; Cooper and Stevens, 1996). Because retromer is required for the stability of both Vps10p and Fet3p–Ftr1p, we reasoned that Fet3p–Ftr1p contains at least one endosome-to-Golgi sorting signal. This served as the basis of an assay aimed at identifying Fet3p–Ftr1p sequences, which can restore function to a Vps10p mutant lacking the cytoplasmic tail. We focused our analysis on Ftr1p because experiments, which are described in the following sections, indicated that Fet3p does not contain any post-Golgi sorting signals. Ftr1p contains four cytoplasmic portions; three of these are loops connecting membrane-spanning segments and the fourth is a C-terminal tail (Fig. 2A). The third loop of Ftr1p, connecting the fifth and sixth membrane-spanning segments, was not investigated because it is predicted to consist of, at most, four amino acids. Fusion proteins in which the cytoplasmic tail of Vps10p was replaced with sequences encoding the remaining cytoplasmic regions of Ftr1p were constructed and integrated at the endogenous VPS10 locus. All constructs also contained 13 copies of the Myc epitope tag (13xMyc) at the C terminus. As controls, full-length Vps10p was tagged with a 13xMyc tag at its C terminus, and a deletion mutant that removed the entire cytoplasmic domain of Vps10p was constructed by integrating a 13xMyc epitope tag after Arg1421 (Fig. 2B).

Two different methods were used to analyze the functionality of the Vps10p-Ftr1p chimeric proteins relative to the quantitative immunoblotting of cell extracts to determine steady-state levels of Ftr1p-GFP in all of these strains, in addition to the double mutant strains grd19Δ vps5Δ and grd19Δ vps29Δ. In all the retromer and grd19 mutant strains, the amount of Ftr1p-GFP was significantly reduced compared with a wild-type control extract, and the end4-1 mutation substantially restored levels of detected Ftr1p-GFP. These results indicate that, in these mutants, Ftr1p-GFP must first be delivered to the plasma membrane to be subsequently delivered to the vacuole and implicate retromer and Grd19p in a recycling pathway that maintains Fet3p–Ftr1p on the plasma membrane. These results further suggest that retromer and Grd19p are likely functioning together and do not act independently or in a sequential manner, as the Grd19p-retromer double mutants have reduced steady-state levels of Ftr1p-GFP that are essentially identical to the single mutants.
control constructs. Vps10p mutants that are not recycled, such as Vps10pΔtail, are delivered to the vacuole and degraded, resulting in low steady-state levels of the protein (Cereghino et al., 1995; Cooper and Stevens, 1996). Therefore, we compared the steady-state levels of the Vps10p-Ftr1p fusion proteins to full-length Vps10p and Vps10pΔtail in a wild-type strain and a pep4Δ strain that lacks the predominant vacuolar protease, and thereby demonstrates that this Ftr1p truncation mutant is trafficked properly to the plasma membrane and vacuole lumen, although mis-sorting of this internal deletion mutant was not as severe as the Ftr1pΔ318 mutant (Fig. 3 A), suggesting that other portions of the Ftr1p C-tail may also play a role in recycling. Importantly, none of the truncated proteins accumulated in the ER, indicating that their folding and association with Fet3p was not grossly affected by removal of these sequences.

