P4-ATPase Requirement for AP-1/Clathrin Function in Protein Transport from the trans-Golgi Network and Early Endosomes

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Drp2 is a resident type 4 P-type ATPase (P4-ATPase) and potential phospholipid translocase of the trans-Golgi network (TGN) where it has been implicated in clathrin function. However, precise protein transport pathways requiring Drp2p and how it contributes to clathrin-coated vesicle budding remain unclear. Here we show a functional codependence between Drp2p and the AP-1 clathrin adaptor in protein sorting at the TGN and early endosomes of Saccharomyces cerevisiae. Genetic criteria indicate that Drp2p and AP-1 operate in the same pathway and that AP-1 requires Drp2p for function. In addition, we show that loss of AP-1 markedly increases Drp2p trafficking to the plasma membrane, but does not perturb retrieval of Drp2p from the early endosome back to the TGN. Thus AP-1 is required at the TGN to sort Drp2p out of the exocytic pathway, presumably for delivery to the early endosome. Moreover, a conditional allele that inactivates Drp2p phospholipid translocate (flippase) activity disrupts its own transport in this AP-1 pathway. Drp2p physically interacts with AP-1; however, AP-1 and clathrin are both recruited normally to the TGN in drp2Δ cells. These results imply that Drp2p acts independently of coat recruitment to facilitate AP-1/clathrin-coated vesicle budding from the TGN.

INTRODUCTION

Clathrin mediates protein transport between the plasma membrane, endosomes, and TGN through association with pathway-specific adaptors that link integral membrane cargo proteins to the clathrin lattice during vesicle formation. In mammalian cells, the ubiquitously expressed heterotetrameric AP-1A clathrin adaptor, composed of γ, β1, μ1A, and σ1-adaptins, appears to mediate both anterograde transport of proteins from the TGN to early endosomes, as well as the retrograde trip back to the TGN (Hinners and Tooze, 2003; Robinson, 2004). For its role in anterograde transport of lysosomal enzymes, AP-1A collaborates with monomeric adaptors called GGAs (Golgi localized, γ-ear–containing, Arf-binding proteins) to recruit the mannose-6-phosphate receptor into clathrin-coated vesicles (CCVs; Doray et al., 2002). The AP-1B complex, which differs from AP-1A by an alternate μ1B subunit, has a functionally distinct role in sorting cargo from the TGN to the basolateral membrane of polarized epithelial cells (Folsch et al., 1999). In budding yeast, there is evidence that AP-1 mediates early endosome to TGN retrograde transport (Valdivia et al., 2002; Foote and Nothwehr, 2006), but no anterograde role for AP-1 has yet been described. The yeast GGAs appear to function independently of AP-1 to mediate delivery of cargo from the TGN to the late endosome (Black and Pelham, 2000). Deletion of genes encoding AP-1 subunits or GGAs has little consequence to growth of yeast. However, the combined deletion of AP-1 and GGA causes a severe growth defect, suggesting that these two adaptors mediate parallel transport pathways and that yeast cannot tolerate loss of both pathways (Costaguta et al., 2001; Hirst et al., 2001).

The mechanism of AP-1/clathrin-coated vesicle (AP-1/CCV) formation is incompletely understood. A commonly held view is that ARF-dependent recruitment of AP-1 and clathrin to membranes induces localized assembly of clathrin triskelia into a lattice, and the intrinsic curvature of the growing clathrin lattice provides sufficient energy for the membrane deformation needed for vesicle budding. Consistent with this possibility is the observation that clathrin and AP-1 in cytosolic fractions can induce vesicle formation from liposomes in vitro, suggesting that AP-1/CCVs can be produced without assistance from integral membrane proteins (Zhu et al., 1999). However, it is not clear if CCVs bud in vitro from liposomes at a rate that could sustain protein trafficking pathways in vivo. Therefore, whether or not integral membrane proteins contribute to AP-1/CCV biogenesis in vivo remains an important unanswered question. In addition, it is possible that clathrin self-assembly alone does not provide enough energy to drive bending of biological membranes without assistance from accessory proteins (Nossal, 2001). The epsins, for example, are thought to facilitate membrane bending to assist clathrin in forming vesicles (Ford et al., 2002).

