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GCS1, an Arf Guanosine Triphosphatase-activating Protein in *Saccharomyces cerevisiae*, Is Required for Normal Actin Cytoskeletal Organization In Vivo and Stimulates Actin Polymerization In Vitro

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Recent cloning of a rat brain phosphatidylinositol 3,4,5-trisphosphate binding protein, centaurin α, identified a novel gene family based on homology to an amino-terminal zinc-binding domain. In *Saccharomyces cerevisiae*, the protein with the highest homology to centaurin α is Gcs1p, the product of the *GCS1* gene. *GCS1* was originally identified as a gene conditionally required for the reentry of cells into the cell cycle after stationary phase growth. Gcs1p was previously characterized as a guanosine triphosphatase-activating protein for the small guanosine triphosphatase Arf1, and *gcs1* mutants displayed vesicle-trafficking defects. Here, we have shown that similar to centaurin α, recombinant Gcs1p bound phosphoinositide-based affinity resins with high affinity and specificity. A novel *GCS1* disruption strain (*gcs1*Δ) exhibited morphological defects, as well as mislocalization of cortical actin patches. *gcs1*Δ was hypersensitive to the actin monomer-sequestering drug, latrunculin-B. Synthetic lethality was observed between null alleles of *GCS1* and *SLA2*, the gene encoding a protein involved in stabilization of the actin cytoskeleton. In addition, synthetic growth defects were observed between null alleles of *GCS1* and *SAC6*, the gene encoding the yeast fimbrin homologue. Recombinant Gcs1p bound to actin filaments, stimulated actin polymerization, and inhibited actin depolymerization in vitro. These data provide in vivo and in vitro evidence that Gcs1p interacts directly with the actin cytoskeleton in *S. cerevisiae*.

INTRODUCTION

Inositol lipids are involved in diverse pathways in eukaryotic cells, acting as membrane localization signals, working as cofactors for numerous enzymes, serving as substrates for the production of second messengers, and functioning as bona fide second messengers (for reviews see Lee and Rhee, 1995; De Camilli *et al.*, 1996; Toker and Cantley, 1997). Recent interest has focused on the D-3 phosphoinositides: phosphatidylinositol (PtdIns) 3-phosphate (PtdIns(3)P), PtdIns 3,4-bisphosphate (PtdIns(3,4)P_2), PtdIns 3,5-bisphosphate (PtdIns(3,5)P_2), and PtdIns 3,4,5-trisphosphate (PtdIns(3,4,5)P_3), which are synthesized by constitutively active or receptor-stimulated phosphoinositide 3-kinases. Phosphoinositide 3-kinases are required for

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many fundamental cellular processes, including cell growth and survival, vesicular trafficking, and cytoskeletal organization (reviewed by Vanhaesebroeck *et al.*, 1996; Toker and Cantley, 1997).

Regulation of these cellular processes is presumably mediated by the interaction of D-3 phosphoinositides with specific intracellular targets (Theibert *et al.*, 1997). Numerous candidate targets for D-3 phosphoinositides have now been identified, including protein kinases and proteins involved in the regulation of vesicle trafficking and the actin cytoskeleton (see references in Toker and Cantley, 1997). Our laboratory has identified and cloned a rat brain PtdIns(3,4,5)P_3-binding protein, centaurin α, (Hammonds-Odie *et al.*, 1996), and two related PtdIns(3,4,5)P_3-binding proteins were subsequently identified (Stricker *et al.*, 1997; Tanaka *et al.*, 1997). The deduced amino acid sequence predicts that centaurin α contains a pleckstrin homology (PH) domain and a putative zinc-binding domain (Hammonds-Odie *et al.*, 1996). PH domains have been implicated in phosphoinositide binding in a variety of proteins (Gibson *et al.*, 1994; Klarlund *et al.*, 1997). Within the zinc-binding domain, centaurin α is similar to numerous proteins (Hammonds-Odie *et al.*, 1996), including a rat liver Arf1 guanosine triphosphatase (GTPase)-activating protein (GAP) (Cukierman *et al.*, 1995) and several yeast proteins (Ireland *et al.*, 1994; Zhang *et al.*, 1998).

In *Saccharomyces cerevisiae*, the protein with the highest degree of structural homology to centaurin α is Gcs1p. The *GCS1* gene was originally identified as a cold-sensitive mutant that failed to resume logarithmic growth from stationary phase, a G_0 to G_1 progression (Ireland *et al.*, 1994). Johnston and co-workers have shown that mutant *gcs1* cells lose mitochondrial activity (Filipak *et al.*, 1992) and exhibit vesicle trafficking defects at the nonpermissive 15°C temperature (Wang *et al.*, 1996). Biochemically, Gcs1p displays Arf1p GAP activity (Poon *et al.*, 1996) that has been localized to the zinc-binding domain (Antonny *et al.*, 1997). Deletion of *GCS1* in an *arf1* null background results in a strong synthetic growth defect (Poon *et al.*, 1996). In addition, overexpression of *GCS1* or several related proteins, including *GLO3* and *SAT1*, rescues an *arf1* temperature-sensitive (t.s.) mutant (Zhang *et al.*, 1998).

Arfs are members of the Ras GTPase superfamily that have been implicated in regulation of vesicle trafficking and the actin cytoskeleton in mammalian cells. Arfs have been shown to function in endoplasmic reticulum and Golgi transport, endocytosis, and exocytosis (Boman and Kahn, 1995). In vitro, mammalian Arfs are required for the recruitment of coat proteins in various vesicle-budding assays (Orci *et al.*, 1993; Faundez *et al.*, 1997) and stimulate phospholipase D activity (reviewed by Cockcroft, 1996). In mammalian cells, Arf6 is localized to the plasma membrane, and overexpression leads to alterations in the actin cytoskeleton (Radharkrishna *et al.*, 1996; D'Souza-Schorey *et al.*, 1997). Although several yeast Arf proteins have been characterized and implicated in the secretory pathway (Stearns *et al.*, 1990; Lee *et al.*, 1994), the mechanisms by which these Arfs function in vesicle trafficking and whether they are involved in regulation of the actin cytoskeleton in yeast are unresolved issues.

In addition to the conserved zinc-binding and PH domains, centaurin α and several centaurin homologues contain ankyrin repeats and an ezrin/radixin/moesin (ERM) homology domain, suggesting that they may interact with the actin cytoskeleton (Hammonds-Odie *et al.*, 1996). To investigate whether this protein family may function in vivo via interactions with the actin cytoskeleton, we focused the current study on *GCS1*. In this report, we demonstrate that Gcs1p binds phosphoinositides, consistent with the presence of a PH domain. Next, we provide morphological, pharmacological, genetic, and biochemical evidence that support a role for Gcs1p in regulation of the actin cytoskeleton in *S. cerevisiae*.

MATERIALS AND METHODS

All chemicals, purchased from Sigma Chemical (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA), were of the highest grade available unless otherwise indicated. ENHANCE was from Dupont-New England Nuclear (Boston, MA). Oxalyticase was from Enzogenetics (Corwallis, OR). Latrunculin-B (Lat-B) was from Calbiochem (San Diego, CA), and rhodamine phalloidin was from Molecular Probes (Eugene, OR).

Strains and Growth Medium

The genotypes of the strains used in this study are listed in Table 1. YPD, yeast minimal medium, presporulation medium, and sporulation medium have been described previously (Kaiser *et al.*, 1994). To generate the *gcs1*Δ strain, the 5′- and 3′-regions of *GCS1* were generated from yeast genomic DNA by PCR using the following sets of primers: 1) 5′-GGGAATTCTTATAAGCAGA TCTTTGGGGC-3′ (16A-1) and 5′-GGGGATCCCCCATACGAAGAAGTTCCTCCGG-3′ (16A-2) and 2) 5′-GGGGATCCAGGCCGAGGACAAATGGGACG-3′ (16A-3) and 5′-GGGCATGCATG TCAATAAGTAAGTGCCGC-3′ (16A-4), respectively (identity to the *GCS1* gene is underlined). To generate pGCS-HIS3, the PCR products were cloned sequentially into the pTZ18 vector, after which the *HIS3* gene was cloned into the *Bam*HI site generated by the PCR primers.

Gene disruptions were generated by digesting pGCS-HIS3 with *Eco*RI and *Sph*I. Wild-type yeast was transformed by the lithium acetate method and plated onto selective (−histidine) media plates. Colonies were picked and screened by PCR using a primer whose sequence lies outside of the region disrupted: 5′-TCATGCTGACGACGTAC-3′ and 16A-4. Positive clones were backcrossed three times to an isogenic parental wild-type strain. Tetrads from a heterozygous *GCS1/GCS1::HIS3* diploid strain were analyzed to determine whether the *GCS1::HIS3* disruption segregated with mutant phenotypes (Lat-B sensitivity, NaCl sensitivity, and actin mislocalization). At least 20 tetrads were analyzed from each cross, and it was determined that the *GCS1::HIS3* disruption segregated with the mutant phenotypes. One wild-type (YAT1) and one *gcs1*Δ (YAT2) spore were chosen and used throughout this study.

