The Arf1p GTPase-activating protein Glo3p executes its regulatory function through a conserved repeat motif at its C-terminus

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Summary
ADP-ribosylation factors (Arfs), key regulators of intracellular membrane traffic, are known to exert multiple roles in vesicular transport. We previously isolated eight temperature-sensitive (ts) mutants of the yeast ARF1 gene, which showed allele-specific defects in protein transport, and classified them into three groups of intragenic complementation. In this study, we show that the overexpression of Glo3p, one of the GTPase-activating proteins of Arf1p (ArfGAP), suppresses the ts growth of a particular group of the arf1 mutants (arf1-16 and arf1-17). Other ArfGAPs do not show such a suppression activity. All these ArfGAPs show sequence similarity in the ArfGAP catalytic domain, but are divergent in the rest of molecules. By domain swapping analysis of Glo3p and another ArfGAP, Gcs1p, we have shown that the non-catalytic C-terminal region of Glo3p is required for the suppression of the growth defect in the arf1 ts mutants. Interestingly, Glo3p and its homologues from other eukaryotes harbor a well-conserved repeated ISSxxxFG sequence near the C-terminus, which is not found in Gcs1p and its homologues. We name this region the Glo3 motif and present evidence that the motif is required for the function of Glo3p in vivo.

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Key words: ArfGAP, Glo3p, Glo3 motif

Introduction
ADP-ribosylation factors (Arfs) constitute a ubiquitous family of small GTPases in eukaryotes, and are now recognized as essential components in membrane traffic. There are six Arf proteins in mammals and three Arfs in the yeast Saccharomyces cerevisiae, which are classified into three and two classes, respectively. These Arf proteins regulate formation of vesicles in a variety of steps of membrane traffic by interacting with coat components and also appear to control phospholipid metabolism and cytoskeletal organization in various ways (Donaldson and Jackson, 2000; Kirchhausen, 2000; Nie et al., 2003; Scheiman and Orci, 1996). Different classes of Arfs are thought to function at different places within a cell. Evidence is also present that a single Arf species may have multiple roles (Donaldson et al., 2005; Donaldson and Jackson, 2000). In yeast, among the three Arf proteins, Arf1p and Arf2p are 96% identical in amino acid sequences. They execute redundant essential functions for growth and their double knockout is lethal (Stearns et al., 1990), whereas Arf3p is dispensable.

We previously isolated eight temperature-sensitive (ts) mutants of the yeast ARF1 gene by PCR-based random mutagenesis in the arf2Δ background. These arf1 ts mutants showed a variety of transport defects and morphological alterations in an allele-specific manner. Furthermore, intragenic complementation was observed between certain pairs of mutant alleles, both for cell growth and intracellular transport. These results demonstrated that the single Arf1 protein is indeed involved in many different steps of intracellular transport in vivo (Yahara et al., 2001). However, the mechanism of managing these various steps of transport by Arf1p remained unsolved.

Our next interest was to understand how the single Arf1p can fulfill such divergent functions. Many Arf interactors are known to date, including guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs) and effectors (Donaldson et al., 2005; Nie et al., 2003). ArfGEFs and ArfGAPs contain the Sec7 and the ArfGAP domains, respectively. In the human genome, at least 15 ArfGEFs and 16 ArfGAPs are present, and four ArfGEFs and four ArfGAPs exist in S. cerevisiae, indicating the presence of complicated networks for the regulation of Arfs. The yeast arf1 ts mutants we constructed may have different abilities to interact with such a variety of regulators and targets and thus express different downstream activities.

For the purpose of identifying the molecules that execute the pleiotropic functions downstream Arf1p, our arf1 ts mutants are very useful because the multiple roles of Arf1p can be dissected by different mutant alleles. One straightforward genetic approach is to screen for multicopy suppressors of these arf1 ts mutants. The eight arf1 ts mutants we constructed have been classified into three groups by intragenic
complementation tests. *arf1*-*16* and *arf1*-*17* belong to the same group and show very similar phenotypes in protein transport. In both *arf1*-*16* and *arf1*-*17* alleles, glutamic acid at position 41 is mutated to valine (E41V), and *arf1*-*16* has one additional mutation, D129E. These mutant cells have severe lesions in the Golgi-to-ER retrieval, not only by COPI- and Rer1p-dependent systems but also by the HDEL-receptor-mediated mechanism. They also show retardation of ER-to-Golgi transport of carboxypeptidase Y and secretion of underglycosylated invertase. Here, we report that the temperature sensitivity of *arf1*-*16* and *arf1*-*17* alleles is suppressed by the overexpression of Glo3p, but not by other ArfGAPs. We also describe the presence of a novel sequence motif in the C-terminal region of Glo3p, which is important for its suppression activity.

