

Role for Gcs1p in Regulation of Arl1p at *Trans*-Golgi Compartments^D

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ADP-ribosylation factor (ARF) and ARF-like (ARL) proteins are members of the ARF family, which are critical components of several different vesicular trafficking pathways. ARFs have little or no detectable GTPase activity without the assistance of a GTPase-activating protein (GAP). Here, we demonstrate that yeast Gcs1p exhibits GAP activity toward Arl1p and Arf1p *in vitro*, and Arl1p can interact with Gcs1p in a GTP-dependent manner. Arl1p was observed both on *trans*-Golgi and in cytosol and was recruited from cytosol to membranes in a GTP-dependent manner. In *gcs1* mutant cells, the fraction of Arl1p in cytosol relative to *trans*-Golgi was less than it was in wild-type cells. Increasing Gcs1p levels returned the distribution toward that of wild-type cells. Both Arl1p and Gcs1p influenced the distribution of Imh1p, an Arl1p effector. Our data are consistent with the conclusion that Arl1p moves in a dynamic equilibrium between *trans*-Golgi and cytosol, and the release of Arl1p from membranes in cells requires the hydrolysis of bound GTP, which is accelerated by Gcs1p.

INTRODUCTION

Intracellular membrane trafficking is regulated, in part, by small GTP-binding proteins of the ADP-ribosylation factor (ARF) family. ARFs are critical components of several different vesicular trafficking pathways in all eukaryotic cells and activators of specific phospholipase Ds (reviewed by Moss and Vaughan, 1998). ARF function depends on the controlled binding and hydrolysis of GTP. The conformational changes that accompany the binding of GDP or GTP can lead directly to changes in the affinity of the GTPase for proteins, lipids, and membranes. Binding of GTP activates ARF1, facilitates its association with membranes, and enables it to recruit coat proteins to initiate vesicle budding. Inactivation of ARF by hydrolysis of the bound GTP releases coat proteins (and ARF) from vesicle membranes. Unlike other monomeric small GTP-binding proteins, ARFs do not have detectable GTPase activity (Kahn and Gilman, 1986) and their inactivation requires a GTPase-activating protein (GAP).

In mammalian cells, ARF-GAPs have been found to link aspects of cell signaling and morphogenesis to vesicular transport (Randazzo *et al.*, 2000). Thus, ARF-GAPs may allow temporal as well as spatial coordination of the ARF GTPase cycle (Donaldson, 2000). Several GAPs were identified by their ability to enhance the GTPase activity of ARF

proteins *in vitro* (Cukierman *et al.*, 1995; Poon *et al.*, 1996, 1999; Randazzo, 1997; Brown *et al.*, 1998; Premont *et al.*, 1998; Andreev *et al.*, 1999; Turner *et al.*, 1999). An ARF-GAP localized to the rat liver Golgi complex contains a zinc-finger motif, which was important for GAP activity but had no effect on its subcellular distribution (Cukierman *et al.*, 1995). Structurally, this mammalian GAP, ARFGAP, is very similar to the yeast zinc-finger proteins Gcs1p, Glo3p, and Age2p, which are GAPs for yeast Arf1p *in vitro* (Poon *et al.*, 1996, 1999, 2001). Gcs1p and Glo3p have overlapping functions in endoplasmic reticulum (ER)-Golgi transport and Glo3p is important for retrieval of proteins from the Golgi to the ER (Andreev *et al.*, 1999; Dogic *et al.*, 1999; Poon *et al.*, 1999). The Gcs1p and Age2p ARF GAPs provide overlapping, essential function in transport from the yeast *trans*-Golgi network (Poon *et al.*, 2001). Although Gcs1p also was implicated in the regulation of actin cytoskeletal organization (Blader *et al.*, 1999) and mitochondrial morphology (Huang *et al.*, 2002), its multiple functions in cells remain to be established.

ARF-like (ARL) proteins are a subfamily of the ARF branch of the small G protein superfamily. The ARL proteins are highly conserved through evolution and have diverse functions, including regulation of membrane trafficking (Moss and Vaughan, 1998; Pasqualato *et al.*, 2002). In mammalian cells, ARL1 is localized to the TGN and regulates trafficking in TGN-endosomal pathways (Lowe *et al.*, 1996; Lu *et al.*, 2001; Lu and Hong, 2003). ARL2 regulates the interaction of tubulin-folding cofactor D with native tubulin (Bhamidipati *et al.*, 2000), whereas ARL4 and ARL5 may function in the nucleus (Lin *et al.*, 2000, 2002). *Saccharomyces cerevisiae* has two ARL proteins, Arl1p and Arl3p, both of which seem to play roles in vesicular trafficking (Lee *et al.*, 1997; Huang *et al.*, 1999; Panic *et al.*, 2003). Relatively little is known about biological roles, protein effectors, or the importance of membrane-lipid interactions for the ARLs. Because Arl1p, like Arf1p, lacks detectable GTPase activity (Lee *et al.*, 1997), a GAP should be essential for Arl1p func-

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Abbreviations used: ARF, ADP-ribosylation factor; ER, endoplasmic reticulum; GAP, GTPase-activating protein.

Table 1. Yeast strains used in this study

Strain	Genotype ^a	Source
YPH250	<i>MATa ade2, his3, leu2, lys2, trp1, ura3-52, ARF1, ARF2, ARF3, ARL1, ARL3, GCS1, GLO3</i>	Lee <i>et al.</i> (1997)
YPH250dr1	<i>MATa ade2, his3, leu2, lys2, trp1, ura3-52, arf1, ARF2, ARF3, ARL1, ARL3, GCS1, GLO3</i>	Huang <i>et al.</i> (2003)
YPH250d11	<i>MATa ade2, his3, leu2, lys2, trp1, ura3-52, ARF1, ARF2, ARF3, arl1, ARL3, GCS1, GLO3</i>	Huang <i>et al.</i> (1997)
YPH250dgl1	<i>MATa ade2, his3, leu2, lys2, trp1, ura3-52, ARF1, ARF2, ARF3, ARL1, ARL3, gcs1, GLO3</i>	Huang <i>et al.</i> (2002)
YPH250dgl3	<i>MATa ade2, his3, leu2, lys2, trp1, ura3-52, ARF1, ARF2, ARF3, ARL1, ARL3, GCS1, glo3</i>	This work
YPH250dgl11	<i>MATa ade2, his3, leu2, lys2, trp1, ura3-52, ARF1, ARF2, ARF3, arl1, ARL3, gcs1, GLO3</i>	This work
YEM1 α	<i>MATα trp1, his3, leu2, δops-LEU2, 2ops-LacZ</i>	

^a *ade*, adenine-requiring; *his*, histidine-requiring; *trp*, tryptophan-requiring; *ura*, uracil-requiring; *leu*, leucine-requiring. *Arl1* represents *arl1::hisG*; and *arf1* represents *arf1::hisG*; *gcs1* represents *gcs1::Leu2* and *glo3* represents *glo3::Leu2*.

tion. To obtain additional clues to the physiological role(s) of Arl1p, we investigated a potential GAP regulator for Arl1p.

Here, we provide both *in vivo* and *in vitro* evidence that the yeast Arl1p-GAP, Gcs1p, is also an Arl1p GAP. Arl1p was colocalized with Sft2p at the *trans*-Golgi apparatus in a GTP-dependent manner. Release of Golgi-bound Arl1p to the cytosol seemed to require hydrolysis of GTP by Gcs1p. Mutant cells lacking Gcs1p failed to redistribute Arl1p from *trans*-Golgi structures to the cytosol. Thus, our findings indicate that the movement of Arl1p between *trans*-Golgi and cytosol is regulated in part by Gcs1p acting as its GAP.

MATERIALS AND METHODS

Strains, Media, and Microbiological Techniques

Table 1 lists the yeast strains used in this study. Yeast culture media were prepared as described by Sherman *et al.* (1986). YPD contained 1% Bacto-yeast extract, 2% Bacto-peptone, and 2% glucose. SD contained 0.17% Difco yeast nitrogen base (without amino acids and ammonium sulfate), 0.5% ammonium sulfate, and 2% glucose. Nutrients essential for auxotrophic strains were supplied at specified concentrations (Sherman *et al.*, 1986). Yeast strains were transformed by the lithium acetate method (Ito *et al.*, 1983). Plasmids were constructed according to standard protocols (Sambrook *et al.*, 1989) (Table 2). Gene disruption was carried out as described by Lee *et al.* (1994).