Table 1. Yeast strains used in this study

Strain	Genotype	Source
CTY182	Mat **a,** *ura3-52 Δhis3-200, lys2-801$_{AM}$*	V. A. Bankaitis
CTY3	Mat α, *ura3-52, Δhis3-200, Δtrp1, ade3*	V. A. Bankaitis
YAT1	Mat α, *ura3-52, Δhis3-200, Δtrp1*	This study
YAT2	Mat **a,** *ura3-52, Δhis3-200, Δtrp1, GCS1::HIS3*	This study
YAT3	Mat **a,** *ura3-52, Δhis3-200, Δtrp1, GLO3::HIS3*	This study
W303-1a	Mat **a,** *leu2-3,112, ura3-1, his3-11, trp1-1, ade2-1*	G. C. Johnston
GWK9a	Mat **a,** *leu2-3,112, ura3-1, his3-11, trp1-1, ade2-1, gcs1Δ::URA3*	G. C. Johnston
PPY147.28.2C	Mat α, *leu2-3,112, ura3-1, his3-11, trp1-1, ade2-1, gcs1Δ::URA3,* (pPY805.25)	G. C. Johnston
PPY147.28.2D	Mat **a,** *leu2-3,112, ura3-1, his3-11, trp1-1, ade2-1*	G. C. Johnston
DDY216	Mat α; Δ*sac6::URA3; lys2; trp1; his3; leu2; ura3*	D. G. Drubin
DDY318	Mat α; Δ*sac6::LEU2; lys2; his3; leu2; ura3*	D. G. Drubin
DDY322	Mat α; Δ*abp1::LEU2; Δhis3-200; leu2-3,112; ura3-52*	D. G. Drubin
DDY340	Mat α; *act1-104::HIS3; Δhis3-200; leu2-3,112; ura3-52; can1-1; tub2-201*	D. G. Drubin
DDY342	Mat α; *act1-113::HIS3; Δhis3-200; leu2-3,112; ura3-52; can1-1; tub2-201*	D. G. Drubin
DDY343	Mat α; *act1-115::HIS3; Δhis3-200; leu2-3,112; ura3-52; ade2-101; can1-1; tub2-201*	D. G. Drubin
DDY344	Mat α; *act1-116::HIS3; Δhis3-200; leu2-3,112; ura3-52; can1-1; tub2-201*	D. G. Drubin
DDY351	Mat α; *act1-129::HIS3; Δhis3-200; leu2-3,112; ura3-52; can1-1; tub2-201*	D. G. Drubin
DDY546	Mat α; Δ*sla2::URA3; Δhis3-200; leu2-3,112; lys2-801$_{am}$; ura3-52*	D. G. Drubin
DDY950	Mat α; Δ*rvs167::TRP1; lys2; trp1; leu2; ura3*	D. G. Drubin

Growth Assays

To assess the reentry phenotype, cells were grown for 5 d and then diluted to early-log phase in fresh YPD and shifted to the indicated temperatures. The criteria for assessing stationary phase were consistent with those of Singer and co-workers (Drebot *et al.*, 1987). Wild-type cells were assessed by light microscopy, and 90% of the cells were unbudded and reached maximal density at least 48 h earlier. Culture densities was measured by diluting cell aliquots into sonication buffer (PBS containing 1 mM EDTA and 1 mM EGTA) and sonicating for 10 s to disperse cell clumps (Pringle and Mor, 1975). Dilutions were performed such that the optical density measured 0.1–0.6 U as measured in a spectrophotometer at 595 nm.

Gcs1p Fusion Protein Purification

pQE-GCS1, a plasmid encoding for a His_6-Gcs1p fusion protein, was generated by isolating a full-length *GCS1* PCR product using the following primers: 1) 5′-GGGGATCCATGTCAGATTGGAAAGTGG-3′ and 2) 5′-GGGCATGCTTAGAAATCGTCCCATTTGTCC-3′ (underlined regions indicate identity to *GCS1*). The PCR product was gel purified and ligated in frame into the *Bam*HI/*Sph*I sites of the pQE-30 His tagged vector (Qiagen, Chatsworth, CA).

Overnight cultures of pQE-GCS1 or pQE-40 (His_6-DHFR) were diluted 1:50 into 2 l of LB medium supplemented with 100 μg/ml ampicillin and grown uninduced for a further 8 h at 37°C. Cells were collected by centrifugation, and either native or denatured fusion proteins were batch purified using Ni^+-NTA agarose beads. Denatured fusion protein was purified as described by the manufacturer and dialyzed for 12 h against 4 M urea, 0.1 M sodium phosphate, 0.01 M Tris, pH 8.0, followed by 12 h against 2 M urea, 0.01 M Tris, pH 8.0, and then twice more for 12 h against 0.01 M Tris, pH 8.0. Native protein was purified by lysing cells at 4°C for 90 min in 50 mM sodium phosphate, pH 7.8, 300 mM NaCl (buffer A) supplemented with 5 μg/ml lysozyme and 1% Triton X-100. Lysates were clarified by centrifugation and incubated with Ni^+-NTA agarose beads in binding buffer (10 mM imidazole in buffer A) for at least 2 h at 4°C. Beads were washed extensively with binding buffer followed by stepwise washes with Triton X-100 to a final concentration of 0.1%. Protein was eluted 200 mM imidazole, 0.1% Triton X-100 buffer A and dialyzed twice for 12 h against 10 mM Tris, pH 7.4, 0.1% Triton X-100 final concentration. Protein concentrations were determined by both SDS-PAGE and Coomassie Protein Assay (Pierce Chemical). To determine whether His_6-Gcs1p fusion protein was biologically active, we assessed GAP activity by performing Arf1 GAP assays with recombinant Arf1p as described previously (Poon *et al.*, 1996) and detected GAP activity similar to that reported (our unpublished results).

Polyclonal Antibody Production

Anti-Gcs1p antisera were prepared by immunization with the Ni^{2+}-NTA–purified denatured His_6-Gcs1p fusion protein. The antigen was injected by Southern Biotechnology Associates (Birmingham, AL) into a rabbit using a standard immunization protocol.

Yeast Cell Extract Preparation and Immunoblotting

Yeast whole-cell extracts were prepared as described by Kaiser *et al.* (1994). Briefly, midlogarithmic cells were resuspended in SDS-PAGE sample buffer and boiled for 3 min. Glass beads were added, and the cells were vortexed vigorously for 2 min. Samples were boiled a second time for 3 min and were separated by SDS-PAGE. After transfer to nitrocellulose membranes, the lysates were immunoblotted using a 1:10,000 dilution of the anti-Gcs1p antisera.

Phosphoinositide-Binding Assays

Samples (300 μl) of Ni^{2+}-NTA–purified fusion protein were incubated with 100 μl of a 1:1 slurry of Affigel-conjugated aminopropyl-inositol(1,3,4,5)P_4 (aminopropyl-InsP_4) (Hammonds-Odie *et al.*, 1996) for 1 h at 4°C in binding buffer (10 mM Tris, pH 7.4, 50 mM NaCl). The beads were pelleted, the flow-through collected, and the beads were then washed in 1 ml of binding buffer. Protein was eluted from the resin by incubating the beads with 150 μl of SDS-PAGE sample buffer. Samples were separated by 10% SDS-PAGE, transferred to nitrocellulose, and immunoblotted using the anti–RGS-His_6 antibody (Qiagen). The presence of two additional bands in the "eluate," which were more intense than the total fraction, suggests that His_6-Gcs1p underwent some degradation during the assay. To determine the affinity of His_6-Gcs1p to various phosphoinositides, competition binding assays were performed with the addition of phosphoinositides from a 10× stock to the binding reaction. Phosphoinositides with dipalmitoyl groups, PtdIns(3,5)P_2, and PtdIns(3,4,5)P_3, synthesized as described previously (Chen *et*

al., 1996, 1998; Gu and Prestwich, 1996; Prestwich, 1996; Peng and Prestwich, 1998), were a generous gift from Echelon Research Laboratories (Salt Lake City, UT). PtdIns(3)P and PtdIns(3,4,5)P_3 were from Matraya (Pleasant Gap, PA). PtdIns, PtdIns(4)P, and PtdIns(4,5)P_2 were from Sigma Chemical. Blots were quantified using a Bio-Rad (Richmond, CA) densitometer.

Photolabeling was performed essentially as previously described (Hammonds-Odie *et al.*, 1996) using fusion protein eluted from the aminopropyl-InsP_4 column with 1.5 M NaCl. Briefly, 100 ng of purified fusion protein in 10 mM Tris, pH 7.4, 1 mM EDTA were incubated with 110 nCi of [^{3}H](3-[4-benzoyldihydrocinnamidyl]-propyl)-inositol tetrakisphosphate ([^{3}H]BZDC-Ins(1,3,4,5)P_4) photoprobe (30 Ci/mM) in a final volume of 50 μl. Displacement was determined using the indicated concentrations of unlabeled phosphoinositides. Mixtures were exposed to 360 nm UV light for 1 h on ice, and the reactions were terminated with the addition of SDS sample buffer. Reactions were separated by 10% SDS-PAGE, and the gels were fixed and prepared for fluorography using the Enhance system. Autoradiographs were analyzed using a Bio-Rad densitometer.

Fluorescence Microscopy

Cells were grown overnight at 30°C and then diluted into YPD to early-log phase (0.2 OD/ml). After 6 h (mid-log phase), formaldehyde was added directly to the culture (3% final concentration) and incubated for 30 min at room temperature. Cells were resuspended in sonication buffer + 3% formaldehyde, sonicated for 10 s to disperse cell clumps, and incubated overnight at room temperature. Cells were washed three times in sonication buffer.

The actin cytoskeleton was visualized with rhodamine phalloidin (Molecular Probes) as previously described (Pringle *et al.*, 1989). Cells were visualized with a 100× objective using a Leica DMRB microscope. All fields were photographed for equivalent exposure times at 400 ASA using Ilford δ 400 black and white film with a Leica Wild-MPS52 camera equipped with a 10× multiplier tube. Prints were developed to optimize visualization of the actin patches and cables.

To quantify the number of actin patches, the mother and bud cells of at least 200 randomly selected budding cells were counted by focusing up and down through the cell. Small buds were identified to be no more than 30% the size of the mother cell, and large buds were those larger than 30% of the mother cell. A standard two-tailed Student's *t* test was employed to determine whether there was a significant change in the distribution of the number of patches between wild-type and *gcs1*Δ cells.

Halo Assays

Sensitivity to Lat-B was performed essentially as previously described (Ayscough *et al.*, 1997). Briefly, 10 μl of midlogarithmic growth phase cells were added to 2 ml of 2× YPD or the appropriate selective minimal media, after which 2 ml of 1% agar were added to the cells, and the mixture was poured onto the surface of YPD or selective minimal media plates. Lat-B was diluted into DMSO and 4 μl of vehicle or the indicated concentration of Lat-B were pipetted onto a 6-mm filter disk (Scientific Specialties Group, Mt. Holly, PA), which was placed onto the top agar. Plates were placed at the indicated temperatures for 24 (YPD) to 72 (minimal media) h. Relative sensitivity was calculated as described previously (Reneke *et al.*, 1988).

