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The Gcs1 and Age2 ArfGAP proteins provide overlapping essential function for transport from the yeast trans-Golgi network

Pak Phi Poon,[1,2] Steven F. Nothwehr,[3] Richard A. Singer,[2] and Gerald C. Johnston[1]

[1]Department of Microbiology and Immunology and [2]Department of Biochemistry and Molecular Biology, Dalhousie University, Halifax, Nova Scotia, Canada B3H 4H7
[3]Division of Biological Sciences, University of Missouri, Columbia, MO 65211

Many intracellular vesicle transport pathways involve GTP hydrolysis by the ADP-ribosylation factor (ARF) type of monomeric G proteins, under the control of ArfGAP proteins. Here we show that the structurally related yeast proteins Gcs1 and Age2 form an essential ArfGAP pair that provides overlapping function for TGN transport. Mutant cells lacking the Age2 and Gcs1 proteins cease proliferation, accumulate membranous structures resembling Berkeley bodies, and are unable to properly process and localize the vacuolar hydrolase carboxypeptidase (CPY) and the vacuolar membrane protein alkaline phosphatase (ALP), which are transported from the TGN to the vacuole by distinct transport routes. Immunofluorescence studies localizing the proteins ALP, Kex2 (a TGN resident protein), and Vps10 (the CPY receptor for transport from the TGN to the vacuole) suggest that inadequate function of this ArfGAP pair leads to a fragmentation of TGN, with effects on secretion and endosomal transport. Our results demonstrate that the Gcs1 + Age2 ArfGAP pair provides overlapping function for transport from the TGN, and also indicate that multiple activities at the TGN can be maintained with the aid of a single ArfGAP.

Introduction

Eukaryotic cells have highly regulated processes for the orderly exchange of proteins and lipids among organelles, including the plasma membrane, and for secretion and endocytosis. An important element of this overall process is the generation of transport vesicles that package cargo molecules present in a donor organelle or membrane for delivery to an appropriate target organelle or membrane (for review see Rothman, 1994). Within the early secretory system the flow of transport vesicles is bidirectional, with vesicles created at the ER moving in a forward direction to the Golgi apparatus, and other vesicles created at the Golgi retrieving material by retrograde vesicular transport to the ER and within the Golgi itself (Pelham, 1995). Likewise, transport from the TGN, a compartment that intersects the secretory and endocytic pathways (for review see Lemmon and Traub, 2000), uses transport vesicles that move cargo to the plasma membrane or to the lysosome/vacuole, in some cases through a sorting compartment termed the endosome. Endosomal sorting coordinates membrane transport among the Golgi, plasma membrane, and vacuole. For example, the yeast prevacuolar endosome sorts material for retrieval back to the TGN and directs other material to the vacuole (Piper et al., 1995; Seaman et al., 1997; Nothwehr et al., 1999). The endosomal compartment also participates in the recycling of cargo internalized from the plasma membrane, such as membrane-associated receptors (Chen and Davis, 2000; Lewis et al., 2000; Wiederkehr et al., 2000).

Vesicular transport involves several steps, including the recruitment of a protein complex that forms the coat of a vesicle as it buds from the donor membrane, the packaging of cargo within the developing vesicle, and the fusion of a fully formed vesicle with a target membrane for delivery of cargo (Tanigawa et al., 1993; Letourneur et al., 1994; Rothman and Wieland, 1996; Bremser et al., 1999). For a variety of vesicular transport stages, including retrograde transport from the Golgi to the ER (Letourneur et al., 1994), endocytosis (Lenhard et al., 1992), and exit from the TGN (Lemmon and Traub, 2000), transport vesicle formation depends on several highly conserved proteins,

Address correspondence to Gerry Johnston, Dept. of Microbiology and Immunology, Dalhousie University, Halifax, Nova Scotia, Canada B3H 4H7. Tel.: (902) 494-6465. Fax: (902) 494-5125. E-mail: g.c.johnston@dal.ca

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including the monomeric GTP-binding proteins termed ARF (ADP-ribosylation factor)* (Donaldson and Klausner, 1994). ARF proteins are activated by the binding of GTP and are deactivated through GTP hydrolysis. In the activated, GTP-bound form, ARF proteins recruit cytosolic coat proteins to the membrane site of nascent vesicle budding (Rothman and Wieland, 1996). ARF proteins have been implicated in the recruitment of a variety of vesicle coats, including the COPI coatomer complex that assembles on vesicles destined for retrograde transport from the Golgi to the ER (Letourneur et al., 1994) and the clathrin/adaptor (AP-1) coat that associates with vesicles transporting cargo from the TGN to the endosome (for review see Schmid, 1997). ARF proteins are also involved in recruitment of another adaptor complex, AP-3 (Dittie et al., 1996; Ooi et al., 1998), that does not associate with clathrin (Newman et al., 1995; Dell'Angelica et al., 1997; Simpson et al., 1997). An ARF protein is also a GTPase; hydrolysis by ARF of its bound GTP ultimately leads to inactivation of ARF and the release of associated coat proteins (Antonny et al., 1997; Zhao et al., 1999). Thus, the GTP-binding and GTPase activities of ARF proteins comprise a regulatory switch for a variety of transport vesicles at several different locations.

The involvement of ARF proteins in Golgi and endosomal activities is well established for the yeast *Saccharomyces cerevisiae* (Gaynor et al., 1998; Yahara et al., 2001). Genetic evidence indicates that yeast ARF proteins mediate the formation of COPI-coated vesicles for retrograde transport from the Golgi to the ER (Stearns et al., 1990; Gaynor et al., 1998; Poon et al., 1999) and clathrin-related vesicles for transport from the TGN (Chen and Graham, 1998). Yeast cells also contain the three groups of heterotetramers that comprise the adaptor complexes AP-1, AP-2, and AP-3 (for review see Odorizzi et al., 1998), although, with the exception of AP-3, the involvement of these yeast AP complexes in specific transport pathways is unresolved.

At least two distinct routes for transport of yeast proteins from the TGN to the vacuole have been identified by characterizing the transport of the vacuolar hydrolase carboxypeptidase Y (CPY) and a type II vacuolar membrane protein, alkaline phosphatase (ALP). Genetic studies reveal that the CPY pathway routes CPY through the prevacuolar endosome (Cowles et al., 1997b; Piper et al., 1997) in a clathrin-dependent fashion (Seeger and Payne, 1992; Gaynor et al., 1998), whereas the ALP pathway transports ALP to the vacuole independently of the prevacuolar endosome (Cowles et al., 1997b; Piper et al., 1997). The exit of CPY from the TGN may involve clathrin-coated vesicles (Deloche et al., 2001), whereas ALP has been found to exit the TGN in a vesicle coated with the AP-3 adaptor complex (Cowles et al., 1997a; Stepp et al., 1997).

An equal variety of roles may exist for the GTPase-activating proteins (GAPs) that regulate the GTPase cycle of ARF (Donaldson, 2000). ARF proteins themselves do not possess intrinsic GTPase activity (Kahn and Gilman, 1986), and consequently rely on GAPs for proper regulation. These GTPase-activating proteins are important for ARF-mediated vesicular transport. Formation of a coat for the generation of a transport vesicle and proper packaging of cargo is thought to depend on a priming complex that contains both ARF and ArfGAPs (Springer et al., 1999). In mammalian cells, ArfGAPs have also been found to link aspects of cell signaling and morphogenesis to vesicular transport (Randazzo et al., 2000). Thus, ArfGAPs may allow both temporal as well as spatial coordination of the ARF GTPase cycle (Donaldson, 2000).

