The yeast ADP-ribosylation factor GAP, Gcs1p, is involved in maintenance of mitochondrial morphology

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Summary

Membrane trafficking is regulated, in part, by small GTP-binding proteins of the ADP-ribosylation factor (ARF) family. ARF function depends on the controlled binding and hydrolysis of GTP. In vitro, the GTPase activity of yeast ARF proteins can be stimulated by Gcs1p. Although Gcs1p was implicated in the regulation of retrograde transport from the Golgi to the ER and in actin cytoskeletal organization, its intracellular functions and distribution remain to be established. Following subcellular fractionation of yeast grown in rich medium, Gcs1p was localized in denser fractions than it was in cells grown in minimal medium. In yeast grown in rich or minimal medium, Gcs1p was distributed over the cytoplasm in a fine punctate pattern with more concentrated staining in the perinuclear regions. Overexpressed Gcs1p in yeast was localized partially with mitochondria and partially in perinuclear structures close to mitochondria. The Gcs1p PH-domain was required for localization in mitochondria but not for the perinuclear region. Transport of carboxypeptidase Y and invertase was not significantly altered by disruption of the *gcs1* gene. This mutation did, however, reduce mitochondrial lateral distribution and branching when yeast were grown in rich medium. In yeast overexpressing Gcs1p, mitochondrial morphology was aberrant, with unbranched tubules and large spherical structures. We suggest that Gcs1p may be involved in the maintenance of mitochondrial morphology, possibly through organizing the actin cytoskeleton in *Saccharomyces*.

Key words: Guanine nucleotide-binding protein, GTPase, Membrane trafficking, Cytoskeletal organization

Introduction

Eukaryotic cells are elaborately subdivided into membrane-enclosed organelles. These functionally distinct intracellular compartments communicate constantly and bidirectionally via the flow of small transport vesicles that bud from a donor membrane through the recruitment of cytosolic coat proteins. Before fusion of a vesicle with the target compartment, these coat proteins must be released back to the cytosol. ADP-ribosylation factors (ARF) are a family of 20-kDa GTP-binding proteins that regulate membrane traffic (Boman and Kahn, 1995; Moss and Vaughan, 1998). 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).

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; Randazzo, 1997; Brown et al., 1998; Premont et al., 1998; Andreev et al., 1999; Poon et al., 1999; Turner et al., 1999). An ARF GAP localized to the rat liver Golgi complex was found to contain a zinc-finger motif that was important for GAP activity but had no effect on subcellular distribution (Cukierman et al., 1995). Structurally, this mammalian GAP is very similar to the zinc-finger proteins Gcs1p and Glo3p that are GAPs for yeast ARF1 and ARF2 in vitro (Poon et al., 1996; Poon et al., 1999). These two proteins have overlapping functions in ER-Golgi transport, and Glo3p is important for retrieval of proteins from the Golgi to the ER (Andreev et al., 1999; Poon et al., 1999; Dogic et al., 1999). The KDEL receptor was reported to regulate GAP recruitment to membranes (Aoe et al., 1997). Two tyrosine-kinase-binding proteins, ASAP1 and Pap, also function as ARF GAPs (Brown et al., 1998; Andreev et al., 1999), indicating that GAP activity can be regulated by intracellular proteins as well as by extracellular signals.

GCS1 was identified as a gene required conditionally for re-entry of stationary phase cells into the cell cycle (Filipak et al., 1992; Ireland et al., 1994). Mutant *gcs1* cells lose mitochondrial activity (Filipak et al., 1992) and exhibit vesicular trafficking defects at nonpermissive temperature (Poon et al., 1996). Gcs1 contains putative zinc-binding PH, and ERM-homology domains (Blader et al., 1999). The yeast *gcs1-1* with a mutation in the zinc-finger region had a growth defect similar to that of the *gcs1*-null mutant (Ireland et al., 1994). Gcs1p was also reported to be required for normal actin cytoskeletal organization and for actin polymerization in vitro (Blader et al., 1999).

Despite these observations, the cellular function and distribution of the Gcs1p remain unclear. We found that Gcs1p function in yeast vesicular transport differs from that of ARF1; it was required for efficient transport of alkaline phosphatase but not carboxypeptidase Y or invertase. Endogenous Gcs1p was more concentrated in the perinuclear region when yeast

were grown in minimal synthetic medium and was distributed throughout the cell in a fine punctate pattern in yeast grown in rich medium. *gcs1* mutants appeared to have reduced lateral distribution and branching of mitochondria when grown in rich medium. When Gcs1p was overexpressed in cells, the protein appeared in a mitochondrial distribution and in perinuclear structures close to mitochondria. We suggest that Gcs1p is a mutifunctional molecule, which may be involved in mitochondrial and ER membrane dynamics in *Saccharomyces*.

Materials and Methods

Strains, media and microbiological techniques

Yeast culture media were prepared as described by Sherman et al. (Sherman et al., 1986). YPD medium contained 1% Bacto-yeast extract, 2% Bacto-peptone, and 2% glucose. SD minimal medium contained 0.17% Difco yeast nitrogen base, 0.5% ammonium sulfate and 2% glucose. Nutrients essential for auxotrophic strains were supplied at specified concentrations (Sherman et al., 1986). Yeast strains SEY 6210.5 (MATa/ MATα, his3/his3, leu2/leu2, trp1/trp1, ura3/ura3), YPH250 (MATa ade2, his3, leu2, lys2, trp1, ura3-52), and YPH252 (MATα ade2, his3, leu2, lys2, trp1, ura3-52) (Skirski and Heiter, 1989) were used in this study. Transformation was by the lithium acetate method (Ito et al., 1983). Plasmids were constructed according to standard protocols (Sambrook et al., 1983).

GCS1 gene disruption

GCS1 DNA generated by PCR was ligated into pT7Blue plasmid (Novagen). The yeast Leu2 gene was inserted at the HpaI site of the GCS1 gene, producing pTGCS1L. Gene disruption was accomplished by a one-step gene replacement method (Rothstein, 1983). Briefly, the ~2.7-kb DNA fragment excised from pTGCS1L by NcoI and BamHI cleavage was used to transform various leu2$^-$ strains and leucine prototrophs were selected as described (Huang et al., 1999). Sporulation, growth, and tetrad analysis were carried out as described (Sherman et al., 1986).