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Abbreviations used: ARF, ADP-ribosylation factor; CCV, clathrin-coated vesicles; CW, calcofluor white; GEF, guanine nucleotide exchange factor; GGA, Golgi-localized, γ-ear–containing, ARF-binding protein; P4-ATPase, type IV P-type ATPase; PE, phosphatidylethanolamine; PS, phosphatidylserine; PVC, prevacuolar compartment.
Phospholipid translocases, or flipases, have substantial potential to bend membranes by coupling a source of energy (ATP) to the unidirectional translocation of phospholipid across the bilayer. Flipase action can create an imbalance in phospholipid number between the two leaflets and cause bending of the membrane in the direction of lipid translocation (Graham, 2004). A large body of evidence indicates that P4-ATPases, which include the yeast Drs2, Dnf1, Dnf2, Dnf3, and Neol proteins, catalyze unidirectional phospholipid translocation from the extracellular leaflet of the plasma membrane, or the topologically equivalent lumenal leaflet of the Golgi and endosomes, to the cytosolic leaflet of these membranes (Tang et al., 1996; Gomes et al., 2000; Ujhazy et al., 2001; Pomorski et al., 2003; Natarajan et al., 2004; Saito et al., 2004; Alder-Baerens et al., 2006). Although a flipase activity has not been reconstituted with a purified P4-ATPase, we have shown that inactivation of a temperature-conditioned mutant form of Drs2p (Drs2-ts) present in purified TGN membranes immediately disrupts phospholipid translocate activity measured with a fluorescent phospholipid derivative (Natarajan et al., 2004), supporting the view that Drs2p is a flipase. In these assays, Drs2p has a preferred substrate preference for phosphatidylethanolamine (PS), but also appears to flip phosphatidylycerolamine (PE; Natarajan et al., 2004; Alder-Baerens et al., 2006). This Drs2-dependent flipase activity also appears to contribute to the asymmetric distribution of endogenous PS and PE to the cytosolic leaflet as drs2Δ mutants exhibit a loss of PS and PE asymmetry (Pomorski et al., 2003; Chen et al., 2006).

P4-ATPases also play essential roles in protein transport in the secretory and endocytic pathways (Chen et al., 1999; Hua et al., 2002; Hua and Graham, 2003; Pomorski et al., 2003; Sakane et al., 2006; Furuta et al., 2007). For example, inactivation of Drs2-ts in vivo rapidly blocks formation of a clathrin-dependent class of post-Golgi vesicles carrying exocytic cargo (Gall et al., 2002). These post-Golgi vesicles appear to be equivalent to one of the two classes of exocytic vesicles (the “higher density” class) first described by Har- say and Bretscher (1995). Formation of these exocytic vesicles is only mildly perturbed by deletion of AP-1 subunits (Gall et al., 2002); therefore, they likely differ from AP-1/CCVs proposed to mediate transport between the TGN and endosomes. Other data linking Drs2p to clathrin function include their colocalization to the TGN, a strong synthetic lethal relationship between drs2, arf1, and che1 (clathrin heavy chain) alleles, and drs2 mutant phenotypes such as the accumulation of enlarged Golgi cisternae, a deficiency of CCVs, and the mislocalization of TGN resident proteins. These phenotypes are similar to what is observed in clathrin mutants (Chen et al., 1999).

These observations are consistent with a role for Drs2p in budding CCVs. However, because the relationship of Drs2p to well-known AP-1 and GGA clathrin adaptors has not been characterized, the precise role of Drs2p in the function of clathrin and its adaptors in the TGN–endosomal system is unclear. Clathrin mediates multiple transport pathways and so this information is essential to test if Drs2p contributes to vesicle biogenesis by helping recruit specific clathrin adapter components to membranes or through a novel mechanism. Here we demonstrate a unique functional codependence between Drs2p and AP-1. Drs2p requires AP-1 for its normal trafficking itinerary, and the enzymatic activity of this integral membrane protein is essential for AP-1/clathrin function. This work provides the first example a clathrin accessory factor essential for AP-1 function that acts independently of coat recruitment, and also suggests that an ATP-powered pump provides a critical source of energy to drive AP-1/CCV formation from biological membranes.

**MATERIALS AND METHODS**

**Media and Strains**

Yeast strains were grown in standard rich medium (YPD) or synthetic defined (SD) minimal media. Calcofluor white (CW) sensitivity was tested on YPD 2% agar plates containing 100 μg/ml CW (Sigma-Aldrich, St. Louis, MO). Yeast strains used in this study were generated by standard genetic crosses, gene disruption, or integration methods as previously described and are listed in Supplementary Table 1. The yeast knockout collection and GFP collection were purchased from Invitrogen (Carlsbad, CA). To prevent accumulation in the TGN, Drs2p was cotransformed into yeast strains with a multicopy vector carrying CDC50 (pRS425-CDC50), encoding a chaperone for Drs2p. Strains carrying pchs3-GFP also carried a plasmid overexpressing the CHS7 chaperone (pCRP1). pGFP-drs2-12 (encoding GFP-Drs2-ts) was constructed by replacing the Age1-Clal fragment of pGFP-DRS2 with the Age1-Clal fragment from pS8315-drs2-12. To create pGFP-TLG1, genomic DNA was amplified with primers Tlg1-F (GTACGCTGAAACACAGTTTGAAGATCCGTTTC) and Tlg1-R (ACTCGTGGACTCAAGCAATGAATTGC) and Tgl1-R (ACTCGTGGACTCAAGCAATGAATTGC) and the product was digested with SalI and subcloned into SD medium containing 200 μg/ml matruncan A (Lat A). Brefeldin A (Sigma-Aldrich) was dissolved in ethanol at 10 μg/ml and used to treat cells at 100 μg/ml in imaging buffer.