To interpret these results from the perspective of endocytic sorting signals, it was important to distinguish if the Ftr1pΔ318-GFP–truncated protein was being targeted to the vacuole via endocytosis or the biosynthetic route. We again made use of the end4-1 strain to determine the localization of the Ftr1pΔ318-GFP construct under iron-starvation conditions. In the end4-1 strain, Ftr1pΔ318-GFP was localized exclusively to the plasma membrane (Fig. 3 A), and these results were confirmed by immunoblotting of cell extracts. Less Ftr1p was detected when the cytoplasmic tail of the protein was truncated, and levels of the truncated protein were stabilized in the end4-1 strain, as well as in a pep4Δ strain (Fig. 3 B). The results indicate that this Ftr1p truncation mutant is trafficked properly to the plasma membrane, but it is not maintained there. Together, these results demonstrate that aa 319–328 of Ftr1p are important for endocytic recycling.
The delivery of newly synthesized Fet3p and Ftr1p to the plasma membrane depends on their coexpression, and iron-induced down-regulation of Fet3p parallels Ftr1p, indicating that these two proteins traffic together throughout the endomembrane system (Stearman et al., 1996; Sato et al., 2004; Felice et al., 2005). To determine if the aberrant trafficking of Ftr1p truncation mutants also impacts the trafficking of Fet3p, we examined the localization of Fet3p-GFP in the context of the Ftr1p-tail truncation. We integrated a GFP tag on Fet3p in strains expressing full-length Ftr1p or Ftr1p\(^{Δ318}\) and determined the localization of Fet3p-GFP under iron-limiting conditions by fluorescence microscopy. Fet3p-GFP was restricted to the plasma membrane when coexpressed with full-length Ftr1p, but it was also localized to the vacuole lumen (and a very minor fraction in the ER) when coexpressed with Ftr1p\(^{Δ318}\) (Fig. 4). In a strain with a deletion of most of the Fet3p cytoplasmic tail (deletion of the last 45 amino acids), Ftr1p-GFP was localized to the ER and the plasma membrane (Fig. 4), as expected from a previous study (Singh et al., 2006), suggesting that the Fet3p cytoplasmic tail is required for efficient ER export, but does not influence post-ER trafficking. However, in a strain with deletions of both the Fet3p and Ftr1p C-terminal tails (C-tails), both proteins were localized to the ER, plasma membrane, and the vacuole lumen (Fig. 4), indicating that once exported from the ER, the complex was sorted to the vacuole in the absence of the Ftr1p recycling signal. Collectively, the results indicate that a region of the C-terminal cytoplasmic tail of Ftr1p (aa 319–328; GHLFPTKLNQ) is necessary for endocytic recycling of both Fet3p and Ftr1p under iron-depleted conditions.

Grd19p binds to the Ftr1p recycling signal

We next sought to address whether any of the endosomal sorting factors identified in the initial screen recognize the Ftr1p recycling signal. Previous work has shown that epitope-tagged Grd19p present in cell extract, but not the retromer subunit Vps5p, can be captured by a GST-Ste13p fusion protein containing the entire Ste13p cytoplasmic domain (Voos and Stevens, 1998). We first tested binding of purified recombinant Grd19p to a panel of GST fusion proteins containing each of the cytoplasmic regions of Fet3p and Ftr1p, as well as several smaller portions of the Ftr1p C-tail (Fig. 5 A). Grd19p bound to the Ftr1p C-tail, but not any of the other cytoplasmic portions of Ftr1p or Fet3p. Remarkably, Grd19p also bound to a GST fusion protein consisting of only aa 319–328 of Ftr1p, but did not bind to the Ftr1p C-tail deletion mutant lacking these residues, narrowing the site of Grd19p interaction to aa 319–328 of Ftr1p. A recent study demonstrated that the C-terminal tails of Fet3p and Ftr1p physically associate via a region that includes the Grd19p binding site of Ftr1p (Singh et al., 2006), and we therefore tested if binding of Grd19p to the Ftr1p cytoplasmic tail in vitro is influenced by coinoccubation with the Fet3p cytoplasmic tail, revealing that it was not (unpublished data). Finally, we also tested if the retromer complex binds to the recycling signal in the Fet3p–Ftr1p complex using cell lysate prepared from a strain expressing Vps29p- myc; however, no Vps29p was captured by any of the GST fusion proteins (unpublished data). Although this does not rule out that retromer does not bind Ftr1p, all of the binding experiments indicate that Grd19p specifically recognizes the Fet3p–Ftr1p endocytic recycling signal.
only a very small proportion of Ftr1p-GFP resides within endosomal compartments (Fig. 1 A). To circumvent this issue, we overexpressed the indicated GST-Ftr1p C-tail fusion proteins or GST alone in a yeast strain expressing an endogenous epitope-tagged mutant form of Grd19p, Grd19p-R81A-myc (Fig. 5 B). Arginine81 lies within the highly conserved PtdIns(3)P-binding pocket in the PX domain of Grd19p, and mutation of this residue to alanine results in mislocalization of the protein to the cytosol (Fig. S2 A, available at http://www.jcb.org/cgi/content/full/jcb.200609161/DC1). Cells grown in synthetic media were converted to spheroplasts, the bifunctional membrane-permeable chemical cross-linker dithiobis succinimidyl propionate (DSP) was added before lysis, and the GST fusion proteins were captured on glutathione-Sepharose beads. The purified material was then probed with antibodies to Grd19p-R81A-myc. The results demonstrate that Grd19p-R81A was only coprecipitated from cells expressing the GST-Ftr1p C-tail (315–404) fusion protein, but not from cells expressing the GST-Ftr1p C-tail construct, which lacks the putative endocytic recycling signal. These results indicate that Grd19p can bind the Ftr1p C-tail in cells and further confirms that this interaction requires aa 319–328 of Ftr1p.

