Retrograde transport from the yeast Golgi is mediated by two ARF GAP proteins with overlapping function

Pak Phi Poon^{1,2}, Dan Cassel³, Anne Spang⁴,
Miriam Rotman³, Elah Pick³,
Richard A. Singer^{2,5} and
Gerald C. Johnston^{1,6}

Departments of ¹Microbiology and Immunology, ²Biochemistry and ⁵Medicine, Dalhousie University, Halifax, Nova Scotia, Canada B3H 4H7, ³Department of Biology, Technion-Israel Institute of Technology, Haifa 32000, Israel and ⁴Department of Molecular and Cell Biology, University of California, Berkeley, CA 94720-3204, USA

⁶Corresponding author
e-mail: johnstong@tupdean1.med.dal.ca

ARF proteins, which mediate vesicular transport, have little or no intrinsic GTPase activity. They rely on the actions of GTPase-activating proteins (GAPs) for their function. The *in vitro* GTPase activity of the *Saccharomyces cerevisiae* ARF proteins Arf1 and Arf2 is stimulated by the yeast Gcs1 protein, and *in vivo* genetic interactions between *arf* and *gcs1* mutations implicate Gcs1 in vesicular transport. However, the Gcs1 protein is dispensable, indicating that additional ARF GAP proteins exist. We show that the structurally related protein Glo3, which is also dispensable, also exhibits ARF GAP activity. Genetic and *in vitro* approaches reveal that Glo3 and Gcs1 have an overlapping essential function at the endoplasmic reticulum (ER)–Golgi stage of vesicular transport. Mutant cells deficient for both ARF GAPs cannot proliferate, undergo a dramatic accumulation of ER and are defective for protein transport between ER and Golgi. The *glo3Δ* and *gcs1Δ* single mutations each interact with a *sec21* mutation that affects a component of COPI, which mediates vesicular transport within the ER–Golgi shuttle, while increased dosage of the *BET1*, *BOS1* and *SEC22* genes encoding members of a v-SNARE family that functions within the ER–Golgi alleviates the effects of a *glo3Δ* mutation. An *in vitro* assay indicates that efficient retrieval from the Golgi to the ER requires these two proteins. These findings suggest that Glo3 and Gcs1 ARF GAPs mediate retrograde vesicular transport from the Golgi to the ER.

Keywords: ARF GAP/Gcs1/Glo3/GTPase-activating protein/*Saccharomyces cerevisiae*/vesicular transport

Introduction

The movement of membrane-associated material between organelles and the maintenance of organellar integrity involve the generation of membrane vesicles and packaging of cargo molecules within these vesicles, followed by the regulated movement between membrane compartments (reviewed in Rothman, 1994). The regulated formation and

delivery of vesicles between the endoplasmic reticulum (ER) and Golgi compartments have been studied extensively, and many of the molecular components that mediate this transport have been identified (Kuehn and Schekman, 1997).

Movement of vesicles between membrane compartments of the early secretory system is bidirectional, with anterograde transport directing the forward movement of secretory cargo from the ER, and retrograde transport retrieving material that escapes to a more forward compartment or recycling transport components that are necessary for continued anterograde transport (Pelham, 1995). For both anterograde and retrograde transport, the formation of a transport vesicle includes the recruitment to the donor membrane of cytosolic coat proteins that constitute the COPI and COPII protein complexes. The COPII complex has been shown to play an essential role in the formation of ER-derived vesicles for anterograde movement (Bednarek *et al.*, 1995), while the COPI complex is required for both anterograde and retrograde movement (Bednarek *et al.*, 1995; Cosson *et al.*, 1996; Lewis and Pelham, 1996). Recently, a genetic approach has suggested that the COPI coat proteins may be involved in retrograde transport (Letourneur *et al.*, 1994), a finding which in turn suggests that the anterograde effects of COPI components are due to the necessity for retrograde transport to retrieve limiting components that facilitate continued anterograde movement.

The COPI proteins bind to membranes in association with members of the ARF family of GTP-binding proteins. The activation of ARF proteins by the binding of GTP results in the recruitment of COPI proteins to the membrane to facilitate transport vesicle formation (Rothman and Wieland, 1996). Hydrolysis of the ARF-bound GTP is then required for the efficient delivery of the vesicle to the target membrane. In this way, the GTPase cycle carried out by ARF proteins plays an important role in vesicle traffic (Donaldson *et al.*, 1992; Boman and Kahn, 1995).

Because ARFs do not possess intrinsic GTPase activity (Kahn and Gilman, 1986), a GTPase-activating protein (GAP) is necessary for ARF function. Recently, we identified the *Saccharomyces cerevisiae* Gcs1 protein as a GAP for yeast Arf1 and Arf2 proteins, and presented evidence for a role for Gcs1 in ARF-dependent membrane traffic (Poon *et al.*, 1996). Here we provide both *in vivo* and *in vitro* evidence that a second yeast protein, Glo3 (Ireland *et al.*, 1994), is also an ARF GAP. We demonstrate using several procedures that the Glo3 protein affects vesicular transport within the ER–Golgi shuttle, and that Glo3 and Gcs1 constitute an essential pair of proteins that provides overlapping ARF GAP function for retrograde vesicular transport from the Golgi to the ER.

Fig. 1. Glo3 is an ARF GAP both *in vitro* and *in vivo*. (A) GAP activity of recombinant Glo3 protein was assessed *in vitro* by hydrolysis of GTP bound to recombinant yeast Arf1 (○) and Arf2 (●) proteins. For comparison, the activity of the Gcs1 ARF GAP with Arf1 protein (◇) is also displayed. (B) A *glo3Δ* mutant strain was mated with an isogenic single mutant strain deleted for the *ARF1* gene. Resultant diploids were sporulated and meiotic segregants were incubated on solid medium at 30°C. The genotype of each segregant from six tetrads was determined by replica plating. Arrows indicate representative *glo3Δ arf1Δ* double mutant segregants.