**Coimmunoprecipitation of AP-1 and Drs2p**

For chemical cross-linking experiments, 50 OD600 units of each strain were incubated in 5 ml softening buffer (100 mM Tris-HCl, pH 9.4, 10 mM DTT) at 30°C for 10 min and then converted to spheroplasts by incubating in 1 ml spheroplasting buffer (1 M sorbitol, 0.5% (v/v) glycerol, 10 mM K2PO4·3H2O, 200 μg/ml Zymolase) at 30°C for 30 min. Spheroplasts were washed three times with cross-linking buffer (1 M sorbitol, 0.15 M NaCl, 0.1 M KH2PO4, pH 7.2), resuspended in cross-linking buffer supplemented with either 1 or 2 μM dithiobis(succinimidyl)propionate (DSP, 20 μg/ml stock in dimethylsulfoxide; Pierce, Rockford, IL) and incubated at room temperature for 30 min. Cells were then pelleted, resuspended in 1 ml ice-cold lysis buffer (20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 150 mM NaCl, 1% CHAPS, 1× complete protease inhibitor cocktail lacking EDTA), Roche Diagnostics, Basel, Switzerland, and 1 mg/ml RNase, and incubated on ice for 15 min. The cell lysate was cleared by incubation with 30 μl protein G Sepharose for 1.5 h at 4°C and subsequently with 30 μl protein G Sepharose for 1.5 h at 4°C. The protein G Sepharose beads were washed three times with washing buffer I (20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 350 mM NaCl, 1% Tween-20, 1× complete protease inhibitor cocktail lacking EDTA) and twice with washing buffer II (20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 150 mM NaCl, 1× complete protease inhibitor cocktail lacking EDTA). Bound material was eluted with SDS/urea sample buffer, separated by SDS-PAGE, and subjected to immunoblotting as previously described (Chen et al., 1999).

**RESULTS**

**Drs2p is Required for AP-1 Function in TGN-early Endosome Pathways**

Prior studies implicated Drs2p in protein transport between the TGN and early endosome, suggesting a primary role for Drs2 in AP-1/clathrin function rather than the late endosome pathway mediated by GGAs (Chen et al., 1999). In budding yeast, simultaneous inactivation of both AP-1 and GGA function causes a severe synthetic growth defect (Costaguta et al., 2001; Hirst et al., 2001). If Drs2 is required for AP-1 function, a drs2 null allele (drs2Δ) should cause a strong synthetic growth defect when combined with null
alleles of the two GGA genes (gga1Δ and gga2Δ), comparable to the poor growth of an apl4Δ (γ-adaptoin) gga1Δ gga2Δ strain. In addition, deletion of an AP-1 subunit should have no additional consequence to a drs2Δ strain if AP-1 function is already lost in this strain. To test these predictions, we crossed drs2Δ with gga1Δ gga2Δ and apl4Δ and performed tetrad analyses. The drs2Δ gga1Δ gga2Δ triple mutant progeny were either inviable or grew extremely slowly. In addition, the drs2Δ gga2Δ double mutants grew slower than the single mutant or WT progeny (Figure 1, A and B). By contrast, the drs2Δ apl4Δ mutant exhibited a growth rate identical to the drs2Δ single mutant, even at 32°C, a semipermissive temperature for growth of cold-sensitive drs2Δ strains (Figure 1B). These results suggest that Drs2p acts in parallel with GGA and functions in the same pathway as AP-1.

A well-defined role for AP-1 in yeast is in the trafficking of chitin synthase III (Chs3p) between the early endosomes and TGN (Valdivia et al., 2002), and so we tested if Drs2p is required in this pathway. Chs3p deposits a ring of chitin around emerging buds that remains as a scar after the bud is released. Most Chs3p is retained intracellularly by AP-1–dependent transport between the TGN and early endosome, but a portion is diverted to the plasma membrane in a cell cycle–regulated process that requires the Chs5p and Chs6p coat complex (Trautwein et al., 2006; Wang et al., 2006). Thus, chs6Δ cells retain nearly all Chs3p intracellularly and have reduced chitin incorporation at the bud site. This makes chs6Δ cells resistant to the toxic effects of CW, a chitin-binding fluorescent compound (Figure 2A). In the chs6Δ background, deletion of any AP-1 subunit disrupts intracellular Chs3p retention and allows cell surface transport of Chs3p in constitutive exocytic vesicles, restoring the appearance of chitin rings and CW sensitivity to an AP-1 chs6Δ double mutant (Valdivia et al., 2002). Like the chs6Δ apl4Δ strain, the chs6Δ drs2Δ double mutant failed to grow on the medium containing CW (Figure 2A) and formed chitin rings indistinguishable from those in the WT and drs2Δ cells (Figure 2B).