Yeast cells have six structurally related proteins with the potential to provide GAP activity for the yeast Arf1 and Arf2 proteins (Poon et al., 1996, 1999; Zhang et al., 1998) and to mediate vesicular transport (Poon et al., 1999). Two of these proteins, Gcs1 and Glo3, have been shown by both in vivo and in vitro criteria to be yeast ArfGAPs capable of stimulating the GTPase activity of the yeast Arf1 protein. Because the yeast Arf1 protein has been implicated in many stages of intracellular membrane transport (Yahara et al., 2001), the finding that Gcs1 + Glo3 ArfGAPs can stimulate Arf1 GTPase does not by itself provide insight into the particular stage of vesicular transport that is dependent on the activity of this pair of ArfGAPs. Indeed, the ability of a single Arf protein to mediate many stages of vesicular transport suggests that regulators of Arf function must provide spatial regulation. There is increasing evidence, both from yeast and other systems (Donaldson, 2000; Donaldson and Jackson, 2000), that ArfGAP proteins may be localized in vesicular transport. We have shown that Gcs1 and Glo3 provide overlapping function for Golgi to ER retrograde transport (Poon et al., 1999) without affecting membrane transport from the plasma membrane to the vacuole (unpublished data). Here we describe an analogous functional relationship between the ArfGAP Gcs1 and another structurally related protein, Age2. Like Gcs1 and Glo3, the Gcs1 and Age2 proteins constitute an ArfGAP pair that provides essential overlapping function for vesicular transport (Zhang et al., 1998). In the present study we have addressed the stage of intracellular membrane transport that is dependent on the Gcs1 + Age2 ArfGAP pair, and report that Gcs1 in concert with Age2 facilitates TGN/endosomal transport. Moreover, each member of the Gcs1 + Age2 pair can mediate the multiple transport activities at the TGN. Thus, our studies indicate that at least two stages of vesicular transport in yeast are modulated by pairs of ArfGAPs, with the Gcs1 ArfGAP involved in both stages.

Results

Deletion of the *AGE2* gene is lethal for cells lacking the *GCS1* gene

The Gcs1 and Glo3 ArfGAPs activate the GTPase function of members of the ARF family of monomeric GTP-binding proteins (Poon et al., 1996, 1999). Our previous genetic and molecular analyses revealed that both the Gcs1 and Glo3 proteins provide function for retrograde vesicular transport from the Golgi to the ER. The presence of either of these ArfGAP proteins is sufficient for this retrograde activity, but the simultaneous deficiency in both Gcs1 and Glo3 activities results in impaired

*Abbreviations used in this paper: ALP, alkaline phosphatase; AP, adaptor protein; ARF, ADP-ribosylation factor; CPY, carboxypeptidase Y; DIC, differential interference contrast; GAP, GTPase-activating protein; HA, hemagglutinin; PVC, prevacuolar compartment.

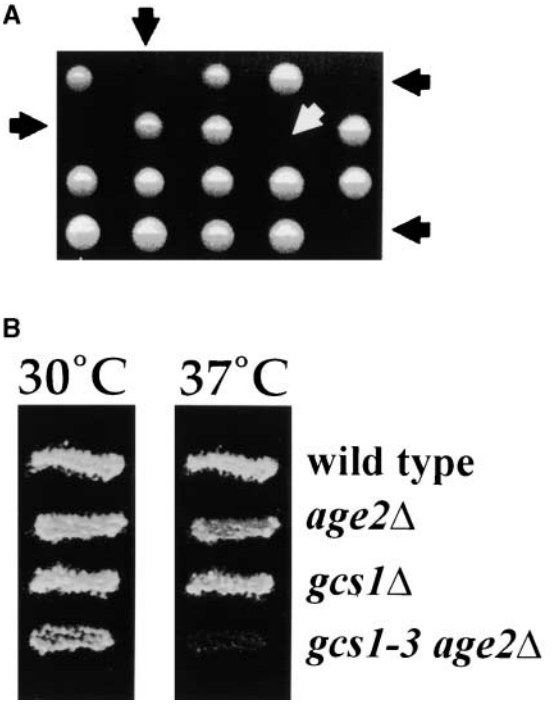

Figure 1. **Absence of both Gcs1 and Age2 proteins is lethal.** (A) A *GCS1/gcs1::URA3 AGE2/ age2::HIS3* heterozygous diploid strain was sporulated, and haploid segregants from resulting asci were incubated on solid medium at 30°C to assess growth. Segregant colonies were also replica plated to selective media to assess genotypes. Arrows indicate the positions of *gcs1::URA3 age2::HIS3* double-mutant segregants. (B) A *GCS1/gcs1::URA3 AGE2/ age2::HIS3* heterozygous diploid strain containing plasmid pPP805-3, bearing the temperature-sensitive *gcs1-3* allele, was sporulated, and the resulting segregants were assessed for growth at 30 and 37°C. Relevant genotypes are indicated.

Golgi-to-ER transport (Poon et al., 1999). Indeed, cells lacking both ArfGAPs are inviable (Zhang et al., 1998; Poon et al., 1999). Thus, Gcs1 and Glo3 proteins have an overlapping essential function for Golgi-to-ER transport. Moreover, Gcs1 may have additional roles in vesicular transport not shared by Glo3, for the absence of Gcs1 imparts a conditional defect in endocytosis (Wang et al., 1996), whereas cells lacking Glo3 are indistinguishable from wild-type cells in that endocytosis assay (unpublished data). To assess the involvement of Gcs1 in other aspects of vesicular transport, we directed our attention to other potential ArfGAP proteins that may also act in concert with Gcs1.

The yeast genome encodes four proteins with structural similarity to the Gcs1 and Glo3 ArfGAPs. The individual deletion of each of the genes encoding these proteins has little effect on cell growth (Zhang et al., 1998, and unpublished data). However, pair-wise deletions reveal that, like the deletion of both *GCS1* and *GLO3* genes, the deletion of *GCS1* and the YIL044c open reading frame (referred to as *SAT2* in Zhang et al., 1998) is lethal (Zhang et al., 1998, and Fig. 1 A). Therefore, we investigated the potentially overlapping functions provided by Gcs1 and the protein encoded by YIL044c, which has been assigned the name Age2 (ArfGAP effector).

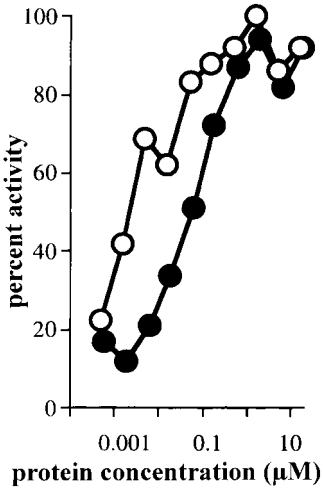

Figure 2. **Age2 is an ArfGAP.** GAP activity of recombinant Age2 protein (●) was assessed by in vitro hydrolysis of radiolabeled GTP bound to recombinant yeast Arf1 protein. For comparison, the activity of the Gcs1 ArfGAP (○) on Arf1 protein is also displayed. Arf-bound GTP was not hydrolysed when assayed with bovine serum albumin over the same protein concentrations (unpublished data). Activity is expressed as percent of maximum radioactivity released from GTP-Arf with the highest level of release expressed as 100%. Recombinant Gcs1 ArfGAP was able to stimulate hydrolysis of >90% of labeled material associated with recombinant Arf1 protein.

The Age2 protein, like Gcs1, is an ArfGAP in vitro

As with Gcs1 and Glo3, the most striking similarity between the Gcs1 and Age2 proteins is in an NH_2-terminal 68-residue region of each protein, including the Zn-binding motif required for ArfGAP activity in vitro (Cukierman et al., 1995; Poon et al., 1996). To determine if the structural similarity among the Gcs1, Glo3, and Age2 proteins extends to function, we assessed the ArfGAP activity of Age2. Recombinant Age2 protein was produced in *Escherichia coli* and assessed in vitro using recombinant yeast Arf1 protein as substrate. As expected based on structural considerations, Age2 protein was able to stimulate the hydrolysis of Arf1-bound GTP to GDP (Fig. 2). Under the assay conditions used here, Age2 ArfGAP activity was approximately tenfold lower than that of Gcs1, similar to the ratio of activity of Glo3 to Gcs1 (Poon et al., 1999). Like Gcs1 and Glo3, the Age2 protein is a yeast ArfGAP.