Plasmid Construction

GCS1 mutant expression clones were constructed and subcloned in pJG4-5 for yeast two-hybrid assay, in pET15b for recombinant protein production, or in pVT101U for immunofluorescence microscopy. To introduce site-specific mutations in *GCS1*, a two-step recombinant PCR procedure was used. In Gcs1zn, two cysteines in the zinc-finger motif were replaced with alanines by primer pairs GCS1.1, GCS1.4 and GCS1.3, GCS1.2 (Table 3). Gcs1dC was generated with GCS1.1 and GCS1.dPH, and Gcs1N was generated with GCS1.1 and GCS1.N. For Myc-tagged Gcs1p expression, primer pairs GCS1.1 and GCS1.myc were used. For construction of Arl1T32N, primer pairs yARL1.1, yL1 T/N.2 and yL1 T/N.1, yARL1.2 (Table 2) were used for the primary PCR. For the construction of Arl1Q72L, primer pairs yARL1.1/yL1 Q/L.2 and yL1 Q/L.1, yARL1.2 were used. For the deletion of N-terminal 17 amino acids, primer yARL1d17N was used. These constructs were subcloned in pEG202 for yeast two-hybrid assay, pGEX4T-1 or pET15b for recombinant protein production, or Ylplac128 for immunofluorescence microscopy. For monomeric red fluorescent protein (mRFP)-tagged proteins, template mRFP1 in pRSETb (kindly provided by Dr. Roger Tsien, University of California at San Diego, La Jolla, CA) was used to generate mRFP-fused Arl1p or Arl1p. mRFP-fused proteins were driven by the ADH promoter in a centromeric vector and expression of these proteins was approximately two- to threefold higher than endogenous Arl1p (Supplemental Figure S1A). These expression constructs should be able to minimize the overexpression defect. *IMH1* was cloned with IMH1.1 and IMH1.2 primer pairs and subcloned into pRS314, containing the green fluorescent protein (GFP) gene under control of the *GAL1* promoter.

Yeast Two-Hybrid Analysis

Yeast two-hybrid analyses were performed using the "interaction-trap" system (Golemis and Khazak, 1997). In this system, bait (ARF or ARL) was fused with the DNA-binding domain of *LexA* in pEG202. *GCS1*, *GLO3*, *AGE2*, *SAT1/AGE1*, *SPS18*, and *GTS1* were made in the vector pJG4-5, which uses the

inducible yeast *GAL1* promoter to express the protein fused to an acidic domain that functions as a transcriptional activation motif. Reporter yeast, YEM1 α , containing interacting proteins, can transactivate two reporter genes, *LacZ* and *Leu2*, which can express β -galactosidase activity and grow on minimal medium lacking leucine.

In Vitro and In Vivo Binding Analysis

For *in vitro* binding analyses, 2 μ g of purified glutathione S-transferase (GST), GST-Arf1Q71Ld17N, GST-Arf3Q71Ld17N, or GST-Arl1Q72Ld17N bound to glutathione-Sepharose beads were incubated with 2 μ g of purified His-tagged Gcs1p in 1 ml of binding buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 10 mM MgCl₂) for 1 h at 4°C. Beads were then washed three times with binding buffer and boiled in sample buffer (50 μ l), and samples of proteins were separated by SDS-PAGE in 10% gel before blotting.

For binding analyses in cells, GST-fused Arl1d17N, Arl1Q72Ld17N, or Arl1T32Nd17N (expressed by pYEX-4T-1) was induced with 0.5 mM Cu²⁺ in *arl1*-deleted yeast, which were then lysed with glass beads in phosphate-buffered saline containing 5 mM MgCl₂, 10 mM dithiothreitol and protease inhibitor cocktail (1 μ g/ml aprotinin, 1 μ g/ml leupeptin, 1 μ g/ml pepstatin, 1 μ M benzamide, and 1 mM phenylmethylsulfonyl fluoride). After centrifugation (500 \times g, 5 min), the cleared lysate (1 ml) was incubated with glutathione-Sepharose (40 μ l) at 4°C overnight, and then washed three times with binding buffer. Bound proteins were analyzed as described for *in vitro* binding assay.

GAP Assays

GAP activity was assessed essentially as described by Huber *et al.* (2001). GAP activity is assayed by a single-round hydrolysis of ARF-bound GTP by using [γ -³²P]GTP and measuring [³²P]Pi release after charcoal absorption of the nucleotides. Recombinant myristoylated Arl1p, Arl1p, or Arl3p protein was incubated in 2 mM EDTA, 1 mM MgCl₂, 50 mM NaCl, 25 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS), pH 7.4, and a 1:10 dilution of a dimyristoylphosphatidyl-choline (DMPC)/cholate mixture, with [γ -³²P]GTP for 15 min at 30°C, resulting in the association of ~50% of the labeled nucleotide with Arl1p, Arl3p, and Arf1p proteins. GAP activity was determined at 30°C in 100 μ l assay systems at different time points (up to 20 min) containing 5 mM MgCl₂, 25 mM MOPS, pH 7.4, and different concentrations (0.1–200 ng) of recombinant Gcs1p or Glo3p. Samples were taken at 0 and 5 min for the typical experiment. Radioactivity is measured by scintillation counter. The GAP assays also show hydrolysis of [γ -³²P]GTP in a time-dependent manner.

Indirect Immunofluorescence

Cells grown to a density of 1–2 \times 10⁷ cells/ml in 3 ml of minimal selective medium with 2% glucose or YPD medium were prepared for indirect immunofluorescence essentially as described previously (Lee *et al.*, 1997), except for replacement of the methanol (6-min incubation)-acetone (30-s incubation) step with a 0.2% SDS solution at –20°C before Arl1p staining (Hagan and Ayscough, 1999). Antibodies included affinity-purified anti-Arl1p, commercial monoclonal anti-myc (BD Biosciences Clontech, Palo Alto, CA) and anti-GFP (Sigma, St. Louis, MO) antibodies. Secondary antibodies Alexa FluorTM 488 goat anti-rabbit IgG and Alexa FluorTM 594 goat anti-mouse IgG (Molecular Probes, Eugene, OR) were used at dilutions of 1:1000 and 1:2000, respectively. Nuclei were stained with H33258 (2 μ g/ml) included in the mounting solution. Preparations were inspected with a Zeiss Axioskop microscope and photographed on Photometric coolSNAP fx, and then processed by Image-ProPlus software (Media Cybernetics, Silver Spring, MD).

Cells containing GFP- or mRFP-tag proteins were imaged live in synthetic medium. Except for GFP-Imh1p, which requires galactose for induction, all fluorescence protein-tagged chimera were cultured in glucose-containing medium. After overnight culture, mid-log phase cells were examined, and images were processed as described above. Quantification of those signals was performed using Axio Vision Rel. 4.2 software.

