Arl1p is involved in transport of the GPI-anchored protein Gas1p from the late Golgi to the plasma membrane

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Summary
The molecular mechanisms involved in the transport of GPI-anchored proteins from the trans-Golgi network (TGN) to the cell periphery have not been established. Arl1p is a member of the Arf-like protein (Arl) subfamily of small GTPases and is localized in the late Golgi. Although Arl1p is implicated in regulation of Golgi structure and function, no endogenous cargo protein that is regulated by Arl1p has been identified in yeast. In this study, we demonstrate that Arl1p is involved in the anterograde transport from the Golgi to the cell surface of the glycosylphosphatidylinositol (GPI)-anchored plasmamembrane-resident protein Gas1p, but not the cell-wall-localized GPI-anchored proteins Crh1p, Crh2p and Cwp1p, or non-GPI-anchored plasma membrane-protein Gap1p. We also show that regulators of Arl1p (Sys1p, Arl3p and Gcs1p) and an effector (Imh1p) all participate in the transport of Gas1p. Thus, we infer that the signaling cascade Sys1p-Arl3p-Arl1p-Imh1p specifically participates in the transport of a GPI-anchored protein from the late Golgi to the plasma membrane.

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Key words: ARF, GTPase, Golgi, GPI-anchored protein

Introduction
Arl-like protein 1 (Arl1p), a member of the ARF-like protein subfamily of small GTPases, is localized in the late Golgi compartment in both mammalian cells and yeast (Lu et al., 2001; Setty et al., 2003; Liu et al., 2005), and has been implicated in the regulation of Golgi structure and function (Lu et al., 2001). In yeast and mammals, Arl1p can recruit the GRIP-domain containing effectors, Imh1p or Golgin-245 and Golgin-97, to the Golgi membrane (Lu and Hong, 2003; Panic et al., 2003; Setty et al., 2003). Mammalian Arl1 and GRIP-domain proteins, Golgin-245 or Golgin-97, have recently been shown to regulate membrane trafficking between the TGN and endosomal system (Lu et al., 2004; Yoshino et al., 2005). Studies also indicated that ARL1 has genetic interaction with YPT6 (Bensen et al., 2001; Tong et al., 2004). Ypt6p, a small GTPase, is associated with the Golgi and required for the fusion of endosome-derived vesicles with the late Golgi (Lewis et al., 2000; Siniossoglou et al., 2000). Overexpression of Arl1p complements temperature sensitivity of a ypt6 mutation, and a double deletion of arl1 and ypt6 in yeast is lethal (Bensen et al., 2001; Tong et al., 2004). Therefore, Arl1p was hypothesized to regulate membrane trafficking between the TGN and endosomal system and may have redundant function with Ypt6p in bringing endosome-derived retrograde vesicles to the TGN (Graham, 2004). These data are consistent with the conclusion that Arl1p functions in transport pathways in the TGN; however, no endogenous cargo of Arl1p-derived vesicles has yet been identified in yeast.

Recently, Arl3p was found to act upstream of Arl1p and regulate membrane binding of Arl1p, which, in turn, regulates Golgi localization of Imh1p (Jackson, 2003; Panic et al., 2003; Setty et al., 2003). This regulation was hypothesized to result from the recruitment of Arl1p’s guanine nucleotide exchange factor (GEF) by Arl3p (Jackson, 2003). In addition, the association of Arl3p with the Golgi membrane is dependent upon the acetylation of Arl3p at its N-terminus by an acetyltransferase and a Golgi-localized integral membrane protein, Sys1p (Behnia et al., 2004; Setty et al., 2004). Thus, a signaling cascade of Sys1p-Arl3p-Arl1p-Imh1p was proposed to regulate trafficking events in the TGN (Graham, 2004; Setty et al., 2004). Moreover, Gcs1p was identified as a GTPase-activating protein (GAP) for Arl1p that regulates Golgi association and activity of Arl1p (Liu et al., 2005). Hence, both Arl3p and Gcs1p regulate Arl1p activity.