Expression and purification of recombinant proteins and polyclonal antibody production

The open reading frame of *GCS1* was obtained by PCR, using primers that incorporated unique *Nco*I and *Bam*HI sites at the initiating methionine and 31 bp downstream of the stop codon, respectively. For the His-tagged Gcs1p, a DNA fragment containing the *GCS1* coding region was generated by amplifying yeast genomic DNA with sequence-specific primers. The PCR product was purified and ligated to the expression vector pET30a (Novagen), yielding pET30aGCS1. The His-tagged fusion protein was synthesized in BL21(DE3) *E. coli* and purified on Ni^{2+}NTA resin (Qiagen, Chatsworth, CA) as described (Huang et al., 1999). Denatured, purified recombinant Gcs1p isolated from an SDS-PAGE gel was used as an antigen to raise polyclonal antibodies in rabbits, essentially as described in Huang et al., 1999 (Huang et al., 1999). Affinity-purified polyclonal antibodies were diluted 1:10 for western blotting and used undiluted for indirect immunofluorescence experiments.

Construction of Gcs1p mutant expression clones

Gcs1p mutants with zinc-finger disruption (Gcs1ΔZn) or PH domain deletion (Gcs1ΔPH) were constructed by amplifying the cloned GCS1 gene with mutant-specific primers. For the zinc-finger-disruption, two-step PCR was used. In the first PCR, overlapping 5′ and 3′ DNA fragments were generated. The 5′-end primer (tagaccatgtcagattggaaa-gtggacc) and 3′-end primer (ggcagcttcaagggcaatgaaagctccaaactt) served as primers for amplification of the 5′ fragment. The 3′ fragment was generated using a 3′-ΔZn oligonucleotide (attgcccttgaagctgccggta-tccatagaggg) primer in combination with the 3′-end anti-sense primer: ttacaagtcttcttcagaaatcagccttttgttcgaaatcgaaatcgtcccatttg, so that the encoded protein would have a Myc sequence EQKLISEEDL at its C-terminus. In the second PCR, appropriate pairs of overlapping fragments were combined with the 5′- and 3′-end primers to generate the full-length Gcs1ΔZn mutant sequence. PCR amplification of GCS1 fragments with primer pairs of the 5′-end primer and an anti-sense PH domain deletion primer (ttatgcagaccgttcttgtggagg) resulted in the Gcs1ΔPH mutant. The PCR fragments were then ligated into pT7Blue plasmid (Novagen), sequenced and subcloned into plasmids pVT 101U or 102U, expression plasmids containing the ADH1 promotor (Vernet et al., 1987).

For amplification of the PH domain of Gcs1p (corresponding to the residues 226-352), primer pairs 5′-ggatccgcagggtccagcaatactttg-3′ and 5′-ggatccttgaatgaatgttgagaaaa-3′ that incorporated *Bam*HI sites were used. The DNA fragment containing the Gcs1-PH domain was amplified from the cloned *GCS1* coding region. The PCR product was ligated to pT7Blue plasmid (Novagen) first and then subcloned into the pCGF1a vector using *Bam*HI cutting sites to obtain a GFP-PH fusion construct. For the induction of GFP-PH fusion protein, yeast cells were grown overnight in selection medium containing galactose, and cells expressing the fusion protein were observed using FITC optics.

Preparation of yeast cell extracts and immunoblotting

Whole yeast extracts were prepared by agitating (using a vortex mixer) yeast cells suspended in TE buffer (10 mM Tris, pH 7.4, 1 mM EDTA) with glass beads for 1 minute followed by incubation on ice for 1 minute, repeated five times. After brief centrifugation to clarify the lysate, protein was quantified by Coomassie blue assay (Pierce). Proteins separated by SDS-PAGE were transferred to PVDF membranes (Millipore Corp.), which were incubated (60 minutes, room temperature) with antibodies in Tris-buffered saline (pH 7.4) containing 0.1% Tween 20 and 5% dried skimmed milk. Bound antibodies were detected with the ECL system (Amersham Pharmacia Biotech).

Protein labeling and immunoprecipitation

Yeast grown overnight at 30°C in minimal medium with 200 mM $(NH4)_2SO_4$ to an A600 of 0.5 were incubated for 30 minutes at 37°C, transferred to sulfate-free minimal medium (final A600=5) and incubated for 15 minutes at 37°C or 30 minutes at 15°C before addition of 30 μCi of Pro-mix L-35S label (blend of [^{35}S] methionine and [^{35}S]cysteine, 14.3 mCi/ml) per A_{600} unit. After 10 minutes (37°C) or 20 min (15°C), labeling was terminated by addition of 5%(v/v) of chase solution containing 0.3% cysteine (w/v), 0.4% of methionine (w/v) and 100 mM $(NH_4)_2SO_4$. Samples (1 ml) were removed at the indicated time thereafter and added to equal volumes of ice-cold 20 mM NaN_3 in double-distilled water. Cells were collected and washed once with 10 mM NaN_3. Glass beads (300 μl) and 300 μl of buffer containing 50 mM Tris-Cl (pH 7.5), 1% SDS, 1 mM EDTA and 1 mM PMSF were added and the mixture was mixed vigorously (vortex mixter) for 90 seconds at room temperature before boiling (95°C) for six minutes. Immunoprecipitation, electrophoresis and autoradiography were done essentially as described previously (Huang et al., 1999) using anti-carboxypeptidase Y or anti-vacuolar alkaline phosphatase antiserum.