Results

Glo3 is an ARF GAP in vitro and in vivo

The Gcs1 protein provides GAP activity for members of the ARF family of monomeric GTP-binding proteins. Within the Gcs1 protein is a novel 'zinc finger' motif that is necessary for ARF GAP activity (Poon *et al.*, 1996). This finger motif is found in another yeast protein, Glo3. Analysis of the predicted amino acid sequences of the two proteins reveals 48% similarity and 28% identity. To determine whether this structural similarity between the Glo3 and Gcs1 proteins extends to ARF GAP activity, recombinant Glo3 protein was produced in *Escherichia coli* and assayed *in vitro* for the ability to stimulate the hydrolysis of ARF-bound GTP. Using recombinant yeast Arf1 and Arf2 proteins, we found that Glo3 does indeed possess ARF GAP activity for both ARF proteins (Figure 1A). Thus Glo3, like Gcs1, is a yeast ARF GAP.

As a test for Glo3 *in vivo* function, we deleted one copy of the *GLO3* gene from a diploid cell, sporulated the resulting diploid cells, and found that haploid segregants lacking the *GLO3* gene proliferated and formed colonies at 30°C. Thus, like Gcs1, Glo3 is not an essential protein. However, *glo3Δ* mutant cells grew more slowly than did wild-type cells (2.4 h doubling time for *glo3Δ* mutant cells, compared with 1.8 h for congenic *GLO3* cells). Moreover, *glo3Δ* mutant cells failed to proliferate at all at 15°C, a temperature that allows growth of wild-type cells. Thus Glo3 has an important function *in vivo*.

The *in vitro* substrates for Glo3 and Gcs1 GAP activity, Arf1 and Arf2, have overlapping functions, making each of these ARF proteins individually dispensable. Therefore, we were able to adopt a genetic strategy to assess the *in vivo* role of Glo3 as a GAP for ARF proteins. We mated a *glo3Δ* mutant with an *arf1Δ* single mutant strain, sporulated the resultant heterozygous diploid strain, and determined the phenotypes of the resulting haploid segregants. The *glo3Δ arf1Δ* double mutant segregants lacking both Glo3 and Arf1 proteins were markedly impaired for colony formation (Figure 1B), although tiny colonies did form after extended incubation. This 'synthetic enhancement' of mutant phenotype is similar to that reported for *gcs1Δ arf1Δ* double mutant cells (Poon *et al.*, 1996) and is consistent with an *in vivo* function for Glo3 as an ARF GAP.

Genetic interactions localize Glo3 to vesicular transport between the ER and Golgi

To assess genetically the role of the Glo3 ARF GAP in vesicular transport, we transformed a *glo3Δ* single mutant strain with a high copy genomic library, and selected transformants at the restrictive temperature of 15°C (see above) to identify yeast genes that, in increased dosage, can compensate for the absence of Glo3 activity and allow colony formation at this temperature. Among the genes we identified in this way were *BOS1*, *BET1* and *SEC22* (Figure 2A), genes that encode a set of interacting proteins that belong to the v-SNARE family of integral membrane proteins (Newman *et al.*, 1990). The Bos1, Bet1 and Sec22 proteins are required for vesicular transport within the ER–Golgi shuttle (Newman and Ferro-Novick, 1987; Shim *et al.*, 1991). To confirm that this copy suppression reflects an involvement of the Bos1 and Glo3 proteins in a common process, we assessed genetic interactions between the temperature-sensitive *bos1-1* and the *glo3Δ* mutations. Sporulation of a diploid strain heterozygous for the *bos1-1* and *glo3Δ* mutations yielded viable single mutant and wild-type segregants, but *bos1-1 glo3Δ* double-mutant segregants were unable to form colonies (data not shown). Microscopic examination showed that these double mutant segregants germinated but went through only a few divisions before ceasing growth. Thus the *glo3Δ* and *bos1-1* mutations display synthetic enhancement, a finding consistent with a significant role for the Glo3 protein in vesicular transport within the ER–Golgi shuttle.

The evidence of a role for Glo3 in the ER–Golgi shuttle led us to investigate the genetic interaction between Glo3 and the COPI proteins that cooperate with ARF proteins to form coated vesicles (Malhorda *et al.*, 1989; Rothman and Orci, 1992). The COPI subunit Sec21 has been implicated in transport between the ER and the Golgi (Hosobuchi *et al.*, 1992; Gaynor and Emr, 1997), based in part on the effects of the *sec21-1* mutation that causes temperature-sensitive function of the Sec21 protein (Letourneur *et al.*, 1994). We found by tetrad analysis that the absence of the *GLO3* gene in *sec21-1* mutant cells is lethal. As shown in Figure 2B, cells harboring both the *glo3Δ* and *sec21-1* mutations were unable to grow, whereas each type of single mutant segregant was viable. These genetic interactions, between Glo3 and the Sec21 COPI protein or the Bos1 v-SNARE protein, suggest that the

Fig. 2. Genetic interactions involving *gcs1Δ* and *glo3Δ* mutations. (A) *glo3Δ* mutants were transformed with a high copy yeast genomic library and grown on defined solid medium without leucine. Transformed cells were replica-plated to 23 and 15°C, and several plasmids were identified that were capable of suppressing the cold sensitivity by the *glo3Δ* phenotype. Characterization of genomic inserts revealed the presence of *SEC22*, *BOS1* and *BET1* genes, and PCR amplification of *SEC22*, *BOS1* and *BET1* confirmed that each of these genes is a high-copy suppressor. The plasmid YEp351 ('vector') and the *GLO3* gene carried on YEp351 served as negative and positive controls, respectively. (B) Meiotic segregants from the sporulation of a *GLO3/glo3Δ::HIS3 SEC21/sec21-1* heterozygous diploid strain were grown on solid medium at 23°C, and replica-plated to selective conditions to determine the genotype of each segregant from 14 tetrads. Arrows indicate representative *glo3Δ sec21-1* double mutant segregants. (C) Meiotic segregants from the sporulation of a *GCS1/gcs1Δ::URA3 SEC21/sec21-1* heterozygous diploid were grown on solid medium at 26°C and then replica-plated to fresh medium and incubated at 26 and 33°C. The genotype of each segregant from 25 tetrads was determined by replica plating to selective conditions, and the boxed regions indicate representative *gcs1Δ sec21-1* double mutant segregants.