### Results

**GLO3 is a multicopy suppressor of arf1-*16* and arf1-*17**

To identify genes that participate in multiple functions of Arf1p, we screened the *S. cerevisiae* genomic DNA library on a multicopy vector for plasmids that suppress the growth defect of *arf1*-*16* at 35°C. In addition to authentic *ARF1* and *ARF2*, several clones containing different genomic loci were obtained. Subcloning and sequencing analysis identified five genes, *GLO3*, *BET1*, *BOS1*, *OLE1* and *GYP6*, as suppressors of *arf1*-*16* (Fig. 1). Among them, *GLO3*, encoding an ArfGAP, showed the strongest suppression activity on *arf1*-*16*. Growth of the *arf1*-*17* allele, which has the same E41V mutation and is -allele specific, was also rescued by *GLO3* (Fig. 1).

Of the ArfGAPs, only *GLO3* can suppress *arf1*-*16* and *arf1*-*17*

In *S. cerevisiae*, at least four genes (*GLO3*, *GCS1*, *AGE1* and *AGE2*) are reported to encode ArfGAP (Poon et al., 1999; Poon et al., 2001b; Poon et al., 1996; Zhang et al., 2003). In contrast to *GLO3*, however, overexpression of *GCS1*, *AGE1* or *AGE2* did not suppress the ts growth of *arf1*-*16* and *arf1*-*17* at all. In the case of *GCS1*, its overexpression even exaggerated the growth defect of *arf1*-*16* and *arf1*-*17*, but had no effect on the wild type (Fig. 1). The expression levels of *GLO3* and *GCS1* in *arf1*-*16* and *arf1*-*17* cells under these conditions were confirmed by immunoblotting of HA-tagged proteins (supplementary material Fig. S1). The temperature sensitivity of other *arf1* ts mutants (*arf1*-11, -13, -14 and -18) was not suppressed by the overexpression of any of these ArfGAPs (supplementary material Fig. S2), indicating that the suppression was *arf1*-allele specific and *GLO3* specific.

*arf1*-*17p* mutant protein shows a severe defect in GDP binding

Because the ts growth of *arf1*-*16* and *arf1*-*17* was suppressed by the overexpression of one ArfGAP, we examined nucleotide-binding activities of these *arf1* mutant proteins. *arf1*-*16* or *arf1*-*17* protein was co-expressed with N-myristoyltransferase in *E. coli*, and purified by DEAE-Sepharose and gel-filtration chromatography as described in Materials and Methods. Some portions of *arf1*-*16p* and *arf1*-*17p* behaved as a much larger protein perhaps due to self-oligomerization. In the case of *arf1*-*16p*, most of the protein was eluted in fractions corresponding to a molecular mass of around 160 kDa (data not shown), therefore the purification procedure of wild-type (WT) Arf1p was not directly applicable to *arf1*-*16p*. The tendency for oligomerization was much smaller in the case of *arf1*-*17p*; 60-70% of *arf1*-*17p* was eluted in fractions of the same molecular size as WT-Arf1p (~20 kDa). So we decided to use *arf1*-*17p* for the biochemical analysis. Stoichiometric myristoylation was confirmed for both WT-Arf1p and *arf1*-*17p*.

![Fig. 1. Suppression activity of ArfGAPs, BET1, BOS1, OLE1, and GYP6 for arf1-16 and arf1-17 ts mutants.](Image 343x328 to 534x719)
The purified Arf1 proteins were then subjected to the analysis of guanine-nucleotide binding activities. Aliquots of Arf1p were mixed with [35S]GTPγS or [3H]GDP and incubated at 23 or 30°C in the presence of distyrylphosphatidylcholine (DMPC)/cholate micelles as described previously (Kahn and Gilman, 1986). The resulting complex was trapped on a nitrocellulose filter and washed, and then the guanine nucleotide bound to Arf1p was quantified by scintillation counting. Fig. 2 shows the time course of GTPγS- and GDP-binding to the WT and arf1-17 proteins. For WT-Arf1p, the binding kinetics was almost the same at 23 and 30°C. By contrast, arf1-17p showed a clear temperature sensitivity in nucleotide binding. The GDP binding of arf1-17p was also lower at 30°C, but not as defective as GDP binding. These results suggest that arf1-17p has a less defective binding to GTP than to GDP at 30°C. If the ts defect of arf1-17 cells is related to such imbalance of GTP/GDP binding at high temperature, it may be that this defect is suppressed by the overproduction of ArfGAP.