Subcellular fractionation

Yeast grown in selective minimal medium or YPD medium were harvested by centrifugation and washed once with 10 mM NaN_3, before Lyticase digestion of cell walls in a solution of 1.2 M sorbitol and 100 mM potassium phosphate, pH 6.5. Spheroplasts were suspended in buffer containing 0.1 M sorbitol, 20 mM HEPES (pH 7.4), 50 mM potassium acetate and 1 mM EDTA with protease inhibitors, and then disrupted on ice with 20 strokes in a Dounce homogenizer. The lysate

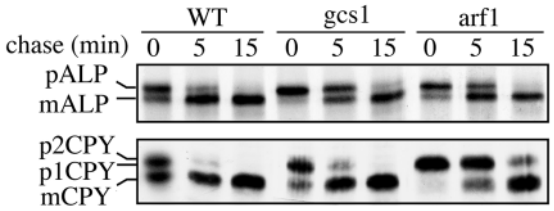

Fig. 1. Maturation of vacuolar enzymes ALP and CPY. Wild-type (*ARF1/GCS1*) and *gcs1* and *arf1* mutant cells were grown, radiolabeled with ^{35}S-labeled methionine and cysteine and incubated for the indicated chase time (min) before immunoprecipitation. p1 CPY is the core-glycosylated form found in the ER, p2 is the outer chain-glycosylated Golgi form and the mature form (M) results from proteolytic processing in the vacuole. pALP is the proenzyme form and mALP is the mature form in the vacuole.

was centrifuged (450 ***g***) to remove debris and unbroken cells. Cleared lysate (0.8 ml) was loaded on top of a manually generated six-step sucrose gradient (0.7 ml each of 60, 50, 40, 30, 20 and 10% sucrose in lysis buffer), which was then centrifuged at 170,000 ***g*** for three hours in a Beckman SW55 rotor at 4°C. Proteins in samples (100 µl) of fractions, collected manually from the top, were precipitated with 10% TCA, separated by SDS-PAGE and analyzed by immunoblotting. Diluted antibodies against mitochondrial porin (1:500) (Molecular Probes Inc.), Kar2 (1:1000), Emp47 (1:5000) and ARF1 (1:5000) (Lee et al., 1997) were used to identify organelles.

Indirect immunofluorescence

Cells grown to a density of 1-2×10^7 cells/ml in 25 ml of minimal selective medium with 2% glucose or 2% galactose or YPD medium were prepared for indirect immunofluorescence essentially as described (Lee et al., 1997). Antibodies included affinity-purified anti-Gcs1p and anti-mitochondrial porin (Molecular Probes Inc.) diluted to 1:100, with secondary antibodies Alexa Fluor™ 488 goat anti-rabbit IgG and Alexa Fluor™ 594 goat anti-mouse IgG (Molecular Probes Inc.) used at 1:1000 and 1:2000 dilutions, respectively. For detection of actin, monoclonal anti-actin (clone C4, ICN Biomedicals, Inc.) was used at a 1:400 dilution. Nuclei were stained with H33258 (2 µg/ml) included in the mounting solution. Preparations were inspected with a Nikon Microphot SA microscope and photographed on Kodak Elite chrome 400 film.

For detection of actin, 3 ml of yeast overnight cultures were fixed with 3.7% formaldehyde at room temperature for one hour. Cells were then pelleted, washed twice with 1 ml of PBS and permeabilized by incubation in 1 ml of 0.2% TritonX-100 in PBS for 10 minutes at room temperature, followed by two washes in PBS. Approximately 2 OD_{600} units of cells were incubated in 40 µl of PBS containing 0.15 µM Alexa Fluor™ 594-phalloidin (Molecular Probes) for one hour at room temperature. The cells were then washed three times with PBS, suspended in mounting solution containing 2 µg/ml Hoechst 33258 and inspected by microscopy.

Invertase assay

Cells grown to a density of 1×10^7 cells/ml in YP medium containing 5% glucose were harvested by centrifugation, washed twice with sterile H_2O and suspended in YP medium containing 0.05% glucose to derepress the expression of invertase, which was measured as described by Novick and Botstein (Novick and Botstein, 1985).

Extraction of Gcs1p from membrane fraction

After overnight growth in YPD medium, yeast were harvested and digested with Lyticase as described above. Spheroplasts were suspended in buffer containing 0.6 M sorbitol, 5 mM MES (pH 6.0), 1 mM KCl and 0.5 mM EDTA with protease inhibitors and disrupted on ice with 20 strokes in a Dounce homogenizer. The lysate was centrifuged (450 ***g***) twice to remove debris and unbroken cells. The cleared lysate was centrifuged (13,000 ***g*** for 10 minutes at 4°C) to pellet fraction P13. P13 was suspended in the same buffer (600 µl). To determine the nature of the association of Gcs1p with membranes, 200 µl of P13 was adjusted to a concentration of 1 M NaCl in the same buffer, and 200 µl of P13 was left untreated on ice for 30 minutes before centrifugation at 150,000 ***g*** for one hour in a Beckman SW55 rotor at 4°C. The remaining 200 µl of P13 were centrifuged, and the pellet was suspended in 10 µl 0.3 M sucrose, 10 mM Tris-HCl, pH 7.4. Ice-cold Na_2CO_3, pH 11.5, was added to a final concentration of 0.1 M. After 30 minutes on ice, the sample was layered on top of 0.5 ml of 0.3 M sucrose, 10 mM Tris-HCl, pH 7.4 and centrifuged at 150,000 ***g*** for one hour in a Beckman SW55 rotor at 4°C. Samples were then analyzed by western blotting as described above.