Glycosylphosphatidylinositol (GPI)-anchored proteins are a diverse class of proteins that are anchored to the membrane by a post-translational lipid modification, the GPI-moiety. Previous studies of GPI-anchored protein trafficking have focused on transport between the endoplasmic reticulum (ER) and Golgi, and sorting and endocytosis at the plasma membrane (Muniz et al., 2000; Chatterjee and Mayor, 2001; Morsomme et al., 2003; Mayor and Riezman, 2004). By contrast, little is known about the molecular mechanisms by which GPI-anchored proteins move from the TGN to the plasma membrane. Gas1p, a GPI-anchored protein, represents the major cell-surface glycoprotein in yeast. In a Sar1p-
dependent process involving Gas1p interacting with a cargo receptor, Emp24p complex, Gas1p is delivered from the ER through COPII vesicles to the Golgi complex (Barlowe et al., 1994; Doering and Schekman, 1996; Muniz et al., 2000). Furthermore, Gas1p is sorted and packaged separately from non-GPI-anchored proteins, such as Gap1p, in the ER membrane (Muniz et al., 2000; Muniz et al., 2001). Since GPI-anchored proteins are localized in the membrane microdomains, lipid rafts (Kubler et al., 1996; Bagnat et al., 2000; Bagnat and Simons, 2002) and membrane microdomains are required for GPI-anchored protein transport in the ER (Skrzypek et al., 1997; Sutterlin et al., 1997), we speculated that the transport of GPI-anchored proteins and non-GPI anchored proteins from the TGN to the plasma membrane is regulated by different sorting and packaging machinery (Simons and van Meer, 1988), which is similar to the separation from the ER. Since a Sar1p/SNARE-based vesicle sorting mechanism has been established for the exit of GPI-anchored proteins from the ER to the Golgi complex, it is possible that GPI-anchored proteins also use an ARF-family-member-based mechanism to traffic from the TGN. In this study, we show that Arl1p and its signaling cascade Sys1p-Arl3p-Arh1p-Imhlp are involved in the transport from the late Golgi to the plasma membrane of the GPI-anchored protein Gas1p, but not the cell-wall-localized, GPI-anchored proteins Crh1p, Crh2p and Cwp1p, or the non-GPI-anchored protein Gap1p.

**Results**

**Arl1p is not directly involved in Snc1p recycling and vacuolar protein transport**

Snc1p is a v-SNARE protein that recycles between the Golgi and plasma membrane, and its endosome-Golgi recycling requires the presence of Ypt6p (Lewis et al., 2000; Siniossoglou et al., 2000). To address whether Arl1p functions in a pathway parallel to the Ypt6p, we examined the recycling of Snc1p in *arl1*-null cells. In *ypt6*-deleted cells, most of the GFP-Snc1p was localized intracellularly, with loss of its plasma membrane and polarized distribution as seen in wild-type cells (Fig. 1A). In an *arl1* mutant, however, unlike the findings in the *ypt6* mutant, GFP-Snc1p was localized to the plasma membrane and polarized to the daughter cells, suggesting that Arl1p is not required for the recycling of GFP-Snc1p from the Golgi to the plasma membrane or the fusion of GFP-Snc1p vesicles from the endosomal system to the Golgi. These data indicate that Arl1p and Ypt6p function in different pathways in the TGN.

Transport of carboxypeptidase Y (CPY) and alkaline phosphatase (ALP) from the TGN to the vacuole occurs by two well-characterized pathways (Cowles et al., 1997). Arl1p and Arl3p were reported to be involved in the transport of CPY, but not ALP, from the Golgi compartment (Bonangelino et al., 2002). In our studies, there was no significant defect in an *arl1* mutant in transport of CPY or ALP from the TGN to vacuole at 30°C and 37°C (our unpublished data) (Huang et al., 2002) (Fig. 1B,C), although, the transport of CPY and ALP in an *arl1* mutant exhibited a moderate delay at 16°C. These data suggest that Arl1p is not directly involved in the transport of these vacuolar enzymes from the TGN to vacuoles.

**Arl1p is involved in a specific transport pathway towards the cell surface**

We next examined the transport pathways towards the periplasmic region and plasma membrane. To observe the transport from the TGN to the periplasmic region, the secretion of biologically active mature α-factor was followed in a halo assay. No significant difference between wild-type and *arl1*-mutant cells was observed (data not shown), indicating that Arl1p is not required for α-factor secretion to the periplasmic region.

Plasma membrane proteins can be divided into lipid-raft associated or non-lipid-raft associated proteins; these two kinds of surface proteins might have distinct transport pathways from the TGN. To examine whether Arl1p is involved in a specific transport of plasma membrane proteins, we examined the distribution of Gas1p- and Gap1p-derived GFP-tagged proteins, GFP-GPI (Gas1p) and Gap1pK9K16-GFP, expressed in *arl1*-deleted yeast. GFP-GPI (Gas1p) is derived from Gas1p, a GPI-anchored protein and the major cell surface glycoprotein in yeast. Gap1p is a general amino-acid permease that does not possess a GPI anchor and does not localize to lipid rafts (Soetens et al., 2001; Bagnat and Simons, 2002). The Gap1pK9K16 mutation abolishes its ubiquitylation, thereby preventing its sorting to the vacuole; thus, the protein remains...
Arl1p regulates Gas1p transport