As a cargo of the AP-1–mediated early endosome to TGN retrograde pathway, Chs3p normally avoids transport to the late endosome, but transits this compartment frequently in AP-1 mutants (Valdivia et al., 2002). This perturbation is revealed in a class E vps AP-1 double mutant (apl4Δ vps27Δ), where Chs3p is trapped in enlarged endosomal compartments adjacent to the vacuole (class E compartment) that also accumulate the endocytic tracer FM4-64. As in apl4Δ vps27Δ cells, Chs3p accumulates in the class E compartment of drs2Δ vps27Δ cells (Figure 2C). The class E compartment in drs2Δ vps27Δ cells has a larger and more diffuse morphology than in vps27Δ or apl4Δ vps27Δ cells. However, the drs2Δ vps27Δ and vps27Δ cells exhibit a similar defect in the kinetics of FM4-64 delivery to the vacuole, and so the drs2Δ mutation, although altering the class E compartment morphology, does not suppress the vps27Δ FM4-64 trafficking defect. In summary, the data shown in Figure 2 indicates that loss of Drs2p disrupts Chs3p trafficking in a manner similar to loss of AP-1.

Missorting of Drs2p to the Plasma Membrane of AP-1 Mutants

Our previous work suggested that Drs2 primarily traffics between the TGN and early endosome in order to maintain its TGN localization (Liu et al., 2007). To test if Drs2 localization requires AP-1, we first examined the distribution of GFP-Drs2p relative to the TGN marker Sec7-RFP in WT and
Deletion of the AP-1 puncta after 30 min of Lat A treatment. In stark contrast, GFP-Drs2p was primarily retained intracellularly in small vesicles, and so any Drs2p molecules that are transported to the plasma membrane are rapidly retrieved through endocytosis and imaged the cells over time (Figure 3B). In WT cells, strains expressing GFP-Drs2p with Lat A to inhibit endocytosis of AP-1 mutant. To test this, we treated WT and AP-1 mutant strains expressing GFP-Drs2p into transport vesicles targeted to the plasma membrane, similarly to WT cells (Figure 3B). We conclude that AP-1 is required, and GGAs dispensable, for efficient exclusion of Drs2p from exocytic vesicles targeted to the plasma membrane. This likely reflects a role for AP-1 in transporting Drs2p from the TGN to early endosomes; however, see Discussion for an alternative model.

Figure 3. Loss of AP-1 perturbs Drs2p trafficking but not its steady-state TGN localization. (A) Colocalization of GFP-Drs2 and Sec7-DsRed at the TGN of WT (KLY1101) and AP-1-deficient (apl2Δ) cells (KLY351). Some of the cells are outlined for clarity. (B) Disruption of AP-1 subunit genes causes misorting of GFP-Drs2p to the plasma membrane, where accumulation was induced by blocking endocytosis with latrunculin A (Lat A) at room temperature for 30 min. WT (BY4741) and AP-1 mutant cells (BY4741 derivatives) were treated with Lat A and imaged as previously described (Liu et al., 2007).

AP-1-deficient cells (Figure 3A). GFP-Drs2p localized to several discrete puncta in WT cells, and this pattern was not altered in cells lacking AP-1 (apl2Δ). Furthermore, 81% of Drs2p-GFP puncta were coincident with Sec7-DsRed puncta in WT cells, whereas 82% of Drs2p-GFP puncta colocalized with Sec7-DsRed puncta in apl2Δ cells. Therefore, the steady-state localization of GFP-Drs2p to the TGN is not noticeably perturbed by AP-1 disruption.

We then addressed whether deletion of AP-1 has an effect on trafficking of GFP-Drs2p. Acute inhibition of endocytosis by treating cells with Lat A, an inhibitor of actin assembly and transport of Drs2p into transport vesicles targeted to the plasma membrane. In addition, Drs2p contains multiple endocytosis signals, and so any Drs2p molecules that are transported to the plasma membrane are rapidly retrieved through endocytosis (Liu et al., 2007). If GFP-Drs2p requires AP-1 for TGN to early endosome transport, we would expect an increased frequency of Drs2p transport to the plasma membrane in an AP-1 mutant. To test this, we treated WT and AP-1 mutant strains expressing GFP-Drs2p with Lat A to inhibit endocytosis and imaged the cells over time (Figure 3B). In WT cells, GFP-Drs2p was primarily retained intracellularly in small puncta after 30 min of Lat A treatment. In stark contrast, deletion of the AP-1 β subunit (apl2Δ), γ subunit (apl4Δ), or σ subunit (aps1Δ) caused accumulation of nearly all GFP-Drs2p on the plasma membrane within 30 min of blocking endocytosis with Lat A.