Genetic Interactions

All procedures were essentially as described by Sherman *et al.*, (1986). Haploid mat α *gcs1*Δ yeast were crossed with haploid mat α yeast containing either mutant alleles of the *ACT1* gene or deletions in the *SLA2, SAC6, ABP1*, or *RVS167* genes (Table 1). Heterozygous diploids were selected on synthetic plates, and two separate colonies from each cross were chosen for culture and subsequent sporulation. The tetrad spores were dissected onto YPD plates and grown at 26°C for 4–5 d. Auxotrophies and temperature sensitivities were tested by frogging onto synthetic media or YPD.

Actin Cosedimentation

To assess the binding of Gcs1p to filamentous actin (F-actin), an actin cosedimentation assay was performed, similar to that described by Yao *et al.* (1996). F-actin was incubated with His_6-Gcs1p (prespun at 270,000 × *g* for 10 min at room temperature) in a total volume of 25 μl for 30 min at room temperature. All buffers and His_6-Gcs1p were supplemented with 0.1 volume of 10× F-buffer, and the Triton X-100 final concentration was 0.06%, which did not effect actin polymerization. The reactions were centrifuged at 270,000 × *g* for 10 min at 25°C. Supernatants were removed and the pellets were resuspended in SDS-sample buffer, and proteins were separated by 10% SDS-PAGE. After transfer to nitrocellulose, the His_6-Gcs1p was detected using the anti-RGS-His_4 antibody (Qiagen).

Light Scattering Assays

Monomeric actin in G-buffer (5 mM Tris [pH 7.4], 0.2 mM $CaCl_2$, 20 μM ATP, 20 μM DTT) was prespun at 270,000 × *g* at 4°C for 1 h; 10× initiation buffer (1 M KCl, 20 mM $MgCl_2$, 5 mM ATP) was added to initiate polymerization in the absence or presence of His_6-Gcs1p, and light scattering was measured at a 90° angle in a PTI Deltascan Fluorescent Spectrophotometer (Piscataway, NJ) at 400 nm. Actin depolymerization was performed by diluting 2 μM F-actin 20-fold into G-buffer in the absence or presence of His_6-Gcs1p.

Pyrene Actin Polymerization Assays

Actin polymerization was performed as described by the manufacturer (Cytoskeleton, Denver, CO). Briefly, 5 μM final concentration of monomeric actin (1:10 pyrene labeled) was incubated on ice for 10 min with the indicated concentrations of His_6-Gcs1p or His_6-DHFR. Samples were then equilibrated 10 min in a fluorescent spectrophotometer (ISS, Champaign, IL), after which polymerization was induced by the addition of KCl, $MgCl_2$, and ATP.

RESULTS

Gcs1p Contains a Putative Zinc-Binding Domain, a PH Domain, and ERM Homology Domain

Centaurin α is a mammalian brain PtdIns(3,4,5)P_3-binding protein that has an N-terminal cysteine-rich putative zinc-binding domain, a C-terminal PH domain, and homology to the actin-binding domain of the ERM family of cytoskeletal proteins (Hammonds-

Figure 1 (facing page). Sequence comparison of *GCS1*. (A) Domain structure of Gcs1p. (B) Comparison of the $CxxCx_{16}CxxC$ amino-terminal zinc-binding domain of *GCS1* to rat brain centaurin α, the yeast proteins *GLO3, GTS1,* and *SPS18,* and the rat liver Arf GAP. (C) Comparison of Gcs1p with merlin and the ERM family of proteins: ezrin, radixin, and moesin. (D) Comparison of the PH domain from *GCS1* to six yeast proteins that contain PH domains according to the Stanford *Saccharomyces* Genome Database (SGD): *NUM1*, *BEM2*, *OSH1*, *BUD4*, *BEM3*, and *BOB1*. Shaded amino acids represent conserved amino acids in a majority of the family members; capital letters are amino acids conserved between *GCS1* and other proteins. Conserved amino acids belong to the same Dayhoff group (GPAST, MILV, KRH, NQED, FWY, C).

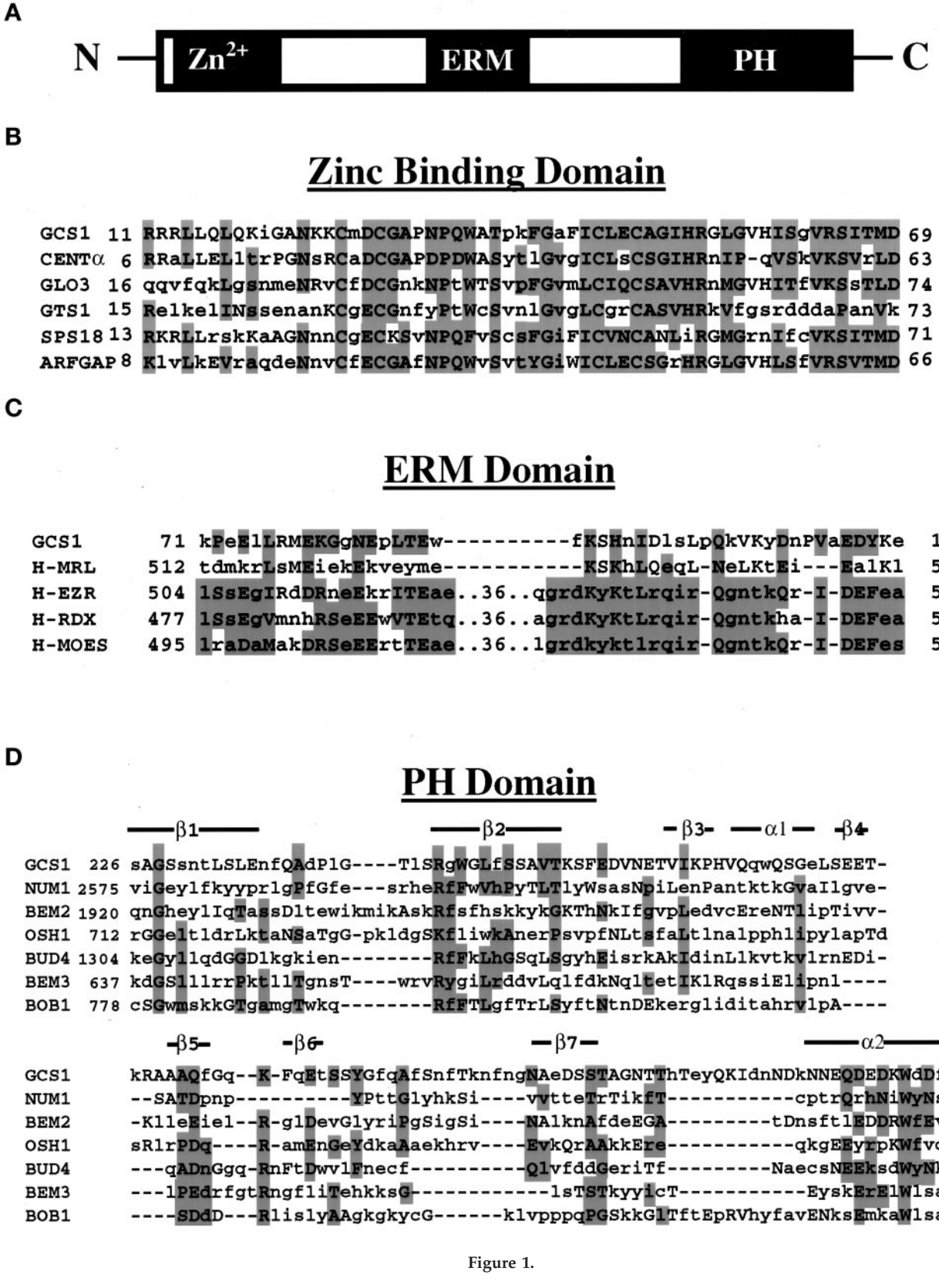

A
N
Zn2+
ERM
PH
C
B
Zinc Binding Domain
GCS1 11 RRRLLQLQKiGANKKCmDCGAPNPQWATpkFGaFICLECAGIHRGLGVHISgVRSITMD 69
CENTα 6 RRaLLELltrPGNsRCaDCGAPDPDWASytlGvgICLsCSGIHRnIP-qVSkVKSVrLD 63
GLO3 16 qqvfqkLgsnmeNRvCfDCGnkNPtWTSvpFGvmLCIQCSAVHRnMGVHITfVKSsTLD 74
GTS1 15 RelkelINssenanKCgECGnfyPtWcSvnlGvgLCgrCASVHRkVfgsrdddaPanVk 73
SPS18 13 RKRLLrskKaAGNnnCgECKSvNPQFvScsFGiFICVNCANLiRGMGrnIfcVKSITMD 71
ARFGAP 8 KlvLkEVraqdeNnvCfECGAfNPQWvSvtYGiWICLECSGrHRGLGVHLSfVRSVTMD 66
C
ERM Domain
GCS1 71 kPeElLRMEKGgNEpLTEw----------fKSHnIDlsLpQkVKyDnPVaEDYKe 116
H-MRL 512 tdmkrLsMEiekEkveyme----------KSKhLQeqL-NeLKtEi---EalKl 557
H-EZR 504 lSsEgIRdDRneEkrITEae..36..qgrdKyKtLrqir-QgntkQr-I-DEFea 585
H-RDX 477 lSsEgVmnhRSeEEwVTEtq..36..agrdKyKtLrqir-Qgntkha-I-DEFea 558
H-MOES 495 lraDaMakDRSeEErtTEae..36..lgrdkyktlrqir-QgntkQr-I-DEFes 576
D
PH Domain
β1 β2 β3 α1 β4
GCS1 226 sAGSsntLSLEnfQAdPlG----TlSRgWGLfSSAVTKSFEDVNETVIKPHVQqwQSGeLSEET-
NUM1 2575 viGeylfkyyprlgPfGfe---srheRfFwVhPyTLTlyWsasNpiLenPantktkGvaIlgve-
BEM2 1920 qnGheylIqTassDltewikmikAskRfsfhskkykGKThNkIfgvpLedvcEreNTlipTivv-
OSH1 712 rGGeltldrLktaNSaTgG-pkldgSKfliwkAnerPsvpfNLtsfaLtlnalpphlipylapTd
BUD4 1304 keGyllqdGGDlkgkien--------RfFkLhGSqLSgyhEisrkAkIdinLlkvtkvlrnEDi-
BEM3 637 kdGSlllrrPktllTgnsT----wrvRygiLrddvLqlfdkNqltetIKlRqssiElipnl----
BOB1 778 cSGwmskkGTgamgTwkq--------RfFTLgfTrLSyftNtnDEkergliditahrvlpA----
β5 β6 β7 α2
GCS1 kRAAAQfGq--K-FqEtSSYGfqAfSnfTknfngNAeDSSTAGNTThTeyQKIdnNDkNNEQDEDKWdDf 352
NUM1 --SATDpnp----------YPttGlyhkSi----vvtteTrTikfT----------cptrQrhNiWyNs 2678
BEM2 -KlleEiel--R-glDevGlyriPgSigSi----NAlknAfdeEGA---------tDnsftlEDDRWfEv 2038
OSH1 sRlrPDq----R-amEnGeYdkaAaekhrv----EvkQrAAkkEre----------qkgEEyrpKWfvq 823
BUD4 ---qADnGgq-RnFtDwvlFnecf----------QlvfddGeriTf---------NaecsNEEksdWyNk 1407
BEM3 ---lPEdrfgtRngfliTehkksG----------lsTSTkyyicT----------EyskErElWlsa 736
BOB1 ----SDdD---RlislyAAgkgkycG------klvpppqPGSkkGlTftEpRVhyfavENksEmkaWlsa 888

Figure 1.