Arl1p is required for the surface targeting of GFP-GPI (Gas1p). (A) The location of GFP-GPI (Gas1p) and Gap1<sup>K9K16</sup>-GFP in arl1-, gcs1- and arl3-mutant cells. GFP-GPI (Gas1p) or Gap1<sup>K9K16</sup>-GFP was transformed into wild-type (WT), and arl1-, gcs1- and arl3-null cells and then induced in selection medium containing 2% galactose for 16 hours. GFP signals were observed as in Fig. 1A. (B) Distribution of GPI-anchored proteins. Crh1-GFP, Crh2-GFP, Cwp1-GFP or mRFP-Gas1 was transformed into wild-type and arl1-null cells. Transformants were grown overnight in selection medium and then transferred to fresh medium. After 3 hours of incubation, GFP signals were observed. (C) The intracellular accumulated mRFP-Gas1 in arl1 mutant cell colocalized with GFP-Sft2. Plasmids expressing mRFP-Gas1 and GFP-Sft2 were co-transformed into wild-type and arl1-mutant cells. GFP and RFP signals were observed as described in B. Arrows indicate colocalization of mRFP-Gas1 and GFP-Sft2.

The Arl3p-Arl1p cascade facilitates the transport of endogenous Gas1p from the Golgi to the plasma membrane

We further investigated whether endogenous Gas1p transport is indeed regulated by Arl1p. Gas1p is a β-1,3-glucanosyltransferase that is important in cell wall integrity (Kopecka and Gabriel, 1992; Mouyna et al., 2000). Disruption of GAS1 caused defects in morphogenesis and cell wall integrity, leading to hypersensitivity to Calcofluor White and Congo Red. Congo Red binds to β-1,3-glucan, a component of the yeast cell wall, and interferes with its assembly into a multi-stranded ribbon in the cell wall. Mutants with cell wall defects show hypersensitivity to Congo Red (Kopecka and Gabriel, 1992; Tomishige et al., 2003). Therefore, we first examined whether an arl1 mutant was hypersensitive to Congo Red. Fig. 3A shows that arf1, arl1, arl3, and gcs1 mutants were all hypersensitive to Congo Red. The hypersensitivity to Congo Red of these mutant yeasts could be rescued by an osmolarity adjustment (addition of 1.2 M sorbitol), indicating that the phenomenon of hypersensitivity to Congo Red in these mutants...
intracellular Gas1p was observed in wild-type cells, indicating that a higher percentage of type cells (42%, 52% and 45% compared with the 10-11% in Fig. 3B,D, the ratio of intracellular Gas1p to that on the surface residual Gas1p was detected by western blotting. As shown in treated with proteinase K to remove cell-surface Gas1p and intracellular Gas1p. Yeast spheroplasts were prepared and treated with proteinase K to remove cell-surface Gas1p and residual Gas1p was detected by western blotting. As shown in Fig. 3B,D, the ratio of intracellular Gas1p to that on the surface in arl1-, gcs1- and arl3-mutant cells is higher than that in wild-type cells (42%, 52% and 45% compared with the 10-11% in wild-type cells), indicating that a higher percentage of intracellular Gas1p was observed in arl1-, gcs1- and arl3-mutant cells than in wild-type cells.

To further demonstrate the role of Arl1p in anterograde transport, we used pulse-chase experiments to track the transport of de-novo-synthesized Gas1p from the TGN to the plasma membrane (Morsomme et al., 2003). After a 10-minute pulse and 80-minute chase, approximately 90% of Gas1p in wild-type cells reached the plasma membrane, whereas in arl1-deleted cells ~50% of Gas1p remained intracellularly (Fig. 3C,E). This intracellular accumulation was also observed in arl1-, arl3- and gcs1-null cells. These results demonstrate that the anterograde transport of de-novo-synthesized Gas1p requires the presence of Arl1p. Accumulation of glycosylated-Gas1p combined with accumulation of GFP-GPI (Gas1p) and mRFP-Gas1 in the late Golgi compartment (Fig. 2) suggests that Arl1p and its regulators (Arl3p and Gcs1p) are involved in Gas1p transport from the late-Golgi to the plasma membrane. Moreover, although arf1-mutant cells accumulate similar levels of Gas1p as arl1-mutant cells (Fig. 3B-E), they are more sensitive to Congo Red (Fig. 3A). Arf1p is the main Arf family member and is important for Golgi-to-ER and intra-Golgi transport. Because deletion of ARF1 leads to transport defects in many transport pathways in yeast (Gaynor et al., 1998; Yahara et al., 2004; Setty et al., 2004). Thus, we examined whether Sys1p is also required for the transport of Gas1p. Like the arl1 mutant, a sys1-null mutant produced Congo Red hypersensitivity assay. Different mutant yeast were serially diluted tenfold as indicated and spotted on plates of YPD, Congo Red (100 μg/ml) and Congo Red (100 μg/ml) with 1.2 M sorbitol to analyze their sensitivity to cell-wall interference. (B) Steady-state distribution of Gas1p in arf1, arl1, gcs1, arl3 and wide-type cells. After mild digestion with lyticase, spheroplasts were treated with or without proteinase K to remove Gas1p that was localized at the plasma membrane. Residual Gas1p was detected with western blotting. For quantification, Gas1p signals were normalized to actin with the proteinase-K-untreated samples defined as 1. The fraction of the proteinase-K-treated samples indicated below each lane represents the intracellular and glycosylated Gas1p in each strain. Arrowheads indicate the ER unglycosylated form (~105 kDa) of Gas1p. (C) Transport kinetics of Gas1p in arf1-, arl1-, gcs1- and arl3-mutant cells. Cells were pulse-labeled for 10 minutes with [35S]-Pro-mix and chased for 80 minutes. After removal of cell-surface proteins by treatment with proteinase K, Gas1p and actin were immunoprecipitated and analyzed by electrophoresis and autoradiography. Intracellular Gas1p was quantified as described in B. (D) Statistical analysis of the ratio of steady-state intracellular Gas1p in different mutant cells. Steady-state Gas1p distribution of three independent experiments was analyzed by plasma membrane proteinase shaving assay as described in B. (E) Statistical analysis of transport kinetics of intracellular Gas1p. Transport kinetics of Gas1p was traced by pulse-chase experiments as described in C. Error bars represent the mean ± s.e.m. of triplicate determinations.
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Red hypersensitivity to the same extent as an arl3 deletion (Fig. 4A). GFP-GPI (Gas1p), but not Gap1<sup>K9K16</sup>-GFP, accumulated in Golgi-like punctate structures in the sys1 mutant (Fig. 4B). The arf3 mutant serves as a control for this experiment because GFP-GPI (Gas1p) can properly localize to the plasma membrane in both the arf3-mutant and wild-type cells. These results are consistent with the current model for a Sys1p-Arl3p-Arl1p cascade in TGN-vesicular transport. The cascade of Arl3p-Arl1p to regulate Gas1p transport was further confirmed with Congo Red hypersensitivity analysis (supplementary material Fig. S1). Although the expression of wild-type Arl1p only partially rescued the Congo Red hypersensitivity of the arl3 mutant, the constitutive active Arl1Q72L fully rescued this hypersensitivity of the arl3 mutant. This observation supports the notion that Arl1p is downstream of Arl3p and the Arl3p-Arl1p cascade functions in Gas1p transport.