Inadequate overlapping activity of the Gcs1 and Age2 proteins leads to rapid growth arrest and impaired invertase transport

The overlapping essential activity indicated for Gcs1 and Age2 suggests that these two proteins mediate a common process. To identify a common site of action for the Gcs1 and Age2 proteins, we created a situation in which the functionally overlapping activity of Gcs1 and Age2 could be rapidly diminished. Cell viability demands that either Gcs1 or Age2 be present under some conditions; therefore, we created cells lacking the Age2 protein that were kept alive by mutant Gcs1 proteins with conditional activity. Several mutant genes encoding temperature-sensitive versions of the Gcs1 protein (Poon et al., 1999) were introduced, as plasmid-borne copies, into diploid cells harboring heterozygous

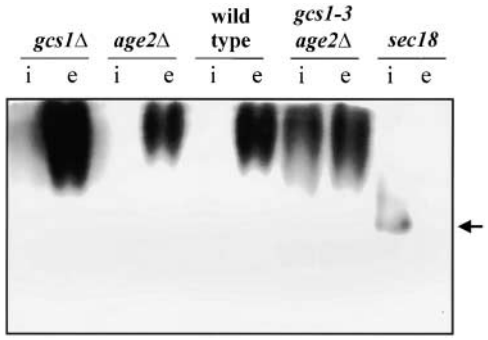

Figure 3. **Secretion of invertase is impaired by inadequate Gcs1 + Age2 activity.** Cells growing at 26°C were transferred to low-glucose medium to induce invertase expression and incubated at 37°C for 45 min. Secreted and intracellular invertase was separated into internal (i) and external (e) fractions and resolved by SDS-PAGE without heat treating the samples. Invertase was detected by an in-gel enzyme assay (Gabriel and Wang, 1969). For reference, a *sec18* mutant was incubated at 38°C before invertase induction as above. The arrow indicates the position of the ER-glycosylated form of invertase. Longer incubations of up to 2 h did not change the pattern or distribution of invertase in mutant cells (unpublished data).

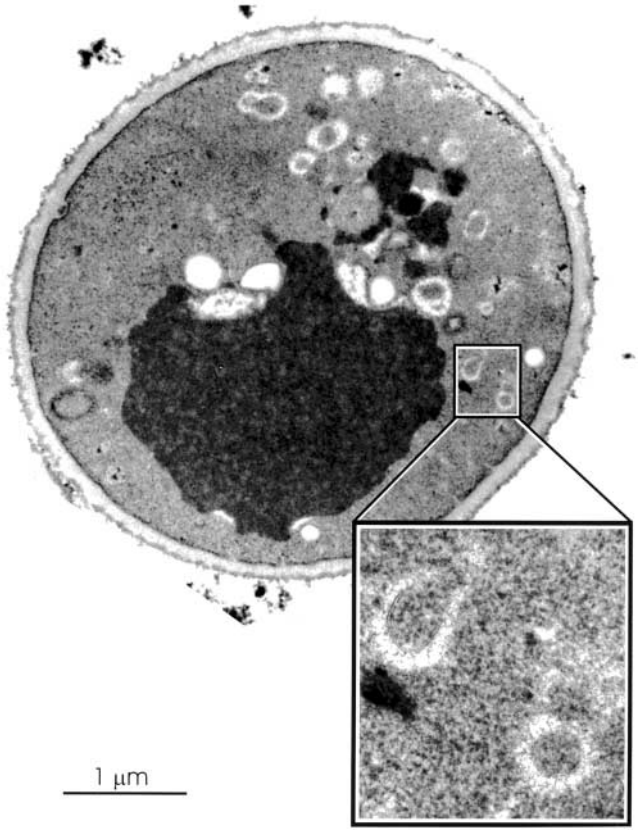

Figure 4. **Cells with inadequate Gcs1 + Age2 activity accumulate membranous structures.** Cells were grown at 30°C or incubated at 37°C for 1 h before processing for electron microscopy. Shown is a representative section through a double-mutant cell processed after incubation at 37°C. The inset is a higher magnification of a region exhibiting membranous structures that resemble Berkeley bodies. Wild-type and single-mutant cells (and double-mutant cells at 30°C) did not display any such structures (unpublished data). In marked contrast, 50% of 113 random sections of double-mutant cells after growth at 37°C displayed these membranous structures.

deletion mutations in the chromosomal *GCS1* and *AGE2* genes. Sporulation of the transformed diploids resulted in *gcs1::URA3 age2::HIS3* double-mutant genetic segregants harboring a plasmid-borne *gcs1-ts* allele. From the same genetic crosses, *gcs1* and *age2* single-mutant cells were chosen as controls. Although the *gcs1-ts age2* haploid cells were able to grow well at 30°C, transfer to 37°C resulted in cessation of cell proliferation due to diminished *gcs1-ts* function at that temperature; in contrast, the *gcs1* and *age2* single-mutant cells grew well at both temperatures (Fig. 1 B). To find a situation that results in rapid depletion of Gcs1 activity in cells lacking Age2 protein, we screened the various double-mutant segregants for a mutant version of the Gcs1 protein that is rapidly degraded upon transfer from 30 to 37°C. The temperature-sensitive Gcs1 protein encoded by the *gcs1-3* mutant allele rapidly disappeared upon shift to 37°C with an estimated half-life of <30 min (unpublished data). The impaired Gcs1 function caused by the *gcs1-3* mutation may be a consequence of this protein instability, because increased dosage of the *gcs1-3* mutant gene (presumably leading to increased protein levels) is able to supply essential function at 37°C (unpublished data). We used *gcs1-3 age2* double-mutant cells for subsequent experiments, so that transfer to the restrictive temperature produced a situation in which mutant cells experienced a rapid depletion of ArfGAP protein for the vesicular transport stage modulated by Gcs1 and Age2.

As an initial indicator of vesicular transport that may be affected by Gcs1 + Age2 activity, we assessed the glycosylation status of the extracellular enzyme invertase and the effectiveness of invertase secretion. Invertase expression is induced in low-glucose medium, and newly synthesized invertase undergoes *N*-linked glycosylation in the ER. These invertase molecules are then transported to the Golgi, where further glycosylation ensues; the extent of glycosylation is reflected in the degree of decrease in invertase mobility during gel electrophoresis. Growing cells were transferred to low-glucose medium to induce the synthesis of invertase, and were incubated at 37°C. For wild-type cells and in *gcs1-3* and *age2* single-mutant cells, invertase was found predominantly in the external fraction, in highly glycosylated form (Fig. 3). In *sec18* mutant control cells, defective for transport out of the ER (Novick et al., 1980), invertase was only core glycosylated and was not secreted (Fig. 3). In marked contrast, *gcs1-3 age2* double-mutant cells had invertase in a highly glycosylated form, indicating exposure to Golgi-resident enzymes (Fig. 3). However, about half of this glycosylated invertase remained inside the cells, indicating that transport from the Golgi to the plasma membrane is impaired.

Inadequate Gcs1 + Age2 activity leads to the elaboration of membranous structures

Cells were examined by electron microscopy after incubation at 37°C. Wild-type cells and *gcs1* and *age2* single-mutant cells did not display any morphological indication of impaired vesicular transport (unpublished data), but the *gcs1-3 age2* double-mutant cells incubated at 37°C con-

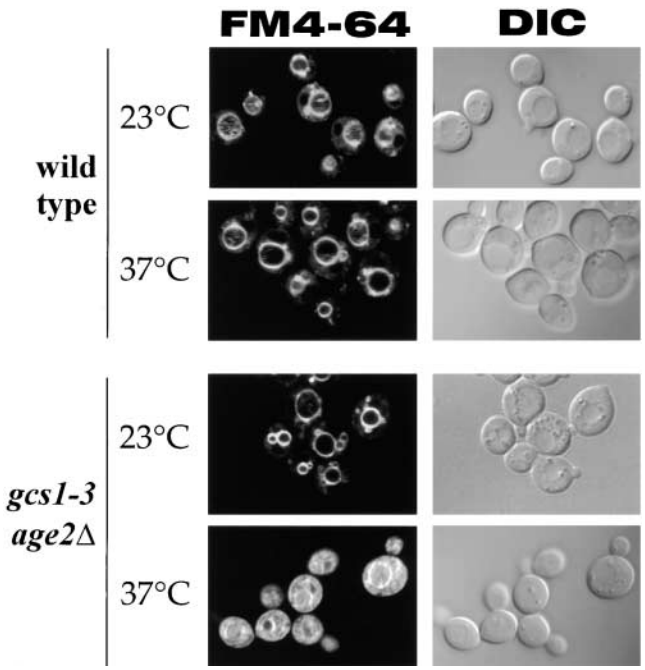

Figure 5. **Cells with inadequate Gcs1 + Age2 activity are impaired for endocytosis.** Cells growing at 23°C were incubated with the membrane dye FM4-64 for 10 min, transferred to fresh dye-free medium for a further 1-h incubation at 23 or 37°C, and visualized by DIC optics and fluorescence microscopy (FM4-64).