Cell surface accumulation of GFP-Drs2p was less pronounced in apm1Δ and nearly absent in apm2Δ cells treated with Lat A. These mutants harbor deletions of the partially redundant AP-1 μ1 subunits (unpublished observations). However, deletion of both APM1 and APM2 leads to plasma membrane accumulation comparable to deletion of other AP-1 subunits (apm1Δ apm2Δ; Figure 3B). These data indicate that Drs2p trafficking primarily relies on Apm1p, although Apm2p can partially contribute. The gga1Δ gga2Δ strain was also treated with Lat A, and these cells slowly accumulated GFP-Drs2p on the plasma membrane, similarly to WT cells (Figure 3B). We conclude that AP-1 is required, and GGAs dispensable, for efficient exclusion of Drs2p from exocytic vesicles targeted to the plasma membrane. This likely reflects a role for AP-1 in transporting Drs2p from the TGN to early endosomes; however, see Discussion for an alternative model.

Missorting of Drs2p to the plasma membrane of AP-1 null cells could be an indirect consequence of chronically perturbing protein transport between the early endosomes and TGN. To distinguish chronic versus acute effects of AP-1 inactivation on Drs2p trafficking, an apl2 temperature-sensitive (ts) mutant was shifted to the nonpermissive temperature (37°C) to inactivate AP-1 just before Lat A addition. The apl2-ts strain accumulated GFP-Drs2p on the plasma membrane within 30 min at 37°C, whereas Lat A–treated cells maintained at the permissive temperature (24°C) or WT cells shifted to 37°C retained significant internal punctate fluorescence (Figure 4A). For other potential anterograde AP-1 cargos tested, a minor fraction of Chs3-GFP but no Kex2-GFP or GFP-Tlg1 accumulated on the plasma membrane of Lat A–treated apl4Δ cells (Figure 4B and unpublished observations). Thus, missorting of Drs2p to the plasma membrane is a specific and immediate consequence of AP-1 inactivation.

To rule out the possibility that missorting of GFP-Drs2 to the plasma membrane is a secondary consequence of inactivating AP-1 retrograde function, we examined the trafficking of GFP-Drs2p in two other mutants that display a defect in the retrograde pathway. The Snx4-Snx41-Snx42 sorting nexin complex and the F-box protein Rcy1p function in early endosome to TGN retrograde transport (Wiederkehr et al., 2000; Hettema et al., 2003). However, disruption of RCY1 only slightly increased GFP-Drs2 accumulation on the plasma membrane upon addition of Lat A, whereas disruption of SNX4 has no apparent impact on Drs2p trafficking (Figure 4C). Therefore, wholesale missorting of GFP-Drs2p to the plasma membrane in AP-1–deficient cells is not a secondary consequence of disrupting early endosome to TGN retrograde transport.

The observation that AP-1 disruption rerouted Drs2p to the plasma membrane without substantially perturbing its steady-state localization to the TGN, suggests that endocytosis and early endosome to TGN transport of GFP-Drs2 was unaffected. To test if AP-1 disruption perturbs retrograde transport of Drs2p similarly to Chs3p, localization of GFP-Drs2p was monitored in apl2Δ vps27Δ cells. In contrast to Chs3-GFP (shown in Figure 2), GFP-Drs2p did not accumulate in the class E compartment marked with FM4-64 (Figure 5A). In addition, GFP-Drs2 was efficiently endocytosed from the plasma membrane and transported to the TGN in AP-1–deficient cells. To show this, the apl2Δ strain expressing GFP-Drs2 and Sec7-DsRed was treated with Lat A to accumulate GFP-Drs2 on the plasma membrane. After washing out the Lat A to lift the endocytosis block, GFP-
Drs2 efficiently returned to Sec7-DsRed–marked TGN cisternae (Figure 5B). In summary, these data indicate that Drs2p requires AP-1 at the TGN for exclusion from exocytic vesicles. However, Drs2p can be retrieved from early endosomes and transported to the TGN in the absence of AP-1.

**Drs2p Activity Drives Its Own Transport in the AP-1 Pathway**

Drs2p is the only cargo thus far characterized in yeast that depends solely on AP-1 in what appears to be a TGN-to-endosome anterograde pathway. To determine if Drs2p activity is required for the function of AP-1 in this pathway, we examined the trafficking of a GFP-tagged Drs2 temperature-sensitive protein (Drs2-ts) at the permissive (24°C) and nonpermissive (38°C) temperatures. Because Drs2-ts is the only source of Drs2p in these cells, we could determine if inactivation of Drs2p ATPase and flippase activities at high temperature disrupts its own trafficking in the AP-1–dependent anterograde direction. GFP-Drs2 and GFP-Drs2-ts were expressed in a strain deficient for multiple P4-ATPases (drs2/H9004 dnf1/H9004 dnf2/H9004 dnf3/H9004), where Drs2p is required to support viability (Hua et al., 2002), and we confirmed that the temperature-sensitive growth phenotype caused by the drs2-ts allele was not perturbed by the GFP moiety (unpublished observation). WT GFP-Drs2 maintained a punctate distribution at both temperatures in the presence or absence of Lat A (Figure 6). This indicates that the Dnf1, Dnf2, and Dnf3 P4-ATPases are dispensable for Drs2p trafficking in the AP-1 pathway.