Retromer and Grd19p colocalize on tubular endosomes

The most straightforward model to explain the dual requirement for Grd19p and retromer in Fet3p–Ftr1p sorting is that they function together at a common sorting step, and this predicts that some portion of Grd19p and retromer colocalize on endosomes. To test this, we constructed strains that expressed functional, C-terminally tagged Grd19p-GFP and Vps17p-RFP, and then imaged the entire volume of these cells by spinning disc confocal microscopy. Three-dimensional reconstructions and two-dimensional maximum projections were generated from the image z stacks (Fig. 6 and Video 1). The majority of labeled organelles contained both Grd19p-GFP and Vps17p-RFP, although some organelles were labeled by only one of the tagged proteins. The three-dimensional representations allowed us to characterize the morphology of the endosomes labeled by Grd19p and Vps17p, many of which had a distinct tubular shape. Retromer proteins have been localized to the tubular domains of early sorting endosomes in cultured mammalian cells (Zhong et al., 2002; Arighi et al., 2004; Carlton et al., 2004; Seaman, 2004; J.G. Carlton et al., 2005), and this is thought to reflect their roles in a geometric sorting mechanism that segregates integral membrane proteins from lumenal content (Maxfield and McGraw, 2004). Furthermore, human Snx3 is also enriched on the tubular domains of endosomes (Xu et al., 2001). Our results clearly indicate that a substantial proportion of Grd19p and the retromer subunit Vps17p colocalize on tubular endosomes, suggesting that they may function together, although the exclusive presence of each protein on some endosomes indicates that they function autonomously as well.

We also tested the idea that retromer might be required for association of Grd19p with endosomal membranes, as many phosphoinositide-binding modules require other interactions for stable association with intracellular membranes (Lemmon, 2003).
Grd19p consists of a PtdIns(3)P-binding PX domain with a short N-terminal extension (Zhou et al., 2003). PtdIns(3)P-binding is critical for endosomal targeting and function, as the R81A point mutant resulted in missorting of Ftr1p-GFP to the vacuole (Fig. S2 B). We examined localization of Grd19p-GFP in \textit{vps29} \textit{Δ} cells and found that it still localized to endosomes, although the compartments decorated by Grd19p appeared smaller and more numerous (Fig. S2 C). We conclude that retromer does not influence recruitment of Grd19p to endosomes from the cytosol, but it may influence the cellular distribution of Grd19p-positive endosomes, and these results further indicate that Grd19p and retromer are targeted to endosomes independently.

In the course of these experiments, we also determined the relative localization of Vps17p to another sorting nexin, Snx4p, which functions in a retromer-independent early endosome-to-Golgi pathway (Hettema et al., 2003) and is not required for Fet3p–Ftr1p recycling. Although yeast retromer is considered to function solely in late endosome-to-Golgi trafficking pathways, a substantial degree of colocalization between Snx4p-GFP and Vps17p-RFP was observed (Fig. 6). These observations suggest that the localization of retromer in the yeast endocytic system is more widespread than currently appreciated. In addition, we visualized fluorescently labeled Grd19p and Snx4p within the same cell (Fig. 6). Grd19p-GFP- and Snx4p-RFP-labeled organelles exhibited little to no colocalization, indicating that Grd19p and Snx4p are localized almost exclusively to distinct subsets of endosomes. This is consistent with published studies reporting that these two sorting nexins function in retrieval pathways from different populations of endosomes (Hettema et al., 2003).

\textbf{Co-purification of retromer and Grd19p from cell extracts}

Based on the results so far, we hypothesized that Grd19p and retromer may physically cooperate, with Grd19p linking cargo recognition to retromer-dependent export from endosomes. To test this, we purified epitope-tagged Vps29p retromer subunit from a cell lysate and determined if Grd19p copurified with it. Strains were constructed that expressed 3xHA epitope-tagged Vps29p and Grd19p tagged with a 13xMyc epitope, and as a control, Vps29p-3xHA and another retromer subunit, Vps17p, tagged with a 13xMyc epitope. Cells were converted to spheroplasts and lysed, and Vps29p was immunopurified under native conditions, and the precipitates were probed with antibodies to Grd19p-myc. Under these conditions, no Grd19p-myc copurified with Vps29p-HA, but Vps17p-myc did (unpublished data). However, when DSP was added to intact spheroplasts before lysing the cells, both Grd19p and Vps17p copurified with Vps29p (Fig. 7). As a control for this experiment, we used a strain that simultaneously expressed Vps29p-HA and Snx4p-myc and found that Snx4p did not copurify with retromer (Fig. 7), despite the fact that substantial amounts of Snx4p and retromer colocalized on endosomal membranes. Furthermore, cross-linking depended on colocalization of Grd19p and retromer on endosomes as the Grd19p-R81A mutant, which is localized to the cytosol (Fig. S2 A), reporting that these two sorting nexins function in retrieval pathways from different populations of endosomes (Hettema et al., 2003).