Endogenous Gas1p is mainly accumulated at the Golgi compartment in the arl1 mutant

To examine whether the subcellular distribution of endogenous Gas1p was affected in the arl1 mutant, we first fractionated spheroplast-homogenized lysates into two fractions, plasma-membrane-rich (P13) and microsome-Golgi-rich and soluble fraction (S13), by sedimentation centrifugation. Fig. 5A shows that the level of Gas1p in the microsome-Golgi-rich and soluble fraction (S13) in the arl1 mutant is considerably higher than in the wild-type cells. We further investigated this by sucrose-density-gradient centrifugation to separate and analyze the fraction S13. Gas1p, Drs2p (late Golgi marker), Emp47p (early Golgi marker), Pgk1p (cytosol marker) and Pep12p (endosome marker) in each fraction were identified by western blot analysis and quantified (Fig. 5B). Although in the arl1 mutant, some Gas1p distributed at lighter fractions (2-6 fractions), most of the Gas1p was at fractions 7-9 and distribution pattern of Gas1p was more similar to that of the late Golgi protein Drs2p (Fig. 5B lower panel). These data indicate that, in

Fig. 4. Sys1p is involved in transport of Gas1p. (A) Congo Red hypersensitivity of the sys1 mutant. Different mutant cells (as indicated) were serially diluted and spotted on plates of YPD, Congo Red (100 μg/ml), and Congo Red (100 μg/ml) with sorbitol. A gas1 mutant was used as a control cell. (B) GFP-GPI (Gas1p) distribution is abnormal in arl1- and sys1-mutant cells. GFP-GPI (Gas1p) or Gap1<sup>K9K16</sup>-GFP were transformed into wild-type, arl1-, sys1- and arf3-null cells and then induced in selection medium containing 2% galactose for 16 hours. Living cells were imaged using a Zeiss microscope.

Fig. 5. Subcellular distribution of endogenous Gas1p. (A) Differential centrifugation analysis. Wild-type or arl1-mutant cells were grown in YPAD medium, subjected to velocity sedimentation and then separated into P13 and S13 fractions as described in Materials and Methods. Proteins in samples of fractions were analyzed by immunoblotting. Gas1p, Pma1p (plasma membrane marker), Pgk1p (cytosol marker) and Kex2p (Golgi marker) were identified with specific antibodies. (B) Sucrose-density-gradient centrifugation analysis. S13 was layered on top of the sucrose gradient (10-30%), which was then subjected to centrifugation as described in Materials and Methods. Twelve fractions were collected from the top and samples were analyzed by western blottting using antibodies against Gas1p, Drs2p (late Golgi marker), Emp47p (early Golgi marker), Pgk1p (cytosol marker) and Pep12p (endosome marker). The lower panel displays the quantification of the western blot results.
the arl1 mutant, endogenous Gas1p mainly accumulated at the Golgi compartment and most likely at the late Golgi.