Glo3p and Gcs1p show similar GAP activities toward arf1-17p in vitro

The above finding led us to speculate that different affinity of Glo3p and Gcs1p to arf1-16p/17p would explain their different effects on the ts growth of the mutants. We tested the interaction between ArfGAPs and arf1-16p/17p using two methods, the two-hybrid analysis and the in vitro GAP assay. It has already been reported that an N-terminal-truncated form (ΔN17) of Arf1p with the Q71L mutation, which is stabilized in the GTP-bound active form, interacts with both Glo3p and Gcs1p in a two-hybrid system (Eugster et al., 2000). We examined the interaction of arf1-16p/17p with these ArfGAPs using the same two-hybrid system. We generated two-hybrid constructs expressing N-truncated (ΔN17) versions of Arf1(Q71L)p, arf1-16(Q71L)p, or arf1-17(Q71L)p as fusions to the LexA DNA-binding domain in the bait vector (pGlida), and then tested for interaction with ArfGAPs (Glo3p and Gcs1p) fused to the B42 activation domain in the prey vector (pJG4-5) (Fig. 3). All chimeric proteins were expressed under the GAL1 promoter and their expression levels were confirmed by immunoblotting (supplementary material Fig. S3). We could observe that both Glo3p and Gcs1p interact with Arf1(Q71L)p as described previously. By contrast, neither of them showed positive interaction with arf1-16(Q71L)p or arf1-17(Q71L)p.

Next we tested the GAP activities of Glo3p and Gcs1p toward arf1-17p in vitro. Recombinant Glo3p and Gcs1p were produced in E. coli and subjected to the in vitro GAP assay using purified WT-Arf1p and arf1-17p as substrates (Fig. 4). Glo3p and Gcs1p stimulated the hydrolysis of GTP to GDP by WT-Arf1p at both 23 and 35°C. The GTPase activity of arf1-17p was also enhanced by these ArfGAPs. The stimulatory effects were almost comparable to those of the wild type at 23°C, but were significantly lower at 35°C. However, what should be noted here is that the effects of Glo3p and Gcs1p on the arf1-17 mutant protein were almost indistinguishable in this in vitro reaction.

The C-terminal domain of Glo3p is important for its suppression activity on arf1-16

How can we then explain the difference between Glo3p and...
Roles of the noncatalytic region of ArfGAPs

Poon et al., 1999. The homology between Glo3p and Gcs1p is limited to their N-terminal region containing the ArfGAP domain, which is known as the minimal domain to execute the ArfGAP activity of rat Arfgap1 in vitro (Cukierman et al., 1995). By contrast, the similarity between Glo3p and Gcs1p is very low in other regions of the molecules. It has also been reported that the C-terminal non-catalytic domain of rat Arfgap1 is required for its recruitment from cytoplasm to membranes and for its interaction with the KDEL receptor (Aoe et al., 1999; Huber et al., 1998). From these aspects, we suspected that the clue to understand the difference between Glo3p and Gcs1p in vivo lies in the C-terminal domains of the proteins.

To examine this possibility, we performed domain swapping of Glo3p and Gcs1p and examined the ability of the chimeric molecules to suppress the growth defect of arf1-16 (Fig. 5). The ArfGAP domain of yeast ArfGAPs contains a functionally important C2C2H2 zinc-finger motif (Zhang et al., 1998), and the similarity between Glo3p and Gcs1p is especially high around this motif. When the C-terminal large portion (residues 60-353) of Gcs1p was replaced by the C-terminal region (residues 65-494) of Glo3p (named Gcs1-A-Glo3p: 1-60/65-494), overexpression of the chimeric protein rescued the temperature sensitivity of arf1-16. However, the reverse chimeric construct, Glo3-A-Gcs1p (1-64/60-353), did not show the suppression activity. Furthermore, the overexpression of Glo3-A-Gcs1p compromised the ts growth of arf1-16, just like Gcs1p itself. Similarly, chimeric proteins fused at a slightly further downstream position, Gcs1-N-Glo3p (1-111/122-494) and Glo3-N-Gcs1p (1-122/113-353), caused suppression or aggravation of the ts growth, depending on which protein the C-terminus was derived from. These results support the differentiated roles of the C-terminal domains of the proteins.