\textbf{Figure 6.} Retromer and Grd19p partially colocalize on tubular endosomal membranes. Live cells coexpressing endogenous C-terminally tagged Grd19p-GFP and Vps17p-RFP, Snx4p-GFP and Vps17p-RFP, and Grd19p-GFP and Snx4p-RFP were imaged by spinning disc confocal microscopy. Images acquired using the individual red and green channels were then merged to determine extent of colocalization. Shown are two-dimensional maximum projections of the entire cell volumes.

\textbf{Figure 7.} Grd19p copurifies with retromer after chemical cross-linking. Strains expressing the indicated epitope-tagged proteins were grown in synthetic medium and converted to spheroplasts, and DSP was added to a final concentration of 5 mM. The cells were lysed, and Vps29p-HA was captured on anti-HA beads. The cross-links were disrupted by reduction in SDS sample buffer, and the samples were probed by immunoblotting with anti-myc (top) or anti-HA antibodies (bottom). Note that the 9E10 (anti-myc) antibody detects a faint 55-kD background band that migrates just above Grd19p-myc, and that Vps17p-myc recovered by immunoprecipitation runs ~10 kD below Vps17p-myc detected in whole-cell extract. T, 2% total (whole-cell extract); IP, immunoprecipitates.
did not cross-link to retromer (Fig. 7). These results indicate that Grd19p and retromer interact in vivo on PtdIns(3)P-containing endosomes.

Discussion

Our results suggest that the sorting nexin Grd19/Snx3p functions as a cargo-specific accessory component of the retromer complex, which is required for endocytic recycling of the Fet3p–Ftr1p iron transporter. In support of this model, we report that in null mutants of grd19 and each of the five retromer subunits, Fet3p–Ftr1p is missorted to the vacuole when cells are grown under conditions that favor recycling, that Grd19p directly binds a sequence in Ftr1p required for endocytic recycling, that Grd19p-GFP and Vps17p-RFP colocalize substantially on tubular endosomes, and that Grd19p can be chemically cross-linked to the retromer complex in vivo. Although the full range of functions and the specific mechanisms by which retromer operates in membrane trafficking have not been elucidated, it is clear that it is a general endosomal sorting factor required for the proper sorting and export of a diverse set of cargo molecules from endosomes. The work presented in this study is relevant for understanding how cargo is identified by retromer, and the results suggest that the repertoire of retromer-dependent cargos is extended by its interaction with Grd19p. We speculate that Grd19p functions with retromer in a manner analogous to vesicle coat protein adapters that link cargo selection to coat protein recruitment.

Recycling of Fet3p–Ftr1p is likely to be initiated through recognition of Ftr1p by Grd19p because the PX domain of Grd19p binds PtdIns(3)P with the highest affinity of all the PX domains encoded in the yeast genome (Yu and Lemmon, 2001). In contrast, the PX domains of the retromer subunits Vps5p and Vps17p bind PtdIns(3)P with at least 100-fold lower affinity than Grd19p (Yu and Lemmon, 2001). Preliminary analysis of the binding interaction between Grd19p and the Ftr1p recycling signal by surface plasmon resonance indicates that the affinity is relatively weak (Kd between 10 and 100 μM; unpublished data), which is consistent with PtdIns(3)P binding providing the driving force for recruitment of Grd19p to endosomes. Because the abundances of Grd19p and Vps17p appear to be similar (Fig. 7), Grd19p is expected to preferentially accumulate on early endosomes containing relatively low amounts of PtdIns(3)P. Retromer will subsequently load onto endosomes as they accrue higher levels of PtdIns(3)P during maturation, facilitated by interactions with other factors, such as Grd19p and cargo molecules. In this manner, Grd19p could serve as both a coincidence sensor that detects the presence of Fet3p–Ftr1p on PtdIns(3)P-containing endosomes and as an adaptor that recruits retromer to cargo to initiate export from the endosome. Grd19p and retromer appear to be sufficient for export from the endosome because we have not observed any recycling defects in other known cargo-sorting factors, including the clathrin adapter AP-1 complex, other sorting nexins, or the recently identified GSE–EGO complex involved in endosome-to-plasma membrane sorting of the general amino acid permease Gap1p (Gao and Kaiser, 2006; Table S1). Once exported from the endosome, Fet3p–Ftr1p is probably delivered to the Golgi for resecretion because deletion of the Golgi Rab GTPase Ypt6p and its regulators also results in defective recycling, and because the Ftr1p C-terminal tail can direct Vps10p from the endosome back to the Golgi.