Glo3 ARF GAP has an important function in vesicular transport at the ER-Golgi.

The Glo3 ARF GAP mediates vesicular transport between the ER and the Golgi

As a biochemical indication of the impairment of vesicular transport in the absence of the Glo3 ARF GAP, we examined the post-translational processing and maturation of the vacuolar hydrolase carboxypeptidase Y (CPY). On its way from the ER to the vacuole, the CPY proenzyme is first core-glycosylated in the ER to produce the P1 form, and subsequently the core oligosaccharides are extended after transport to the Golgi to produce the P2 form (Stevens *et al.*, 1982). The mature (M) form of CPY is generated by proteolytic cleavage only after transport of the P2 form to the vacuole. As shown in Figure 3A,

Fig. 3. CPY transport is defective under conditions of decreased ARF GAP activity. (A) Proliferating cells were incubated at 26 or 37°C for 15 min, exposed to [³⁵S]amino acids for 10 min, incubated further with unlabeled methionine and cysteine, and sampled at intervals. CPY was immunoprecipitated, resolved by SDS-PAGE and detected by autoradiography. (B) Spheroplasts were incubated for 10 min at 37°C, exposed to [³⁵S]amino acids for 15 min, incubated further with unlabeled methionine and cysteine for 30 min, lysed and fractionated by differential centrifugation. CPY was immunoprecipitated, resolved by SDS-PAGE and detected by autoradiography. The hemagglutinin (HA) epitope-tagged Och1 protein (Och1-HA), a Golgi marker that is found in the P13 and P100 fractions (Gaynor and Emr, 1997), was detected by Western blot analysis.

the absence of the Glo3 ARF GAP impaired CPY transport: *glo3Δ* mutant cells displayed a transient accumulation of the P1 form of CPY, although they eventually were able to produce the mature vacuolar form. Likewise, *glo3Δ* mutant cells were also impaired in the processing of the secreted enzyme invertase (Figure 4), a protein that becomes progressively glycosylated during passage through the ER and the Golgi (Esmon *et al.*, 1981). These biochemical findings are consistent with a role for the Glo3 ARF GAP in vesicular transport within the ER-Golgi shuttle.

Glo3 and Gcs1 constitute an essential ARF GAP pair

Neither the Glo3 ARF GAP nor the previously described Gcs1 ARF GAP (Poon *et al.*, 1996) are essential proteins individually: *glo3Δ* and *gcs1Δ* single mutant cells are viable. However, ARF GAP function has been considered to be essential for proper ARF protein function (Cukierman *et al.*, 1995). As a test of the functional relationship between the Glo3 and Gcs1 ARF GAPs, we sporulated a diploid strain heterozygous for the *glo3Δ* and *gcs1Δ* deletion mutations. As also reported recently (Zhang *et al.*,

Fig. 4. Glycosylation and secretion of invertase is affected under conditions of decreased ARF GAP activity. Cells were transferred to low-glucose medium to induce invertase expression and incubated at 26 or 37°C for 1.5 h. Secreted and intracellular material was separated into external (e) and internal (i) fractions, respectively, and resolved by SDS-PAGE without heat treating the samples. Invertase was detected by an in gel enzyme assay (Gabriel and Wang, 1969).

1998), we were unable to obtain viable *glo3Δ gcs1Δ* double mutant haploid segregants (Figure 5), suggesting that at least one member of this ARF GAP pair is needed for spore germination or for mitotic growth. To determine the mitotic requirements for this protein pair, the same heterozygous diploid was first transformed with a *GLO3* plasmid and then sporulated; the presence of this plasmid in the resulting *glo3Δ gcs1Δ* double mutant spores allowed germination and colony growth. Segregants were then tested for the ability to lose the *GLO3* plasmid. Although single deletion segregants readily lost the *GLO3* plasmid, *glo3Δ gcs1Δ* double mutant segregants could not lose the plasmid and remain viable (data not shown). Therefore, the Glo3 and Gcs1 ARF GAPs provide an overlapping essential function.

Genetic interactions localize an overlapping function for Glo3 and Gcs1 to the ER–Golgi

As shown in Figure 2B, the *sec21-1* mutation in combination with the *glo3Δ* mutation is lethal, indicating that Sec21 and Glo3 affect a common vesicular transport process within the ER–Golgi shuttle. By a similar genetic procedure, it was found that *sec21-1 gcs1Δ* double mutant cells were viable at several growth temperatures but unable to grow at 33°C, a temperature that allowed colony formation of *gcs1Δ* and *sec21-1* single mutant cells (Figure 2C). Therefore, the genetic interaction between *sec21-1* and *gcs1Δ*, although less severe than that between *sec21-1* and *glo3Δ*, is significant. These genetic interactions indicate that the Gcs1 and Glo3 ARF GAPs both provide function for vesicular transport within the ER–Golgi shuttle.