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Gcs1p on arf1-16p/17p in vivo? Analysis of the amino acid sequences of Glo3p and Gcs1p reveals 48% similarity and 28% identity (Poon et al., 1999). The homology between Glo3p and Gcs1p is limited to their N-terminal region containing the ArfGAP domain, which is known as the minimal domain to execute the ArfGAP activity of rat Arfgap1 in vitro (Cukierman et al., 1995). By contrast, the similarity between Glo3p and Gcs1p is very low in other regions of the molecules. It has also been reported that the C-terminal non-catalytic domain of rat Arfgap1 is required for its recruitment from cytoplasm to membranes and for its interaction with the KDEL receptor (Aoe et al., 1999; Huber et al., 1998). From these aspects, we suspected that the clue to understand the difference between Glo3p and Gcs1p in vivo lies in the C-terminal domains of the proteins.

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Tandem Glo3 motif at the C-terminus of Glo3p is essential for the suppression of arf1-16

Because Glo3p and Gcs1p showed different effects in vivo depending on their C-termini, we went on to examine whether ArfGAPs from other eukaryotic organisms show any particular similarity to either of them. We searched genome databases of
Homo sapiens, Mus musculus, Caenorhabditis elegans, Drosophila melanogaster and Arabidopsis thaliana using the whole length of amino-acid sequences of Glo3p and Gcs1p independently, and picked up ArfGAP(s) that showed high blast scores in each species. As shown in Table 1, completely separable subgroups could be defined, and we hitherto classify these ArfGAPs as Glo3-type and Gcs1-type, respectively. Interestingly, multiple alignments of the Glo3-type ArfGAPs revealed limited but very high sequence similarity at their C-termini (Fig. 6A). We call this conserved C-terminal region the Glo3 motif. The Glo3 motif generally contains two repeats of the ISSxxxFG sequence, with the exception of D. melanogaster and C. elegans proteins, in which one of the two repeats diverges. K386 and L411 of S. cerevisiae Glo3p in this motif are also well conserved among the Glo3-type ArfGAPs. In contrast, none of Gcs1-type ArfGAPs contain the Glo3 motif. Other types of ArfGAPs such as Age1p or Age2p of yeast do not possess the Glo3 motif either.

To examine the physiological importance of the Glo3 motif, we introduced mutations in this motif of Glo3p and tested its ability to suppress the growth defect of arf1-16 (Fig. 6B-D). Residues in either the first or second repeat of ISSxxxFG were replaced by alanines and subjected to the suppression test. Overexpression of the glo3-11 and glo3-12 proteins, in which only one ISS sequence was mutated, partially suppressed the ts growth of arf1-16. When both ISS sequences were mutated (glo3-13 and 14), the suppression activity was completely lost. This result indicates that the Glo3 motif is critical for the in vivo function of Glo3p.

Discussion

We have been investigating how Arf1p plays multiple roles in living cells. arf1 ts mutants we isolated show a variety of transport defects and morphological alterations in an allele-specific manner. To identify partner molecules that participate in individual functions of Arf1p, we have screened for multicopy suppressors of the arf1 ts mutants. In the present study, we have demonstrated that the overexpression of GLO3, one of the ArfGAPs, suppresses the ts growth of arf1-16 and arf1-17 mutants, which are classified into the same intragenic complementation group, whereas other ArfGAPs (GCS1, AGE1 and AGE2) do not. The overproduction of Gcs1p is inhibitory for the growth of arf1-16 and arf1-17 cells. This result raises the possibility that different ArfGAPs act differently on Arf1p to allow it to fulfill divergent functions.