In yeast, five proteins have been identified that are sorted via the Grd19p–retromer pathway. These include native proteins that cycle between the Golgi and endosomes, Ste13p, Kex2p, and Pep12p (Nothwehr et al., 2000; Hettema et al., 2003), and Fet3p–Ftr1p, which uses the Grd19p–retromer pathway to be sorted back to the plasma membrane. The Golgi retrieval signals in Ste13p and Kex2p contain key aromatic residues, although their relevance to Grd19p-mediated sorting is not clear because Grd19p (supplied in a cell extract) still bound well to the cytoplasmic tail of Ste13p, even when the aromatic residue-based retrieval signal had been deleted (Voos and Stevens, 1998). Consistent with this, mutation of the single aromatic residue within the Ftr1p recycling signal (Phenylalanine323 to Alanine) did not affect recycling (unpublished data), so a more systematic analysis of this signal is required to identify its key features. It is also interesting that vacuolar targeting of the Ftr1p mutant lacking the Grd19p binding site (Ftr1pΔ319-328) was not so robust, perhaps implying that other sorting determinants are present within the C-terminal tail of Ftr1p. Inasmuch as the available data suggest that the sequences of the signals which confer Grd19p- and retromer-dependent trafficking are diverse, Grd19p probably recognizes structural features of cargo proteins rather than a strict linear amino acid sequence.

Importantly, our results showing that Grd19p directly recognizes Ftr1p and Ste13p and links them to retromer establish for the first time how a sorting nexin (other than Snx1) and retromer cooperate to recognize cargo. Although direct interactions between cargo and any subunit of retromer have not yet been confirmed using purified proteins, the current view posits that cargo is recognized by Vps35p, although other sorting nexins, including human Snx1, also have the capacity to bind cytoplasmic regions of some endocytic cargo proteins (Wang et al., 2002). Moreover, the recent discovery that Vps26 has an arrestin-like structure raises the possibility that Vps26p, like arrestins, may also interact with cargo (Shi et al., 2006). Another possible function of retromer is suggested by the crystal structure of Vps29, which has a protein fold resembling that of phosphoesterases (Collins et al., 2005; Wang et al., 2005), and recent studies have shown that Vps29p exhibits protein phosphatase activity (Damen et al., 2006). Regardless of the specific functions of the individual retromer subunits, it is clear that multiple cargo recognition mechanisms must contribute to the general function of retromer in endosomal sorting because Grd19p is not required for all retromer-dependent trafficking.

In mammalian cells, the early endosomal system is comprised of vacuolar domains connected to an extensive network of tubules, which are enriched in integral membrane cargo proteins that are subsequently sorted to a variety of different organelles (Bonifacio and Rojas, 2006). On the basis of the large surface area/volume ratio of tubes compared with spherical structures, it has been proposed that the packaging of integral membrane proteins into tubes is a highly efficient geometry-based mechanism for segregating membrane and lumenal components (Helenius et al., 1983; Marsh et al., 1986; Geuze et al., 1987).
Human retromer appears to be involved in this sorting mechanism through its cargo-binding activities and the ability of the BAR domain—containing Snx1 subunit to sense regions of high membrane curvature (Carlton et al., 2004). The role of this geometric sorting mechanism in yeast is not known due, in large part, to the difficulty in visualizing cargo within domains of yeast endosomes by light microscopy. However, visualization of yeast endosomes by electron microscopy has provided clear evidence that a subset of them is indeed tubular in shape (Prescianotto-Baschong and Riezman, 1998; Quenneville et al., 2006). These results support the notion that retromer-mediated sorting in yeast and mammalian cells involves the same mechanisms, and they open the door to evaluating the roles of various retromer proteins and auxiliary factors, such as Grd19p in the biogenesis of these organelles. We expect that the interaction between Grd19p and retromer in yeast holds true for the human orthologues, as this interaction could explain the observation that overexpression of human Snx3 in cultured cells leads to a huge expansion of tubular early endosomal compartments through enhanced recruitment of the BAR domain—containing Snx1 component of retromer (Xu et al., 2001).