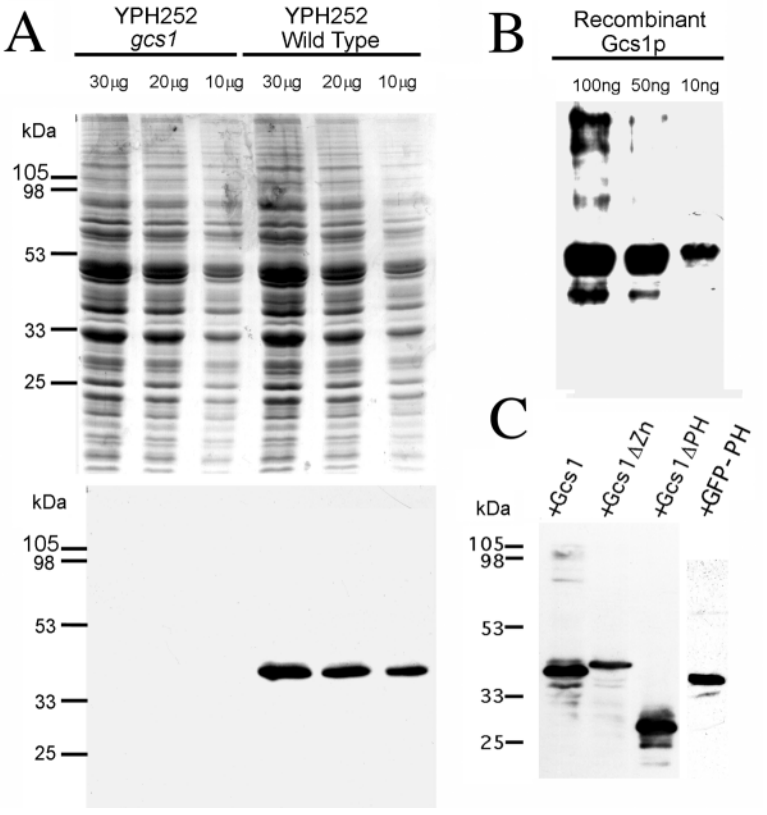

Fig. 2. Detection of Gcs1p and mutated forms in yeast by western blot analysis. The indicated amounts of proteins from (A) YPH 252 wild-type or *gcs1* mutant cells and (B) purified His-tagged Gcs1p or (C) ~20 µg of proteins from *gcs1* mutant cells overexpressing wild-type Gcs1p, HA-tagged zinc-finger-mutated (ΔZn) Gcs1p, PH domain-deleted (ΔPH) Gcs1p or GFP-fused PH domain (GFP-PH) were separated by SDS-PAGE. Proteins were stained with Coomassie blue (A) or transferred to PVDF, reacted with affinity-purified antibodies against Gcs1p and detected using the ECL system. Positions of protein standards (kDa) are at the left.

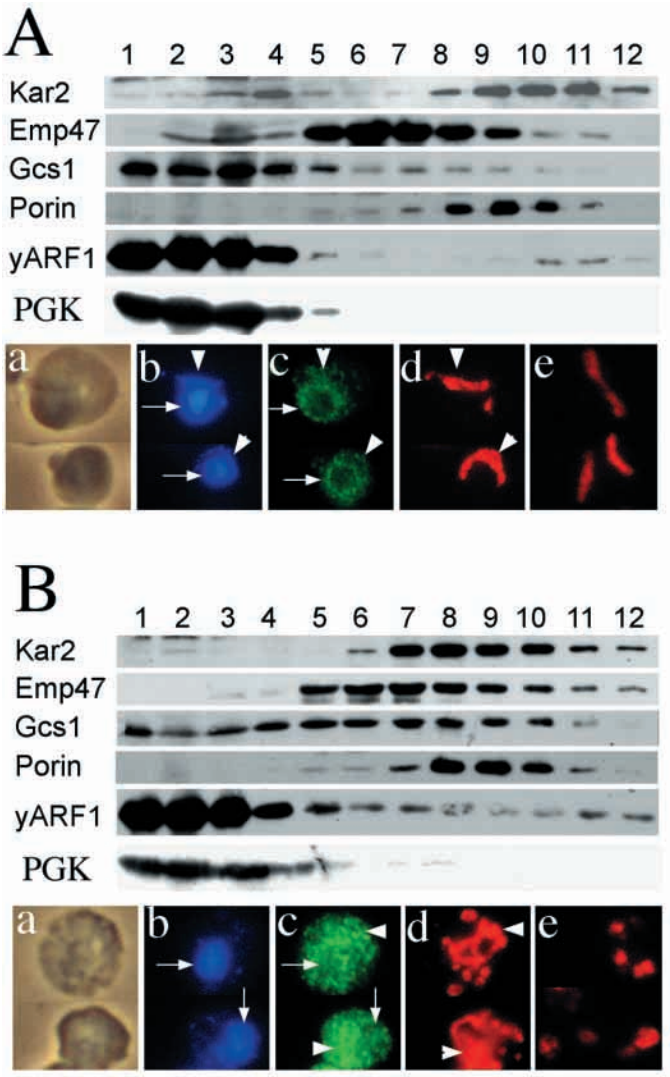

Fig. 3. Subcellular localization of endogenous Gcs1p. Yeast YPH 252 cells were grown in minimal medium (A) or YPD medium (B). Lysates of spheroplasts from cells were fractionated by sucrose gradient (10-60%) centrifugation. Proteins in samples (100 μl) of fractions were precipitated, separated by SDS-PAGE and analyzed by immunoblotting. Gcs1p, yARF1, Emp47 (Golgi marker), porin (mitochondrial marker), Kar2 (an ER marker), and PGK (cytoplasmic marker) were identified with specific antibodies and detected using the ECL system with exposure to Hyper-film MP. Gradient fractions were numbered from the top. Below the immunoblots in each panel are results of indirect immunofluorescence staining of YPH 252 wild-type (a, b, c, d) or *gcs1* mutant (e) cells. Cells were fixed with formaldehyde; spheroplasts were prepared and reacted with purified anti-Gcs1p antibodies (c) or anti-porin antibody (d, e) followed by secondary antibodies. In (a) are phase images of the cells in b, c, and d. Nucleic acids were stained with H33258 (b). Arrows indicate perinuclear localization and arrowheads mitochondria.