Imh1p, but not Ypt6p, is partly involved in Gas1p transport
We next examined whether an effector of Arl1p, Imh1p, is involved in Gas1p transport. In the Congo Red hypersensitivity assay, imh1-null cells did not show a growth defect compared with the growth of arl1 mutant yeast on YPD and Congo Red plates (Fig. 6A). However, it did cause a mild intracellular accumulation of Gas1p (Fig. 6B,C), suggesting that Imh1p is involved in Gas1p transport.

Although anterograde transport of Gas1p is affected in the arl1 mutant, it is possible that this is an indirect effect resulting from a defect in retrograde transport (Graham, 2004; Lu et al., 2004). Therefore, we examined whether ypt6-null cells exhibit a similar defect as seen with the arl1 mutant. The ypt6-null cells did not show hypersensitivity to Congo Red when the growth of ypt6-null cells on YPD and Congo Red plates are compared (Fig. 6A). Furthermore, ypt6-null yeast did not exhibit an obvious defect in Gas1p transport in the plasma membrane protease shaving assay (Fig. 6B,C), indicating that the deficiency of retrograde transport mediated by Ypt6p did not cause a Gas1p transport delay.

Regulation of Arl1p activity is important for Gas1p transport
To examine the effect of Arl1p activity on Gas1p transport, we introduced wild-type, constitutively active (Arl1Q72L), and constitutively inactive (Arl1T32N) Arl1p into arl1-null or wild-type cells. Ectopically expressed Arl1p, but not Arl1Q72L or Arl1T32N, repressed the Congo Red hypersensitivity observed in arl1 cells (Fig. 7A). In wild-type cells, overexpression of Arl1T32N, but not Arl1Q72L, caused hypersensitivity to Congo Red (Fig. 7B). The inability of constitutively active Arl1Q72L to complement the loss of endogenous Arl1p was also observed in our previous report (Liu et al., 2005). This result is consistent with the observation that a gcs1 deletion also disturbs Gas1p transport (Fig. 2A and Fig. 3). We next examined the localization of GFP-GPI (Gas1p) in wild-type or arl1-null cells overexpressing different forms of Arl1p. Consistent with Fig. 7A, in the arl1 mutant, only wild-type Arl1p could rescue the plasma membrane targeting of GFP-GPI. The expression of Arl1Q72L even caused GFP-GPI (Gas1p) accumulation in enlarged Golgi structures (Fig. 7C, upper panel). In wild-type cells, expression of Arl1T32N caused GFP-GPI (Gas1p) to accumulate in punctate structures in the absence of plasma membrane signals (Fig. 7C lower panel). Similar to Arl1T32N, expression of Arl1Q72L also caused accumulation of some GFP-GPI (Gas1p) in Golgi-like and unknown structures. However, it did not appear to thoroughly block the transport of GFP-GPI (Gas1p) to the plasma membrane. This result suggests that although Arl1Q72L is unable to hydrolyze GTP for proper vesicle trafficking in wild-type yeast, endogenous Arl1p still sustains basal level transport of its cargo molecules. Together, our data indicate that the transport of Gas1p needs functional regulation of Arl1p involving GTP binding, Golgi targeting and GTP hydrolysis.

Arl1Q72L suppresses the Congo Red hypersensitivity in an arl3 mutant
Although Arl1Q72L could not restore the Congo Red hypersensitive phenotype in an arl1 mutant (Fig. 7A), it interestingly could rescue hypersensitivity in an arl3 mutant (supplementary material Fig. S1). Therefore, we asked what is different regarding the effect of Arl1Q72L in arl3 and arl1 mutant. Consistent with previous findings (Panic et al., 2003), we observed that endogenous Arl1p or ectopically expressed Arl1p lost its Golgi localization in the arl3 mutant (Fig. 8A). Our previous report (Liu et al., 2005) and Fig. 8B (upper panel) showed that Arl1Q72L caused the enlargement of late Golgi when expressed in ARL3 wild-type cells. However, Arl1Q72L
maintained the Golgi-like distribution in the arl3 mutant (Fig. 8A) and these Golgi-like structures appeared normal both in size and number. We next examined the late Golgi structure of Arl1Q72L expressed in an arl3 mutant by co-expressing GFP-Sft2p and Arl1Q72L in an arl3 mutant (Fig. 8B). The Arl1Q72L partially colocalized with the late Golgi marker Sft2p in an arl3 mutant and, unlike that in ARL3 cells, did not cause enlargement of the Golgi. These results suggest that the proper location of Arl1Q72L and its normal morphology at the late Golgi in an arl3 mutant is correlated to its function in suppressing the Congo Red hypersensitivity in the arl3 mutant.