**Acute inactivation of Drs2p enzymatic activity causes its missorting to the plasma membrane.** Strains KLY921 and KLY931, deficient for Dnf P4-ATPases and expressing GFP-Drs2 or GFP-Drs2-ts (pGFP-drs2-12) as the sole source of Drs2p, were incubated at the permissive (24°C) or nonpermissive (38°C) temperature for 1 h before a 30-min incubation with or without Lat A at the same temperature. Plasma membrane fluorescence is only observed with Lat A–treated cells expressing GFP-Drs2-ts at 38°C.

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**Figure 4.** GFP-Drs2p missorting to the plasma membrane is an immediate and specific consequence of AP-1 deficiency. (A) GFP-Drs2p is missorted to the plasma membrane of an apl2-ts (β1-adaptin) strain preincubated 30 min at the nonpermissive temperature (37°C) before Lat A addition. Partial plasma membrane accumulation of GFP-Drs2p was also observed at the permissive temperature suggesting a partial loss of AP-1 function at 24°C. Lat A incubations were for 30 min at the indicated temperature. (B) Localization of GFP-Drs2 in rcy1Δ and snx4Δ cells treated with Lat A for 30 min at 30°C. Localization of GFP-Tlg1 (C) and Chs3-GFP (D) in WT and AP-1–deficient (apl4Δ) cells treated with Lat A as in B.

**Figure 5.** Drs2p does not require AP-1 for early endosome-to-TGN retrograde transport. (A) An apl4 Δvps27Δ strain (KLY711) expressing GFP-Drs2 was stained with FM4-64 to mark the class E compartment and imaged. Arrowheads are within the vacuole region and point to the class E compartments. (B) An apl2Δ strain (KLY551) expressing GFP-Drs2 and Sec7-DsRed was treated with Lat A for 1 h to completely accumulate GFP-Drs2p on the plasma membrane. The cells were washed twice, resuspended in fresh medium without Lat A (Lat A washout) and incubated for 1 h at 30°C before imaging.
**Figure 7.** Drs2p interacts with AP-1 but is not required for AP-1 or clathrin recruitment to Golgi membranes. Coimmunoprecipitation of AP-1 and Drs2p. Cells expressing epitope tagged Drs2p (A), Apl4p (B), or control cells without the tag were treated with chemical cross-linker before lysis and immunoprecipitation (IP) with tag antibodies. 0.5% of the initial lysate was loaded in the “lysate” lanes, and the indicated proteins were detected by immunoblot. (C) WT (KLY791, KLY792) and 

**DISCUSSION**

The results of this work provide a number of unique observations with significant mechanistic implications for clathrin-mediated protein transport from the TGN and early endosomes. We find that an integral membrane P-type ATPase called Drs2p is essential for AP-1/clathrin function in vivo. The strong genetic interaction between drs2 and gga1/gga2 alleles, the complete lack of a genetic interaction between drs2 and AP-1 alleles, and the phenotypic similarities between drs2 and AP-1 mutants strongly suggest that AP-1 function is lost in drs2 cells. In fact, Drs2p and AP-1 exhibit a novel functional codependency as we show that AP-1 is
required at the TGN to prevent Drs2p transport to the plasma membrane. In addition, Drs2 flipase activity is required to drive its own trafficking in the AP-1 pathway that excludes Drs2p from exocytic vesicles. Importantly, we also show that Drs2p is not required for recruitment of AP-1 and clathrin to the TGN, thus providing the first example of an essential AP-1/clathrin accessory protein that acts independently of coat recruitment.