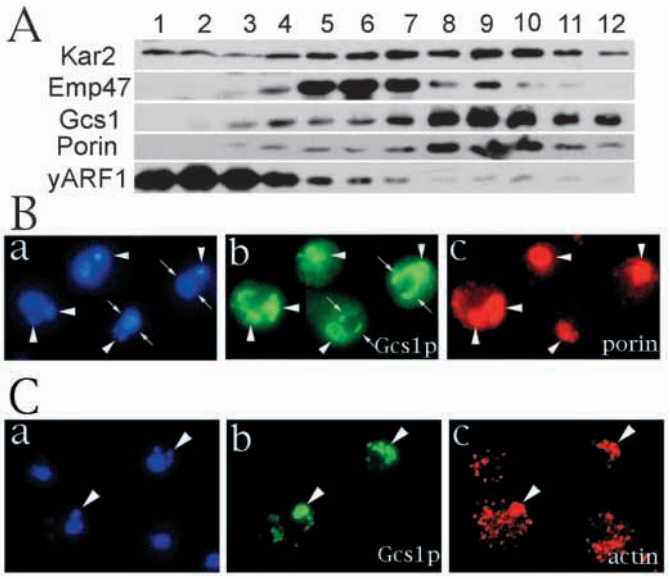

Fig. 4. Effect of overexpression of Gcs1p on mitochondrial morphology. Data are presented as in Fig. 3. (A) lysates of spheroplasts from *gcs1* cells overexpressing wild-type recombinant Gcs1p under the control of the *ADH1* promoter and grown in minimal medium were fractionated for analysis by immunoblotting. (B) and (C) indirect immunofluorescence staining of *gcs1* cells overexpressing wild-type Gcs1p. (B) spheroplasts were reacted with purified anti-Gcs1p antibodies (b) or anti-porin antibody (c) or stained with H33258 (a). Arrows indicate perinuclear localization and arrowheads mitochondria. Panel C, spheroplasts were reacted with purified anti-Gcs1p antibodies (b) or anti-actin antibody (c) or stained with H33258 (a). Arrowheads indicate colocalization of Gcs1p and actin structures.

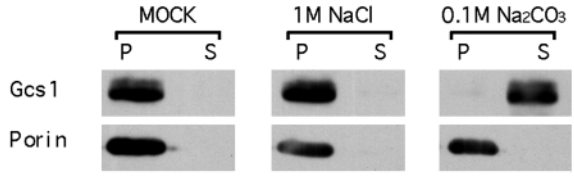

Fig. 5. Gcs1p is weakly associated with membrane fraction. The ER/mitochondria-enriched fraction (P13) from wild-type yeast was treated with 1 M NaCl, 0.1 M Na_2CO_3 or buffer (Mock) on ice for 30 minutes, followed by centrifugation at 150,000 ***g*** for one hour. The resulting supernatant and pellet fractions were analyzed by western blotting using anti-Gcs1p or anti-porin antibodies.

Results

Function of Gcs1p differs from that of ARF1 in vesicular transport

To assess the cellular functions of Gcs1p, the *GCS1* open reading frame was disrupted using the *Leu2* marker gene, which did not result in significant growth defects at 37°C, 30°C or 15°C (data not shown). In addition, unlike a previous report (Ireland et al., 1994), Gcs1p was not required for stationary phase cells to re-enter the cell cycle at the 15°C nonpermissive temperature (data not shown).

To evaluate processing of two vacuolar hydrolases, pulse-chase labeling with ^{35}S-labeled cysteine and methionine of wild-type and *arf1*, and *gcs1* mutant cells was followed by immunoprecipitation of carboxypeptidase Y (CPY) and vacuolar alkaline phosphatase (ALP) (Fig. 1), which follow different maturation pathways (Cowles et al., 1997). CPY is a soluble vacuolar hydrolase that is core-glycosylated in the ER to produce the p1 form, followed by oligosaccharide extension

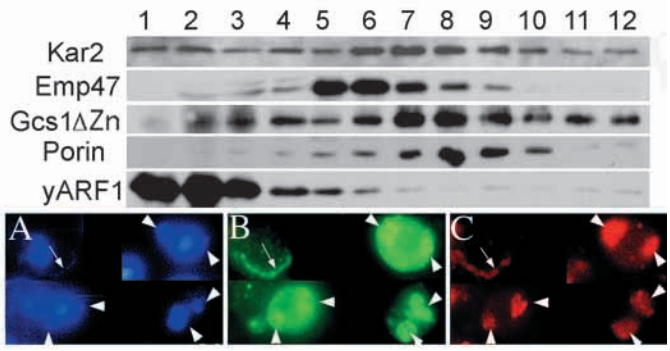

Fig. 6. Effect of overexpression of Gcs1p on mitochondrial morphology does not require its zinc-finger motif. Data are presented as in Fig. 3. Upper panel, lysates of spheroplasts from *gcs1* cells overexpressing Gcs1ΔZn and grown in minimal medium were fractionated for analysis by immunoblotting. Lower panel, indirect immunofluorescence staining of *gcs1* cells overexpressing Gcs1ΔZn. Spheroplasts were reacted with purified anti-Gcs1p antibody (B) or anti-porin antibody (C) or stained with H33258 (A). Arrows indicate tubular mitochondria and arrowheads mitochondria.

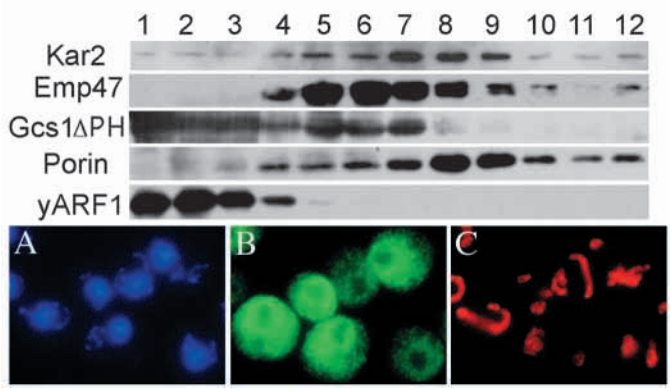

Fig. 7. The C-terminal PH domain of Gcs1p is required for mitochondrial localization. Data are presented as in Fig. 3. Upper panel, lysates of spheroplasts from *gcs1* cells overexpressing Gcs1ΔPH and grown in minimal medium were fractionated. Lower panel, indirect immunofluorescence staining of spheroplasts reacted with purified anti-Gcs1p antibody (B) or anti-porin antibody (C) or stained with H33258 (A).

in the Golgi complex (p2 form) and proteolysis in the vacuole lumen to generate mature CPY. Unlike the *arf1* mutation, the *gcs1* mutation did not block CPY processing (Fig. 1, lower panel. Compare five and 15 minute chase times with 0 minutes).