tained obvious membranous structures (Fig. 4). These structures resembled the Berkeley bodies that were originally observed in cells harboring mutations such as *sec7* or *sec14*, which impede membrane traffic through the Golgi apparatus, and these characteristic membranous structures are thought to be derived from Golgi compartments (Novick et al., 1980). Therefore, the altered morphology resulting from inadequate Gcs1 + Age2 activity may reflect impaired Golgi-related transport.

gcs1-3 age2 double-mutant cells are impaired for endocytosis

To assess whether endosomal trafficking is affected in *gcs1-3 age2* double-mutant cells, we determined the ability of cells to transport the lipophilic dye FM4-64 from the plasma membrane to the vacuole (Vida and Emr, 1995), a transport process that involves routing of membrane-bound FM4-64 through the endosomal compartment. Wild-type cells (Fig. 5) and single-mutant cells (unpublished data) at 37°C showed a significant localization of FM4-64 to the vacuole, as indicated by a high concentration of the dye outlining the vacuolar membrane. In marked contrast, although *gcs1-3 age2* double-mutant cells were able to internalize the dye, they displayed a diffuse and granular cytoplasmic staining pattern, with indistinct vacuolar membrane staining (Fig. 5). Even after extended incubation, mutant cells remained impaired in the delivery of the FM4-64 dye to the vacuole (unpublished data). Thus, inadequate Gcs1 + Age2 activity also impairs endocytosis, suggesting that the Gcs1 + Age2 proteins provide overlapping function for vesicular transport affecting endosomal traffic.

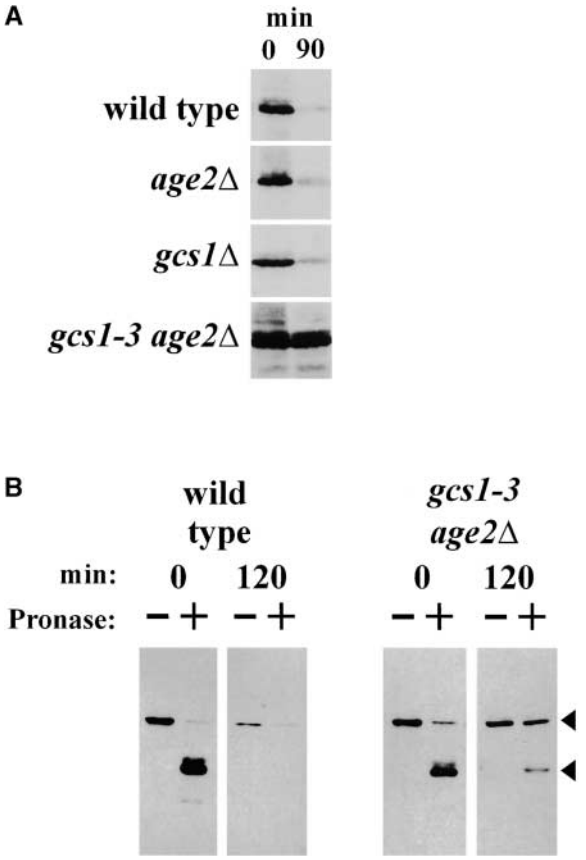

Figure 6. **The overlapping activity of Gcs1 and Age2 is necessary for transport of the α-factor receptor, Ste3, to the vacuole.** (A) Cells growing at 26°C were incubated at 37°C for 30 min, at which time cycloheximide was added, and were then either harvested immediately (0 min) or incubated at 37°C for a further 90 min. Cell extracts were resolved by SDS-PAGE and Ste3 protein was visualized by Western blot analysis. (B) Cells growing at 26°C were incubated at 37°C in medium containing cycloheximide. Portions of samples taken immediately or after a 120-min incubation were treated with Pronase to degrade cell surface proteins before Western blot analysis. The upper arrow indicates the position of intact Ste3, and the lower arrow indicates the position of Pronase-mediated breakdown products.

As a biochemical measure of endocytosis, we assessed the internalization and degradation of the α-factor mating–pheromone receptor Ste3. In wild-type cells, the Ste3 protein is constitutively removed from the plasma membrane, and is delivered via the endocytic pathway to the vacuole where it is degraded (Davis et al., 1993). In our assessment, cells were shifted to 37°C and incubated in the presence of the protein synthesis inhibitor cycloheximide to prevent further synthesis of Ste3 protein. As shown in Fig. 6 A, wild-type and single-mutant cells treated in this way showed a rapid loss of Ste3 protein, whereas in double-mutant cells the Ste3 protein was stable. The increased stability of Ste3 in double-mutant cells could indicate that the Ste3 protein was not internalized, or that Ste3 was internalized but not delivered to the vacuole for degradation. To resolve this issue, we incubated cells as above and then treated them with Pronase, a mixture of protein-degrading enzymes. Ste3 protein that remained at the plasma membrane would be exposed to enzymatic degradation, whereas Ste3 protein that had been

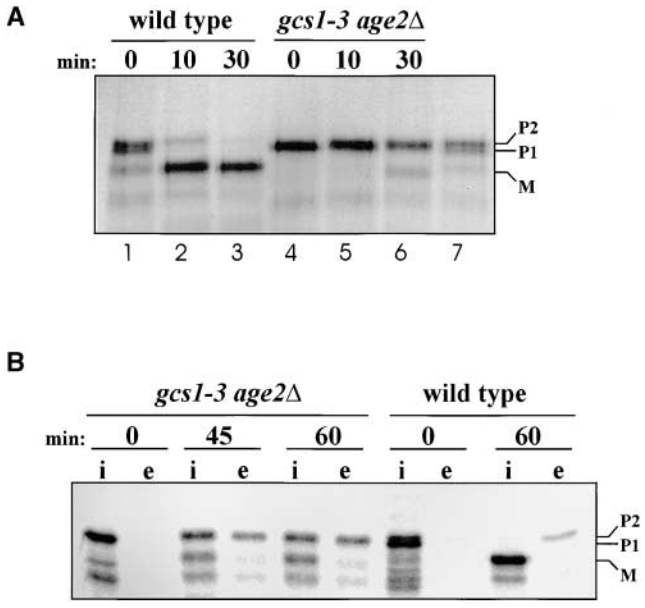

Figure 7. **CPY transport from the TGN is aberrant under conditions of diminished Gcs + Age2 activity**. (A) Cells growing at 26°C were incubated at 37°C for 15 min, exposed to radiolabeled amino acids for 7 min, and incubated further with unlabeled amino acids. CPY was immunoprecipitated from samples removed at the indicated times, resolved by SDS-PAGE, and detected by autoradiography. The locations of the P1, P2, and mature (M) forms of CPY are indicated. After 30 min, wild-type cells had >90% of CPY in the mature form (lane 3), whereas the majority (68%) of CPY in double-mutant cells was in the P2 form (lane 6). For reference, lane 7 contains another 0-time sample to aid identification of the P1 and P2 forms of CPY. (B) Cells were treated as in panel A, but fractionated into external (e) and internal (i) fractions before immunoprecipitation. Although wild-type cells retained >90% of CPY internally, double-mutant cells secreted 35% of the CPY during the incubation.

successfully internalized but had failed to reach the vacuole would be expected to remain intact. As shown in Fig. 6 B, the Ste3 protein of double-mutant cells was resistant to Pronase digestion. We interpret this finding to indicate that Gcs1 + Age2 activity is not necessary for initial stages of endocytosis, but is required for effective delivery of endosomal cargo to the vacuole. These results are consistent with the internalization but cytoplasmic pattern of staining of membrane-bound FM4-64 in double-mutant cells, and suggest that Gcs1 + Age2 activity mediates effective endosomal transport after the internalization stage.