In the nucleotide-binding assay, the arf1-17 protein showed lower binding of GDP than GTP at 30°C. If this imbalance of GDP/GTP binding was a cause of the mutant phenotype of arf1-17 cells, it seemed reasonable that the overproduction of the Glo3p ArfGAP suppressed it. The difference in the suppression activity between Glo3p and Gcs1p might be explained by their different affinities to the mutant Arf1p. However, the GAP activities of Glo3p and Gcs1p on the arf1-17 protein turned out to be almost identical in vitro. The key to solve this
Roles of the noncatalytic region of ArfGAPs

An apparent disparity was revealed to lie in the non-catalytic regions of ArfGAPs. All known ArfGAPs contain the ArfGAP domain, which executes the ArfGAP catalytic activity, but the amino-acid sequences in the rest of molecules are different from each other. When the non-catalytic C-terminal portions of Glo3p and Gcs1p were exchanged, their effects on arf1-16 were also switched, suggesting that this region is required for their individual functions in vivo. Interestingly, we found a novel motif at the C-terminus of Glo3p (residues 386-422 of Glo3p), which is conserved among Glo3-type ArfGAPs in many eukaryotes. We named this region the Glo3 motif. The characteristic feature of this motif is one or two repeats of the ISSxxxFG sequence. Point mutations of the conserved motif resulted in the loss of the suppression activity in arf1-16, indicating that the Glo3 motif is required for the proper activity of Glo3p in living cells. These Glo3-motif mutant proteins are supposed to retain the ArfGAP activity, because the N-terminal ArfGAP domain remains intact. Therefore, the activity of Glo3p is probably modulated by the third molecule(s) through the interaction with the Glo3 motif in vivo. arf1-16/17 mutant proteins presumably have a problem in interacting with such modulators.

What could be the third molecule(s) then? Glo3p has been thought to play an important role in the Golgi-to-ER retrograde transport in cooperation with the coatomer complex. Dogic et al. (Dogic et al., 1999) showed in an analysis of yeast disruptants of the ArfGAP genes that the deletion of GLO3 alone resulted in a defect of the retrieval of dilysine-tagged proteins from the Golgi. Glo3p was also reported to interact with Sec21p (γ-COP) and Sec27p (β'-COP), whereas Gcs1p failed to bind to coatomer both in a two-hybrid assay and in immunoprecipitation analysis (Eugster et al., 2000; Lewis et al., 2004). The Glo3 motif at the C-terminus may be required for the interaction with the coatomer complex. In an attempt to support this possibility by a genetic analysis, however, none of the COPI genes could suppress the ts growth of arf1-16 by their single overproduction (our unpublished results).

Another class of molecules we are interested in are Bet1p and Bos1p. Recent studies have shown that the ArfGAP activity is required not only to remove the protein coat but also for concentration of cargo into vesicles (Lanoix et al., 1999; Lanoix et al., 2001; Nickel et al., 1998; Pepperkok et al., 2000). In the case of a related GTPase, Sar1p, we have also shown

Table 1. Glo3-type and Gcs1-type ArfGAPs from various species

| Species     | Glo3 type | Gcs1 type |
|-------------|-----------|-----------|
| S. cerevisiae | Glo3p     | Gcs1p     |
| H. sapiens  | ZNF289    | Arfgap1   |
| M. musculus | Zfp289    | Arfgap3   |
| D. melanogaster | CG6838 | CG4237   |
| C. elegans  | F07F6.4   | K02B12.7  |
| A. thaliana | AT5G46750.1 | AT3G53710.1 |
|             | AT4G17890.1 | AT2G37550.1 |
|             | AT2G35210.1 |