Table 2. Plasmids used in this study

Plasmid	Description	Source or reference
pIG4-5	2 μ m TRP1 P _{GAL} acid bolb B42	Gyuris <i>et al.</i> (1993)
pEG202	2 μ m HIS3 P _{ADH} LEX A DNA binding domain	Gyuris <i>et al.</i> (1993)
pET30a	f1 ori Kan P _{T7} His.Tag	Novagen (Madison, WI)
pGEX4T-1	f1 ori Amp P _{T7} GST.Tag	Amersham Biosciences
pYEX4T-1	2 μ m URA3 P _{CUP1} GST.Tag	BD Biosciences Clontech
pRS314	CEN6 TRP1	Sikorski and Hieter (1989)
pRS315	CEN6 LEU2	Sikorski and Hieter (1989)
pVT101U	2 μ m URA3 P _{ADH}	Verent <i>et al.</i> (1987)
YIplac128	LEU2	Gietz <i>et al.</i> (1988)
GFPST2	GFPST2 in pRS406	Hugh R. B. Pelham
EMP47MYC	EMP47MYC in YIplac128	Stephan Schroder-Kohne
GCS1pJG4-5	GCS1 in pJG4-5 2 μ m TRP1 P _{GAL}	This study
GCS1znpJG4-5	GCS1zn in pJG4-5 2 μ m TRP1 P _{GAL}	This study
GCS1dPHpJG4-5	GCS1dPH in pJG4-5 2 μ m TRP1 P _{GAL}	This study
GCS1NpJG4-5	GCS1N in pJG4-5 2 μ m TRP1 P _{GAL}	This study
GCS1pET30a	GCS1 in pET30a kan	Huang <i>et al.</i> , 2002
GLO3pJG4-5	GLO3 in pJG4-5 2 μ m TRP1 P _{GAL}	This study
ARL1d17NpEG202	ARL1d17N in pEG202 2 μ m HIS3 P _{ADH}	This study
ARL1Q/Ld17NpEG202	ARL1Q72Ld17N in pEG202 2 μ m HIS3 P _{ADH}	This study
ARL1T/Nd17NpEG202	ARL1T32Nd17N in pEG202 2 μ m LEU2 P _{ADH}	This study
ARF1Q/Ld17NpEG202	ARF1Q71Ld17N in pEG202 2 μ m HIS3 P _{ADH}	This study
ARF3Q/Ld17NpEG202	ARF3Q71Ld17N in pEG202 2 μ m HIS3 P _{ADH}	This study
ARL1Q/Ld17NpGEX4T	ARL1Q72Ld17N in pGEX4T-1 Amp	This study
ARF1Q/Ld17NpGEX4T	ARF1Q71Ld17N in pGEX4T-1 Amp	This study
ARF3Q/Ld17NpGEX4T	ARF3Q71Ld17N in pGEX4T-1 Amp	This study
ARL1d17NpYEX	ARL1d17N in pYEX4T-1 2 μ m URA3 P _{CUP1} GST.Tag	This study
ARL1Q/Ld17NpYEX	ARL1Q72Ld17N in pYEX4T-1 2 μ m URA3 P _{CUP1} GST.Tag	This study
ARL1T32Nd17NpYEX	ARL1T32Nd17N in pYEX4T-1 2 μ m URA3 P _{CUP1} GST.Tag	This study
ARL1mRFP	ARL1mRFP in pRS315 CEN6 LEU2 P _{ADH}	This study
ARF1mRFP	ARF1mRFP in pRS315 CEN6 LEU2 P _{ADH}	This study
GCS1GFP	GCS1GFP in pVT101U 2 μ m URA3 P _{ADH}	This study
GCS1MYC	GCS1MYC in pVT101U 2 μ m URA3 P _{ADH}	This study
ARL1YIplac128	P _{ADH} -ARL1 in yYIplac128 LEU2	This study
ARL1Q/LYIplac128	P _{ADH} -ARL1Q72L in YIplac128 LEU2	This study
ARL1T/NYIplac128	P _{ADH} -ARL1T32N in YIplac128 LEU2	This study
GFPIMH1pRS314	P _{GAL} -GFPIMH1 in pRS314 CEN6 TRP1	This study

Preparation of Yeast Cell Extracts and Immunoblotting

Whole yeast extracts were prepared by agitating (vortex mixer) yeast cells suspended in TE buffer (10 mM Tris, pH 7.4, 1 mM EDTA) with glass beads for 1 min followed by incubation on ice for 1 min, repeated five times. After brief centrifugation (3300 \times g; 5 min) to clarify the lysate, protein was quantified by Coomassie Blue assay (Pierce Chemical, Rockford, IL). Proteins separated by SDS-PAGE were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA), which were incubated (60 min; room temperature) with antibodies in Tris-buffered saline, pH 7.4, containing 0.1% Tween 20 and 5% dried skim milk. Bound antibodies were detected with the ECL system (Amersham Biosciences, Piscataway, NJ). Anti-LexA antibody was used in 1:5000 (Invitrogen, Carlsbad, CA), and anti-hemagglutinin (HA) was used in 1:5000 (Roche Diagnostics, Indianapolis, IN); other antibodies used in this study were generated from our laboratory.

RESULTS

Arl1p Interacts with *Gcs1p* in a Nucleotide-dependent Manner

The yeast genome contains six genes that encode polypeptides with similarity to ARF GAPs. Four of these (*Gcs1p*, *Glo3p*, *Age2p*, and *Sat1p/Age1p*) are known to be Arf1p GAPs (Antonny *et al.*, 1997; Poon *et al.*, 1999, 2001; Zhang *et al.*, 2003). Among the others, *Sps18p* is expressed only in sporulating cells, and *Gts1p* is likely a transcription factor (Coe *et al.*, 1994; Mitsui *et al.*, 1994).

Our initial attempt to identify an Arl1p GAP using full-length, wild-type Arl1 or Arl1Q72L, a putative active form of Arl1p, in a yeast two-hybrid system was unsuccessful

(our unpublished data). It was known that deletion of the first 17 N-terminal hydrophobic amino acids of the active GTP-form of Arf1 protein could enhance its interaction with GAP proteins in a two-hybrid system (Eugster *et al.*, 2000; Figure 1A). Therefore, we tested whether Arl1Q72Ld17N could interact with any of the six ARF GAP-related proteins. We found that Arl1Q72Ld17N interacted with *Gcs1p* but not with *Glo3p* or other ARF GAP-related proteins (Figure 1A; our unpublished data). The putative GDP-form Arl1T32Nd17N and wild-type Arl1d17N did not interact with *Gcs1p* (Figure 1A). Thus, Arl1p associated with *Gcs1p* in a nucleotide-dependent manner. Our data also showed that *Gcs1p* could interact with Arf1Q71Ld17N but not with Arf3Q71Ld17N and Arl3Q78Ld17N (Figure 1A and Supplemental Figure S2), suggesting that *Gcs1p* could be a GAP for both Arl1p and Arf1p but not Arf3p or Arl3p. Consistent with previous report (Eugster *et al.*, 2000), *Gcs1p* seemed to not interact with Arf1d17N in yeast two-hybrid analysis (Supplemental Figure S2).

Gcs1p binds to phospholipids vesicles and contains a putative zinc-binding motif and ERM-homology domains (Antonny *et al.*, 1997; Blader *et al.*, 1999). To identify the Arl1p-interacting domain of *Gcs1p*, we generated yeast two-hybrid constructs expressing mutated and truncated *Gcs1p*: *Gcs1-zn* with two cysteine residues in the zinc-finger motif replaced by alanine, *Gcs1dC* lacking 125 amino acids at the

Table 3. Primers used in this study

Primer	Sequence (5'-3')
GCS1.1	GAATTCATGTCAGATTGGAAAGTGGACC
GCS1.2	TGAAC TTCCTTGAATGTTGAGAAAA
GCS1.3	CCCTCTATGGATAACCGGCAGCTTCAAGGGCAAT
GCS1.4	ATTGCCCTTGAAGCTGCCGGTATCCATAGAGGG
GCS1.dPH	TTATGCAGACCGTCTTGTGGAGG
GCS1.N	AAACTGATCCATAGTGATAGATCTTA
GCS1.myc	TTACAAGTCTTCTCAGAAATCAGCTTTTGTTC
yARL1.1	GAATTCATGGGTAACATTTTTAGTTCAATG
yARL1.2	ATTCGGATCCATTTAAAAAAGTATGCATCTAC
yL1 T/N.1	GGTGCAGTAAAAATACCATCTTATATCG
yL1 T/N.2	TAAGATGGTATTTTTACCTGCACCATC
yL1 Q/L.1	GGTGCAGTAAAAATACCATCTTATATCG
yL1 Q/L.2	TAAGATGGTATTTTTACCTGCACCATC
yARL1d17N	GAATTCATGGAATTGCGTATATTGATTTTGGG
IMH1.1	GGATCCATGTTCAAACAGCTGTCACAAATTG
IMH1.2	CGTATCTTCTGCTTCAGCTAC

C terminus, and Gcs1N lacking both ERM domains and the C terminus (Figure 1B). These constructs were expressed in roughly equal amounts, but Gcs1-zn and Gcs1dC exhibited twofold higher expression (Figure 1C). We detected interactions of Arl1Q72Ld17N with wild-type Gcs1 and Gcs1dC but not Gcs1-zn or Gcs1N (Figure 1B). Thus, structural elements within the zinc-finger and ERM domains and/or intervening sequence may be essential for Gcs1p-Arl1p interaction. The interaction between Arl1Q72Ld17N and Gcs1dC seemed to be much less than that of wild-type Gcs1, indicating a possible role for the C terminus of Gcs1p for this interaction. Arf1Q71Ld17N interacted with wild-type Gcs1, Gcs1dC, and Gcs1-zn, but not Gcs1N (Figure 1B, bottom), consonant with interactions of Arl1Q72Ld17N and Arf1Q71Ld17N with different regions of Gcs1p.