Inadequate Gcs1 + Age2 activity results in missorting of CPY

Material transported to the vacuole includes newly synthesized vacuolar proteins that move from the ER through the Golgi, and that are sorted in the late Golgi compartments to be directed to the vacuole through the endosomal system. Therefore, a defect in vesicular transport that affects Golgi and/or endosomal function can also affect this pathway. A useful marker of this transport from the ER through the Golgi to the vacuole is a vacuolar hydrolase, CPY. In the ER, glycosylation of CPY gives rise to the precursor form p1CPY. Transit to the Golgi and extension of core oligosaccharides results in the Golgi-specific precursor p2CPY. Finally, transport of p2CPY to the vacuole via an endosomal compartment allows proteolytic maturation to yield the mature form of CPY. Impairment of transport to the vacuole can lead to defective targeting so that the vacuolar maturation step does not occur and the precursor p2CPY is exported out of the cell (Rothman and Stevens, 1986). To determine the role of Gcs1 + Age2 activity in the transport of CPY, we transferred cells to 37°C and assessed CPY processing at this temperature. Wild-type (Fig. 7 A) and single-mutant cells (unpublished data) displayed a rapid maturation of newly made CPY to the mature form, indicating normal transit of CPY to the vacuole. However, *gcs1-3 age2* double-mutant cells were defective in CPY maturation (Fig. 7 A), such that the majority of newly synthesized CPY remained in the p2CPY form. This maturation blockage is consistent with impaired delivery from the TGN to the vacuole.

In addition to the impaired maturation of p2CPY, there was also a decrease in the yield of CPY isolated from *gcs1-3 age2* double-mutant cells (Fig. 7 A, compare lanes 5 and 6). This deficit may reflect the export of CPY from the cell that is seen when vacuolar targeting is affected (Rothman and Stevens, 1986; Robinson et al., 1988; Marcusson et al., 1994). Indeed, as shown in Fig. 7 B, resolution of external and internal cell fractions showed that a significant amount of CPY was externalized by mutant cells compared with wild-type cells treated in the same manner (Fig. 7 B). Moreover, the form of CPY that was exported by mutant cells was p2CPY. Thus, impaired Gcs1 + Age2 activity leads to missorting of CPY. This missorting and the accumulation of p2CPY are consistent with defective transport from the TGN.

The CPY sorting receptor Vps10 is aberrantly localized in *gcs1-3 age2* cells

Entry of CPY into a TGN-to-vacuole pathway is facilitated by a sorting receptor, Vps10, that binds CPY at the TGN (Marcusson et al., 1994; Cooper and Stevens, 1996). Transport vesicles deliver the Vps10–CPY complex to a late endosomal compartment, the prevacuolar compartment (PVC), where Vps10 is thought to uncouple from CPY. Vps10 is then packaged into vesicles for retrieval to the TGN in a manner dependent on the retromer complex (Seaman et al., 1998). In cells mutated for the PVC retrieval apparatus, Vps10 is mislocalized to the vacuole, where it is degraded (Nothwehr and Hindes, 1997; Seaman et al., 1997). Therefore, the transport block for p2CPY in *gcs1-3 age2* double-mutant cells may reflect altered transport or defective localization of Vps10. To test these ideas, cells were transferred to 37°C and Vps10 stability was assessed by a pulse–chase immunoprecipitation procedure (Fig. 8 A). In *gcs1-3 age2* double-mutant cells, the stability of Vps10 was indistinguishable from that in wild-type or single-mutant cells, suggesting that Vps10 is not mislocalized to the vacuole.

As another measure of Vps10 localization, we used indirect immunofluorescence microscopy to visualize Vps10 tagged with three copies of the influenza hemagglutinin (HA) epitope. This Vps10-HA protein fully complements a *vps10* mutation, and thus appears to be functional (Nothwehr et al., 2000). As previously observed, in wild-type cells,

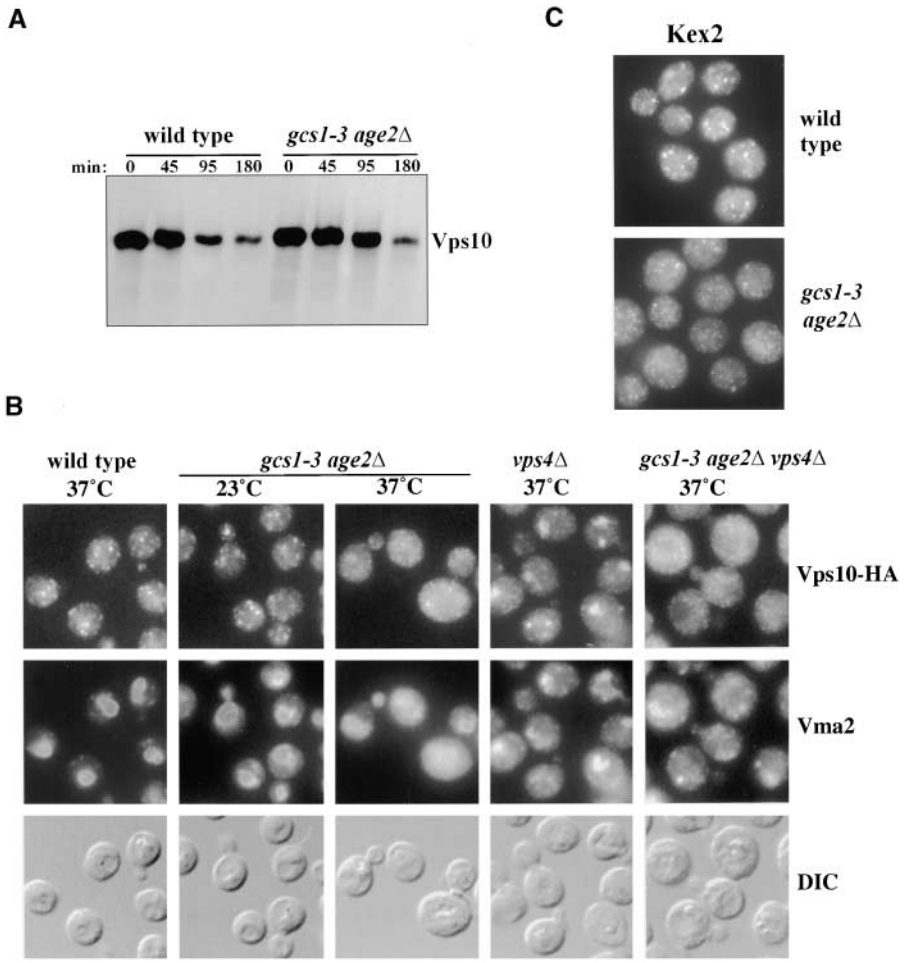

Figure 8. **The Gcs + Age2 proteins mediate normal trafficking of the CPY receptor Vps10.** (A) Cells growing at 23°C were incubated at 37°C for 15 min, exposed to radiolabeled amino acids for 10 min, and incubated further with unlabeled amino acids. Vps10 was immunoprecipitated from samples removed at the indicated times, resolved by SDS-PAGE and detected by autoradiography. (B) Cells carrying the *VPS10::3xHA* allele growing at 23°C were incubated at 37°C for 2 h, fixed, stained with both anti-HA and anti-Vma2 antibodies, and visualized by DIC optics and fluorescence microscopy. (C) Wild-type and double-mutant cells growing at 23°C were incubated at 37°C for 2 h, fixed, stained with anti-Kex2 antibodies, and visualized by fluorescence microscopy.