Depletion of both Glo3 and Gcs1 results in accumulation of ER and early secretory blockage

For a more direct assessment of the overlapping effects of Glo3 and Gcs1 function, we constructed a yeast strain

Fig. 5. Genetic interaction between *glo3Δ* and *gcs1Δ* mutations. A *GLO3/glo3Δ::HIS3 GCS1/gcs1Δ::URA3* heterozygous diploid strain was sporulated and the segregants were incubated on solid medium. Segregants from 15 tetrads were replica-plated to selective media to assess genotypes. Arrows indicate representative *glo3Δ::HIS3 gcs1Δ::URA3* double mutant cells.

lacking the chromosomal *GLO3* and *GCS1* genes and kept alive by a plasmid carrying a functional *GLO3* gene under the control of the repressible *GALI* promoter. As shown in Figure 6A, *glo3Δ gcs1Δ* double-mutant cells harboring the *GAL-GLO3* plasmid were able to proliferate using galactose as the carbon source, but ceased cell division when transferred to glucose-containing medium, which represses transcription from the *GALI* promoter and prevents further Glo3 production. By electron microscopy, proliferating mutant cells appeared morphologically indistinguishable from proliferating wild-type cells (data not shown), and wild-type cells transferred to glucose medium continued to proliferate and displayed a morphology typical of proliferating cells (Figure 6B). In contrast, mutant cells that had ceased cell division in glucose medium contained highly elaborated ER (Figure 6C). This accumulation of ER is an indication of disrupted vesicular transport between the ER and Golgi membranes.

As shown in Figure 6A, the depletion of ARF GAP activity by the repression of *GAL-GLO3* transcription requires a significant incubation period to affect cell proliferation. To determine the effects of a more rapid impairment of ARF GAP function, we generated a conditional ARF GAP mutant, by creating temperature-sensitive versions of the *GCS1* gene and introducing a temperature-sensitive *gcs1* allele (*gcs1-28*) on a plasmid into *glo3Δ gcs1Δ* double mutant cells. Transfer of these *glo3Δ gcs1-28* cells to 37°C caused a rapid arrest of cell proliferation (Figure 6D), and these arrested mutant cells displayed a significant accumulation of ER (Figure 6E), similar to the effect of depleting ARF GAP function by glucose-mediated repression of *GAL-GLO3* expression. Thus, morphological effects of inadequate activity by Glo3 and Gcs1 suggest a role for this essential pair of ARF GAPs in ER–Golgi vesicular transport.

As a biochemical measure of vesicular transport, we examined the maturation of the secreted enzyme, invertase. These experiments showed that invertase is secreted by *glo3Δ gcs1-28* double mutant cells, even at the growth-

Fig. 6. Cells depleted for Glo3 and Gcs1 ARF GAP activity accumulate endoplasmic reticulum. (A) A *glo3Δ gcs1Δ* double mutant strain (●) and an isogenic wild-type strain (○), both harboring the pGAL-GLO3 plasmid, were transferred from galactose to glucose medium at t_0 and incubated for 10 h. At t_0 and after 10 h of incubation (arrow), cells were harvested and processed for electron microscopic analysis. (B) Wild-type and (C) *glo3Δ gcs1Δ* double mutant cells 10 h after transfer to glucose medium. (D) Cells of a *glo3Δ gcs1-28* double mutant strain (●) or an isogenic wild-type strain (○), both harboring plasmid pPP805.28 (carrying the temperature-sensitive *gcs1-28* allele), were transferred from 26 to 37°C. At t_0 and after 1 h (arrow), cells were harvested and processed for electron microscopic analysis. (E) *glo3Δ gcs1-28* cells after 1 h at 37°C.

inhibitory temperature of 37°C (Figure 4). However, the invertase that was secreted was markedly hypoglycosylated. Therefore, inadequate activity of Glo3 and Gcs1 imposes a Golgi-related defect that is manifested by perturbed glycosylation.

As another measure of vesicular transport, we examined the maturation of CPY, a process that is slowed in the *glo3Δ* single mutant situation (Figure 3A). As shown in Figure 3A, the transfer of *glo3Δ gcs1-28* double-mutant cells to 37°C caused a complete blockage of CPY processing so that mutant cells accumulated the P1, ER-glycosylated form of CPY. To localize the P1 form of CPY that accumulates in *glo3Δ gcs1-28* double-mutant cells, we subjected cell extracts to a differential centrifugation procedure (Gaynor and Emr, 1997). As shown in Figure 3B, the cell fractionation of *glo3Δ gcs1-28* mutant cells after 37°C incubation showed marked accumulation of the P1 form of CPY in the ER (P13) fraction. This finding indicates that the impairment in vesicular transport imposed by the absence of both Glo3 and Gcs1 ARF GAPs leads to an inability to transport CPY out of the ER to the Golgi.

Glo3 and Gcs1 mediate Golgi-ER retrograde transport in vitro

ARF proteins are involved in several transport steps throughout the secretory system; our genetic, molecular and biochemical findings indicate that the Glo3 and Gcs1 ARF GAPs facilitate vesicular transport between the ER and the Golgi. To confirm a role for these proteins in the early secretory pathway, we employed an *in vitro* assay for retrograde transport from the Golgi to the ER (Spang and Schekman, 1998). This assay has already implicated the yeast Arf1 protein in Golgi-ER transport (Spang and Schekman, 1998). For this assay, the HDEL sequence that mediates protein retrieval (Semenza *et al.*, 1990; Lewis and Pelham, 1996) was fused to the α -factor precursor protein (pp α F) and the resultant fusion protein was 35 S-labeled (Dean and Pelham, 1990). The [35 S]pp α F-HDEL was then translocated to the ER of perforated spheroplasts, where the fusion protein becomes *N*-glycosylated (gp α F-HDEL). Normally the *N*-glycan on ER-localized proteins is subjected to enzymatic trimming of the terminal glucose residues; to prevent this trimming, we used a mutant defective for glucosidase I activity (see Spang and Schekman, 1998), but otherwise unimpaired