To confirm the direct interactions between Arl1p and Gcs1p, His-tagged Gcs1p was synthesized, purified, and incubated with purified GST, GST-Arl1Q72Ld17N, GST-Arf1Q71Ld17N, or GST-Arf3Q71Ld17N proteins immobilized on glutathione-Sepharose (Figure 2A). Retained His-Gcs1p was detected by immunoblotting (Figure 2A, top). Similar amounts of GST, GST-Arl1Q72Ld17N, GST-Arf1Q71Ld17N, or GST-Arf3Q71Ld17N were present in each reaction, as shown by Coomassie staining (Figure 2A, bottom). GST-Arl1Q72Ld17N and GST-Arf1Q71Ld17N, but not GST or GST-Arf3Q71Ld17N, adsorbed significant amounts of His-Gcs1p. To examine their interaction *in vivo*, we expressed GST-Arl1d17N, GST-Arl1Q72Ld17N, or GST-Arl1T32Nd17N in *arl1* mutant yeast. GST-Arl1 proteins were pulled down on glutathione Sepharose, and the presence of bound endogenous Gcs1p was assessed by immunoblotting (Figure 2B). Arl1Q72Ld17N and to a lesser extent Arl1d17N, but not Arl1T32Nd17N, had bound endogenous Gcs1p, suggesting that Arl1p directly interacted with Gcs1p in a GTP-dependent manner.

Gcs1p Possesses GAP Activity for Arl1p and Arf1p *In Vitro*

To test whether Gcs1p has GAP activity for Arl1p, different amounts of purified recombinant Gcs1p and Glo3p were assayed *in vitro* for their ability to stimulate the hydrolysis of GTP bound to recombinant-Arl1p, Arf1p, or Arl3p. Figure 3 shows that Gcs1p exhibited GAP activity for both Arl1p and Arf1p. The GAP activity of Glo3 is considerably greater

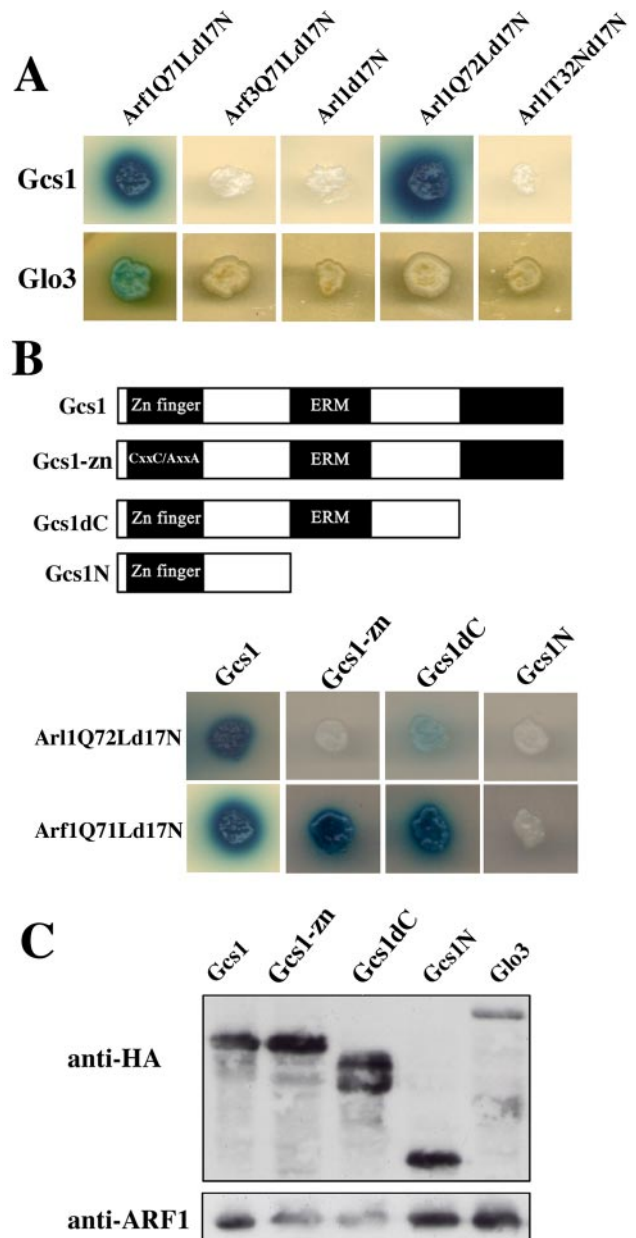


Figure 1. Interaction of Arl1Q72Ld17N with Gcs1p in a two-hybrid assay. (A) Arl1Q72Ld17N specifically interacts with Gcs1p but not Glo3p. The yeast reporter strain YEM1 α cotransformed with the indicated ARFpEG202 or pEG202-ARL constructs and Gcs1pJG4-5 or Glo3pJG4-5 were plated on synthetic medium lacking histidine and tryptophan (His-Trp- plate). Colonies from His-Trp- plates were assayed for β -galactosidase activity by spotting on His-Trp- plates containing X-gal to test for specificity. (B) Arl1Q72Ld17N and Arf1Q71Ld17N specifically interacts with different domains of Gcs1p. Top, diagram of Gcs1p and deletion constructs. Bottom, yeast reporter strain YEM1 α cotransformed with Arf1Q71Ld17NpEG202, or Arl1Q72Ld17NpEG202 and the indicated Gcs1pJG4-5 construct was plated and colonies were assayed for β -galactosidase activity. (C) Expression of HA-tagged fusion proteins. Proteins ($\sim 20 \mu\text{g}$) from lysates of the cells expressing Gcs1pJG4-5 or Glo3pJG4-5 constructs, as indicated, were separated by SDS-PAGE in a 10% gel, transferred to PVDF membranes, and reacted with anti-HA antibodies, or anti-Arf1p antibodies as indicated, followed by detection using the ECL system. Because the expression of HA-tagged proteins was not the same, different amounts of lysates were loaded for Western blotting. Arf1p was used as loading control.