ALP is a type II membrane protein, delivered as a proenzyme to the vacuole and cleaved rapidly near the C-terminus to yield mature enzyme. Like the *arf1* mutation, the *gcs1* mutation delayed ALP processing from the precursor to mature vacuolar form (Fig. 1, upper panel. Compare 5 minutes of chase time with 0 minutes). Unlike ARF1, however, Gcs1p was not required for efficient export of invertase at non-permissive (15°C) or permissive (30°C) temperatures (data not shown). Wang et al. (Wang et al., 1996) reported that endocytosis of the vital dye FM4-64 was impaired in the *gcs1* mutant at non-permissive temperature (15°C), but neither FM4-64 nor Lucifer yellow uptake was blocked in our *gcs1*-null mutants at 15°C or 30°C (data not shown). Thus, in the *gcs1* mutant, the kinetics of vesicular trafficking of ALP was specifically disturbed, without apparent alteration of ER to Golgi or endocytic pathways.

Subcellular localization of endogenous Gcs1p

Among total cellular proteins, antibodies prepared against Gcs1p reacted only with a protein of ~39 kDa, the expected size for Gcs1p (Fig. 2A, lower panel). This protein was not detected in a *gcs1* mutant (Fig. 2A) or by the preimmune serum (not shown). Immunoblotting with this antiserum detected nanogram amounts of Gcs1p (Fig. 2B) as well as various mutant forms of Gcs1p (Fig. 2C).

Gcs1p, ARF1, Kar2 (ER marker), Emp47 (Golgi marker), porin (mitochondrial marker) and PGK (cytoplasmic marker) in subcellular fractions separated by sucrose density gradient centrifugation were identified by western blot analysis (Fig. 3). Most of the Gcs1p in yeast grown in minimal medium was near the top of the gradient – and was apparently cytoplasmic – as was the majority of ARF1 and PGK (Fig. 3A). However, much of the Gcs1p in yeast grown in YPD medium was found in denser fractions, which contained ER, Golgi and mitochondrial markers (Fig. 3B). By immunofluorescence microscopy, endogenous Gcs1p in yeast grown in minimal medium was concentrated largely in the perinuclear region (Fig. 3A,C, arrowheads), whereas in yeast grown in YPD medium, it was more widely distributed through the cell in a fine punctate pattern (Fig. 3B,C). Although, distribution of the mitochondrial marker porin appeared very similar in wild-type and *gcs1* mutant cells grown in minimal medium (Fig. 3A,D,E), it differed

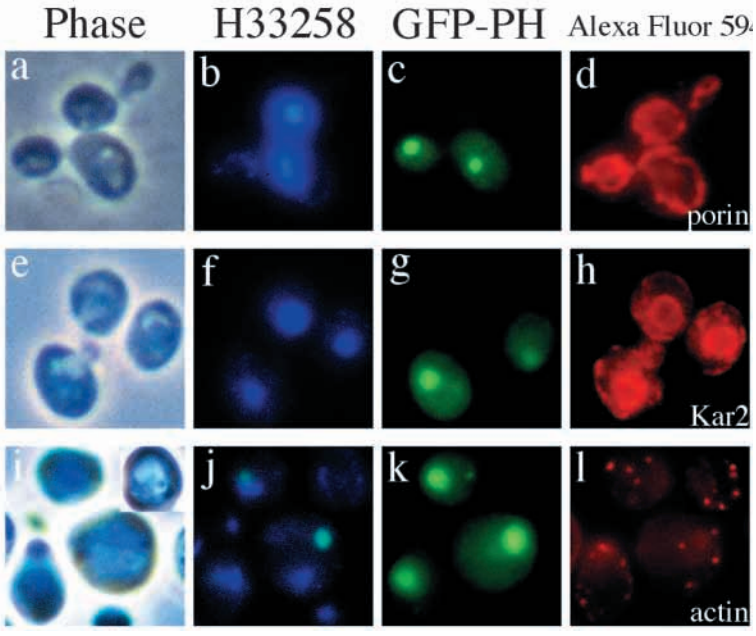

Fig. 8. Overexpression of GFP-fused PH domain of Gcs1p did not affect distribution of mitochondrial or ER markers or actin. Immunofluorescence staining of *gcs1* cells overexpressing GFP-fused PH domain and reacted with purified anti-Gcs1p antibody (c, g and k), anti-porin antibody (d), anti-Kar2 antibody (h) or Alexa Fluor™ 594-phalloidin (l), or stained with H33258 (b, f and j).

markedly when cells were grown in YPD medium (Fig. 3B,D,E). Of the endogenous Gcs1p, only a minor part appeared to be localized to mitochondrial regions (Fig. 3, arrows).

Mitochondrial morphology was altered in yeast overexpressing Gcs1p

Using gradient fractionation of the lysate of *gcs1* mutant yeast expressing recombinant wild-type *GCS1* under the control of the *ADH*1 promoter, Gcs1p was significantly more concentrated in the denser fractions (Fig. 4) than it was in wild-type cells (Fig. 3A). Most of the Gcs1p apparently comigrated with mitochondrial marker (porin) and ER marker (Kar2) proteins. Using a Gcs1p-specific antibody, we observed that overexpressed Gcs1p in yeast was localized partially with the mitochondria (Fig. 4B, arrowheads). Staining nucleic acids with H33258 showed that some of the Gcs1p had perinuclear localization (Fig. 4B,a, arrows), indicating that it might localize to the ER as well as the mitochondrial membrane.