Odie *et al.*, 1996). Database comparisons indicated that *GCS1* is the yeast gene that shares the highest degree of structural homology to centaurin α (Figure 1A). Gcs1p contains an amino-terminal $CxxCx_{16}CxxC$ putative zinc-binding domain (Figure 1B), which is 53% identical and 73% similar to centaurin α. Gcs1p is also 56% identical and 71% similar to a recently cloned rat liver Arf GAP (Cukierman *et al.*, 1995) in this region, which has been shown to contain the Arf GAP domain (Antonny *et al.*, 1997). A number of other yeast proteins, such as Glo3p and Sat1p, are homologous to Gcs1p in this region (Zhang *et al.*, 1998). In addition, Gcs1p contains a region homologous to an actin-binding domain in the ERM protein family (Figure 1C) (Turunen *et al.*, 1994). The ERM proteins function to link the actin cytoskeleton to the plasma membrane by binding actin via the C terminus to plasma membrane proteins, such as CD44, via the N terminus (Tsukita *et al.*, 1997). Furthermore, a PH domain consensus sequence is present in Gcs1p (Figure 1D). PH domains are functional motifs found in many signal-transduction and cytoskeletal proteins and have been shown to mediate phosphoinositide and protein interactions (Gibson *et al.*, 1994; Lemmon *et al.*, 1996; Klarlund *et al.*, 1997). Numerous yeast proteins, including several that regulate small GTPases, such as *BEM2*, *BEM3*, and *ROM2*, contain PH domains. The PH domain in Gcs1p is also related to the PH domain found in centaurin α; however, *GLO3* and the rat liver Arf GAP do not appear to contain this domain.

Gcs1p Binds Phosphoinositide-based Probes With High Affinity

The structural homology between centaurin α and *GCS1*, as well as the presence of a PH domain in Gcs1p, suggested that Gcs1p may bind phosphoinositides. The identification of phosphoinositide binding in centaurin α, the clathrin adaptor/assembly protein AP-2, and α-COP of the Golgi coatomer COPI complex was facilitated using phosphoinositide-based affinity probes such as aminopropyl-$InsP_4$ affigel (Hammonds-Odie *et al.*, 1996; Prestwich, 1996; Chaudhary *et al.*, 1998). The inositol polyphosphate head group is conjugated via an aminopropyl moiety to the matrix, which results in these probes having a higher hydrophobic character than the free inositol polyphosphate, and therefore presumably mimic the structure of a phosphoinositide (Hammonds-Odie *et al.*, 1996). Purification of recombinant His_6-Gcs1p from bacterial lysates yielded a major protein band at 45 kDa and two proteolytic fragments, all of which were recognized by antibodies against the fusion tag. This purified His_6-Gcs1p specifically and efficiently interacted with the aminopropyl-$InsP_4$ resin (Figure 2A). Approximately 80% of the total His_6-Gcs1p bound to the resin, while ~20% of the His_6-Gcs1p immunoreactivity was present in the "flow through" and "wash" fractions. Efficient recovery of the recombinant protein ("eluate") from the resin was effected using high-ionic strength buffer conditions that had been previously established for the recovery of centaurin α from the same affinity resin. No detectable bacterial proteins interacted with the resin under the binding conditions used, and the recombinant Gcs1p comprised all of the protein in the eluate fraction.

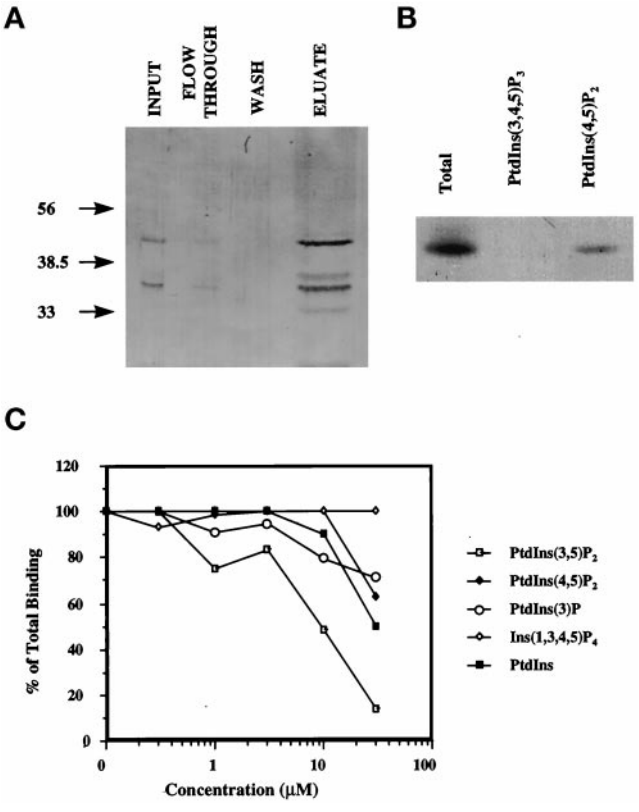


Figure 2. His_6-Gcs1p binding to PtdIns(3,4,5)P_3 analogs. (A) Ni^{2+}-NTA agarose-purified His_6-Gcs1p (total) was incubated with the Affigel-aminopropyl-$InsP_4$ resin. The flow through was collected (flow through), and the resin was washed (wash) and eluted with 2× SDS-sample buffer (eluate). Fractions were separated by SDS-PAGE and subjected to immunoblot analysis using an anti-RGS-His_4 antibody. (B) His_6-Gcs1p, eluted from the Affigel-aminopropyl-$InsP_4$ resin with 1.5 M NaCl buffer, was dialyzed, and then incubated with 110 nCi [^{3}H]BZDC-Ins(1,3,4,5)P_4 photolabel, in the absence (total) or presence of 10 μM unlabeled phosphoinositide shown. Proteins were separated by 10% SDS-PAGE, and the gels were fixed, dried, and fluorographed. (C) Competition of His_6-Gcs1p binding to the Affigel-aminopropyl-$InsP_4$ resin by including in the binding assay increasing concentrations of unlabeled phosphoinositides. The resin was washed, and bound protein was eluted with sample buffer and separated by SDS-PAGE. Gels were transferred to nitrocellulose, and the His_6-Gcs1p was detected with the anti-RGS-His_4 antibody. Blots were densitized, and the results are presented as a percentage of total binding in the absence of unlabeled phosphoinositide; each value represents at least three independent determinations.