The results presented in this work demonstrate that recognition of recycling protein cargo by retromer can be initiated by a sorting nexin (Grd19p) that functions as an adaptor to link cargo to the cellular recycling machinery. With the identification of the endocytic recycling machinery and insight into how it mediates recycling of Feh3p–Pfr1p, the opportunity now exists to explore how, in response to changes in extracellular iron concentration, the iron transporter is channeled into either recycling or degradative pathways.

Materials and methods

Yeasts, media, and growth conditions

Unless otherwise indicated, all yeast strains were constructed by integration using recombination of gene-targeted, PCR-generated DNAs using the method of Longtine et al. (1998) to ensure expression from the native loci. The strain background expressing the endA1 allele is YEp6210 (MAFa ura3-52, his3-200, trp1-901, lys2-801, sul2-9, and leu2-3) and the strain background for yeast strains expressing Ftr1p-Vps10p chimeric proteins is BHY10 (SEY6210 [MAT a leu2-3, trp1-901, lys2-801, ::pBHY11 [CPY-Inv 13xMyc]) to check the presence of the GFP tag by microscopy or the 13Xmyc tag by immunoblotting. Growth on plates that lacked histidine, for G418 sensitivity, and for the selectable marker, was amplified from genomic DNA of strain TSY30 (FTR1::13xMyc-HIS3MX6), and the PCR DNA was used to transform a BamHI–SalI fragment and cloned into vector pRS416. Site-directed mutagenesis (QuikChange Site-Directed Mutagenesis kit [Stratagene]) was used to generate the R81A mutation in this plasmid, and this mutation was confirmed by sequencing. A GRD19:KANMX strain was then cotransformed with the BamHI–SalI fragment released from this plasmid along with GFP::HIS3MX or 13xmyc::HIS3MX PCR DNA, with flanking sequences targeted to the GRD19 ORF (Longtine et al., 1998). Transforms were screened for growth on plates that lacked histidine, for G418 sensitivity, and for the presence of the GFP tag by microscopy or the 13xMyc tag by immunoblotting using the 9E10 antibody.

Antibodies and reagents

Enzymes used in DNA manipulations were purchased from New England Biolabs or Promega. Standard molecular biological and microbiological techniques were used. Repetitive PCR DNAs were used as templates for PCR-generated DNAs used in these studies included: 9E10 anti-myc [1:10,000; University of Pennsylvania Cell Center], anti–human α-T (1:1,000; Covance), anti–PGK (1:10,000; Invitrogen), anti–T7 (1:10,000; Novagen), anti–CPY (1:1,000; Invitrogen), and anti–GFP (1:2,000; Covance). Secondary sheep anti–mouse HRP-conjugated antibodies (GE Healthcare) were used at 1:5,000.

CPY secretion assay

This assay is based on the method of Conibear and Stevens (2002), with the following exceptions. Filters were incubated with AP-conjugated goat anti–mouse IgG (1:1,000; Sigma), with two PCR DNAs; one encoding the indicated sequences. These PCR DNAs were then sequenced to confirm the constructs. Control strains to compare the functionality of the Vps10-Ftr1p fusion proteins included a Vps10p truncation mutant in which a 13xMyc-epitope tag was integrated after codon 1,421 of the VPS10 ORF, and full-length Vps10p tagged on its C terminus with the 13xMyc epitope. Both control strains were constructed using transformation of PCR-generated DNAs using pAAda-13xMyc-HIS3MX6 as the template (Longtine et al., 1998).

Strains expressing mutant and tagged integrated copies of Grd19p were constructed as follows. The GRD19 ORF including promoter and terminator regions was amplified by PCR as a BamHI–SalI fragment and cloned into vector pRS416. Site-directed mutagenesis (QuikChange Site-Directed Mutagenesis kit [Stratagene]) was used to generate the R81A mutation in this plasmid, and this mutation was confirmed by sequencing. A GRD19::KANMX strain was then cotransformed with the BamHI–SalI fragment released from this plasmid along with GFP::HIS3MX or 13xmyc::HIS3MX PCR DNA, with flanking sequences targeted to the GRD19 ORF (Longtine et al., 1998). Transforms were screened for growth on plates that lacked histidine, for G418 sensitivity, and for the presence of the GFP tag by microscopy or the 13xMyc tag by immunoblotting using the 9E10 antibody.