**Discussion**

Here, we report that Arl1p participates in a class of GPI-anchored protein trafficking from the late Golgi to the plasma membrane. This is the first identification of an endogenous cargo molecule of Arl1p in yeast. Our study also supports the notion that regulators of Arl1p, such as Sys1p, Arl3p and Gcs1p, and effector Imh1p, all participate in the transport of Gas1p. The transport of Gas1p requires functional regulation of Arl1p involving GTP binding, Golgi targeting and GTP hydrolysis. Based on our findings, we conclude that the signaling cascade Sys1p-Arl3p-Arl1p-Imh1p facilitates the transport of a subset of GPI-anchored proteins from the late Golgi to the plasma membrane.

**Arl1p plays a distinct role from Ypt6p**

Our results indicate that Arl1p plays a role in the anterograde transport of Gas1p from the late Golgi to the plasma membrane, and rule out a redundant function of Arl1p with Ypt6p on the retrograde transport. Arl1p, Gcs1p and Arl3p (our unpublished data) are not required for Snc1p recycling that is mediated by Ypt6p. Although we could not exclude the possibility that Arl1p is involved in retrograde transport from the endosomal system to the TGN for cargo proteins other than Snc1p, we demonstrated that Ypt6p and Arl1p do not mediate the same cargo protein trafficking. Therefore, the genetic interaction and synthetic lethality of **ARL1** and **YPT6** might not come from redundant functions in bringing endosome-derived retrograde vesicles to the TGN. However, the observation that deletion of **YPT6** did not retard the transport of Gas1p or show Congo Red hypersensitivity further supports the hypothesis that Arl1p and Ypt6p have distinct functions.

**Arl1p is not directly involved in vacuolar protein transport**

Although Arl1p and Arl3p were reported to be involved in the transport of CPY from the Golgi compartment (Bonangelino et al., 2002), our observation and another report have indicated that CPY is processed with kinetics similar to wild-type cells, but with moderate delay or mis-sorting to the extracellular medium in **arl1** mutant (Lee et al., 1997; Jochum et al., 2002). The partial secretion of CPY (Jochum et al., 2002; Rosenwald et al., 2002) and mild delay in CPY and ALP processing (Fig. 1B,C) suggest that Arl1p is not directly involved in vacuolar protein transport. The discrepancy between different reports of CPY processing in **arl1**-mutant cells might result from differing extents of indirect effects produced from Golgi function deficiencies, although the use of yeast strains with different genetic background may also be responsible for these differences.

**Arl1p facilitates the transport of a subset of GPI-anchored proteins**

Initially, we showed that Arl1p is required for GFP-tagged, GPI-anchored protein transport, but not non-GPI-anchored protein transport, from the Golgi to the plasma membrane. To analyze endogenous Gas1p...
The Sys1p-Arl3p-Arl1p-Imh1p cascade is involved in the transport of Gas1p to the plasma membrane

Using Gas1p distribution as a cargo marker, we further demonstrate that the Sys1p-Arl3p-Arl1p-Imh1p cascade is involved in a potential trafficking pathway for Gas1p from the late Golgi to the plasma membrane. Although the deletion of IMH1 could partially affect the transport of Gas1p from the late Golgi to the plasma membrane. Although the deletion of IMH1 could partially affect the transport of Gas1p from the late Golgi to the plasma membrane. Although the deletion of IMH1 could partially affect the transport of Gas1p from the late Golgi to the plasma membrane. Although the deletion of IMH1 could partially affect the transport of Gas1p from the late Golgi to the plasma membrane. Although the deletion of IMH1 could partially affect the transport of Gas1p from the late Golgi to the plasma membrane.

Proper GTP hydrolysis is crucial for the function of Arl1p

With the expression of ectopic mutant Arl1p in wild-type and arl1-null cells, the importance of GTP hydrolysis by Arl1p was observed (Fig. 7). The constitutively active Arl1p mutant Arl1Q72L could not rescue the Congo Red hypersensitivity in the absence of endogenous Arl1p. In addition, Arl1Q72L even caused substantial intracellular accumulation of Gas1p in wild-type and arl1-mutant cells, indicating that GTP hydrolysis is crucial for the function of Arl1p. Because GTP hydrolysis by Arl1p is decreased in the gcs1 mutant (Liu et al., 2005), we also observed a Gas1p transport defect in a gcs1 mutant (Fig. 3). It has been reported that GTP hydrolysis in the early stage of vesicle formation is important (Presley et al., 2002). Therefore, it is reasonable that the GTP-hydrolysis-deficient mutant Arl1Q72L cannot restore the transport defect of Gas1p in an arl1 mutant.

However, Arl1Q72L seems to have normal function in the arl3 mutant and lose its effect on late Golgi morphology. We propose that although Arl1Q72L can target to late Golgi in the arl3 mutant, it may dissociate from membranes more easily than in ARL3-positive cells. Thus, Arl1Q72L maintains the transport function on the late Golgi in the arl3 mutant and will not cause the enlargement of the late Golgi.