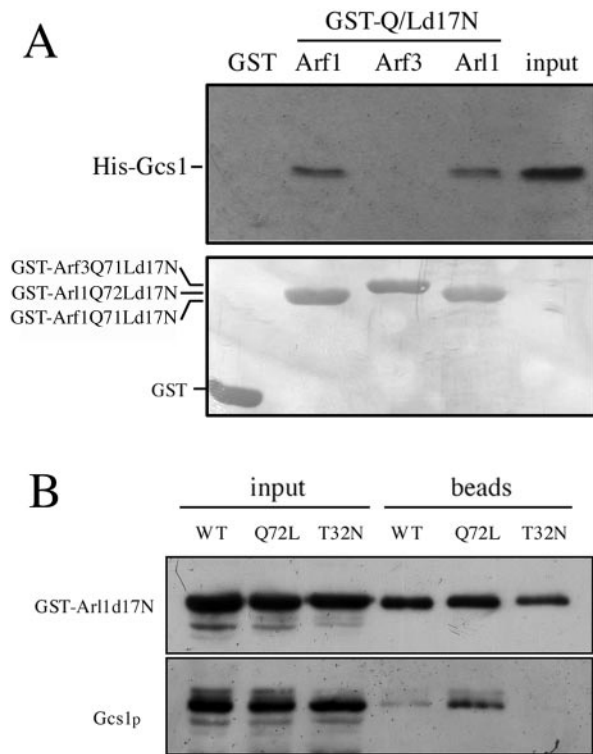


Figure 2. Arl1Q72Ld17N interacts with Gcs1p in vitro and in vivo. (A) In vitro interaction of His-Gcs1p with GST-Arl1Q72Ld17N, GST-Arf1Q71Ld17N, or GST-Arf3Q71Ld17N constructs. Purified recombinant His-Gcs1p (2 μ g) was mixed with 2 μ g of purified recombinant GST, GST-Arl1Q72Ld17N, GST-Arf1Q71Ld17N, or GST-Arf3Q71Ld17N immobilized on glutathione-Sepharose beads and incubated at 4°C for 1 h. After three times wash, bound proteins were eluted by boiling in 20 μ l of 2 \times sample buffer, and separated by SDS-PAGE in a 12% gel. Input lane contained 1% of the amount added to beads. Proteins were stained with Coomassie Blue (bottom) to assess equal loading, and bound proteins were visualized by Western blotting with anti-Gcs1p antibody (top). (B) In vivo interaction of Arl1Q72Ld17N with endogenous Gcs1p in a GTP-dependent manner. Expression of GST-fused Arl1d17N (WT), Arl1Q72Ld17N (Q72L), or Arl1T32Nd17N (T32N) from pYEX-4T-1 was induced, and yeast were lysed with glass beads. After centrifugation, the cleared lysates were incubated with glutathione-Sepharose beads at 4°C overnight and then washed three times with binding buffer before analysis of bound proteins as described in A. Antibodies used are indicated to the left of the panel.

with Arf1p than Arl1p. GAP activity of Arl3p was little affected by Gcs1p or Glo3p. In addition, Gcs1p-zn lost GAP activity for both Arl1p and Arf1p (our unpublished data). These data suggest that Gcs1p could be a GAP for Arl1p in vitro.

Arl1p Localizes to Trans-Golgi Compartment

Our previous report suggested that Arl1p is localized to the Golgi (Lee *et al.*, 1997); however, the precise location of Arl1p has yet to be determined. Initially, three Golgi resident proteins, Emp47p (*cis*-Golgi), Sec7p (late Golgi), and Sft2p (late Golgi), were used to determine the localization of endogenous Arl1p by immunofluorescence microscopy. Constructs encoding GFP-tagged Sec7p and Sft2p, and myc-tagged Emp47p were integrated into wild-type yeast genomic DNA. The coding sequence of GFP was inserted in the chromosome immediately upstream of the translational

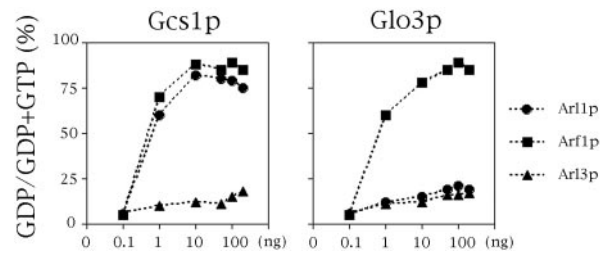


Figure 3. GAP activity of Gcs1p for Arl1p in vitro. GAP activity of the different concentrations of purified recombinant Gcs1p or Glo3p (as indicated at bottom) was assayed using Arl1p (square), Arf1p (circle), or Arl3p (triangle) with [γ - 32 P]GTP bound as substrate as described in *Materials and Methods*.

stop codon for SEC7. Like Emp47p, Sec7p, and Sft2p, Arl1p occurred in typical Golgi-like punctate structures (Figure 4A). It was partially colocalized with Sft2p, but not with Emp47p or Sec7p (Figure 4A, arrows), suggesting that Arl1p is concentrated in a subregion of *trans*-Golgi, which differs from that occupied by Emp47p or Sec7p. However, our result is inconsistent with a previous report, which suggested that Arl1p was colocalized with Sec7-GFP (Setty *et al.*, 2003). To clarify this difference, we generated Arl1-mRFP to examine localization of Arl1p in live cells. As shown in Figure 4B, Arl1-mRFP colocalized with GFP-Sft2p, but less with Sec7-GFP. Moreover, Arl1-mRFP distributes from cytosol to Golgi, similar to the observation from indirect immunofluorescence staining. Our data suggest that Arl1p is localized to a subdomain of *trans*-Golgi compartments containing Sft2p.

Localization of Arl1p on Trans-Golgi Is Nucleotide Dependent

To determine whether localization of Arl1p was guanine nucleotide dependent, we constructed nontagged wild-type Arl1p, GTP binding-defective Arl1T32N, and GTP hydrolysis-deficient Arl1Q72L mutants under the control of the *ADH* promoter in Yiplac128 (Giets and Sugino, 1988). After linearization with *EcoRV*, these plasmids were integrated into the *leu2* locus of the *arl1*-deleted yeast genome. The expression levels of these integrated wild-type and mutated Arl1p were approximately three- to fivefold higher than the level of endogenous Arl1p (Supplemental Figure S1B). The integrated wild-type Arl1p could rescue the *arl1* deleted phenotype, suggesting that it possesses at least some of the properties of endogenous Arl1p (our unpublished data). Figure 5 shows that Arl1Q72L and some Arl1p wild-type were colocalized with the late Golgi marker GFP-Sft2p, whereas Arl1T32N staining was, for the most part, distributed through the cytoplasm. In addition, expression of Arl1Q72L seemed to cause the enlargement of *trans*-Golgi structures. Although the enlarged Golgi is adjacent to the vacuole, the morphology of the vacuole seems normal (Supplemental Figure S3). Our results indicate that the distributions of the GTP-bound and GDP-bound forms of Arl1p were distinct from each other and resembled, in part, those of the corresponding ARL1 in mammalian cells (Lu *et al.*, 2001).

Gcs1p Affects Localization of Arl1p

It is thought GTP-bound active ARF associates with membrane and disassociates when GTP is hydrolyzed to GDP. Thus, the localization of ARF may reflect the identity of the bound guanine nucleotide. If Gcs1p has GAP activity for

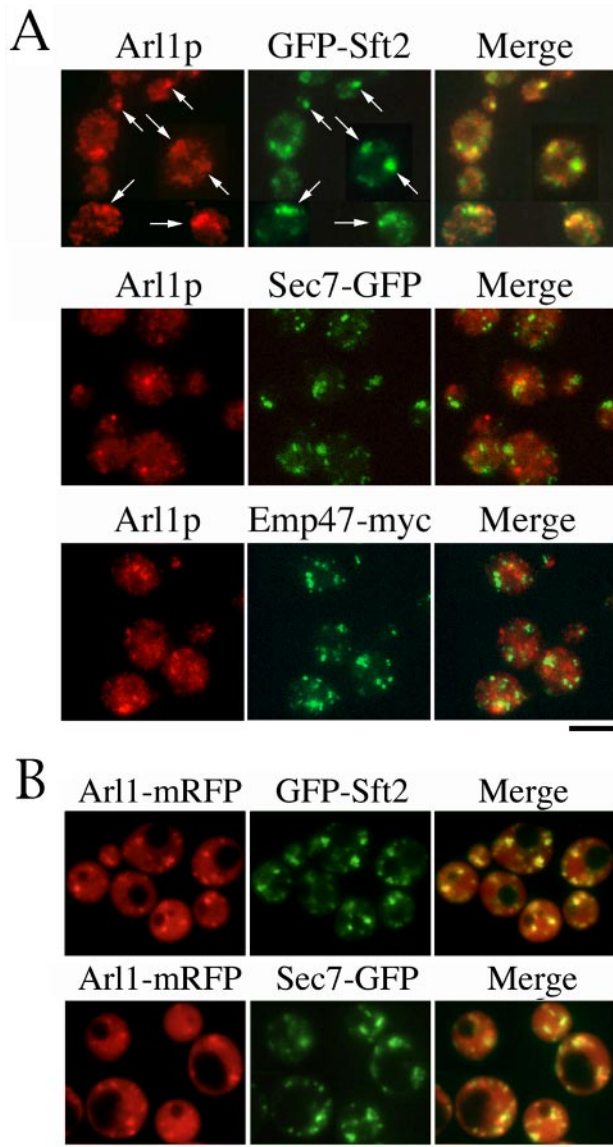


Figure 4. Arl1p partially colocalizes with GFP-Sft2p. (A) Endogenous Arl1p partially colocalizes with Sft2p. Myc-tagged Emp47p (*cis*-Golgi), or GFP-tagged Sec7p (late Golgi), or Sft2p (late Golgi) was used to identify the location of Arl1p by immunofluorescence microscopy, as described in *Materials and Methods*. Cells were fixed with formaldehyde; spheroplasts were prepared and reacted with purified anti-Arl1p, anti-GFP, or anti-myc antibodies, followed by appropriate secondary antibodies. Arrows indicate colocalization of Arl1p and Sft2p. Observations were replicated in three experiments. (B) Arl1-mRFP on centromeric vector, pRS315, under ADH promoter was transformed into *arl1*-deleted yeast containing Sec7-GFP or GFP-Sft2. Mid-log phase cells were imaged live with microscopy.