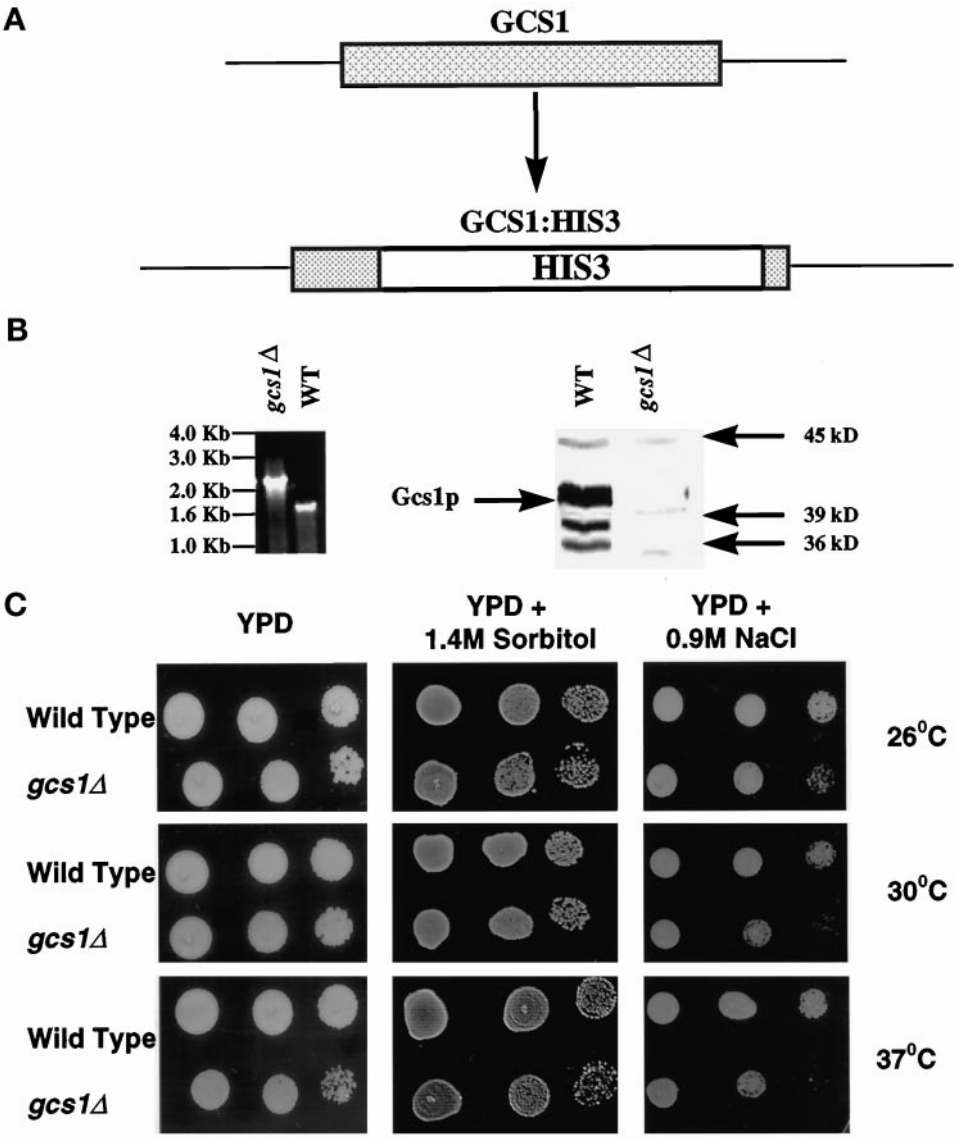


Figure 3. Disruption of *GCS1*. (A) *GCS1* was disrupted by replacing nucleotides 224-1021 with the *HIS3* gene by homologous recombination. (B) Genomic DNA isolated from wild-type and *gcs1*Δ was screened by PCR (left). Whole-cell lysates were prepared from mid-logarithmic wild-type and *gcs1*Δ cells grown at 30°C were separated by SDS-PAGE and transferred to nitrocellulose. Gcs1p was detected by immunoblot analysis using antisera generated against full-length His_6-Gcs1p fusion protein (right). (C) Serially diluted cell suspensions (10 μl) were spotted onto YPD and YPD plates supplemented with either 1.4 M sorbitol or 0.9 M NaCl. The plates were incubated for 48–72 h at the indicated temperatures.

His_6-Gcs1p was also efficiently photoaffinity labeled with a [[^{3}H]]BZDC-Ins(1,3,4,5)P_4 photoprobe (Figure 2B). The labeling was displaced by addition of 10 μM PtdIns(3,4,5P)$_3$ (a lipid containing the Ins(1,3,4,5)P_4 head group of the photoprobe) or PtdIns(4,5)P_2 to the binding reaction. In yeast, the phosphoinositides identified to date are PtdIns, PtdIns(3)P, PtdIns(4)P, PtdIns(3,5)P_2, and PtdIns(4,5)P_2 (Dove *et al.*, 1997). To characterize the binding specificity of His_6-Gcs1p, increasing concentrations of these physiologically relevant phosphoinositides were added to the aminopropyl-InsP_4 resin–binding assays. His_6-Gcs1p was displaced from the affinity resin by increasing concentrations of phosphoinositides. PtdIns(3,5)P_2 was the most potent displacer, followed by PtdIns, PtdIns(4,5)P_2, and PtdIns(3)P (Figure 2C). The IC_{50} for displacement by PtdIns(3,5)P_2 was approximately 7 μM, similar to the IC_{50} obtained with photoaffinity labeling (our unpublished data). These data demonstrate that Gcs1p binds phosphoinositide analogues with high affinity and specificity.

*gcs1*Δ *Deletion Mutant Strains Display Mutant Growth and Morphological Phenotypes*

A *gcs1* deletion mutant strain (*gcs1*Δ), in which the majority of the *GCS1* gene was deleted, was generated by replacing nucleotides 224-1021 with the *HIS3* gene cassette (Figure 3A). PCR analysis indicated that the deletion construct was properly targeted to the *GCS1* locus. By immunoblot analysis, using anti-Gcs1p antisera, no detectable Gcs1p protein was present in the

*gcs1*Δ strain while the wild-type stain expressed Gcs1p during vegetative growth (Figure 3B). Similar to the reentry phenotype originally described for *gcs1*, this *gcs1*Δ strain also did not progress at 15°C from stationary phase to logarithmic growth. Although normal for growth at the 30°C permissive temperature, vegetative cells displayed several morphological defects (see below), suggesting that *GCS1* may function not only in the transition from stationary phase, but also during vegetative growth. To determine whether *gcs1*Δ had any other growth phenotypes, cells were grown in the presence of elevated sorbitol or NaCl concentrations. *gcs1*Δ grew normally in YPD medium with high sorbitol concentrations (≤1.4 M), but grew slowly at 26°C, or not at all at 30 or 37°C on YPD medium containing 0.9 M NaCl (Figure 3C). In addition, *gcs1*Δ was unable to grow in YPD with 40 mM NaF (our unpublished data), a previously reported *gcs1* mutant phenotype (Poon *et al.*, 1996). These data demonstrate that Gcs1p is required for growth in various stress conditions at the permissive temperature.

Previous reports show that *gcs1* mutant strains exhibit endosomal and exocytic trafficking defects at the 15°C nonpermissive temperature, consistent with its reported role as an Arf1 GAP (Poon *et al.*, 1996; Wang *et al.*, 1996). At the permissive temperature, maturation of carboxypeptidase Y (a resident vacuolar enzyme) and secretion of invertase in *gcs1*Δ were normal (our unpublished results), indicating that Gcs1p is not essential for protein trafficking to the vacuole or the plasma membrane. In ultrastructural analysis by electron microscopy, no accumulation of intracellular membranous structures, such as 50-nm vesicles or collapsed Golgi, was observed in *gcs1*Δ cells. However, in contrast to wild-type cells that contain one to two large vacuoles, *gcs1*Δ cells exhibited numerous (>4) small membrane-bound vacuolar-like structures, suggesting that Gcs1p is required for normal vacuolar morphology (our unpublished results). The presence of aberrant vacuolar structures is a pleiotropic phenotype observed in strains with mutations in genes required for maintenance of the actin cytoskeleton and/or various vesicle-trafficking pathways.

Gcs1p Is Required For Normal Actin Cytoskeleton Distribution

Examination of *gcs1*Δ cells by phase microscopy indicated that many of the mutant cells were larger than the wild-type strain, were multibudded, and/or displayed elongated bud neck structures (Figure 4C). These morphological characteristics, as well as sensitivity to high-ionic strength medium and vesicle-trafficking defects, are phenotypes that are frequently observed in strains with mutations in genes required for organization of the actin cytoskeleton (Drubin *et al.*, 1993; Ayscough and Drubin, 1996). In *S. cerevisiae*, the actin cytoskeleton shows a characteristic polarization as the cell progresses through the cell cycle. In unbudded cells, actin is concentrated in cortical actin patches at points juxtaposed to the membrane, from which the next daughter cell emerges. As a bud emerges, the cortical actin patches accumulate in the bud, followed by the emergence of actin cables, which are oriented along an axis between the mother and daughter cells. Strains harboring mutations in genes associated with the actin cytoskeleton can display changes in actin cables and/or cortical actin patch number and polarization (Welch *et al.*, 1994).

To assess the actin cytoskeleton, F-actin was examined in midlogarithmic wild-type and *gcs1*Δ cells grown at 30°C by staining fixed cells with rhodamine phalloidin (Figure 4). Staining in wild-type cells was consistent with the actin distributions described above. In contrast, an increased number of actin patches in the mother cell were present in the *gcs1*Δ strain. In addition, although actin cables were evident in *gcs1*Δ cells, the cables often appeared misaligned. To determine whether the apparent increase in the number of actin patches in *gcs1*Δ was statistically significant, we quantified and compared the number of patches in mother and daughter cells of the wild-type and mutant strains. Whereas only 25% of budding wild-type mother cells had >3 actin patches, ~80% of the *gcs1*Δ budding cells had >3 actin patches ($p < 0.001$). The number of actin patches in the daughter cell was similar between the wild-type and *gcs1*Δ cells. This phenotype did not appear to be the result of a cell cycle-progression defect since both small and large budded *gcs1*Δ cells displayed an increase in the number of actin patches in the mother cell (our unpublished results). Together, these data suggest that Gcs1p is required for the normal organization of the actin cytoskeleton.

GCS1 Is Required In Vivo to Stabilize the Actin Cytoskeleton

Yeast strains carrying mutations in genes encoding proteins implicated in regulation of the actin cytoskeleton also exhibit altered sensitivity to reagents that disrupt actin polymerization or depolymerization. Lat-B, a cell-permeant marine toxin binds to monomeric G-actin and inhibits actin polymerization (Coue *et al.*, 1987). Hypersensitivity to latrunculin, which is indicative of an unstable filamentous actin network, has been reported for mutant alleles in the actin-binding proteins *sla2*, *cap2*, *srv2* (adenylyl cyclase-associated protein), and *sac6* (a yeast fimbrin homologue) and in specific actin alleles, such as *act1–111*, *act1–108*, and *act1–136* (Ayscough *et al.*, 1997). To investigate the sensitivity of *gcs1*Δ cells to Lat-B, a halo sensitivity assay was employed (Ayscough *et al.*, 1997). Com-