Fig. 7. Glo3 and Gcs1 facilitate retrograde transport *in vitro*. Retrograde transport assays were performed as described in Materials and methods. Wild-type and mutant membranes and cytosol fractions were added to the COPII vesicle fraction of the stage II reaction as indicated, and incubated at 30°C. The trimmed form of [³⁵S]ppαF-HDEL identifies fusion protein that was retrieved to the acceptor ER during stage III of the *in vitro* retrieval reaction. The untrimmed form represents a contaminating species in the ER membrane fraction.

for vesicular transport. In such mutant cells, the terminal sugar residues on the [³⁵S]gpαF-HDEL protein are retained. Upon subsequent addition of purified Sar1 protein, Sec23/24 complex and Sec13/31 complex, COPII vesicles containing this untrimmed form of [³⁵S]gpαF-HDEL as cargo were formed. The newly formed COPII vesicles were then isolated and incubated with fresh perforated spheroplasts from wild-type or mutant cells and with cytosol from various sources (Figure 7). In all cases, cells used as a source of spheroplasts or cytosol had the wild-type glucosidase I protein to allow trimming of proteins in the ER. The cytosol fractions and perforated spheroplasts provide the specific factors necessary for vesicle fusion so that the COPII vesicles can fuse with the Golgi (Barlowe, 1997); the ARF GAPs studied here are not necessary for this fusion (unpublished observations). After delivery of the glycosylated, untrimmed cargo protein into the Golgi, recognition of the HDEL signal on the [³⁵S]gpαF-HDEL protein by the HDEL receptor (Dean and Pelham, 1990) leads to inclusion of untrimmed [³⁵S]gpαF-HDEL protein into COPI retrograde transport vesicles. Fusion of these COPI vesicles with the ER and delivery of untrimmed [³⁵S]ppαF-HDEL exposes these proteins to ER-resident glucosidases. After incubation, ER membranes were isolated by centrifugation, and retrieval of [³⁵S]gpαF-HDEL was monitored by the trimming of [³⁵S]gpαF-HDEL, which increases its electrophoretic mobility (Latterich and Schekman, 1994; Spang and Schekman, 1998).

Using perforated wild-type spheroplasts containing Golgi and ER (the acceptor membranes) and cytosol from wild-type cells, retrieval of [³⁵S]gpαF-HDEL could be observed (Figure 7, lane 1) although some untrimmed [³⁵S]gpαF-HDEL was always present in the isolated ER membrane fraction, which is most likely due to vesicles that have docked but not fused with the membrane or to contaminating Golgi fractions (Spang and Schekman, 1998). The amount of trimmed material was significantly decreased when the acceptor membranes were derived from the *glo3Δ gcs1-28* double mutant strain (Figure 7, lanes 2–4) regardless of the source of the cytosol. This finding suggests that a significant amount of Glo3 and Gcs1 ARF GAP activity resides on membranes. The defect in retrieval was less pronounced when acceptor membranes from single mutant cells were tested. When Glo3 was the only ARF GAP present (using a *glo3Δ* single mutant), the level of retrieval was decreased only slightly compared

Fig. 8. The rat ARF GAP functions in yeast. *glo3Δ gcs1Δ* transformants carrying the pGAL-GLO3 and a plasmid expressing the indicated proteins were replica-plated to solid enriched medium containing galactose (Gal) or glucose (Glu) and incubated at 30°C.

with the wild-type situation (Figure 7, lane 5). In contrast, when Gcs1 was the only ARF GAP, there was a more severe impairment of retrieval (Figure 7, lane 6). Nevertheless, in the absence of Glo3, Gcs1 can provide substantial ARF GAP function for Golgi–ER retrieval (Figure 7, compare lanes 4 and 6). Thus, although both Gcs1 and Glo3 provide GAP function for Golgi–ER transport, Glo3 may be the predominant ARF GAP within this interval.

A mammalian ARF GAP can substitute for the function common to Glo3 and Gcs1

The mammalian ARF GAP protein described previously (Cukierman *et al.*, 1995) has also been shown to be associated with the Golgi. We therefore tested the ability of this mammalian ARF GAP to restore vesicular transport in the absence of the yeast Glo3 and Gcs1 ARF GAPs. A plasmid was constructed in which the mammalian ARF GAP cDNA (Cukierman *et al.*, 1995) was expressed from the *GCS1* promoter; as shown in Figure 8, this plasmid provided sufficient ARF GAP activity in *glo3Δ gcs1Δ* (pGAL-GLO3) mutant cells to allow growth on glucose medium. This finding shows that this mammalian protein can serve as an ARF GAP for yeast vesicular transport between the Golgi and the ER.

Discussion

Using both *in vivo* and *in vitro* approaches, we have identified a second yeast GAP for ARF proteins, and show that this ARF GAP protein, Glo3, facilitates vesicular transport. Furthermore, our results show that Glo3 and Gcs1, a previously characterized yeast ARF GAP (Poon *et al.*, 1996), are each sufficient for vesicular transport between the ER and the Golgi apparatus, and that the two proteins provide a redundant function. Genetic and biochemical evidence suggests that Glo3 and Gcs1 directly affect retrograde transport at this stage. Both *glo3Δ* and *gcs1Δ* mutations display genetic interactions with the *sec21-1* mutation that affects the α-COPI component of the COPI coat complex. Others have shown that this COPI component may not be required for transport of material out of the ER but that inactivation of COPI by mutation of the α-subunit results in a defect in regulated retrograde transport from the Golgi to the ER (Letourneur *et al.*, 1994; Gaynor and Emr, 1997). Indeed, at least one member of the Glo3 + Gcs1 ARF GAP pair is necessary for retrograde movement from the Golgi to the ER in a

specific *in vitro* retrieval assay. This stage of retrograde transport has been characterized as being mediated by ARF proteins (Gaynor *et al.*, 1998). Moreover, we find that at least the Glo3 protein is localized on COPI vesicles (unpublished data). Recently, a genetic screen for mutants defective in retrieval of dilysine proteins from the Golgi to the ER resulted in the identification a mutant allele of the *GLO3* gene (F. Letourneur, personal communication). Our findings therefore suggest that the Glo3 and Gcs1 ARF GAPs have redundant function for COPI-mediated vesicular transport from the Golgi to the ER.