Arl1p in cells, the amount of Gcs1p in a cell might affect the GTP-binding equilibrium of Arl1p. To investigate whether absence of Gcs1p in a cell affects the intracellular localization of Arl1p, endogenous Arl1p in wild-type, *gcs1*-deleted, or *glo3*-deleted yeast was examined (Figure 6). In the *gcs1*-null mutant, Arl1p was colocalized with GFP-Sft2p to a much greater extent than it was in cells containing Gcs1p, 45 and 13%, respectively. This effect on Arl1p localization was not observed in the *glo3* mutant. This phenomenon was also observed when we examined Arl1-mRFP in *arl1*- or *arl1gcs1*-

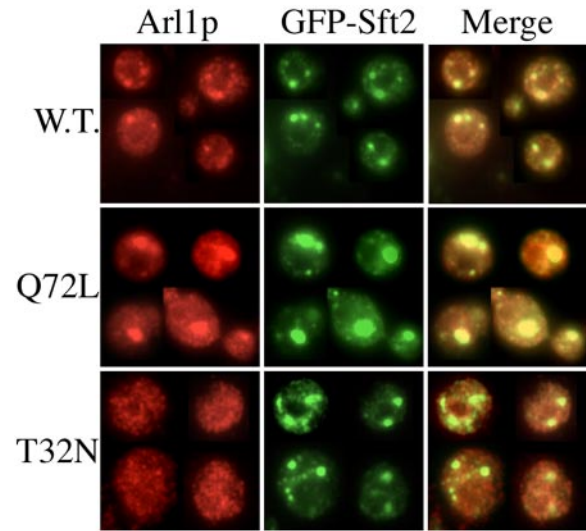


Figure 5. Localization of Arl1p on *trans*-Golgi is nucleotide dependent. Wild-type Arl1, Arl1Q72L, or Arl1T32N from integrated plasmid YIplac128 under the control of the *ADH* promoter was transformed into *arl1* (YPH250dl1) cells expressing GFP-Sft2p from pRS314 vector. Logarithmically growing cells were fixed and spheroplasts were prepared for reaction with affinity-purified anti-Arl1p antibody and monoclonal anti-GFP antibody. Indirect immunofluorescence staining was done as described in *Materials and Methods*. Observations were replicated in at least three experiments.

deleted yeasts (Supplemental Figure S4). Our findings suggest that failure to hydrolyze GTP on Arl1p in the *gcs1* mutant could result in accumulation of Arl1p-GTP on *trans*-Golgi membranes, thus inhibiting vesicular trafficking.

Next, we determined whether overexpression of Gcs1p altered the intracellular localization of Arl1p. We used indirect immunofluorescence staining to observe the effect of Gcs1-myc overexpression on endogenous Arl1p. Figure 7 shows that, in *gcs1* cells overexpressing Gcs1p-myc, Arl1p, but not Arf1p, was no longer in a punctate pattern, whereas localization displayed a cytoplasmic pattern. In contrast, localization of GFP-Sft2p seemed normal, suggesting that the Golgi structure is intact. Thus, this effect of Gcs1p seemed to be specific for Arl1p. Similar results also were observed when plasmids encoding C-terminal GFP-tagged Gcs1p and Arl1-mRFP or Arf1-mRFP were cotransformed into *gcs1* mutants (Supplemental Figure S5A). We also used Gcs1-zn to demonstrate that GAP activity is important for Gcs1p in regulation of Arl1p at *trans*-Golgi compartments. The overexpression of Gcs1-zn-GFP seemed normal like Gcs1p-GFP, but it showed no effect on Arl1p localization (Supplemental Figure S5A). Overexpression of Gcs1-zn-myc protein also was attempted; however, it seemed lumpy (possibly due to aggregation of the recombinant protein) and could not be used in this experiment (our unpublished data). In addition, Arl1p was not dissociated from its punctate patterns in *glo3* cells overexpressing Glo3p-myc (Supplemental Figure S5B). Age2p, a GAP protein, which provides overlapping function with Gcs1p (Poon *et al.*, 2001), also had no effect on Arl1p distribution (Supplemental Figure S5B). Our findings suggest that the effect of Gcs1p on intracellular localization of Arl1p might be anticipated from its acceleration of GTP hydrolysis on Arl1p.

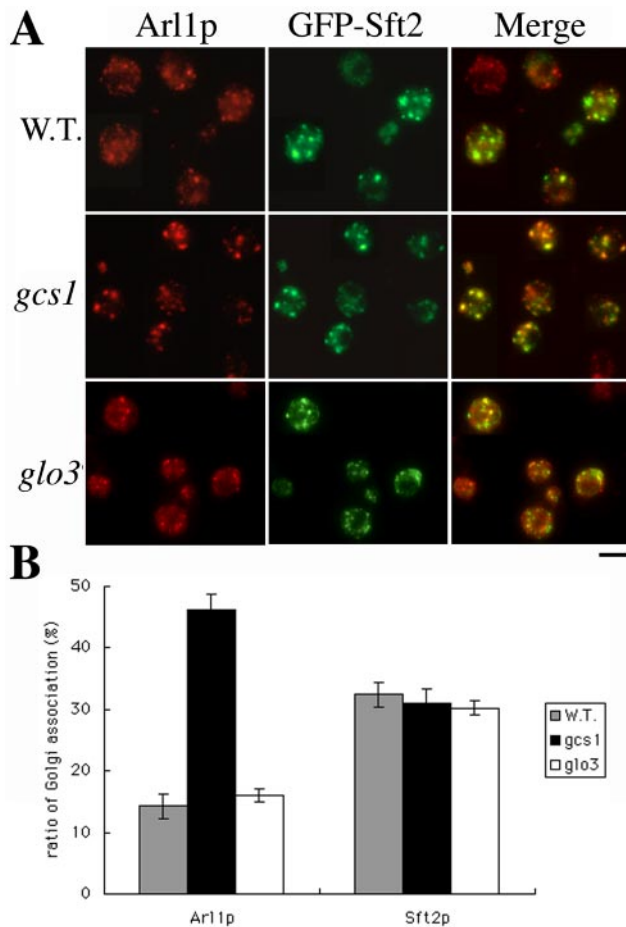


Figure 6. More Arl1p is present in *trans*-Golgi of *gcs1* mutant than wild-type yeast. (A) Wild-type, *gcs1*-, or *glo3*-deleted yeast were stained with anti-GFP and affinity-purified anti-Arl1p antibodies to monitor the Golgi association of Arl1p. GFP-Sft2p was the late-Golgi marker. (B) Quantification of Golgi association ratio of Arl1p. Signals in A were quantified by Axio Vision Rel. 4.2 software. Punctate signals were summed and divided with whole cell signals (n = 50 and $p < 0.05$).