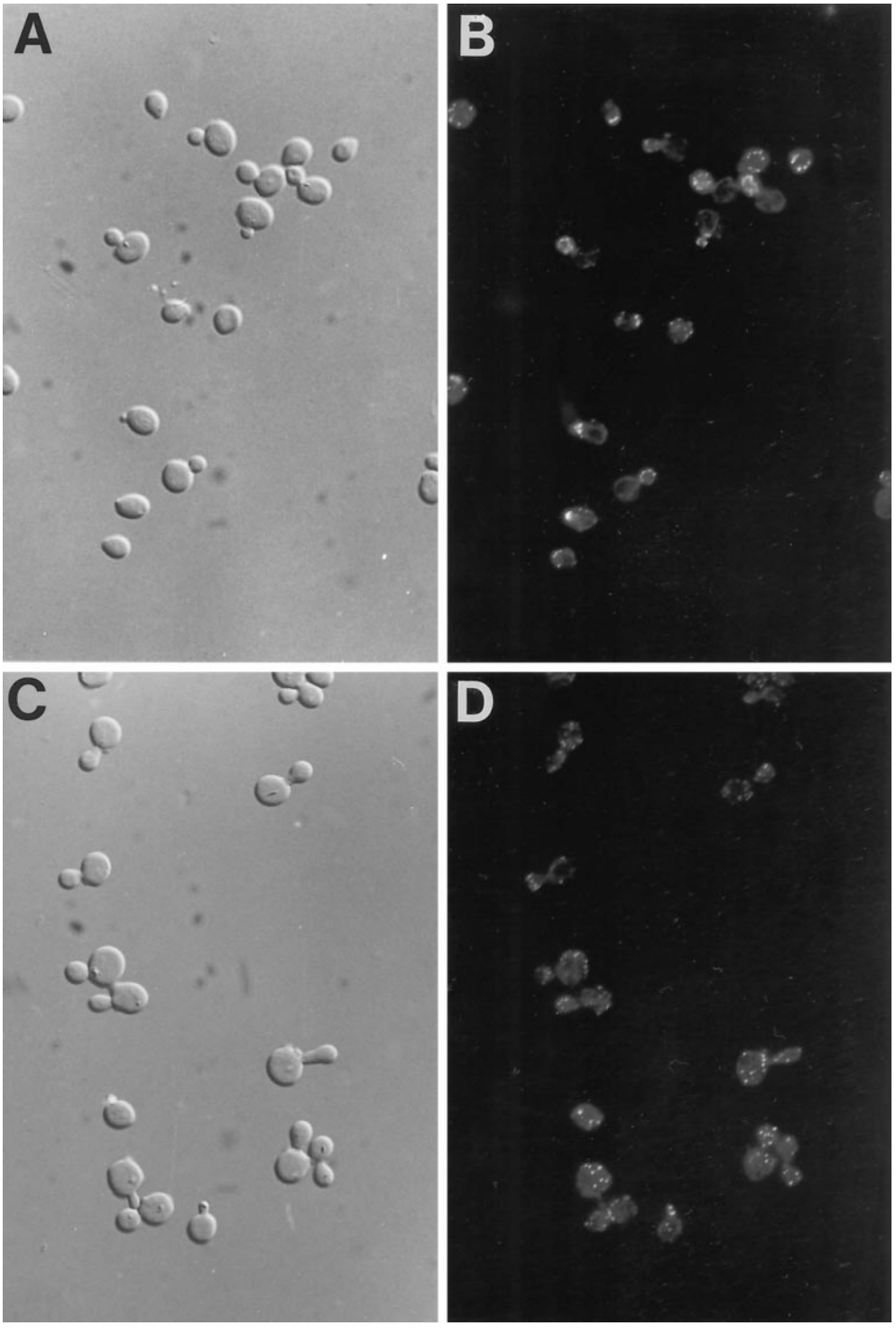


Figure 4. Actin cytoskeleton distribution of log-phase wild-type and*gcs1*Δ cells. Wild-type (A and B) and *gcs1*Δ (C and D) cells grown at 30°C were fixed, and stained with rhodamine-conjugated phalloidin as described in MATERIALS AND METHODS. Cells were viewed with Nomarski optics (left) and fluorescence rhodamine filter (right) with a 100× oil immersion objective. Representative fields are shown; note the increase in the number of actin patches in *gcs1*Δ. Focal planes were chosen to maximize the number of actin patches observed.

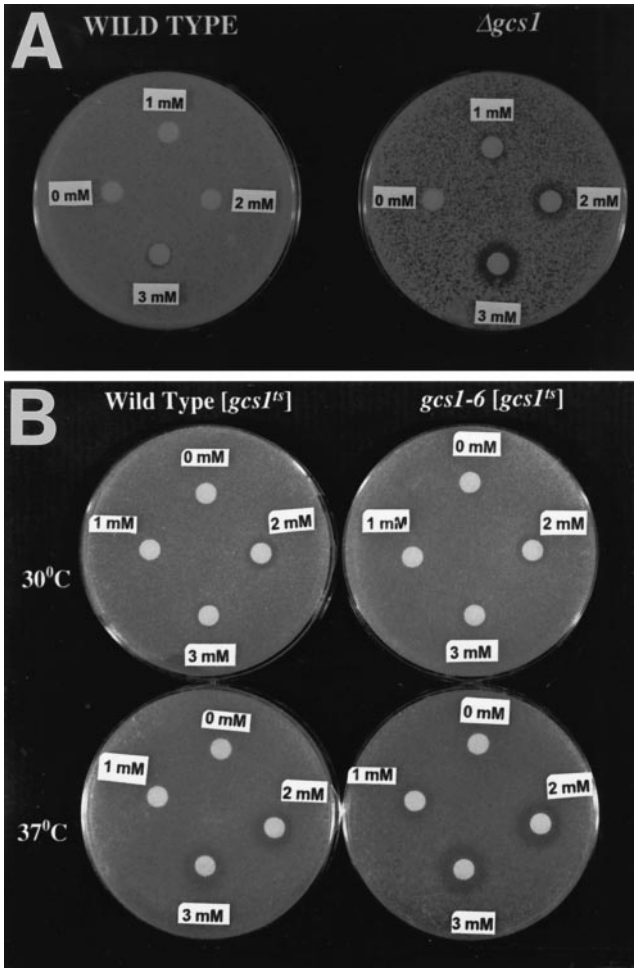


Figure 5. Sensitivity of *gcs1* mutants to Lat-B. (A) Halo assays were used to determine the sensitivity of wild-type (left) and *gcs1*Δ (right) cells to the indicated concentration of Lat-B. (B) Sensitivity of wild-type (left) and *gcs1–6* (right) cells expressing the $gcs1^{ts}$ plasmid at either 30 or 37°C.

pared with the parental wild-type strain, *gcs1*Δ cells were ~2.5 times more sensitive to Lat-B (Figure 5A). A *gcs1* null strain (*gcs1–6)* generated in an unrelated background strain (Ireland, *et al.*, 1994) displayed a similar hypersensitivity to Lat-B. To verify that the increased sensitivity was the direct result of the loss of Gcs1p, halo assays were performed with both a wild-type and *gcs1* null strain bearing a loss-of-function $gcs1^{ts}$ plasmid. At the permissive 30°C temperature, both strains were equally sensitive to Lat-B, whereas at 37°C the null *gcs1* strain harboring the $gcs1^{ts}$ plasmid was ~1.5 times more sensitive to Lat-B (Figure 5B). These data show that *gcs1*Δ cells are hypersensitive to Lat-B, a phenotype that points to a role for Gcs1p in stabilizing the actin cytoskeleton.

Genetic Interactions between Mutants in GCS1, SLA2, and SAC6, Genes That Encode Actin-associated Proteins

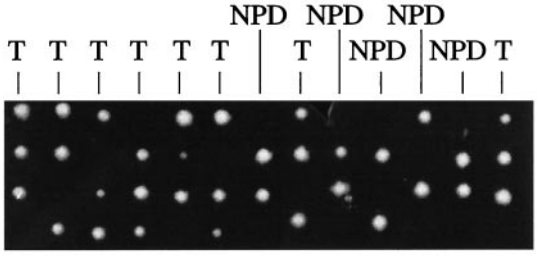
The above results suggest that Gcs1p is important for organization of the actin cytoskeleton. To further establish an in vivo role for Gcs1p in regulation of the actin cytoskeleton, we tested for genetic interactions between *gcs1*Δ and 1) deletions of proteins known to be associated with the actin cytoskeleton in yeast or 2) mutants in the single-yeast actin gene, *ACT1*. Gene knockouts and actin mutants with a variety of phenotypes and genetic interactions were chosen. For example, mutants in the *SLA2* gene are defective in actin polarization, endocytosis, and are temperature sensitive. (Ireland *et al.*, 1994), whereas mutants in the *ABP1* gene, encoding the actin-binding protein Abp1p, behave in a manner similar to wild-type cells (Drubin *et al.*, 1993). Similarly, the *act1–104* allele is not t.s. and has no reported synthetic genetic interactions with actin-associated proteins, whereas the *act1–129* allele is temperature sensitive and is synthetic lethal in combination with *sac6*Δ, *abp1*Δ, and *sla2*Δ (Holtzman *et al.*, 1994).

Haploid progeny that were determined to harbor both *gcs1*Δ and *sla2*Δ were inviable, indicating a synthetic lethality (Figure 6). In addition, *gcs1*Δ mutants, when combined with a deletion of the *SAC6* gene (encoding fimbrin, an actin cross-linking protein) (Adams *et al.*, 1991), demonstrated an inability to grow at 20 and 34°C. No synthetic effects were observed when the *gcs1*Δ mutation was combined with null mutants in the *ABP1* or *RVS167* genes or with any of the five *ACT1* mutant alleles tested (Wertman *et al.*, 1992). This suggests that deletion of the *GCS1* gene results in defects in actin cytoskeleton regulation that are mild in an otherwise wild-type background, but severe in combination with certain mutations that themselves disrupt actin organization, specifically *sla2*Δ (Wertman *et al.*, 1992) and *sac6*Δ (Adams *et al.*, 1991). These genetic data are consistent with Gcs1p interacting in vivo with the actin cytoskeleton in *S. cerevisiae*.