Abnormal mitochondrial morphology was seen in cells overexpressing Gcs1p. Yeast mitochondria can form branched networks distributed evenly around the circumference of the cell in the peripheral cytoplasm (Yaffe, 1999). In cells overexpressing Gcs1p, mitochondria appeared as unbranched tubules or in several small patches (Fig. 4B,c, arrowheads). Moreover, enlarged mitochondria in yeast overexpressing Gcs1p did not have ER marker staining (data not shown), indicating that the mitochondria with which Gcs1p was associated were not wrapped in ER.

Gcs1p has been implicated in normal actin cytoskeletal organization in vivo and stimulates actin polymerization in vitro (Blader et al., 1999). Using Gcs1p-specific antibodies and monoclonal anti-actin antibodies, we observed that overexpressed Gcs1p was concentrated in patches in yeast and colocalized partially with the actin structures (Fig. 4C, arrowheads), suggesting that its overexpression affects mitochondrial morphology as a consequence of perturbing actin structures.

To evaluate the stability of the association of Gcs1p with membranes, the ER/mitochondria-enriched fraction (P13) from velocity sedimentation was extracted with 1 M NaCl or with 0.1 M Na_2CO_3, which solubilizes peripheral, but not integral, membrane proteins (Fujike et al., 1982). Gcs1p, like a peripheral membrane protein, was solubilized by Na_2CO_3 (pH 11), whereas porin, a mitochondrial outer membrane integral protein, remained membrane associated (Fig. 5).

C-terminal PH Domain, but not the zinc-finger motif, of Gcs1pis required for mitochondrial localization and the effect of overexpression on morphology

A mutant (Gcs1ΔZn) in which two cytsein residues in zinc-finger motiff had been replaced by alanine was expressed under the ADH promoter in the *gcs1* mutant cells. Similar to overexpressed wild-type Gcs1p (Fig. 4), the mutant was recovered in higher density fractions of the cell lysate that contained Kar2 and porin (Fig. 6). By immunofluorescence microscopy, Gcs1ΔZn, like overexpressed Gcs1p, was present in some tubular or spherical structures that also stained with anti-porin antibody (Fig. 6). Thus, the zinc-finger motif of Gcs1p was apparently not required for the effects of overexpressed wild-type Gcs1p on mitochondrial localization and morphology.

The C-terminal one-third of Gcs1p, reported to be important for progression from the stationary to G1 phase of the cell cycle (Ireland et al., 1994), was characterized as a PH domain homologue (Blader et al., 1999). Because PH domains mediate phospholipid and protein interactions (Gibson et al., 1994) that can be important for protein-membrane interactions, we suspected that deletion of the PH domain from Gcs1p might interfere with its mitochondrial localization. After expression in the *gcs1* mutant yeast, Gcs1ΔPH lacking 125 amino acids at the C-terminus corresponding to the PH domain was recovered in the least dense fractions of the lysate. Less Gcs1ΔPH was found than with the overexpressed wild-type Gcs1p (Fig. 4) in fractions containing ER and Golgi markers (Fig. 7). In cells, most of the Gcs1ΔPH mutant was concentrated in the perinuclear region (Fig. 7B), and mitochondrial morphology was similar to that in wild-type cells (Fig. 7C). To learn whether overexpression of the PH domain alone would reproduce the effects of overexpression of wild-type Gcs1p on mitochondrial morphology, GFP fused to the N-terminus of the PH domain was overexpressed in *gcs1* mutant yeast. GFP-PH did not colocalize with or affect the distribution of mitochondrial or ER markers or actin (Fig. 8).

Discussion

As reported here, the functions of Gcs1p and ARF1 in yeast vesicular transport were different, and overexpression of Gcs1p altered the mitochondrial morphology. Endogenous Gcs1p exhibited a fine punctate distribution in the perinuclear region when cells were grown in minimal medium, whereas it was distributed unevenly over the cytoplasm in a punctate pattern in cells grown in rich YPD medium, indicating that subcellular localization of Gcs1p could be altered by growth conditions. Mitochondria in *gcs1* mutant cells appeared to be more limited in lateral distribution and branching than in wild-type when yeast were grown in rich YPD medium (Fig. 3). Although endogenous Gcs1p could not be determined to be unequivocally localized to mitochondria, overexpressed Gcs1p was clearly associated in part with mitochondria and partially with perinuclear structures in close proximity to mitochondria. On the basis of these observations, it appears that the subcellular localization and perhaps function(s) of Gcs1p may depend on the physiological conditions of the cell.

Gcs1p functions as a GAP for ARF1 in *Saccharomyces cerevisiae* (Poon et al., 1996), although effects of the *gcs1* mutation on vesicular trafficking are still controversial (Poon et al., 1996; Poon et al., 1999; Blader et al., 1999). Some *gcs1* mutants exhibited defects in endocytic and exocytic pathways at the non-permissive temperature of 15°C (Poon et al., 1996; Wang et al., 1996). Poon et al. (Poon et al., 1999) later reported that Gcs1p was required for efficient maturation of CPY from the ER to the Golgi to the vacuole at the permissive temperature, although this phenotype was not observed by Blader et al. (Blader et al., 1999). We found that transport of the vacuolar enzyme ALP, but not CPY, was slowed in the *gcs1*-null mutant. Processing of both ALP and CPY was defective in the *arf1* and *glo3* mutants, indicating that ARF1 and Glo3p function in the same transport steps (our unpublished results). In contrast to earlier findings (Wang et al., 1996), fluid-phase endocytosis was normal in our *gcs1*-null mutant. The minor vesicular transport defect in the *gcs1* mutant, together with the report that Glo3p is the predominant GAP required for retrieval of ER proteins (Poon

et al., 1996; Dogic et al., 1990), suggests that Gcs1p is not critically involved in ARF1-mediated trafficking. The recent report that COPI subunits can interact with the ARF-GAP Glo3p, but not with Gcs1p (Eugster et al., 2000), is consistent with our conclusion.