Gcs1p and Arl1p Interact Functionally

Previous report showed that GFP-Imh1, an effector of Arl1p, lost its Golgi localization signals in *arl1* null cells (Panic *et al.*, 2003; Setty *et al.*, 2003). We next examined whether the localization of Imh1p also was affected in the *gcs1* mutant. We constructed a *GFP-IMH1* that was driven by a *GAL1* promoter in a centromeric plasmid. The expression level of GFP-Imh1p was similar to that of endogenous Imh1p (Supplemental Figure 1C). Figure 8 shows that GFP-Imh1p displays a Golgi-like distribution in wild-type cells and a diffuse pattern in *arl1*-deleted yeast. Unexpectedly, substantial amounts of GFP-Imh1p became diffuse in *gcs1*-null mutants (Figure 8). The diffuse GFP-Imh1p in the *gcs1*-null mutant is specific because GFP-Imh1p was concentrated at punctate Golgi-structures in *arf1*- or *glo3*-null mutants.

Because more Arl1p proteins were retained in Golgi structures in *gcs1*-null cells, we speculated that GTP-hydrolysis-defective Arl1p in *gcs1*-null cells might cause diffusion of the GFP-Imh1p from Golgi. Therefore, we examined the localization of GFP-Imh1p in *arl1*-deleted cells containing integrated wild-type, constitutively active Arl1 (Arl1Q72L) or inactive Arl1 (Arl1T32N) (Figure 9). Interestingly, wild-type

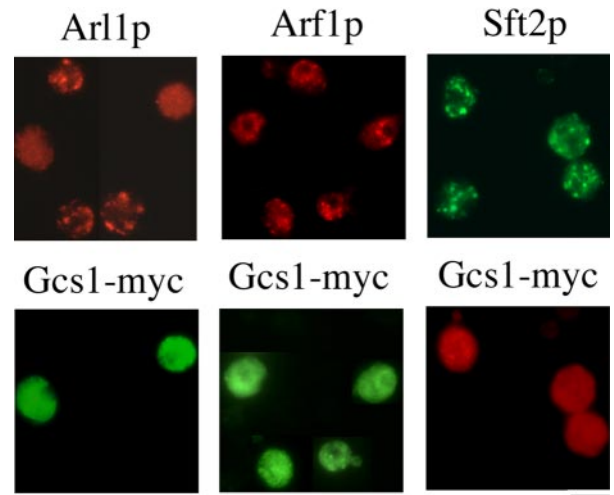


Figure 7. Overexpression of Gcs1-myc, but not Gcs1-zn-myc, caused dissociation of endogenous Arl1p from *trans*-Golgi. Gcs1-myc overexpression affected the distribution of Arl1p. Gcs1-mycpVT101U was transformed into *gcs1* cells with or without GFP-Sft2pRS314. Mid-log phase cells overexpressing Gcs1-myc were stained with anti-myc, anti-GFP, affinity-purified anti-Arl1p, or affinity-purified anti-Arf1p antibody.

Arl1p, but not Arl1Q72L or Arl1T32N, could rescue the Golgi-association of GFP-Imh1p. These data suggest that proper GTP-GDP recycling of Arl1p is required for its function on effector recruitment.

DISCUSSION

Yeast cells contain six structurally related proteins with the potential to provide GAP activity for the yeast Arf family proteins and to participate in vesicular transport (Poon *et al.*, 1996, 1999; Zhang *et al.*, 2003). Three of these proteins, Gcs1p, Glo3p, and Age2p have been shown to be Arf GAPs capable of stimulating the GTPase activity of yeast Arf1p (Poon *et al.*, 1996, 1999, 2001). In this study, we demonstrate

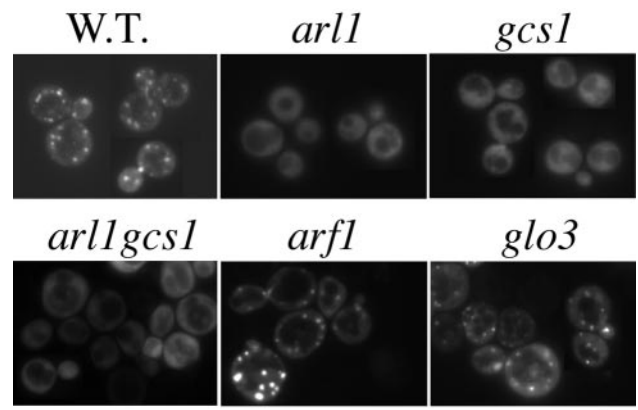


Figure 8. GFP-Imh1p substantially loses its Golgi association in *arl1*- and *gcs1*-null cells. GFP-IMH1 under the control of the *Gal1* promoter (centromeric plasmid) was transformed into different deletion yeast as indicated. After overnight induction (~12 h) with 2% galactose and 0.1% glucose, GFP-Imh1p was observed in live cells and photographed.

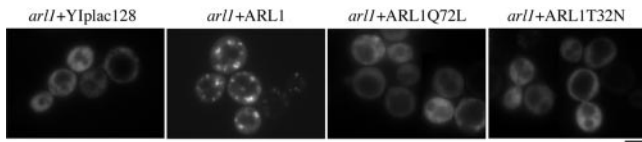


Figure 9. Constitutive active and inactive mutant forms of Arl1p did not complement the loss of Arl1p. GFP-IMH1pRS314 was transformed into *arl1*-deleted cells containing integrated wild-type, constitutively active (Q72L), constitutively inactive (T32N), Arl1p, or empty vector control (YIplac128). After induction with 2% galactose and 0.1% glucose medium, live cells were observed with microscopy.

that Gcs1p is also a GAP for Arl1p. Several types of evidence indicate that in yeast Gcs1p may play a crucial role in regulating Arl1p at *trans*-Golgi compartments.

The extensive sequence identity of Arl1p and Arf1p led us to hypothesize that they would interact similarly with the Gcs1p molecule. Surprisingly, our data showed that although all three Gcs1p domains seemed to be important for Arl1p interaction (Figure 1B), the zinc-binding domain and C terminus were not essential for Arf1Q71Ld17N interaction (Figure 1B). The zinc-finger mutation eliminates Arl1p binding; therefore, the general structure for interaction must contain these two cysteine residues. Other elements may contribute to establish a stronger interaction between the two proteins. Alternatively, the deletions or truncations may alter the structure of the zinc-finger domain sufficiently to disrupt interaction. In the crystal structure of the Arf1-ARF-GAP1 complex determined by Goldberg (1999), the zinc-finger residues do not make direct contacts with Arf1, consistent with the interaction we observed between Arf1Q71Ld17N and Gcs1-zn. Moreover, a recent report (Lee *et al.*, 2005) also has shown that ARFGAP1R50K, a mutant ARFGAP1 without GAP activity, could interact with ARF1 *in vitro*.

From many studies, it has been suggested that for ARF-GAP1 to exert its GAP activity on Arf1 participation of coatamer, GGA and membrane curvature are necessary (Goldberg, 1999; Szafer *et al.*, 2000; Jacques *et al.*, 2002; Bigay *et al.*, 2003). Our data showed that Gcs1p has GAP activity for both Arl1p and Arf1p. Ding *et al.* (1996) also reported that purified spleen ARF GAP accelerated hydrolysis of GTP bound to recombinant mammalian ARL1. From the crystal structure study, it is thought that the N-terminal helix and myristic acid of Arf1p should leave the core of the protein and insert into the membrane when it is activated (GTP-bound). Therefore, it is reasonable that the interaction between active Arf1p or Arl1p and Gcs1p should occur on the membrane structure. Because the interaction of these proteins in the yeast two-hybrid experiments does not occur on a membrane structure, an N-terminal deletion seems to be required. In contrast, the *in vitro* GAP assay is performed in reaction buffer containing micelles (DMPC/cholate); thus, Gcs1p could exhibit activity with both the full-length Arf1p and Arl1p. Bigay *et al.* (2003) reported that the GAP activity of ARFGAP1, the mammalian homologue of Gcs1p, is greatly increased by highly curved liposomes and that Gcs1p shares with ARFGAP1 a preference for binding to conical lipids. Their data showed that ARFGAP1 stimulates the GTPase reaction of ARF1, and the rate constant of GTP hydrolysis on small liposome was in the subsecond range ($k_{cat} > 1 \text{ s}^{-1}$). Randazzo (1997) also showed that the GAP activity of rat liver GAP2 and GAP1 is phospholipids dependent; and, when using ARF1 as a substrate, GAP activity

was 7.2 and 1.92 s^{-1} for GAP2 and GAP1, respectively. Our study showed that the average GAP activity of Gcs1p was 0.18 and 0.22 s^{-1} for Arl1p and Arf1p, respectively, suggesting that membrane curvature or different phospholipids may be required to increase GAP activity. Additional studies of Arl1p and Gcs1p with their interacting adaptor protein(s) or phospholipids are needed to characterize the activity Gcs1p for Arl1p and Arf1p. Thus, we suspect that limitation of those factors may reduce the efficiency of Gcs1p GAP activity for GTP hydrolysis by Arf1p relative to that by Arl1p.