Gcs1p Binds to F-Actin and Stimulates Actin Polymerization In Vitro

Homology with the actin-binding domain in the ERM protein family, in addition to the in vivo data described above, suggested that Gcs1p may interact directly with actin. To test for such interaction, an in vitro actin cosedimentation assay was performed by incubating purified recombinant His_6-tagged Gcs1p (Figure 7A) with polymerized F-actin (Figure 7B). In the absence of actin, the majority of His_6-Gcs1p remained in the supernatant. Addition of F-actin resulted in His_6-Gcs1p association with the F-actin– containing pellet. Addition of BSA, a protein that does not bind actin, to the cosedimentation reaction did not inhibit His_6-Gcs1p binding to F-actin, suggesting

A



B

*gcs1*Δ x ...	strain: DDY...	#tetrads tested	#tetratype tetrads	*synthetic* temp. sens. of double knockout
*abp1*Δ	988	8	4	none
*sla2*Δ	546	14	8	synthetic lethal
*sac6*Δ†	216	8	4	t.s. @ 20°C, 34°C†
*rvs167*Δ*	8	8	4	none
*act1-104**	10	10	5	none
*act1-113**	8	8	4	none
*act1-115**	13	13	9	none
*act1-116**	10	10	5	none
*act1-129**	13	13	6	none

Figure 6. Genetic interactions between *GCS1*, actin-associated proteins, and actin mutants. *gcs1*Δ yeast were crossed with strains containing mutations in the single actin gene, *ACT1*, and with strains containing null mutants of a number of actin-associated proteins. (A) *GCS1* and *SLA2* are synthetic lethal. Shown are tetrads that were tetratype (T) or nonparental ditype (NPD), based on auxotrophies. In all cases, the haploid progeny predicted to be *gcs1*Δ, *sla2*Δ failed to grow at 25°C. (B) A table indicating the crosses made and the resulting temperature sensitivity of the double-mutant progeny. Note that certain of the mutants to which the *gcs1*Δ strain was crossed were already temperature sensitive. The only synthetic effect observed, other than with *sla2*Δ, was with *sac6*Δ. †, *gcs1*Δ, *sac6*Δ strains showed some variability in phenotype; some predicted double mutants failed to grow. The least severe temperature sensitivity is indicated. *, These crosses were made using GWK9A (*gcs1*Δ::*URA3*) provided by Dr. G.C. Johnston (see also Table 1).

that the binding of His_6-Gcs1p to actin was specific. Moreover, a control His_6-DHFR fusion protein did not cosediment with F-actin, even at the highest F-actin concentrations tested (our unpublished data) demonstrating that actin did not bind the His_6 fusion tag. To characterize the binding of His_6-Gcs1p to F-actin, we incubated increasing concentrations of His_6-Gcs1p with F-actin (Figure 7, C and D). Binding was linear with increasing concentrations of His_6-Gcs1p and was saturable. Fifty percent maximal actin binding was observed with ~75 nM His_6-Gcs1p, and at saturation the stoichiometry of His_6-Gcs1p:actin binding was 1:50.

Numerous proteins that bind F-actin modulate actin polymerization dynamics. To examine whether Gcs1p regulates actin polymerization in vitro, a light scattering assay (Figure 8A) was employed. Two major stages of polymerization, nucleation and elongation, can be assessed with this assay as a time-dependent increase in light scattering. Addition of His_6-Gcs1p resulted in a dose-dependent stimulation of actin polymerization, leading to a decrease in the lag phase of polymerization, as well as an increase in the net polymerization rate and the steady-state level of actin polymerization. To test whether the increase in polymerization was a result of actin bundling, we also performed a polymerization assay using pyrene-labeled rabbit skeletal muscle actin. This assay measures actin polymerization as an increase in fluorescence intensity and is insensitive to the distribution of the filaments. His_6-Gcs1p stimulated actin polymerization in this assay with similar effects as seen in the light scattering assay (Figure 8B). The addition of His_6-DHFR did not stimulate actin polymerization, indicating that the effect was not due to the fusion tag or a bacterial contaminant.

Shortening of the lag phase and enhanced rate of polymerization have been previously observed for proteins that nucleate actin polymerization. Another feature of several actin nucleating proteins is their ability to decrease the rate of actin depolymerization. Therefore, we examined whether addition of His_6-Gcs1p affected the rate of actin depolymerization after dilution of F-actin. Addition of His_6-Gcs1p inhibited actin depolymerization in a dose-dependent manner (Figure 9). Addition of heat-inactivated His_6-Gcs1p had no effect on actin polymerization or depolymerization (our unpublished data). These data show that Gcs1p can directly interact with actin, stimulate actin polymerization, and inhibit actin depolymerization in vitro. The ability of Gcs1p to both stimulate actin polymerization and block depolymerization suggests that it may function to stabilize actin filaments.

DISCUSSION

In this report, we show that that Gcs1p is involved in regulating the actin cytoskeleton in *S. cerevisiae*. Five independent lines of evidence support this conclusion. First, Gcs1p contains a region of homology with the actin-binding domain of the ERM family of actin-binding proteins. Second, a *gcs1*Δ strain displayed mutant morphological and growth phenotypes, including mislocalized cortical actin patches, sensitivity to hyperionic conditions, and aberrant budding, that are consistent with defects in the actin cytoskeleton. Third, *gcs1* mutant strains were hypersensitive to Lat-B, an actin monomer-sequestering drug. Fourth, synthetic growth defects were observed in strains in which *gcs1*Δ was combined with null mutations in either *SLA2* or *SAC6*, whose gene products encode proteins implicated in the stabilization of the actin cytoskeleton. Fifth, Gcs1p bound F-actin, stimulated actin polymerization, and inhibited actin depolymer-

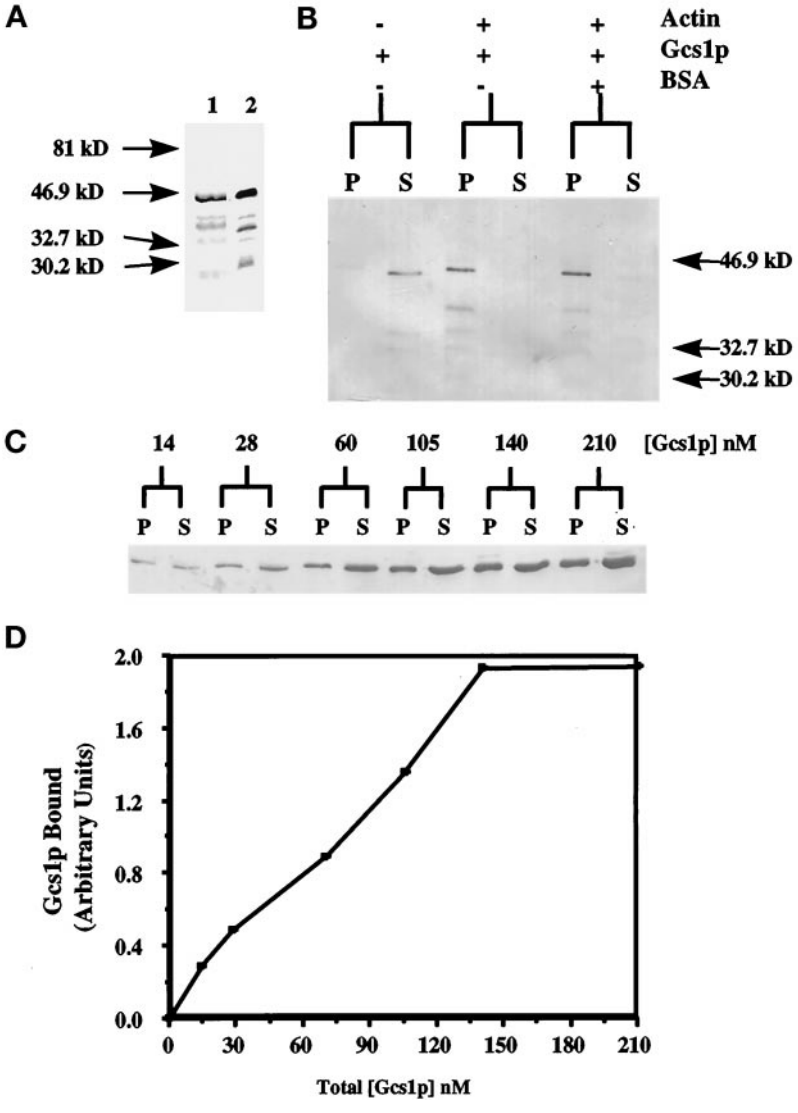


Figure 7. Gcs1p interacts with actin in vitro. (A) His_6-Gcs1p was purified using Ni^{2+}-NTA resin. The purified protein was analyzed by Coomassie stain (lane 1) and by immunoblot analysis using an anti-RGS-His_4 antibody (lane 2). (B) Purified His_6-Gcs1p was incubated with 3 μM polymerized yeast F-actin in the presence or absence of BSA and centrifuged at 270,000 $\times$ *g*. The pellets (P) and supernatants (S) were separated by SDS-PAGE and subjected to immunoblot analysis using an anti-RGS-His_4 antibody. (C) Increasing concentrations (14–210 nM) of purified His_6-Gcs1p were incubated with 3 μM polymerized yeast F-actin and centrifuged at 270,000 $\times$ *g*. The pellets (P) and supernatants (S) were separated by SDS-PAGE and subjected to immunoblot analysis using an anti-RGS-His_4 antibody. (D) Quantification of immunoblot in panel C using a densitometer.

ization in vitro. Furthermore, we have identified a candidate mechanism for regulation of Gcs1p: interaction with phosphoinositides.

In addition to inhibiting depolymerization, Gcs1p shortened the lag phase and increased the net rate and steady-state level of actin polymerization. A protein that stimulates actin polymerization in a similar manner is the mammalian actin-binding protein talin (Kaufmann *et al.*, 1991). Talin is a membrane-linking, F-actin–binding protein of the band 4.1 superfamily (McCann and Craig, 1997), proposed to function by promoting actin filament nucleation and elongation (Kaufmann *et al.*, 1991). It is noteworthy that numerous actin-binding proteins, including talin, also bind phosphoinositides. Phosphoinositide binding has been shown to inhibit the interactions between actin and gelsolin or profilin (Janmey, 1994; Kandzari *et al.*, 1996). Actin polymerization, however, can be modulated by several classes of actin-binding proteins, including those that sever, cap, nucleate, sequester, and bundle actin (Pollard and Cooper, 1986). Thus, although the in vitro biochemical data presented here suggest that Gcs1p may bind/cap the end of actin filaments, it is possible that Gcs1p stabilizes actin filaments by another mechanism. The precise manner by which Gcs1p interacts with actin awaits a detailed biochemical analysis.