Cells lacking either the Glo3 or Gcs1 protein are viable, whereas the absence of both of these ARF GAP proteins is lethal. This conclusion stems from the 'synthetic lethality' seen for the *glo3Δ* and *gcs1Δ* mutations, which we interpret to indicate that Glo3 and Gcs1 provide a redundant essential function for a vesicular transport stage. Recently, this usual interpretation of synthetic lethality, or synthetic enhancement of a mutant phenotype, has been challenged by experiments demonstrating for one particular situation that one protein can supply the function of another (missing) protein in a manner that does not reflect normal function (Madhani and Fink, 1998). However, for the situation described here, we also showed that each single *glo3Δ* or *gcs1Δ* deletion mutation also displays synthetic enhancement in combination with a mutation that affects a component of the COPI complex. These *sec21-1 glo3Δ* and *sec21-1 gcs1Δ* double mutant cells are more impaired than each single mutant cell, indicating that the Glo3 and Gcs1 ARF GAPs each participate in a process related to the Sec21 protein, a COPI component that is essential for retrieval from the Golgi to the ER (Hosobuchi *et al.*, 1992). From these observations, we infer that the ability of each of the Glo3 and Gcs1 ARF GAPs to provide essential activity in the absence of the other ARF GAP is indeed likely to reflect a normal function for each ARF GAP protein in the ER-Golgi shuttle.

In addition to a role for the Glo3 and Gcs1 ARF GAPs in retrieval of material from the Golgi, these proteins also facilitate anterograde transport from the ER to the Golgi: depletion of the Glo3 and Gcs1 proteins, or the absence of Glo3 alone, impairs the export of CPY from the ER. The effect of these ARF GAPs on anterograde vesicular transport may not reflect a direct role for these proteins in this process, and may instead be a consequence of a primary defect in the recycling of transport components that are necessary for efficient anterograde movement.

The perturbation of anterograde transport in cells depleted for Glo3 and Gcs1 proteins shows a degree of cargo selectivity. Unlike CPY, the invertase protein is still transported effectively to the cell surface in these double mutant cells, albeit in an hypoglycosylated state, while transport of CPY is blocked, leading to an accumulation of the P1 form of CPY in the ER fraction. Analogous cargo-selective transport has also been seen in cells with a mutated Sec21 component of the COPI complex. A temperature-sensitive *sec21* mutation also prevents anterograde movement of CPY, leading to the accumulation of the P1 form of CPY characteristic of blockage in exit from the ER, while allowing the continued anterograde transport of invertase (Gaynor and Emr, 1997). One reasonable explanation for such a cargo-selective transport defect is that some types of cargo interact with transport

factors (or receptors) for packaging and delivery to a more forward compartment; in the absence of effective retrograde transport these receptors are not returned, leading to depletion of the receptor pool and blockage of further delivery of cargo dependent on these receptors. The dramatic impairment of *in vitro* retrograde transport when cells are depleted for the Glo3 and Gcs1 ARF GAPs supports the contention that cargo-selective effects are a consequence of inefficient recycling of components that are necessary for the export from the ER of certain cargo molecules, including CPY.

Our findings here suggest that the Glo3 and Gcs1 proteins do not provide equivalent function within the ER-Golgi shuttle. Mutant cells lacking Gcs1 protein have only a moderate phenotype with respect to ER-Golgi traffic. The *gcs1Δ* single mutant cells, with intact Glo3 function, grow at a normal rate and exhibit only minor defects in the transport of CPY and invertase. The genetic interactions of the *gcs1Δ* mutation are also mild. These observations suggest that the Glo3 ARF GAP function remaining in *gcs1Δ* mutant cells is significant for transport between the Golgi and the ER. In contrast, *glo3Δ* single mutant cells with intact Gcs1 function grow more slowly than wild-type cells and display marked effects on the processing of both CPY and invertase. The genetic interactions of the *glo3Δ* mutation are also more severe than those of the *gcs1Δ* mutation. These observations suggest that the Gcs1 protein may normally supply only a minor portion of the ARF GAP activity for transport from the Golgi to the ER. The mutant phenotype of *glo3Δ* mutant cells is not suppressed by the presence of extra, plasmid-borne, copies of the *GCS1* gene (unpublished observations), suggesting that the effectiveness of vesicular transport in *glo3Δ* mutant cells is not limited by the amount of Gcs1 protein. The molecular basis for the apparent difference in effectiveness of Glo3 and Gcs1 for transport within the ER-Golgi shuttle remains to be elucidated.

The Glo3 and Gcs1 proteins may have additional and distinct roles in vesicular transport. In addition to the role in Golgi-ER transport shown here, the Gcs1 ARF GAP also mediates endocytosis: under certain conditions of growth at 15°C, mutant cells lacking Gcs1 are unable to transport the membrane-active fluorescent dye FM4-64 from the cell surface to the vacuole (Wang *et al.*, 1996). Similarly, the *gcs1Δ* mutation interacts genetically with the *YCK1* + *YCK2* gene pair (Wang *et al.*, 1996) whose gene products, functionally redundant isoforms of casein kinase I (Robinson *et al.*, 1992), have been implicated in endocytosis (Panek *et al.*, 1997). Gcs1 may therefore provide ARF GAP function for at least two vesicular transport stages, one for endocytosis and another, as shown here, for retrograde transport from the Golgi to the ER. Despite the overlapping function provided by Glo3 for Golgi-ER transport, the Glo3 and Gcs1 redundancy may not extend to endocytosis: *glo3Δ* single mutant cells can transport the FM4-64 dye efficiently under all conditions tested, indicating that endocytosis is unaffected by the absence of the Glo3 ARF GAP, and the *glo3Δ* mutation shows no genetic interaction with the *YCK1* + *YCK2* casein kinase I genes (unpublished observations). Indeed, efficient endocytosis can still take place even in *glo3Δ gcs1-28* double mutant cells incubated at 37°C and thus

lacking Glo3 and Gcs1 ARF GAP activities (unpublished observations). The evidence for distinct but partially overlapping functions of the Glo3 and Gcs1 proteins suggests that each of these ARF GAPs may function at several vesicular transport stages, and interact with distinct sets of proteins characteristic of these different stages.