AP-1 was initially found to localize to the TGN of mammalian cells and was assumed to mediate protein transport from the TGN to the endosomal system. However, the finding that mutations of AP-1 subunits in mice and yeast appeared to cause trafficking defects in endosome to TGN retrograde transport raised questions as to whether AP-1 plays a primary role in retrograde or anterograde transport (reviewed in Hinners and Tooze, 2003). Our results show that AP-1 is required at the TGN to sort Drs2p out of the AP-1/clathrin and further work is needed to distinguish between these two models. Regardless of the vesicle destination, the current studies, combined with previously published data (Valdivia et al., 2002; Foote and Nothwehr, 2006), support a direct role for AP-1/clathrin in maintaining their TGN localization as an alternative model that is consistent with our data, it is possible that AP-1/CCVs containing Drs2p bud from the TGN and are targeted directly to an earlier Golgi cisterna to facilitate the maturation of trans cisternae to TGN compartments (Figure 8A, pathway designated “AP-1?”). An intra-Golgi retrograde pathway would be a novel role for AP-1/clathrin and further work is needed to distinguish between these two models. Regardless of the vesicle destination, the current studies, combined with previously published data (Valdivia et al., 2002; Foote and Nothwehr, 2006), support a direct role for AP-1 in selecting cargo for inclusion into CCVs at the TGN (Drs2p) and early endosome (Chs3p and Ste13p). Most Chs3p is rerouted to the late endosome of AP-1 null cells, where it can be trapped behind a vps27 block. Pathways that require Drs2p activity for vesicle formation are indicated with a red star. (B) In the absence of Drs2p (drs2a), AP-1 and clathrin are recruited to the membrane, but vesicles are not produced because the coat components cannot induce sufficient curvature in the membrane. (C) Enzymatically active Drs2p (yellow), modeled with a similar structure as SERCA1(Toyoshima et al., 2000), is required for budding AP-1/clathrin-coated vesicles. Interaction of Drs2p with Arf guanine nucleotide exchange factors (Arf GEF; Chanta-
protein transport factors operating in the late secretory and early endocytic pathways. We originally linked Drs2p to clathrin-mediated transport through the recovery of several drs2 alleles in an arf1 synthetic lethal screen and the observation that drs2 is also synthetically lethal with clathrin heavy-chain temperature-sensitive alleles but not with mutations in COPI or COPII subunits (Chen et al., 1999). Subsequently, genetic interactions were discovered between Drs2p and ARF guanine nucleotide exchange factors (Gea1p and Gea2p), an ARF guanine nucleotide activating protein (Gcs1p), GGAs, Ypt31p (a rab protein), TRAPPII subunits, and a phosphatidylinositol 4-kinase (Pik1p; Chantalat et al., 2004; Sciorra et al., 2005; Sakane et al., 2006). In addition, the Drs2-Cdc50 complex physically interacts with Gea2p, AP-1, and Rcy1p, providing a direct link to ARF/AP-1/clathrin pathways as well as the Rcy1-dependent early endosome-to-TGN recycling pathway (this work and Chantalat et al., 2004; Furuta et al., 2007).

Consistent with these genetic and physical interactions, drs2 mutants exhibit protein transport defects specific to the TGN and early endosomal system (the red stars in Figure 8A indicate pathways that require Drs2p). As shown here, AP-1/clathrin-dependent pathways associated with the TGN and early endosome are abrogated in cells deficient for Drs2p. In addition, Drs2p is required for the early endosome to TGN retrograde transport of the SNARE protein Snc1 (Hua et al., 2002; Furuta et al., 2007), which is independent of AP-1 (our unpublished observations) but requires Rcy1p (Galan et al., 2001). Cdc50p, presumably in a complex with Drs2p, accumulates in the early endosomes of rcy1 mutants (Furuta et al., 2007). Therefore, Drs2 activity likely drives its own retrieval back to the TGN in the Rcy1p pathway. Because the majority of Drs2p localizes to the TGN, inactivation of Drs2p activity initially causes its missorting to the early endosome rather than accumulation in the early endosome. The precise role of the F-box protein Rcy1 is unclear, although it functions with the Ypt31/32 rab pair, Gcs1p (Arf-GAP) and a COPII subcomplex in this recycling pathway (Chen et al., 2005; Robinson et al., 2006; Furuta et al., 2007).

In addition to the AP-1 and Rcy1 pathways, we previously described a role for Drs2p and clathrin in the formation of one class of post-Golgi exocytic vesicles (Gall et al., 2002). AP-1 mutations partially perturb formation of these exocytic vesicles, although it was not clear at the time whether the small defect observed reflected a primary or secondary consequence of AP-1 disruption. On the basis of the current studies, we suggest that AP-1 mutations influence the exocytic vesicles indirectly by altering the trafficking patterns of Drs2p (and perhaps other TGN residents as well). Drs2p is required to form the dense exocytic vesicles and AP-1 retrograde vesicles, but does not appear to be significantly incorporated into these vesicles. This is consistent with a role for Drs2p in priming the membrane of the TGN and early endosome to produce a structure that coats more efficiently mold into a vesicle.