Several yeast proteins have been identified and are proposed to function in modulating the actin cytoskeleton. These include Sla2p, a protein associated with cortical actin patches that is necessary for actin nucleation in vitro (Li *et al.*, 1995), Cap2p, an actin-capping protein (Amatruda *et al.*, 1992), Sac6p, a fimbrin homologue that bundles actin filaments (Adams *et al.*,

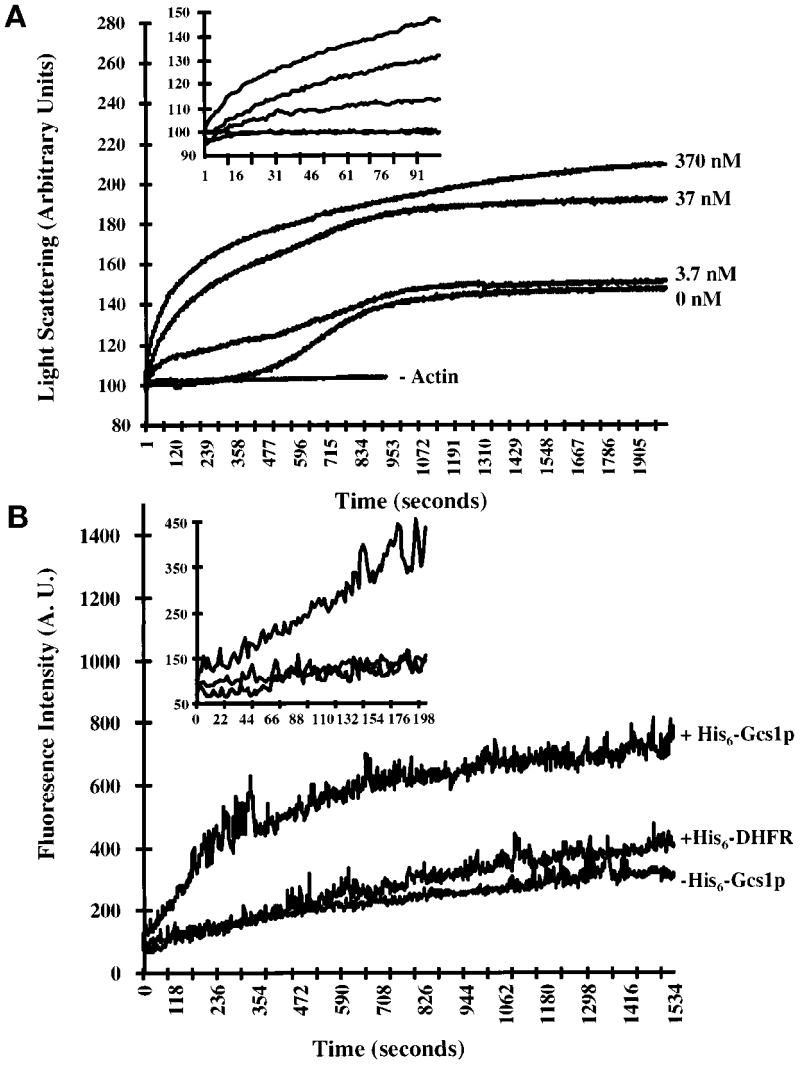


Figure 8. Time course for actin polymerization in the presence of His_6-Gcs1p. (A) Monomeric yeast G-actin (2 μM) was polymerized in the absence or presence of increasing concentrations of His_6-Gcs1p. Actin polymerization was monitored as an increase in light scattering as described in MATERIALS AND METHODS. Inset trace is a magnification of the first 105 s of the polymerization assay. In addition, 370 nM His_6-Gcs1p was incubated in the absence of actin to determine the intrinsic light scattering of His_6-Gcs1p. (B) Monomeric 1:10 pyrene-labeled rabbit muscle actin (5 μM): muscle actin was polymerized in the absence or presence of 0.2 μM His_6-Gcs1p or His_6-DHFR. Polymerization was monitored as an increase in fluorescence intensity as described in MATERIALS AND METHODS. Inset trace is a magnification of the first 300 s of the polymerization assay.

1991), and Tpm1p, yeast tropomyosin (Liu and Bretscher, 1992). Strains harboring loss-of-function mutations in these genes are believed to have a destabilized actin cytoskeleton, which results in an increased microfilament-turnover rate, leading to hypersensitivity to the actin monomer-binding drug latrunculin (Ayscough *et al.*, 1997). Hence, the *gcs1*Δ phenotypes, including hypersensitivity to Lat-B, are consistent with a role for Gcs1p in stabilizing the actin cytoskeleton.

*gcs1*Δ*/sla2* and *gcs1*Δ*/sac6* double mutants showed synthetic growth defects, whereas *GCS1* did not interact genetically with *ABP1*, *RVS167*, or any of the actin alleles tested. In addition to stabilization of the actin cytoskeleton, Sla2p and Sac6p have been implicated as regulators of vesicle trafficking (Kubler and Riezman, 1993; Wesp *et al.*, 1997). Gcs1p is also required for secretion and endocytosis at the nonpermissive temperature (Poon *et al.*, 1996; Wang *et al.*, 1996) and for normal vacuolar morphology at the permissive temperature. This raises the question of whether the genetic interactions result from combining defects in the actin cytoskeleton, in vesicle trafficking, or both. We currently do not know the nature of the genetic interactions between *GCS1* and *SLA2* or *SAC6*. However, these data are in agreement with a large body of evidence showing that the actin cytoskeleton is an important component of vesicle trafficking in yeast (Wendland *et al.*, 1998). In addition to Sla2p and Sac6p, the myosin family of molecular motors, tropomyosin, and actin itself are required for vesicle trafficking (Novick and Botstein, 1985; Liu and Bretscher, 1992; Kubler and Riezman, 1993; Welch *et al.*, 1994; Brown, 1997). Conversely, several of the endocytosis mutants (*end 4* [*sla2*], *end5* [*vpr1*], *end6* [*rvs161*], *end7* [*act1*], and *end14* [*srv2*]) are allelic to proteins known to be di-

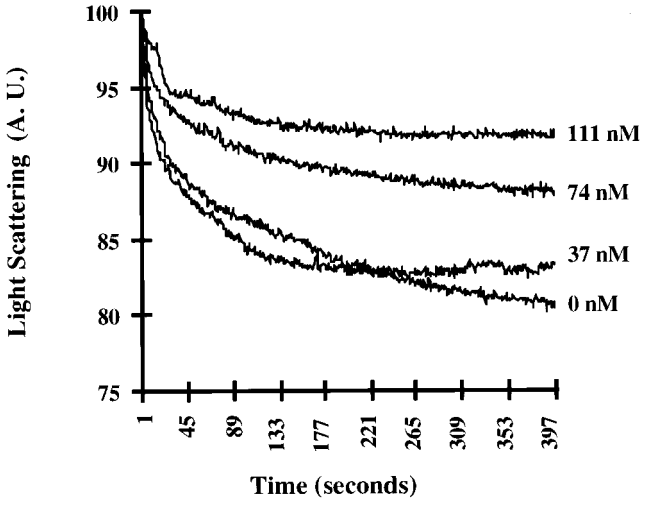


Figure 9. Time course for actin depolymerization in the presence of His_6-Gcs1p. F-actin (2 μM) was diluted 30-fold into G-buffer containing increasing concentrations of His_6-Gcs1p. Actin depolymerization was monitored as an decrease in light scattering as described in MATERIALS AND METHODS.

rectly associated with the actin cytoskeleton (Munn *et al.*, 1995; Wesp *et al.*, 1997). Moreover, late-acting secretory mutants such as *sec1, sec3, sec6,* and the Rab GTPases *sec4 and ypt1* have a depolarized actin cytoskeleton (Segev and Botstein, 1987; Lillie and Brown, 1994; Haarer *et al.*, 1996; Mulholland *et al.*, 1997).

In addition to its role in actin cytoskeletal dynamics reported herein, Gcs1p has been shown to act as an Arf1 GAP in vitro and interacts with *ARF1 in vivo* (Poon *et al.*, 1996). Arf1p is required for secretion in yeast (Stearns *et al.*, 1990). Additional pathways that involve Arfs have been proposed based upon the finding that overexpression of *GCS1* and other members of this gene family, including *GLO3*, *SAT1*, and *SAT2*, rescue a loss of function *arf1*–3^{ts} mutant, via a pathway that appears to be independent of the secretory function of Arf1p (Zhang *et al.*, 1998). Our finding that Gcs1p is involved in regulation of the actin cytoskeleton, together with the data showing that Gcs1p interacts with Arf1, provide an intriguing possibility that Gcs1p may link the Arf and actin cytoskeletal pathways in yeast.

Gcs1p binds to phosphoinositide-based affinity probes, potentially through an identified PH domain. Although the physiological role of phosphoinositide binding has not been determined, the fact that deletion of the PH domain in Gcs1p yielded a phenotype similar to the null strain (Ireland *et al.*, 1994) indicates that the PH domain is important for Gcs1p function in vivo. Based on its proposed function in other proteins (Toker and Cantley, 1997), phosphoinositide binding may act as a membrane localization signal and/or as a modulator of interactions with other proteins such as Arf or actin. Of the physiologically relevant yeast phosphoinositides tested, Gcs1p bound PtdIns(3,5)P_2 with the highest affinity. Present at low levels under normal growth, PtdIns(3,5)P_2 is synthesized rapidly upon shift to hyperosmotic conditions (Dove *et al.*, 1997) and requires Vps34p, the PtdIns 3-kinase, and Fab1p, a PtdIns(3)P 5-kinase (Dove *et al.*, 1997; Gary *et al.*, 1998). Mutations in genes encoding proteins involved in phosphoinositide metabolism share similar phenotypes with *gcs1* mutants. For example, mutations in *VPS34*, *FAB1, PIK1*, the PtdIns 4-kinase, and the PtdIns polyphosphate 5-phosphatase genes have mutant growth, actin cytoskeleton, and vesicle-trafficking phenotypes (Banta *et al.*, 1988; Robinson *et al.*, 1988; Garcia-Bustos *et al.*, 1994; Yamamoto *et al.*, 1995; Cutler *et al.*, 1997; Srinivasan *et al.*, 1997).

In summary, the data presented here suggest that Gcs1p is involved in the regulation of the actin cytoskeleton. Furthermore, the in vitro biochemical data suggest that Gcs1p can modulate actin dynamics directly, indicating a second functional pathway in addition to its Arf1 GAP activity. How specific interactions with Arf1p, actin, and phosphoinositides are integrated with Gcs1p function will provide important clues in understanding the role(s) of Gcs1p in cytoskeletal regulation in yeast and the functional activities of the potential *GCS1* homologue, centaurin α, in mammalian brain.

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