ARF proteins have also been shown to influence a number of vesicular transport activities (Gaynor *et al.*, 1998). Using a conditional *arf1* mutation, Gaynor *et al.* (1998) demonstrated a role for ARF protein in both ER–Golgi transport and endocytosis. Unlike the situation for a *sec21* COP1 mutation or for the defective ARF GAP function described here, impaired ARF activity causes general rather than cargo-specific defects in vesicular transport. This observation along with a morphological assessment of the effects of impaired ARF activity has led to the conclusion that the primary role for ARF may be as a more general regulator of membrane composition that in turn affects organelle integrity (Gaynor *et al.*, 1998). Although we show here that depletion of the Gcs1 and Glo3 pair of ARF GAP proteins affects Golgi–ER vesicular transport, other vesicular transport shuttles are largely unaffected (unpublished data). Thus, ARF GAPs may be more specialized than their ARF protein substrates for vesicular transport between membrane compartments and, as such, ARF GAPs could impart specificity for vesicular transport pathways.

Materials and methods

Strains, plasmid constructions and growth conditions

All strains are isogenic with the wild-type diploid yeast strain W303 (*leu2-3,112 ura3-1 his3-11,15 trp1-1 ade2-1*) and its isogenic haploid derivatives W303-1a (*MATa*) and W303-1b (*MATα*) (Archambault *et al.*, 1992). Standard procedures were used for cell growth, transformation and genetic analysis. The *gcs1Δ::URA3* and *gcs1Δ::LEU2* disruption–deletion alleles have been described (Ireland *et al.*, 1994; Poon *et al.*, 1996). To delete the *GLO3* gene, the *HIS3* gene from plasmid YDpH (Berben *et al.*, 1991) was used to replace a *ScaI*–*SalI* fragment within the library copy of the *GLO3* gene so that 30 bp upstream of the *GLO3* open reading frame (ORF) and 488 bp within the ORF itself were removed. A *SmaI*–*SalI* fragment from the resultant plasmid pP141 containing the *HIS3* gene flanked by *GLO3* chromosomal sequences was used to transplace the *GLO3* gene in strain W303.

To express the rat ARF GAP protein in yeast, a fragment starting at the *HpaII* site within the coding region was placed downstream of the *AhaII* site within the *GCSI* gene carried on a multicopy plasmid derived from YEp352 (Hill *et al.*, 1986). The resulting construct encodes a protein consisting of the first 12 amino acids of the Gcs1 protein and amino acids 14–415 of the rat ARF GAP (Cukierman *et al.*, 1995). Expression of the fusion protein was regulated by a 337 bp region of the *GCSI* promoter.

To generate temperature-sensitive versions of the Gcs1 protein, PCR and gap repair were employed. The *GCSI* sequence between positions –337 and +1591 (with +1 the A residue of the start codon) was amplified by mutagenic PCR. The PCR product and a linearized, gapped plasmid lacking sequences between positions –175 and +1222 of *GCSI* were co-transformed into the yeast strain PPY135 (*glo3Δ gcs1Δ* pGAL–*GLO3*) and the transformation mix was spread on solid selective medium containing galactose as the carbon source. Transformed cells were then screened for the ability to grow on glucose medium at 26 and 37°C. The gap-repaired plasmids were isolated from cells able to grow at 26°C but not at 37°C. To regulate expression of the *GLO3* gene, a *ScaI*–*ClaI* *GLO3* fragment was inserted downstream of the *GALI* promoter within the multiple cloning site of plasmid pEMBLyex4 (Cesareni and Murray, 1987). The *GALI*–*GLO3* gene fusion was then transferred to the low copy vector pRS315 (Sikorski and Hieter, 1989) to produce the plasmid pGAL–*GLO3*.

Electron microscopy

Cells were harvested by centrifugation and fixed in buffered glutaraldehyde essentially as described previously (Byers and Goetsch, 1975; Johnston *et al.*, 1991).

Bacterial expression of proteins

Recombinant Glo3 protein was expressed in *E. coli* under control of the T7 promoter on plasmid pET21b (Novagen). Before transfer to the expression vector, a fragment of *GLO3* was generated by PCR using primers to create an upstream *BamHI* site and a downstream *HindIII* site. The resultant product was placed between the T7 promoter and sequences encoding a polyhistidine tract to produce plasmid pPPL42. The recombinant Glo3, Gcs1, Arf1 and Arf2 proteins were similarly produced as described (Poon *et al.*, 1996).

GAP assays

GAP activity was assayed as described (Cukierman *et al.*, 1995; Makler *et al.*, 1995). Recombinant myristoylated Arf proteins were produced in bacteria co-expressing Arf proteins and *N*-myristoyltransferase (Weiss *et al.*, 1989). Yeast Arf1 and Arf2 proteins were loaded with [α -³²P]GTP in the presence of dimyristoyl-phosphatidylcholine and cholate, causing ~50–60% of nucleotide to associate with Arf protein. Results are expressed as the percentage of Arf-bound [α -³²P]GTP converted to [α -³²P]GDP. In the absence of GAP protein there was no hydrolysis of Arf-bound GTP.