Fig. 6. (A) Alignment of C-terminal amino acid sequences from various Glo3-type ArfGAPs. The ClustalW algorithm was used to create the sequence alignment. Identical amino acid positions for each Glo3-type ArfGAP are marked with asterisks (*). Sc, S. cerevisiae; Hs, H. sapiens; Mm, M. musculus; Ce, C. elegans; Dm, D. melanogaster; At, A. thaliana. Note that one or two repeats of ISSxxxFG sequence exist in every Glo3-type ArfGAP. K386 and L411 of Glo3p are also conserved (shaded box). (B) Mutational analysis of the Glo3 motif. Various amino acid mutations were introduced into this motif of the chimeric protein 3HA-Glo3p. (C) NY16-1 cells (arf1-16) were transformed with pYO324 (vector), pNY14-3HA-GLO3 (CEN 3HA-GLO3), pNY24-3HA-GLO3 (2 μ 3HA-GLO3), pNY24-glo3-m2HA (2 μ 3HA-glo3), pNY24-glo3-m1HA (2 μ 3HA-glo3), and pNY24-glo3-m5HA (2 μ 3HA-glo3). Cells were grown, diluted and spotted on MCD (–Trp) plates, and incubated at the indicated temperatures for 2 days. Integrants of wild-type ARF1 (NYY0-1: WT) transformed with pYO324 were used as a control. (D) Immunoblotting of 3HA-Glo3p to examine expression levels. The transformants that were used in C were grown to an early log phase at 23°C, and incubated at 35°C for 1 hour. Total cell lysates (60 μg) were prepared and analyzed by immunoblotting using the anti-HA monoclonal and anti-Sec61p antibodies. Lane numbers correspond to the transformant numbers used in C.
that the regulation of GTP hydrolysis by Sec23p GAP is important for cargo selection during COPII vesicle budding (Sato and Nakano, 2005). If Glo3p promotes the sorting of a particular set of cargo molecules, such cargo could be isolated as multicopy suppressors of \textit{arf1-16}. \textit{BET1} and \textit{BOS1}, which have been isolated as multicopy suppressors of \textit{arf1-16/17} in the present study, encode SNARE molecules functioning during the vesicular recycling between the ER and the Golgi apparatus (Martinez-Menarguez et al., 1999; Rexach et al., 1994). \textit{BET1} and \textit{BOS1} were also isolated as multicopy suppressors of the temperature sensitivity of \textit{Δglo3} mutant cells (Poon et al., 1999). Furthermore, Rein et al. (Rein et al., Gcs1p (our unpublished result). Binding to Bet1p than the wild-type Arf1p, but this binding was preliminary experiment, arf1-16p/17p showed rather elevated ability of responsible ArfGAP to select cargo. In our preliminary experiment, arf1-16p/17p showed rather elevated binding to Bet1p than the wild-type Arf1p, but this binding was not significantly affected by the addition of either Glo3p or Gcs1p (our unpublished result).

Why does Gcs1p exaggerate the ts defect of \textit{arf1-16} and \textit{arf1-17} cells? Glo3p and Gcs1p are known to play essential overlapping functions in retrograde vesicular transport (Poon et al., 1999). Indeed, \textit{glo3Δ} \textit{gcs1Δ} double deletion mutant cells are inviable (Poon et al., 1999; Zhang et al., 1998). However, there is also evidence suggesting that Glo3p and Gcs1p have additional and distinct roles. For example, the function of Gcs1p overlaps with Age2p for transport from the yeast trans-Golgi network (Poon et al., 2001b). The simultaneous overexpression of \textit{GLO3} and \textit{GCS1} resulted in weaker suppression of \textit{arf1-16/17} than that of \textit{GLO3} alone (data not shown), suggesting that these ArfGAPs are competing for something. Arf1p itself is not a likely candidate because the overexpression of other ArfGAPs, \textit{AGE1} and \textit{AGE2}, have no effect on the growth of the mutants. One attractive model may be that Glo3p and Gcs1p function on the same membrane and compete with each other for the binding site on the membrane.