P4-ATPases in yeast (Drs2p, Dnf1p, Dnf2p, Dnf3p, and Neo1p) have a general function in vesicle-mediated protein transport in the Golgi–endosomal system (Chen et al., 1999; Hua et al., 2002; Hua and Graham, 2003). Although some vesicle classes budding from the TGN and early endosomes primarily require Drs2p (e.g., AP-1, Rcy1, and the dense exocytic vesicles), other pathways are less discriminate for their P4-ATPase requirement. For example, AP-3–dependent protein transport from the TGN to the vacuole requires either Drs2p or Dnf1p, such that a severe defect in this pathway is only observed in the drs2Δ dnf1Δ double mutant (Hua et al., 2002). Likewise, transport of carboxypeptidase Y from the TGN to late endosomes, perhaps carried in GGA/clathrin-coated vesicles, is kinetically delayed in the drs2Δ dnf1Δ double mutant (Hua et al., 2002). Importantly, not all vesicles budding from the Golgi require Drs2p. The “lighter density” class of exocytic vesicles appears to form normally in drs2Δ cells, and cargos normally secreted in the dense vesicles, such as invertase, get rerouted into the light exocytic vesicles (Chen et al., 1999; Gall et al., 2002; Hua et al., 2002). In fact, the rate of protein transport from the ER to the Golgi and subsequently to the cell surface in drs2Δ or dnf1Δ is similar to that of WT cells (Chen et al., 1999; Gall et al., 2002; Hua et al., 2002). These data also indicate that COPI and COPII function in the early secretory pathway is unperturbed by loss of Drs2 and Dnf P4-ATPases. However, conditional alleles of the essential P4-ATPase NEO1 cause transport defects that are similar to COPI mutants and also perturb ARL-dependent transport from endosomes (Hua and Graham, 2003; Wicky et al., 2004). The proposed phospholipid flippase activity for P4-ATPases is best supported by studies showing a temperature-conditional loss of flippase activity in purified TGN membranes carrying a temperature conditional form of Drs2p (Drs2-ts; Natarajan et al., 2004). Inactivation of Drs2-ts in vivo causes an immediate defect in the budding of dense exocytic vesicles (Gall et al., 2002), and rerouting of Drs2-ts to the plasma membrane, implying a defect in budding AP-1/CCVs from the TGN (this work). Therefore, Drs2p flippase activity appears to drive formation of vesicles from the TGN.

Two models have been proposed to explain how the phospholipid flippase activity of Drs2p contributes to vesicle biogenesis (Chen et al., 1999; Gall et al., 2002; Graham, 2004). One possibility is that Drs2p recruits coat proteins to the membrane through protein–protein interactions, and/or by increasing the concentration of specific phospholipids on the cytosolic leaflet. However, our results rule out this possibility by showing that membrane association of ARF, AP-1, and clathrin does not require Drs2p (Figure 8B, right), in spite of the Drs2p requirement for AP-1 function. Therefore, our data argue that membrane recruitment of ARF, AP-1, and clathrin is insufficient to bud AP-1/CCVs in the absence of Drs2p. Even though epsins have been implicated in membrane deformation and AP-1 function (Ford et al., 2002; Hirst et al., 2003; Mills et al., 2003; Costaguta et al., 2006), the yeast Golgi epsins (Ent3p and Ent5p) also fail to support AP-1/CCV function in the absence of Drs2p.

The model best supported by our data is that ATP-dependent transbilayer movement of lipids catalyzed by Drs2p imparts curvature to the membrane that is required to facilitate vesicle budding (Figure 8B, left). The bilayer-couple hypothesis predicts that an increase in surface area of one leaflet relative to the other will spontaneously induce membrane curvature (Sheetz and Singer, 1974). As little as a 1–2% difference in surface area between leaflets of a bilayer can impart curvature to the membrane that is required to facilitate transbilayer movement of lipids catalyzed by Drs2p. In fact, the rate of protein transport from the ER to the Golgi and subsequently to the cell surface in drs2Δ or dnf1Δ is similar to that of WT cells (Chen et al., 1999; Gall et al., 2002; Hua et al., 2002). These data also indicate that COPI and COPII function in the early secretory pathway is unperturbed by loss of Drs2 and Dnf P4-ATPases. However, conditional alleles of the essential P4-ATPase NEO1 cause transport defects that are similar to COPI mutants and also perturb ARL-dependent transport from endosomes (Hua and Graham, 2003; Wicky et al., 2004). The proposed phospholipid flippase activity for P4-ATPases is best supported by studies showing a temperature-conditional loss of flippase activity in purified TGN membranes carrying a temperature conditional form of Drs2p (Drs2-ts; Natarajan et al., 2004). Inactivation of Drs2-ts in vivo causes an immediate defect in the budding of dense exocytic vesicles (Gall et al., 2002), and rerouting of Drs2-ts to the plasma membrane, implying a defect in budding AP-1/CCVs from the TGN (this work). Therefore, Drs2p flippase activity appears to drive formation of vesicles from the TGN.

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expense of the lumenal leaflet and bend the membrane toward the cytosol. This could provide a critical priming step to produce a membrane substrate that coat proteins can more efficiently mold into a vesicle. We propose that Drs2p imparts curvature to the membrane through a bilayer-cou-ple mechanism that is subsequently captured and molded by AP-1/clathrin to produce vesicles.

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REFERENCES


Drs2p Requirement for AP-1 Function