Vps10-HA exhibited a cytosolic punctate staining pattern consistent with that of TGN-resident proteins (Nothwehr et al., 2000, and Fig. 8 B); a similar pattern was seen in *gcs1* and *age2* single-mutant cells (unpublished data). In contrast, in *gcs1-3 age2* double-mutant cells, Vps10-HA exhibited a diffuse cytosolic staining pattern that was clearly nonvacuolar. Therefore, inadequate Gcs1 + Age2 activity affects Vps10 localization but does not cause Vps10 to be delivered to the vacuole.

Vps10 is normally transported from the TGN to the PVC, therefore the diffuse localization of Vps10-HA in *gcs1-3 age2* double-mutant cells could reflect a functional defect in the PVC. To assess PVC function under conditions of inadequate Gcs1 + Age2 activity, we employed a class E *vps* mutation, *vps4*, that blocks transport out of the PVC to the vacuole and to the TGN, such that *vps4* mutant cells accumulate material in the PVC (Babst et al., 1997). In cells lacking Vps4, localization was visualized for Vps10-HA as well as for the vacuolar protein Vma2, a subunit of the vacuolar membrane H^+ pump. As shown in Fig. 8 B, the absence of Vps4 protein caused both Vps10-HA and Vma2 to become trapped in the PVC; Vps10-HA was not retrieved back to the TGN and Vma2 was not delivered to the vacuole. In *gcs1-3 age2 vps4* triple-mutant cells at 37°C, Vma2 remained trapped in the PVC (Fig. 8 B). Thus, in *gcs1-3 age2 vps4* triple-mutant cells, the PVC clearly remains intact. In contrast to Vma2, Vps10-HA was not found within the PVC but instead exhibited the diffuse cytoplasmic localization similar to that seen for *gcs1-3 age2* double-mutant cells. These observations suggest that inadequate Gcs1 + Age2 activity may lead to fragmentation of the TGN. We also observed in *gcs1-3 age2* double-mutant cells a marked decrease in the intense punctate staining pattern that characterizes the TGN-resident protein Kex2 (Fig. 8 C) and the chimeric TGN reporter protein A-ALP (Nothwehr et al., 1993, and unpublished data). These results suggest that inadequate Gcs1 + Age2 activity leads to a fragmented TGN and a block in TGN-to-PVC vesicular transport.

Inadequate Gcs1 + Age2 activity impairs vacuolar delivery of ALP

The transport of a type II vacuolar membrane protein, ALP, from the TGN to the vacuole proceeds by a different route than that followed by CPY. Whereas the TGN-to-vacuole pathway for CPY involves passage through the prevacuolar endosome and is dependent on clathrin (Seeger and Payne, 1992), the TGN-to-vacuole pathway for ALP bypasses the prevacuolar endosome, is clathrin-independent, and relies on the AP-3 adaptor protein complex (Cowles et al., 1997a; Stepp et al., 1997). To assess the effects of Gcs1 + Age2 activity on the transport of ALP to the vacuole we used the same pulse–chase immunoprecipitation procedure as above, with transport of newly synthesized ALP to the vacuole indi-

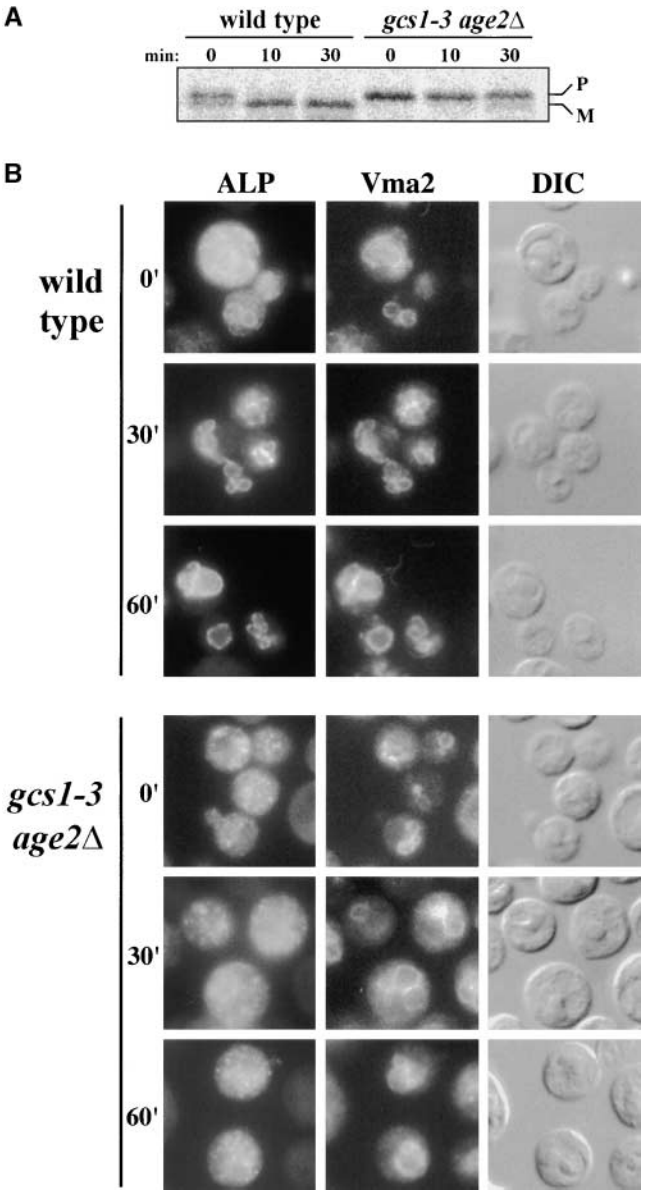

Figure 9. **Transport of ALP to the vacuole is impaired in cells with inadequate Gcs1 + Age2 activity.** (A) Cells growing at 26°C were incubated at 37°C for 15 min, exposed to radiolabeled amino acids for 7 min, and incubated further with unlabeled amino acids. ALP was immunoprecipitated from samples removed at the indicated times, resolved by SDS-PAGE, and detected by autoradiography. The precursor (P) and mature (M) forms are indicated. After 30 min, wild-type cells had >90% of ALP in the mature form, whereas double-mutant cells had 75% of ALP in the precursor form. (B) Cells carrying plasmid pSN351, harboring the ALP gene *PHO8* expressed from the inducible *GAL1* promoter, were grown at 23°C in raffinose medium and transferred to 37°C. After a 15-min incubation, ALP synthesis was induced by the addition of galactose. After a 40-min induction period, glucose was added to repress further *GAL1*-regulated ALP synthesis, and at times thereafter cells were processed for microscopy.

cated by the accumulation of the mature form of ALP. Although both wild-type and mutant cells were equally effective at processing ALP to the mature form at a permissive temperature (unpublished data), at 37°C the *gcs1-3 age2* double-mutant cells were unable to process ALP to the mature vacuolar form (Fig. 9 A). This finding suggests that Gcs1 + Age2 activity is also needed for the exit of ALP from the TGN via the ALP pathway.

Next, we localized the unprocessed ALP in *gcs1-3 age2* double-mutant cells. For this assessment, the *PHO8* gene that encodes ALP was placed under control of the regulatable *GAL1* promoter. Cells carrying this *GAL1-PHO8* gene, growing at a permissive temperature in raffinose-containing medium to prevent ALP expression from the *GAL1* promoter, were transferred to 37°C and 15 min later galactose was added to induce high-level ALP expression. Glucose was then added to stop further ALP expression from the *GAL1* promoter and incubation was continued, with cells harvested for indirect immunofluorescence microscopy to detect ALP and Vma2 (Fig. 9 B). In wild-type and single-mutant cells, a mixed ER/vacuole pattern of ALP-specific immunofluorescence was initially seen, but from 15 min onward the staining pattern was clearly vacuolar, as indicated by the colocalization of ALP and Vma2 immunofluorescence. In contrast, for *gcs1-3 age2* double-mutant cells the ALP immunofluorescence showed a diffuse cytosolic pattern throughout the entire time course (Fig. 9 B), with vacuolar staining only occasionally observed (unpublished data). This ALP staining resembled that described above for Vps10 and Kex2 in *gcs1-3 age2* double-mutant cells, and reinforces the notion that Gcs1 + Age2 activity prevents TGN fragmentation.