Protein expression and purification

GST fusion proteins were constructed by amending the indicated sequences by PCR as BamHI–SalI fragments using wild-type genomic DNA as a template. The amplified products were cloned in-frame into the corresponding restriction sites of vector pGEXAKGKAN (Novagen) for expression in baculovirus or vector pEG(KG) [Mitchell et al., 1993] for expression in yeast. All constructs were sequenced to ensure that no mutations were present.

For bacterial expression, plasmids were transformed into E. coli BL21 (DE3; Novagen). Expression was induced by the addition of 1 mM IPTG at 37°C for 3 h. Cell pellets were resuspended in lysis buffer (PBS [1 mM KH2PO4, 10 mM Na2HPO4, 137 mM NaCl, and 2.7 mM KCl] pH 7.4 saturated with 4 mM DTT) and lysed by sonication.
Table S1 displays the yeast deletion mutants screened for mislocalization of Fe(III)-GFP in retromer and grd19∆mutant cells, as well as the localization of Ffr1p-GFP and Fet3p-GFP in cells deleted for components of the Ypt6p GTPase module. Figure S2 shows the cytotoxic localization of Grd19p-R81A-GFP, the localization of Ffr1p-GFP in cells expressing wild-type Grd19p vs. Grd19p-R81A, and the localization of Grd19p-GFP in vps29∆ cells. The video displays a three-dimensional reconstruction of live cells coexpressing Grd19p-GFP and Vps17p-RFP.
Ekena, K., and T.H. Stevens. 1995. The Saccharomyces cerevisiae MVP1 gene interacts with VPS1 and is required for vacuolar protein sorting. Mol. Cell. Biol. 15:1671–1678.

Felice, M.R., I. De Domenico, L. Li, D.M. Ward, B. Bartok, G. Musci, and J. Kaplan. 2005. Post-transcriptional regulation of the yeast high affinity iron transport system. J. Biol. Chem. 280:22181–22190.

Galan, J.M., A. Wiederkehr, J.H. Sro1, R. Hauenger-Taspis, R.J. Deshaies, H. Riezman, and M. Peter. 2001. Skp1p and the F-box protein Roc1p form a non-SCF complex involved in recycling of the SNARE Snap1p in yeast. Mol. Cell. Biol. 21:3105–3117.

Gao, M., and C.A. Kaiser. 2006. A conserved GTPase-containing complex is required for intracellular sorting of the general amino-acid permease in yeast. Nat. Cell Biol. 8:657–667.

Geuze, H.J., J.W. Slot, and A.L. Schwartz. 1987. Membranes of sorting organelles display lateral heterogeneity in receptor distribution. J. Cell Biol. 104:1715–1723.

Haft, C.R., M. de la Luz Sierra, R. Bafford, M.A. Lesniak, V.A. Barr, and S.I. Taylor. 2000. Human orthologs of yeast vacuolar protein sorting proteins Vps26, 29, and 35: assembly into multimeric complexes. Mol. Biol. Cell. 11:4105–4116.

Helenius, A., I. Mellman, D. Wall, and A. Hubbard. 1983. Endosomes. Trends Biochem. Sci. 78:245–250.

HetteMA, E.H., M.J. Lewis, M.W. Black, and H.R. Pelham. 2003. Retromer and the sorting nexin SNc14/41/42 mediate distinct retrieval pathways from yeast endosomes. EMBO J. 22:548–557.

Horazdovsky, B.F., B.A. Davies, M.N. Seaman, S.A. McLaughlin, S. Yoon, and S.D. Emr. 1997. A sorting nexin-1 homologue, Vps5p, forms a complex with Vps1p and is required for recycling the vacuolar protein-sorting receptor. Mol. Cell. Biol. 8:1529–1541.

LeMonn, M.A. 2003. Phosphoinositide recognition domains. Traffic. 4:201–213.

LeMonn, S.K., and L.M. Traub. 2000. Sorting in the endosomal system in yeast and animal cells. Curr. Opin. Cell Biol. 12:457–466.

Lewis, M.J., B.J. Nichols, C. Pescianotto-Baschong, H. Riezman, and H.R. Pelham. 2000. Specific retrieval of the exocytic SNARE Snap1p from early yeast endosomes. Mol. Biol. Cell. 11:23–38.

Longtime, M.S., A. McKenzie III, D.J. Demarini, N.G. Shah, A. Wach, A. Brachat, P. Philispenn, and J.R. Pringle. 1998. Additional modules for versatile and economical PCR-based gene deletion and modification in Saccharomyces cerevisiae. Yeast. 14:953–961.