Antonny's group showed that rat Arfgap1 and Gcs1p bind preferentially to liposomes containing conical lipids but not those of cylindrical lipids, and the preference for conical lipids correlated with its GAP activity on liposome-bound Arf1p-GTP (Antonny et al., 1997; Bigay et al., 2003). More recently, they proposed that the central region of Arfgap1 contains a sequence that acts as a lipid packing sensor and named it the ALPS motif. Arf1p probably uses these different kinds of ArfGAPs to compete with each other for the binding site on the membrane. Glo3p and Gcs1p, as cargo into COPI vesicles, then the defects of these SNAREs as cargo into COPI vesicles, then the defects of Sec22p. If one hypothesizes that arfGAP has a role to enrich something. Arf1p itself is not a likely candidate because the overexpression of \textit{GCS1} overlaps with \textit{Age2p} for transport from the yeast trans-Golgi. An excess amount of Gcs1p might fill up the binding sites of Glo3p fused to 113-353 of Gcs1p (pNY24-GLO3-N-GCS1), and residues 1-64 from pYT11 (Takita et al., 1995) containing three tandem copies of the hemagglutinin epitope (HA) was inserted at these sites. The resulting plasmids, pNY24-GLO3, pNY24-GCS1, pNY24-AGE1, and pNY24-AGE2, respectively. The epitope-tagged 3HA-GLO3 and GCS1-3HA were constructed as follows. Site-directed mutagenesis was used to create one Spel site adjacent to the start codon of \textit{GLO3} in pNY24-GLO3 and another Spel site just before the stop codon of \textit{GCS1} in pNY24-GCS1. The Nhel-Nhel fragment from pYT11 (Takita et al., 1995) containing three tandem copies of the hemagglutinin epitope (HA) was inserted at these Spel sites. The resulting plasmids, pNY24-3HA-GLO3 and pNY24-3CS1-3HA, were confirmed for functionality by their effects on \textit{arf1-16} (supplementary material Fig. S1). To make chimeric constructs of Glo3p and Gcs1p, ApaI or Nrl1 sites were introduced in appropriate regions of \textit{GLO3} and \textit{GCS1}. BglII-ApaI and BglII-Nrl1 regions of \textit{GLO3} plasmid were replaced by SacI-ApuAI and SacI-Nrl1 fragments of \textit{GCS1}, and the resulting constructs expressed residues 1-59 of Gcs1p fused to 5-353 of Glo3p (pNY24-GCS1-159-GLO3) and residues 1-111 of Gcs1p fused to 122-494 of Glo3p (pNY24-GCS1-111-GLO3) under the \textit{GCS1} promoter, respectively. EcoRI-Nrl1 and EcoRI-ApaI regions of \textit{GCS1} plasmid were also replaced by EcoRI-Nrl1 and EcoRI-ApaI fragments of Glo3p, and the resulting constructs expressed residues 1-122 of Glo3p fused to 113-353 of Gcs1p (pNY24-GLO3-113-GCS1), and residues 1-64 of Glo3p fused to 60-353 of Gcs1p (pNY24-GLO3-A-GCS1) under the \textit{GCS1} promoter, respectively. In all constructions, DNA sequences were confirmed.

Isolation of multicopy suppressors of \textit{arf1-16}

The \textit{S. cerevisiae} genomic DNA library based on YEp13, a multicopy vector with