Discussion

Previously, we demonstrated by both in vitro and in vivo criteria that the Gcs1 protein provides ArfGAP activity (Poon et al., 1996). Here we show that the structurally related protein Age2 also has ArfGAP activity, and that Gcs1 and Age2 form an essential protein pair for effective vesicular transport and cell growth. Inadequate Gcs1 activity in cells lacking Age2 protein leads to cessation of cell growth, with cells displaying a phenotype consistent with impaired transport from the TGN, although other aspects of vesicular transport such as trafficking between the ER and Golgi compartments appear unaffected. Inadequate Gcs1 + Age2 activity leads to the accumulation of membranous bodies resembling Golgi-derived structures, termed Berkeley bodies, that are characteristic of certain mutant cells blocked in transport from the Golgi complex (Novick et al., 1980). Indeed, inadequate Gcs1 + Age2 activity compromises both the CPY and ALP pathways that transport cargo from the TGN to the vacuole (Marcusson et al., 1994; Cowles et al., 1997b; Piper et al., 1997). These findings suggest that Gcs1 and Age2 have overlapping roles in vesicular transport involving the TGN.

The hypothesis that the Gcs1 + Age2 ArfGAP pair primarily influences transport at the TGN is consistent with the known roles for ARF proteins at the Golgi complex. ARF proteins mediate the recruitment of both AP-1 adaptor complexes and AP-3 complexes onto TGN membranes for transport-vesicle formation (Stamnes and Rothman, 1993; Ooi et al., 1998), so that defective regulation of the ARF cycle of GTP binding and hydrolysis, as expected for the situation investigated here in which an overlapping es-

sential activity of the Gcs1 and Age2 ArfGAPs is diminished, would affect the formation and/or function of both AP-1 and AP-3 transport vesicles. AP-1 vesicles assembled at the TGN are thought to be targeted to endosomal compartments (Deloche et al., 2001), such that impaired vesicle formation at the TGN would lead to defective delivery of vesicular transport components needed for endosomal trafficking. Therefore, the effects that we observe on endosomal function and movement of material from the plasma membrane to the vacuole may be indirect consequences of impaired TGN export. Consequently, we propose that the Gcs1 + Age2 ArfGAP pair mediates the formation and/or function of TGN-derived transport vesicles for both endosomal (CPY) and AP-3–dependent nonendosomal (ALP) pathways (Fig. 10).

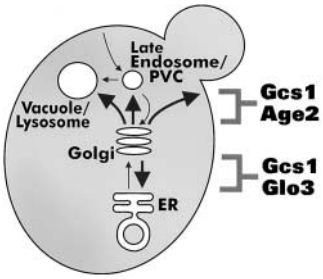

Figure 10. **Vesicular transport stages mediated by ArfGAP pairs.** Large arrows indicate vesicular transport pathways from the TGN mediated by the Gcs1 + Age2 ArfGAP pair and the retrograde Golgi-to-ER pathway mediated by the Gcs1 + Glo3 ArfGAP pair.

The suggestion that the Gcs1 + Age2 ArfGAP pair facilitates transport from the TGN is consistent with other findings. Under certain physiological conditions, a *gcs1* mutation can cause cold sensitivity for growth (Drebot et al., 1987; Ireland et al., 1994) and endosomal trafficking (Wang et al., 1996). This physiologically delimited form of *gcs1* cold sensitivity is consistent with the TGN/endosomal effects reported here for cells depleted for Gcs1 activity. This *gcs1* cold sensitivity may be linked functionally to the AP-3 adaptor complex and transport out of the TGN: both *gcs1* cold sensitivity and endosomal-transport deficiency can be suppressed by increased dosage of the *YCK1* or *YCK2* genes that encode functionally redundant isoforms of casein kinase I (Robinson et al., 1992; Wang et al., 1992, 1996), whereas the effects of decreased casein kinase I activity can in turn be alleviated by mutating the AP-3 complex (Panek et al., 1997). AP-3 activity is also implicated in the studies of overlapping Gcs1 + Age2 activity reported here, for impaired Gcs1 + Age2 activity prevents delivery of ALP from the TGN to the vacuole, a process mediated by AP-3 (Cowles et al., 1997a; Stepp et al., 1997).

At least two types of nonsecretory transport vesicles are formed at the TGN. Transport vesicles assembled with the AP-1 adaptor complex and a clathrin coat are targeted to the endosome, whereas vesicles assembled with the AP-3 adaptor complex in a clathrin-independent manner bypass the endosome in the transport of cargo to the vacuole (Cowles et al., 1997a; Piper et al., 1997; Stepp et al., 1997; Gaynor et al., 1998). In many types of cells, including yeast, ARF proteins have been implicated in the assembly of each of these types of vesicles at the TGN (Stamnes and Rothman, 1993; Ooi et al., 1998; Yahara et al., 2001). The formation of two types of ARF-dependent transport vesicles, coupled with the two ArfGAPs Gcs1 and Age2 that our findings implicate in TGN function, allows the speculation that ArfGAPs may perform more specialized roles in the assembly and/or function of each vesicle type. Evidence suggesting interactions among an ArfGAP, a coat protein, and activated ARF (Goldberg, 1999) provides a mechanism to allow a single ARF isoform to participate in specific transport-vesicle formation and cargo selection at several stages by interacting with additional proteins, including ArfGAPs. ARF proteins also interact with members of the GGA family of modular adaptor-related proteins (Boman et al., 2000; Dell'Angelica et al., 2000), which have recently been shown to influence vesicle formation through interactions with both a coat protein and an ArfGAP (Puertollano et al., 2001). Therefore, combinations of protein interactions involving ArfGAPs may impose specificity on vesicle formation and cargo. The absence of deleterious TGN/endosomal effects for *gcs1* or *age2* single-mutant cells (under the growth conditions used here) indicates that the Gcs1 and Age2 ArfGAP proteins each have the ability to maintain appropriate transport from the TGN, resulting in functionally overlapping activities.

Despite the findings that each of the Gcs1 and Age2 proteins is sufficient to maintain the formation of specific transport vesicles and proper cargo selection at the TGN, our recent genetic findings indicate that these two ArfGAPs have distinct as well as overlapping functions in TGN/endosomal transport (unpublished data). While investigating the functions of the phosphatidylinositol transfer protein Sec14, we found that *gcs1* and *age2* mutations display distinct (nonoverlapping) genetic interactions. Sec14 is the major phosphatidylinositol transfer protein in yeast and is essential for vesicular transport (Bankaitis et al., 1989). Mutations in several genes minimize the need for Sec14 activity, and consequently can restore both growth and vesicular transport to *sec14* mutant cells. Two of these *sec14* suppressor mutations maintain suppression in *age2 sec14* double-mutant cells, but are unable to provide suppression in *gcs1 sec14* double-mutant cells; therefore, these suppressor mutations need Gcs1, but not Age2, for effective suppression. In contrast, other *sec14* suppressor mutations need Age2, but not Gcs1. The demonstrated function of Gcs1 at a different stage of vesicular transport, Golgi-to-ER retrograde transport, which Gcs1 mediates along with the ArfGAP Glo3 (Poon et al., 1999), complicates interpretation of these observations. However, *glo3* and *gcs1* mutations were found to have no such complementary genetic interactions involving *sec14*: all of the *sec14* suppressor mutations maintain suppression in the absence of Glo3 (unpublished data). Therefore, these observations suggest that the effects of the *gcs1* mutation in these *sec14* suppression experiments reflect Gcs1 activity, along with that of Age2, in TGN/endosomal transport. The nature of the distinct activities of Gcs1 and Age2, which are individually dispensable in the presence of Sec14 activity (Ireland et al., 1994; Zhang et al., 1998), remains to be determined.