Recent reports have found that the Arl1p homologues in Trypanosoma brucei (TBcArl1, and the GRIP-domain-containing proteins Golgin-245 and Golgi-97) are involved in the exocytosis of GPI-anchored variant surface glycoprotein (VSG) transport of GPI-anchored marker protein (YFPSP-GPI) from the TGN to the cell periphery and transport of E-cadherin out of the TGN (Kakimura et al., 2004; Price et al., 2005; Lock et al., 2005). Intriguingly, E-cadherin, like GPI-anchored proteins, is also lipid-raft-associated (Seveau et al., 2000; Muniz et al., 2001), we conclude that different classes of GPI and non-GPI-anchored proteins would be sorted into different vesicles from the Golgi to the plasma membrane.
All of these reports indicate a potential function of Arl1p in regulation of lipid-rafts-associated protein transport from the TGN. However, the localization of Pma1p, another lipid-raft-associated membrane protein, is not affected by ARLI disruption (our unpublished data), suggesting that not all lipid raft-associated plasma membrane protein transport is mediated by Arl1p.

In this study, we found that the molecular machinery used to transport the GPI-anchored protein Gas1p is Arl1p dependent. We further demonstrated that the signaling cascade Sys1p-Arl1p, another lipid-raft-associated protein transport is captured using a Zeiss Axioskop microscope equipped with a cool Snap fx camera, overnight culture or induction, mid-log-phase cells were examined and images were provided by J. Arroyo (Universidad Complutense de Madrid, Spain). mRFP-Gas1 provided by J. Arroyo (Universidad Complutense de Madrid, Spain). mRFP-Gas1 was kindly provided by K. Simons (MPI-CBG, Dresden, Germany). Cbh-GFP, Cbh2-GFP and Cwp1-GFP were kindly provided by Hugh R. B. Pelham (MRC-LMB, Cambridge, UK). The anti-Cbh-GFP antibody was a gift from K. Simons (MPI-CBG, Dresden, Germany). CBG, Dresden, Germany). Cbh1-GFP, Cbh2-GFP and Cwp1-GFP were kindly provided by Hugh R. B. Pelham (MRC-LMB, Cambridge, UK). The anti-Cbh-GFP antibody was a gift from K. Simons (MPI-CBG, Dresden, Germany). CBG, Dresden, Germany). CBG, Dresden, Germany).

Microscopy
Images of live cells containing GFP-tagged or mRFP-tagged proteins were obtained after growth in synthetic medium to mid-log phase. All fluorescence protein-tagged chimeras were cultured in selection medium with 2% glucose, except GFP-GPI (Gas1p) and GplK66/GFP, which were induced with 2% galactose. After overnight culture or induction, mid-log-phase cells were examined and images were captured using a Zeiss Axioskop microscope equipped with a cool Snap fx camera, and then processed with Image-Pro Plus software.

Materials and Methods
Strains, media and microbiological techniques
Table 1 lists the yeast strains used in this study. Yeast culture media were prepared according to standard protocols (Sambrook et al., 1989). GFP-GPI (Gas1p) (MBQ31) and Gap1K66.GFP (TPQ88) were kindly provided by K. Simons (MPI-CBG, Dresden, Germany). Cbh-GFP, Cbh2-GFP and Cwp1-GFP were kindly provided by Hugh R. B. Pelham (MRC-LMB, Cambridge, UK). The anti-Cbh-GFP antibody was a gift from K. Simons (MPI-CBG, Dresden, Germany). CBG, Dresden, Germany). CBG, Dresden, Germany).

Plasmids and antibodies
Plasmids used in this study are listed in Table 2. Plasmids were constructed according to standard protocols (Sambrook et al., 1989). GFP-GPI (Gas1p) (MBQ31) and Gap1K66.GFP (TPQ88) were kindly provided by K. Simons (MPI-CBG, Dresden, Germany). Cbh-GFP, Cbh2-GFP and Cwp1-GFP were kindly provided by Hugh R. B. Pelham (MRC-LMB, Cambridge, UK). The anti-Cbh-GFP antibody was a gift from K. Simons (MPI-CBG, Dresden, Germany). CBG, Dresden, Germany).