Materials and Methods

**Strains and plasmids**

Saccharomyces cerevisiae strains, NYOY-1 (WT), NYY16-1 (arf1-16) and NYY17-1 (arf1-17), were described previously (Yahara et al., 2001). The yeast single-copy plasmid, pRS314, and multicopy plasmids, pYO324 and pYO325, have been described elsewhere (Ohya et al., 1991; Sikorski and Hieter, 1989). \textit{GCS1}, \textit{AGE1} and \textit{AGE2} genes were amplified from genomic DNA by PCR using gene-specific primers. After the PCR fragments were cloned into plasmids, the regions of ORF and ~500 bp each of 5'-upstream and 3'-downstream were re-obtained by homologous recombination. For \textit{GLO3}, we subcloned the gene from one of the multicopy suppressor clones of \textit{arf1-16}. The DNA fragments we cloned into the pYO324 plasmid were: \textit{GLO3}, upstream 700 bp/ORF 1482 bp/downstream 622 bp; \textit{GCS1}, upstream 871 bp/ORF 1059 bp/downstream 1593 bp; \textit{AGE1}, upstream 941 bp/ ORF 1449 bp/downstream 388 bp; and \textit{AGE2}, upstream 676 bp/ORF 897 bp/downstream 241 bp. They were named pNY24-GLO3, pNY24-GCS1, pNY24-AGE1 and pNY24-AGE2, respectively. The epitope-tagged 3HA-GLO3 and GCS1-3HA were constructed as follows. Site-directed mutagenesis was used to create one Spel site adjacent to the start codon of \textit{GLO3} in pNY24-GLO3 and another Spel site just before the stop codon of \textit{GCS1} in pNY24-GCS1. The Nhel-Nhel fragment from pYT11 (Takita et al., 1995) containing three tandem copies of the hemagglutinin epitope (HA) was inserted at these Spel sites. The resulting plasmids, pNY24-3HA-GLO3 and pNY24-3CS1-3HA, were confirmed for functionality by their effects on \textit{arf1-16} (supplementary material Fig. S1). To make chimeric constructs of Glo3p and Gcs1p, ApaI or Nrl1 sites were introduced in appropriate regions of \textit{GLO3} and \textit{GCS1}. BglII-ApaI and BglII-Nrl1 regions of \textit{GLO3} plasmid were replaced by SacI-ApuAI and SacI-Nrl1 fragments of \textit{GCS1}, and the resulting constructs expressed residues 1-59 of Gcs1p fused to 5-353 of Glo3p (pNY24-GCS1-159-GLO3) and residues 1-111 of Gcs1p fused to 122-494 of Glo3p (pNY24-GCS1-111-GLO3) under the \textit{GCS1} promoter, respectively. EcoRI-Nrl1 and EcoRI-ApaI regions of \textit{GCS1} plasmid were also replaced by EcoRI-Nrl1 and EcoRI-ApaI fragments of Glo3p, and the resulting constructs expressed residues 1-122 of Glo3p fused to 113-353 of Gcs1p (pNY24-GLO3-113-GCS1), and residues 1-64 of Glo3p fused to 60-353 of Gcs1p (pNY24-GLO3-A-GCS1) under the \textit{GCS1} promoter, respectively. In all constructions, DNA sequences were confirmed.
the LEU2 marker (Yoshishia and Anraku, 1989), was introduced into the arf1-16 mutant (NY1Y16-1). Transformants were grown on MVD (minimal glucose medium) lacking leucine at 23°C for 1 day and then incubated at 35°C for 3 days. Colonies that grew at 35°C were re-streaked on MVD plates and confirmed for growth at 35°C. Plasmids were recovered from the candidate clones and reintroduced into arf1-16 to verify the suppression activity. Out of approximately 50,000 transformants screened, 78 positive clones were obtained. Among strong suppressors, four clones contained either arf1/ or arf2. In addition, we found that 12, 6, 4, and 14 and 2 clones repeatedly contained genomic fragments harboring GLO3, BET1, RPS1, OLE1 and GYP6, respectively. They were subcloned into pY3025, a multicopy vector with the LEU2 gene, to confirm that the suppression activities depended on these five genes.

Two-hybrid assay

The bait plasmid pGilda, the prey plasmid pG4-5, and the yeast reporter strain EGY48 were obtained from OriGene Technologies, Inc. (Rockville, MD, USA). Plasmid constructions and the examination of interaction between (ΔN17)ARF1/QT1Lp and ArfGAPs were performed as previously described except that the bait pEG202 was replaced with pGilda (Euster et al., 2000).

Purification of myristoylated-Arf1 proteins

Yeast N-myristoyltransferase (NMT1) expression vector (pACYC177-ET3d-NMT1) was constructed as previously described (Hart et al., 1993) and its activity was confirmed by using [1-14C]myristic acid (data not shown). The full-length ARF1 and arf1-17 genes were cloned into NdeI-XhoI sites of pET2A and E. coli BL21 (DE3) was co-transformed with the resulting plasmids (pET-ARF1 and pET-arf1-17, respectively) and pACYC177-ET3d-NMT1. Transformed cells were selected for growth at 35°C. Plasmids were recovered from the candidate clones and reconfirmed using [1-14C]myristic acid (data not shown). The full-length ARF1 or arf1-17 proteins were expressed at 30°C to 35°C and were purified by guanidine extraction followed by Ni-NTA purification, as described before, and the yield of myristoylation was performed as described previously (Poon et al., 2001a). The ARF1 or ARF1 mutant (NYY16-1) was expressed at 30°C to 35°C and was purified by guanidine extraction followed by Ni-NTA purification, as described before.

Immunoblotting and antibodies

Cells were cultured to a middle logarithmic phase. Total cell lysates were prepared by agitation with glass beads in 62.5 mM Tris-HCl (pH 6.8), 2.35% SDS, 10 mM EDTA, 50 mM PMSF, and treated at 65°C for 10 minutes. They were subjected to immunoblotting by standard procedures and visualized by the ECL-plus system (Amersham). The anti-Arf1 antibody was described previously (Yahara et al., 2001). The anti-Sec61p antibody was a gift from R. Schekman. Monoclonal anti-HA antibodies, HA7 and 12CA5, were obtained from Sigma (MO, USA) and Roche (Basel, Switzerland), respectively.

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