Table I. ***Saccharomyces cerevisiae*** **strains**

Strain	Genotype	Source
W303-1a	*MAT*a *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*	Poon et al. (1999)
W303-1b	*MATα ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*	Poon et al. (1999)
GWK-9B	W303-1b *gcs1Δ::URA3*	Ireland et al. (1994)
ARY19.2.99B	W303-1b *age2Δ::HIS3*	A. Rowley
PPY164-5D	W303-1b *gcs1Δ::URA3 age2Δ::HIS3* [pPP805-3]	This study
SNY36-9A	MATa *his3-200 leu2-3,113 pho8Δ::ADE2 suc2-Δ9 trp1-901 ura3-52*	Nothwehr et al. (1995)
SHY35	SNY36-9A switched to *MATα*	This study
SNY142-3C	SHY35 *gcs1Δ::kanMX age2Δ::HIS3* [pPP805-3]	This study
SNY131	SNY36-9A *VPS10::3xHA pep4Δ::LEU2*	This study
SNY144	SNY35 *gcs1Δ::kanMX age2Δ::His3MX6 VPS10::3xHA pep4Δ::TRP1* [pPP805-3]	This study
SNY146	*SNY36-9A vps4::natMX4 VPS10::3xHA pep4Δ::LEU2*	This study
SNY147	SHY35 *vps4::natMX4 gcs1Δ::kanMX age2Δ::His3MX6 VPS10::3xHA pep4Δ::TRP1*[pPP805-3]	This study
SNY98	W303-1a *pho8Δ::ADE2*	This study
SNY145	PPY164-5D *pho8Δ::ADE2*	This study
HMSF176	MATa *sec18-1*	American Type Culture Collection

Materials and methods

Strains, plasmids, and growth conditions

Strains used in this study are listed in Table I. Mating-type switching was accomplished by transforming cells with a YCp50-based plasmid carrying the *HO* gene. The creation of temperature-sensitive *gcs1* alleles by PCR has been described (Poon et al., 1999). The *gcs1::kanMX* allele was created by a PCR-based approach that removed >95% of the *GCS1* ORF. Likewise, the *age2::His3MX6* allele was generated by a PCR-based strategy replacing >95% of the *AGE2* ORF with the *Schizosaccharomyces pombe* gene derived from plasmid pFA6a-His3MX6. The *age2::HIS3* deletion mutation, provided by Adele Rowley (Cell zome, Mill Hill, UK), was introduced into strain W303-1b by multiple backcrosses. The *pep4::LEU2* allele was created as described (Nothwehr et al., 1996). The *VPS10::3HA* allele was carried on plasmid pLC115 (Conibear and Stevens, 2000). The *vps4::natMX4* allele was introduced using a PCR-based approach and plasmid pAG25 (Goldstein and McCusker, 1999) as a template. Use of the *pho8::ADE2* allele has been described (Nothwehr et al., 1995). Standard procedures were used for cell growth, transformation and genetic manipulation.

Microscopy

For electron microscopy, cells were harvested by centrifugation and fixed in buffered glutaraldehyde essentially as described (Byers and Goetsch, 1975; Johnston et al., 1991). To assess endocytosis, cells were stained with the lipophilic dye FM4-64 (Vida and Emr, 1995). Staining of Vps10-HA, Vma2, ALP and Kex2 used fluorochrome-conjugated secondary antibodies (Spelbrink and Nothwehr, 1999; Nothwehr et al., 2000). Cells were visualized using a Zeiss Laser Scanning Confocal (LSM510) microscope or an Olympus BX60 epifluorescence microscope.

Bacterial expression of proteins

To express recombinant Age2 protein, the *AGE2* gene was amplified by PCR using oligonucleotides with convenient restriction enzyme recognition sites and subcloned into plasmid pET21b (Novagen) to generate plasmid pPPL71. Age2, Gcs1, and Arf1 proteins were expressed in *E. coli* strain BL21(DE3) as described (Poon et al., 1996). *N*-myristoyltransferase was expressed from plasmid pACYC/ET3d/yNMT (Haun et al., 1993), a gift from Joel Mass (National Heart, Lung, and Blood Institute, Bethesda, MD).

ArfGAP assays

ArfGAP activity was assessed essentially as described by Huber et al. (2001). Recombinant myristoylated yeast Arf1 protein was incubated with [γ- ^{32}P]GTP for 10 min at 30°C; the radiolabeled GTP-Arf mixture was then subjected to centrifugation for 10 min at 4°C (4000 *g*). GTP-Arf binds to the lipids (Franco et al., 1995) in the assay reaction mixture and is found in the pellet fraction. ArfGAP activity was assessed in 50-μl reactions.

Assays of vesicular transport

Invertase was assayed as described (Poon et al., 1999). To assess Ste3 trafficking, cells growing in rich medium at 26°C were harvested by centrifugation, resuspended in prewarmed 37°C medium, and incubated at 37°C for 30 min, at which time cycloheximide was added to 10 μg/ml and incubation was continued for sampling. Sodium azide was added to samples to a final concentration of 10 mM and the cells were pelleted and frozen at −70°C. Protein extracts were prepared for immunoblot analysis as described (Davis et al., 1993), resolved by SDS-PAGE, and transferred to PVDF membrane (Bio-Rad Laboratories). Ste3 was detected by ECL chemiluminescence with anti-Ste3 monoclonal as the primary antibody, a gift from G. Sprague, University of Oregon, Eugene, OR). To assess the presence of Ste3 protein on the cell surface, cells growing in rich medium at 26°C were mixed with an equal volume of 48°C medium containing cycloheximide (20 mM), and at times thereafter, samples (5×10^7 cells) were made to 10 mM NaN_3, pelleted, resuspended in 10 mM NaN_3, incubated on ice for 30 min, and then pelleted and resuspended in 1 ml DB (Davis et al., 1993) containing 0.5% 2-mercaptoethanol and 5 mM $CaCl_2$ and incubated at 37°C for 30 min. 300-μl samples were then treated with 100 μl DB with or without Pronase (4 mg/ml; Boehringer Mannheim), incubated for 1 h at 32°C, and diluted with DB containing 10 mM EDTA and 1 mM PMSF. Protein extracts were prepared by vortexing cells along with glass beads for 10 min at 4°C, and Ste3 was detected by Western blotting.

The processing of CPY was assessed by immunoprecipitation from whole-cell extracts after a pulse–chase procedure (Poon et al., 1999). To measure externalized CPY, cells were treated as described (Poon et al., 1999), except that during the chase period BSA was added to 0.1% Gaynor and Emr, 1997). Samples (0.5 ml) were made 20 mM in NaN_3 and NaF, placed on ice, and diluted with an equal volume of 2.8 M sorbitol containing 100 mM Tris, pH 7.5, 20 mM NaN_3, 20 mM NaF, and 60 mM mercaptoethanol. Zymolyase 100T was then added to 1 mg/ml, the mixture was incubated at 37°C for 30 min, and the resulting spheroplasts were harvested by centrifugation, with the supernatant retained as the external fraction. The spheroplast fraction was resuspended in Laemmli buffer, boiled for 5 min, and treated as described for whole-cell extracts (Poon et al., 1999). The supernatant fraction was mixed with 0.2 vol of Laemmli buffer and boiled for 5 min, then mixed with an equal volume of 300 mM NaCl containing 50 mM Tris, pH 7.5, 8 mM EDTA, and 2% Triton X-100, and subjected to immunoprecipitation (Poon et al., 1999). Samples were resolved by SDS-PAGE, and visualized and quantitated by phosphorimager analysis. Vsp10 stability was assessed (Nothwehr and Hindes, 1997) using anti-Vsp10 antibody provided by Tom Stevens (University of Oregon, Eugene, OR).

To assess ALP processing, cells were radiolabeled (Poon et al., 1999), suspended in 100 μl of 8 M urea containing 1% SDS, 10 mM EDTA, 60 mM Tris, pH 6.8, and 1% 2-mercaptoethanol, and disrupted by vortexing along with glass beads for 10 min at 4°C. Extracts were then treated for 5 min in boiling water, cooled to 23°C, diluted tenfold with immunoprecipitation buffer (Franzusoff et al., 1991), and incubated with 50 μl of Pansorbin for 20 minutes at 23°C. Supernatant material after centrifugation was then incubated with rabbit anti-ALP for 1 h at 4°C followed by 30 μl protein A agarose overnight. Samples were resolved electrophoretically and bands were visualized and quantitated by phosphorimager analysis.

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