Nothwehr, S.F., P. Bruinsma, and L.A. Strawn. 1999. Distinct domains within the yeast VPS5/GRD2 gene encode a sorting nexin-1-like protein required for localizing membrane proteins to the late Golgi. J. Biol. Chem. 274:17577–17586.

Nothwehr, S.F., S.A. Ha, and P. Bruinsma. 2000. Sorting of yeast membrane proteins depends on Rer1p-mediated retrieval from the yeast plasma membrane: orientation, topology and structure-function relationships. Biochem. J. 380:487–496.

Sheff, M.A., and K.S. Thorn. 2004. Optimized cassettes for fluorescent protein tagging in Saccharomyces cerevisiae. Yeast. 21:661–670.

Shi, H., R. Rojas, J.S. Bonifacino, and J.H. Hurley. 2006. The retromer subunit Vps26 has an arrestin fold and binds Vps35 through its C-terminal domain. Struct. Nat. Mol. Biol. 13:540–548.

Singh, A., S. Severance, N. Kaur, W. Willits, and D.J. Kosman. 2006. Assembly, activation, and trafficking of the Fet3p/Ftr1p high affinity iron permease complex in yeast. J. Cell Biol. 281:13355–13364.

Siniossoglou, S., S.Y. Peak-Chew, and H.R. Pelham. 2000. Ric1p and Rgp1p form a complex that catalyzes nucleotide exchange on Ypt6p. EMBO J. 19:4885–4894.

Steinrauwr, R., D.S. Yuan, Y. Yamaguchi-Iwai, R.D. Klausner, and A. Dancis. 1996. A permease-oxidase complex involved in high-affinity iron uptake in yeast. Science. 271:1552–1557.

Teasdale, R.D., D. Locis, F. Houghton, L. Karlsson, and P.A. Gleeson. 2001. A large family of endosome-localized proteins related to sorting nexin 1. Biochem. J. 358:7–16.

Verge, M., F. Futam, C. Gruber, F. Tienmann, L.G. Reinders, L. Huang, A.L. Burlingame, C.R. Haft, and K.E. Mostow. 2004. The mammalian retromer regulates transcytosis of the polymeric immunoglobulin receptor. Nat. Cell Biol. 6:763–769.

Voos, W., and T.H. Stevens. 1998. Retrieval of resident late-Golgi membrane proteins from the prevacuolar compartment of Saccharomyces cerevisiae is dependent on the function of Grp1p. J. Biol. Chem. 140:577–590.

Wang, D., M. Guo, Z. Liang, J. Fan, Z. Zhu, J. Zhang, Z. Xu, X. Li, M. Teng, L. Niu, et al. 2005. Crystal structure of human vacuolar protein sorting protein 29 reveals a phosphodiesterase/nuclease-like fold and two protein-protein interaction sites. J. Biol. Chem. 280:22962–22967.

Wang, Y., Z. Zhou, K. Szabo, C.R. Haft, and J. Trejo. 2002. Down-regulation of proteinase-activated receptor-1 is regulated by sorting nexin 1. Mol. Biol. Cell. 13:1965–1976.

Worthy, C.A., and I.E. Dixon. 2002. Sorting out the cellular functions of sorting nexins. Nat. Rev. Mol. Cell Biol. 3:919–931.

Xu, Y., H. Hortsman, L. Seet, S.H. Wong, and W. Hong. 2001. SNX3 regulates endosomal function through its PX-domain-mediated interaction with PtdIns(3)P. Nat. Cell Biol. 3:658–666.

Yu, J.W., and M.A. Lemmon. 2001. All phox homology (PX) domains from Saccharomyces cerevisiae specifically recognize phosphatidylinositol 3-phosphate. J. Biol. Chem. 276:44179–44184.

Zhong, Q., C.S. Lazar, H. Tronchere, T. Sato, T. Meelroo, M. Ye, Z. Songyang, S.D. Emr, and G.N. Gill. 2002. Endosomal localization and function of sorting nexin 1. Proc. Natl. Acad. Sci. USA. 99:6767–6772.

Zhou, C.Z., L.L. de La Sierra-Gallay, S. Quevillon-Cheruel, B. Collinet, P. Manard, K. Blondau, G. Henches, R. Aufrere, N. Lecliat, M. Graille, et al. 2003. Crystal structure of the yeast Phox homology (PX) domain protein Grd19p complexed to phosphatidylinositol 3-phosphate. J. Biol. Chem. 278:50371–50376.