Table 1. Yeast strains used in this study

| Strain          | Genotypea | Source or reference                  |
|-----------------|-----------|--------------------------------------|
| YPH250          | MATa ade2, his3, leu2, lys2, trp1, ura3–52, ARF1, ARF2, ARF3, ARL1, ARL3, GCS1 | (Lee et al., 1997)                  |
| YPH250dr1       | MATa ade2, his3, leu2, lys2, trp1, ura3–52, ARF1, ARF2, ARF3, ARL1, ARL3, GCS1 | (Huang et al., 2003)               |
| YPH250dl1       | MATa ade2, his3, leu2, lys2, trp1, ura3–52, ARF1, ARF2, ARF3, ARL1, ARL3, GCS1 | (Lee et al., 1997)                  |
| YPH250dl2       | MATa ade2, his3, leu2, lys2, trp1, ura3–52, ARF1, ARF2, ARF3, ARL1, ARL3, GCS1 | (Huang et al., 2002)               |
| BY4741          | MATa his3, leu2, met15, ura3, ARL1, GCS1, ARL3, ARF3, YPT6, IMH1, SYS1, GAS1 | Invitrogen                        |
| BY4741ar1       | MATa his3, leu2, met15, ura3, ARL1, GCS1, ARL3, ARF3, YPT6, IMH1, SYS1, GAS1 | Invitrogen                        |
| BY4741V6       | MATa his3, leu2, met15, ura3, ARL1, GCS1, ARL3, ARF3, YPT6, IMH1, SYS1, GAS1 | Invitrogen                        |
| BY4741ys1       | MATa his3, leu2, met15, ura3, ARL1, GCS1, ARL3, ARF3, YPT6, IMH1, SYS1, GAS1 | Invitrogen                        |

a ade, adenine-requiring; his, histidine-requiring; trp, tryptophan-requiring; ura, uracil-requiring; leu, leucine-requiring; met, methionine-requiring; lys, lysine-requiring.

Table 2. Plasmids used in this study

| Plasmids          | Description                        | Source or reference                  |
|-------------------|------------------------------------|--------------------------------------|
| GFP-Sft2          | GFP-Sft2 in pRS406 under TPI promoter       | Hugh R. B. Pelham (Lewis et al., 2000) |
| GFP-Snc1          | GFP-Snc1 in pRS406 under TPI promoter     | (Bagnat and Simons, 2002)            |
| MBQ31             | GFP-GPI in p416 under GAL1 promoter      | K. Simons                            |
| TPQ88             | Gap1K66.GFP under GAL1 promoter          | I. Arroyo                            |
| pIV40G            | Cbh1-GFP in YEp352                   | J. Arroyo                            |
| pAR14             | Cwp1-GFP in pRS416                    | J. Arroyo                            |
| pMF608            | mRFP-Gas1 in pRS305                   | Y. Jigami                            |
| Yplac12           |                                    | (Gietz and Sugino, 1988)            |
| AR1L1/Yplac12     |                                    | (Liu et al., 2005)                  |
| ARL1G/Yplac12     |                                    | (Liu et al., 2005)                  |
| Yps1-GFP          |                                    | This study                           |
autodigestion were performed essentially as described (Huang et al., 1999), using anti-CPY or anti-ALP antiserum.

Plasma membrane proteinase shaving assay

A plasma membrane proteinase shaving assay was performed as described previously (Morsomme et al., 2003). After a 10-minute incubation with 10 nM MESNA (Sigma), the cell wall was digested in a 20-minute treatment with lyticase (100U/ml) at room temperature and spheroplasts were incubated with 200 μg/ml proteinase K (Sigma) for 30 minutes. After stopping the digestion with PMSF (1 mM) at 0°C for 10 minutes, proteins were TCA-precipitated and analyzed by immunoblotting. The signals on X-ray film were quantified using the Alphalager.

Pulse-chase and plasma membrane proteinase shaving experiments

Experiments were performed as previously described with some modifications (Morsomme et al., 2003). Briefly, proteins were labeled with 30 μCi of Pro-mix L-35S label per A600 unit and chased with 1% chase solution (0.3% cysteine, 0.4% methionine, and 100 mM (NH4)2SO4) for 80 minutes, followed by the plasma membrane proteinase shaving assay procedure. After digestion, cells were lysed and Gas1p and actin were immunoprecipitated using specific antibodies. Proteins were then detected by autoradiography after separation by SDS-PAGE.

Subcellular fractionation by velocity or sucrose-density gradient

Cells were harvested by centrifugation from cultures (50 ml) grown in YPD medium (2% glucose or YPD medium were prepared for indirect immunofluorescence (2002). Cell surface polarization during yeast mating. Experiments were performed as previously described with some modifications (Morsomme et al., 2003). Briefly, proteins were labeled with 30 μCi of Pro-mix L-35S label per A600 unit and chased with 1% chase solution (0.3% cysteine, 0.4% methionine, and 100 mM (NH4)2SO4) for 80 minutes, followed by the plasma membrane proteinase shaving assay procedure. After digestion, cells were lysed and Gas1p and actin were immunoprecipitated using specific antibodies. Proteins were then detected by autoradiography after separation by SDS-PAGE.

Indirect immunofluorescence

Cells grown to a density of A600=0.5-1 in 3 ml of minimal selective medium with 0.4% methionine, and 100 mM (NH4)2SO4 for 80 minutes, followed by the plasma membrane proteinase shaving assay procedure. After digestion, cells were lysed and Gas1p and actin were immunoprecipitated using specific antibodies. Proteins were then detected by autoradiography after separation by SDS-PAGE.

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