Although Gcs1p was identified as a gene product required for re-entry into the cell cycle from the stationary phase at the nonpermissive temperature of 15°C (Ireland et al., 1994), our *gcs1*-null strains did not exhibit this phenotype, perhaps because of different yeast genetic backgrounds. The W303 strain used by Ireland (Ireland et al., 1994) was more dependent on Tlg1p for Golgi function than was the SEY6210 strain (Lewis et al., 2000). The W303 cells were also more sensitive to disruption of *YPT6*, a gene whose mutant phenotype is strikingly similar to that of *tlg1*. This is due to mutation of the SSD1 locus in W303 (Li and Warner, 1996; Tsukada and Gallwitz, 1996). Slight inhibition of invertase secretion by mutant yeast at non-permissive temperature could be strain-dependent (Tsukada et al., 1999). If Gcs1p functions in the secretory pathway, cells lacking this GAP would be expected to display a secretion defect also at a permissive growth temperature, but secretion defects in Gcs1p-lacking cells were not reported (Poon et al., 1999; Dogic et al., 1999) or found in our studies.

Because endogenous Gcs1p was found mainly in a punctate distribution in the cytoplasm, and relatively little was associated with mitochondria, it was surprising that the overexpressed Gcs1p was clearly localized partially with mitochondria and partially in the perinuclear region. Based on its solubilization with Na_2CO_3, the overexpressed Gcs1p appeared to be associated with the mitochondrial outer membrane as a peripheral membrane protein. The Gcs1ΔZn mutant, but not Gcs1ΔPH, was also partially localized to mitochondria, indicating that the PH domain, but not the zinc-finger motif, of Gcs1p could be important for mitochondrial association. Most of the overexpressed Gcs1ΔPH was found in the perinuclear region, indicating that structural elements required for the perinuclear distribution must reside outside of the PH domain. Several types of evidence suggest that there may be a hitherto unrecognized protein trafficking pathway between the ER and the mitochondria (Soltys et al., 1999). Mitochondrial matrix proteins were found at extra-mitochondrial locations, which raised the possibility of integrating mitochondria into vesicular trafficking pathways (Soltys et al., 1999). A glycoprotein synthesized in the rat liver ER was subsequently present in mitochondria (Chandra et al., 1998).

In yeast overexpressing Gcs1p or Gcs1ΔZn, the mitochondrial reticulum either collapsed into spherical organelles or formed an unbranched tubular structure at one side of the cell. In *gcs1* mutant yeast overexpressing Gcs1ΔPH or the PH domain of Gcs1p, the mitochondria appeared to be normal. The mitochondria also appeared to be normal in yeast overexpressing Glo3p, suggesting that Gcs1p and Glo3p do not have overlapping functions, at least in regard to mitochondrial morphology (C.-F.H., C.-C.C., L. T., L.-M.B. and F.J.S.L., unpublished). Mitochondria in wild-type yeast can form branched networks located in the peripheral cytoplasm and distributed evenly around the circumference of the cell (Hermann and Shaw, 1998). Mitochondria that have lost cytoskeletal attachments collapse into spherical organelles (Yaffe, 1999). Gcs1p contains a region with a sequence that resembles an actin-binding domain in the ERM protein family (Mangeat et al., 1999) and interacts with actin in vitro (Blader et al., 1999). Blader et al. (Blader et al., 1999) reported that Gcs1p inhibited the depolymerization of actin filaments. Overexpression of Gcs1p might, therefore, perturb the normal actin polymerization-depolymerization cycle with resulting effects on organelle morphology. Thus, the abnormal mitochondrial morphology observed in cells overexpressing Gcs1p may be due to an indirect effect on the actin cytoskeleton rather than a direct action on the mitochondria. Our understanding of the mechanism(s) responsible for this altered morphology awaits further investigation.

Yeast two-hybrid analysis revealed that Gcs1p could also interact with Akr1p, another cytoskeletal protein that may be involved in endocytosis (Kao et al., 1996). Thus, Gcs1p could be a peripheral membrane protein that interacts with parts of the cytoskeletal network to maintain mitochondrial structure. Mutations in the conserved GTP-binding motif in dynamin-related proteins, such as Drp1 and DNM1, induced rearrangement of the mitochondrial networks into large perinuclear tubules or a linear bundle of tubules aligned along one side of the cell (Smirnova et al., 1998; Otsuga et al., 1998). Overexpression of Gcs1p in yeast may similarly interfere with the normal dynamics of mitochondrial organization, producing phenotypes that resemble those associated with mutation of dynamin-like GTPases.

Gcs1p is structurally and functionally related to centaurin-α, a mammalian phosphatidylinositol 3,4,5-trisphosphate-binding protein, which has also been implicated in normal actin cytoskeletal organization (Venkateswarlu et al., 1999). Phosphatidylinositol phosphates have fundamental cell functions in signal transduction, membrane trafficking and cytoskeletal remodeling (De Camilli et al., 1996). Growing evidence suggests that phosphorylation-dephosphorylation of phosphoinositides in specific intracellular locations signals either the recruitment or the activation of proteins essential for vesicular transport. Crosstalk between phosphatidylinositol metabolites and GTPases is an important property of these regulatory mechanisms. ARF was reported to mediate recruitment of phosphatidylinositol 4-kinase and stimulate synthesis of phosphatidylinositol 4,5-bis-phosphate on the Golgi complex, independently of its activities on coat proteins and PLD (Godi et al., 1999).

Although specialized ER-like membranes complexed to mitochondria have been described (Rusinol et al., 1994), the regulation of interactions between the ER and the mitochondria is still unclear. Recent findings by Prinz et al. (Prinz et al., 2000) suggest that the membranes of the ER and mitochondria in yeast are linked at certain sites and that the ER may have an effect on the structure of mitochondria. Identification of Gcs1p localized to the perinuclear region and mitochondria, as well as its effects on mitochondrial morphology, leads us to speculate that Gcs1p is a mutifunctional molecule (like ARF GEFs), which may also act at different intracellular membranes to regulate actin cytoskeleton organization. Integration of these unexpected findings into an understanding of the role of Gcs1p in overall actin cytoskeletal organization and intracellular membrane dynamics remains a challenge for future studies.

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