Assays of vesicular transport

To assess transport and processing of CPY, cells were grown at 26°C in defined medium to a cell concentration of 5×10^6 cells/ml, then shifted to 37°C for 15 min and labeled with Trans³⁵S-label (ICN Biomedicals) for 10 min. Additional methionine, cysteine and yeast extract were then added to final concentrations of 0.5, 0.5 and 0.4%, respectively, and samples were removed at intervals to tubes containing NaN₃ and NaF at final concentrations of 10 mM. Cells were disrupted by agitation with glass beads and the CPY was immunoprecipitated from cell extracts as described (Franzoso *et al.*, 1991). Proteins within the immunoprecipitated samples were resolved electrophoretically in a 7.5% SDS–polyacrylamide gel.

For cell fractionation to localize CPY, cells were grown to 5×10^6 cells/ml in defined medium at 26°C, collected by centrifugation and converted to spheroplasts as described (Gaynor and Emr, 1997). The spheroplasts were incubated at 37°C for 10 min, labeled with Trans³⁵S-label for 15 min and then exposed to additional unlabeled precursors for 30 min as described above. The spheroplasts were then lysed and subjected to differential centrifugation as described (Gaynor and Emr, 1997). CPY was immunoprecipitated from the resulting fractions and resolved electrophoretically. The Och1 protein, tagged with the HA epitope, was detected by Western analysis.

For assessment of invertase secretion, cells were grown to 5×10^6 cells/ml in defined medium, collected by centrifugation and transferred to medium with 0.1% glucose to induce invertase expression. These cells were incubated at 37°C for 1.5 h and NaN₃ was then added to a final concentration of 10 mM. Cells were harvested and converted to spheroplasts by incubation in 1.4 M sorbitol, 5 mM NaN₃, 0.5 mg/μl zymolyase and 50 mM Tris, pH 7.5, at 37°C for 30 min. After centrifugation of the samples, internal and external pools of invertase were separated by harvesting the spheroplast pellet and supernatant, respectively, after centrifugation. The samples were diluted in Laemmli buffer (without heat denaturation) and resolved electrophoretically in a 5% SDS–polyacrylamide gel. Invertase was detected in the gel as described (Gabriel and Wang, 1969). The gel was rinsed with water and incubated with 0.1 M sucrose, 0.1 M sodium acetate (pH 5.2) for 1 h at 37°C, rinsed with water and treated with a solution of 0.1% 2,3,5-triphenyl tetrazolium chloride, 0.5 M NaOH in a tray floating in a boiling-water bath. Color development was terminated by repeated washes with 7.5% acetic acid.

Preparation of perforated spheroplasts and cytosol

Perforated yeast spheroplasts (semi-intact cells) were prepared as described by Rexach *et al.* (1994). For cytosol preparations, cells were grown to early or mid-log phase in YEPD medium. Wild-type cells were grown at 30°C and temperature-sensitive mutant cells were grown at 23°C. Cells were harvested by centrifugation, washed twice with water, resuspended in a minimal volume of buffer B88 (20 mM HEPES pH 6.8, 250 mM sorbitol, 150 mM KOAc, 5 mM MgOAc) and frozen rapidly in liquid nitrogen. The frozen pellet was pulverized under liquid nitrogen using either a blender (Worthington) or a mortar. The cell powder was

thawed in an ice bath in the presence of ATP (1 mM), dithiothreitol (DTT; 1 mM) and protease inhibitors. The lysate was centrifuged (5 min at 3000 g, 15 min at 20 000 g, 1 h at 100 000 g) and the final supernatant was collected, avoiding the pellet and the lipids that had floated to the top.

In vitro round-trip retrieval assay

Reagents used for the retrieval assay were prepared as described by Baker *et al.* (1988) unless otherwise indicated.

Stage I: translocation. The translocation reaction using [³⁵S]ppα-factor-myc-HDEL (Dean and Pelham, 1990) and donor membranes from cells lacking glucosidase I (Spang and Schekman, 1998) was performed as described by Rexach *et al.* (1994), except that the scale was increased 2-fold, and a high-salt treatment and incubation in B88 buffer for 10 min at 4°C was included. The membranes were then washed twice in B88 and resuspended in B88 to a volume of 220 µl.

Stage II: budding. To the membranes of the stage I reaction were added 25 µg/ml Sar1, 25 µg/ml Sec23/24 complex, 75 µg/ml Sec13/31 complex, 50 µM GTP and an ATP regeneration system (without GDP-mannose) (Baker *et al.*, 1998). The reaction mixture was incubated for 30 min at 20°C, chilled on ice (5 min) and centrifuged (12 000 g, 30 s) to retain COPII vesicles in the supernatant.

Stage III: fusion and retrieval. The medium-speed supernatant from stage II was supplemented with an ATP-regenerating system (without GDP-mannose), 50 µM GTP, 2 mg/ml cytosol and 600 µg/ml perforated spheroplast membranes from a wild-type strain which had been washed twice with B88, or with 2.5 M urea in B88, prior to addition. Reactions (100 µl) were incubated for 30 min at 30°C and subsequently chilled on ice for 5 min. The acceptor ER was isolated by centrifugation at 12 000 g (30 s), and the pellet was washed once with 2.5 M urea in B88 (10 min on ice) and once with B88. Fusion with the acceptor ER was measured by precipitation of protease-protected [³⁵S]gpαF-HDEL with concanavalin A–Sephacryl (Rexach and Schekman, 1991) followed by separation of untrimmed [³⁵S]gpαF-HDEL from trimmed [³⁵S]gpαF-HDEL by SDS–PAGE. The amount of retrieved [³⁵S]gpαF-HDEL was correlated to the amount of [³⁵S]gpαF-HDEL in the medium-speed supernatant of the stage II reaction.

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