We reported previously in 1997 that Arl1p is associated in part with the Golgi complex (Lee *et al.*, 1997). Moreover, a recent study (Setty *et al.*, 2003) showed that an Arl1p-GFP fusion protein colocalized with RFP-Imh1p-GRIP, and RFP-Imh1p-GRIP colocalized with Sec7p-GFP. Based on these observations, it was suggested that Arl1p colocalized with Sec7p. Here, we demonstrate that Arl1p partially colocalizes with the *trans*-Golgi integral membrane protein Sft2p, but not with Sec7p. The differences between these two experiments perhaps result from the fact that the overexpression of RFP-Imh1p-GRIP may affect the subcellular distribution of the *trans*-Golgi network. In Setty's report (Setty *et al.*, 2003) and our unpublished observation, the Golgi punctate structures in cells expressing RFP-Imh1p-GRIP or GFP-Imh1p were larger than those in normal cells. Moreover, overexpression of RFP-GRIP results in the recruitment of Arl1-GFP to Golgi (Setty *et al.*, 2003), which differs from distribution of wild-type Arl1p (Huh *et al.*, 2003). Thus, our data provide evidence that Arl1p localizes at one subdomain of the *trans*-Golgi compartment containing Sft2p.

From rescue experiments involving fragmented vacuole and localization of GFP-Imh1p observed with *arl1*-null mutant, we found that C-terminal-tagged Arl1p, with either GFP or mRFP, would lose part of its function, although its localization seems normal (our unpublished observation). This phenomenon also was observed by Trautwein *et al.* (2004) who reported that cyan fluorescent protein- or yellow fluorescent protein (YFP)-tagged Arf1 was partly defective in its function. Thus, the use of a GFP- or mRFP-tag may be a convenient approach to track Arf1p and Arl1p location, but it is not an appropriate way to identify their cellular function.

We also found that the Golgi association of Arl1p is nucleotide dependent. The GTP-bound form, Arl1Q72L, seems to colocalize with Sft2p and is associated with the aggregation of the *trans*-Golgi apparatus. This phenomenon is reminiscent of the Golgi expansion caused by overexpression of active human Arl1p (Lu *et al.*, 2001). These results suggest that the GTPase cycle of Arl1p determines its distribution between Golgi and cytosol in a manner that is fundamentally similar to that seen for Arf1p. Moreover, Arl1Q72L seemed not to alter vacuole morphology and vacuole enzyme trafficking was not substantially affected in *arl1*- and *gcs1*-deleted cells (our unpublished observation), suggesting that Arl1p and Gcs1p are not directly involved in Golgi-to-vacuole transport.

The GTPase cycle of ARF proteins is regulated by their GAPs, which, in turn, leads to the dissociation of ARFs from membranes. When we examined the localization of Arl1p in cells containing different amount of Gcs1p, we found that eliminating Gcs1p from cells prevented Arl1p release from the *trans*-Golgi to the cytosol. We also found that increasing amounts of Gcs1p-myc facilitated the release of Golgi-bound Arl1p to the cytosol. However, no phenotypic differences were observed in cells overexpressing the zinc-finger mutant, Gcs1-zn-GFP. The latter observations support the idea

that the pattern of Arl1p distribution upon elimination (or overexpression) of Gcs1p resulted from decreased (or increased) GAP catalytic activities. These data also fit the general model of ARF proteins, namely, that the GTP-bound active ARF localizes to membranes and subsequently disassociate from membranes when the GTP is hydrolyzed to GDP. Again, although Gcs1p possesses GAP activity for both Arl1p and Arf1p *in vitro*, distribution of Arf1p was not affected by the level of Gcs1p. These results suggest that Gcs1p GAP activity toward Arf1p in cells needs other cofactors, or perhaps equally, Arl1p and Arf1p interact with membranes in different ways.

Our data showed that GFP-Imh1p became diffuse in *gcs1*-null mutants (Figure 8), suggesting that GTP-hydrolysis defective Arl1p in *gcs1*-null cells might cause diffusion of the GFP-Imh1p from Golgi. Although the current model for recruiting Imh1p to Golgi is in favor of having an active form of Arl1p in Golgi (Panic *et al.*, 2003), our data showed that constitutively active Arl1p not only could not complement Arl1p function but also seemed to cause the disassociation of GFP-Imh1p from Golgi. These data suggest that a proper GTP-GDP recycling of Arl1p as well as other factors in Golgi membrane may be required for Imh1p recruitment.

Gcs1p has been implicated in mitochondrial function, vesicular trafficking, and actin cytoskeletal organization (Filipak *et al.*, 1992; Wang *et al.*, 1996; Poon *et al.*, 1996; Blader *et al.*, 1999). Overexpressed Gcs1p was concentrated in patches in yeast and colocalized partially with actin structures, suggesting that its overexpression affects mitochondrial morphology as a consequence of perturbing actin structures (Huang *et al.*, 2002). The effect of Gcs1p overexpression on mitochondrial structure does not require Arl1p or Arf1p, because the effect on mitochondria can be observed in the *arl1* or *arf1* mutants (our unpublished data). The zinc-finger motif of Gcs1p is required for its functional interaction with Arl1p but not for its effects on mitochondria (Huang *et al.*, 2002).

Consistent with our findings, the studies of Eugster *et al.* (2000) indicate Glo3p to be the major Arf1 GAP *in vivo*. They reported that Arf1Q71Ld17N could interact with Glo3p, Gcs1p, and COPI subunits and only Glo3p, but not Gcs1p, could interact with COPI subunits. In addition, *gcs1* is synthetic lethal with *arf1* or *glo3* (Zhang *et al.*, 2003) but not with *arl1* (this work). Analysis of knockout strains of all six GLO3-related proteins in yeast revealed that the *glo3* mutant alone results in a defect in KKXX retrieval from the Golgi (Dogic *et al.*, 1999). The effectiveness of vesicular transport in *glo3* mutant cells is not limited by the amount of Gcs1 protein. The *glo3* mutant, but not the *gcs1* mutant, showed strong synthetic lethality with *sec21-1*, a mutant of γ -COP (Poon *et al.*, 1999). These observations are consistent with the notion that the Gcs1p may normally supply only a minor portion of the ARF GAP activity for transport from the Golgi to the ER in the cells.

The Gcs1p and Age2p ARF GAPs provide overlapping functions for transport from the *trans*-Golgi network (Poon *et al.*, 2001). Consistent with our results, it seems that Gcs1p may provide the major GAP activity for Arl1p in *trans*-Golgi vesicular trafficking. Thus, distinct but partially overlapping functions of the Gcs1p, Glo3p, and Age2p suggest that each of these GAPs may serve in several vesicular transport pathways and interact with distinct sets of proteins characteristic of the different loci.

Identification of the Gcs1p interaction with Arl1p, as well as with Arf1p, led us to infer that Gcs1p is a multifunctional molecule, which may act at different intracellular membranes to regulate intracellular membrane dynamics and/or

actin cytoskeletal organization. Consistent with the previous model for ARF function, our findings also suggest that Arl1p-GTP is tethered in a relatively immobile complex on the surface of the Golgi membrane and does not come off until after the hydrolysis of GTP has occurred (Rothman, 1994). Integration of our unexpected findings into an understanding of the role of Gcs1p and Arl1p in the overall intracellular membrane trafficking remains a challenge for future studies.

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