

Functional Interactions between the p35 Subunit of the Arp2/3 Complex and Calmodulin in Yeast

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The *end9-1* (*arc35-1*) mutant was identified as an endocytosis mutant and is a mutant allele of *ARC35* that encodes a subunit of the Arp2/3 complex. As for other mutants in the Arp2/3 complex, *arc35-1* is defective for endocytosis and organization of the actin cytoskeleton. Both defects can be suppressed by overexpression of calmodulin. Analysis of a collection of temperature-sensitive *cmd1* mutants for their ability to suppress either the endocytic defect and/or the actin defect indicates that the two defects are tightly coupled. We demonstrate that Arc35p and Cmd1p interact and that Arc35p is required for cortical localization of calmodulin. This is the first report linking Arp2/3 complex function with calmodulin through which it exercises at least one of its endocytic functions.

INTRODUCTION

Endocytosis is the process whereby cells internalize part of their own plasma membrane along with extracellular material facilitating uptake of nutrients, down-regulation of receptors, and removal of damaged or surplus proteins. Endocytosis is mediated by vesicles budding from the cell surface and the delivery of endocytosed material to the lysosome via at least two intermediate compartments (Singer-Krüger *et al.*, 1993; Mukherjee *et al.*, 1997; Clague, 1998). Work in yeast (Riezman *et al.*, 1996; Wendland *et al.*, 1998) indicates that the actin cytoskeleton plays a fundamental role in endocytosis. Recent evidence, obtained mainly by using the inhibitors cytochalasin D and latrunculin A (latA), suggests an involvement of the actin cytoskeleton in the uptake step of endocytosis in animal cells (Gottlieb *et al.*, 1993; Jackman *et al.*, 1994; Durrbach *et al.*, 1996; Shurety *et al.*, 1996; Lamaze *et al.*, 1997; Freedman *et al.*, 1999; Valentijn *et al.*, 1999).

The development of quantitative assays for receptor-mediated endocytosis in the yeast *Saccharomyces cerevisiae* (Dulic *et al.*, 1991) combined with genetics has led to the identification of numerous genes involved in the internalization step of endocytosis (Munn and Riezman, 1994). Genes regulating lipid metabolism and various genes involved in building or regulating the actin cytoskeleton were isolated in the screen. In addition, calmodulin (Kübler *et al.*, 1994) and the actin-related protein Arp2 (Moreau *et al.*, 1997), which regulate the actin cytoskeleton, were shown to be involved in the uptake step of endocytosis. Other screens have also led to the isolation of endocytosis mutants affecting actin

dynamics (Wendland *et al.*, 1996; Singer-Krüger and Ferro-Novick, 1997).

Calmodulin is a major calcium sensor conserved from yeast to human. One single essential gene (*CMD1*) encodes calmodulin in *Saccharomyces cerevisiae* (Davis *et al.*, 1986). Calmodulin plays a fundamental role in endocytosis in yeast (Kübler *et al.*, 1994; Geli *et al.*, 1998). The calmodulin requirement may be calcium-independent because the mutant *cmd1-3*, impaired in calcium binding *in vitro* (Geiser *et al.*, 1991), allowed virtually wild-type internalization kinetics (Kübler *et al.*, 1994). Calmodulin has been purified from clathrin-coated vesicles and was shown to interact with clathrin light-chain (Salisbury *et al.*, 1980; Pley *et al.*, 1995). Calmodulin antagonists were shown to stimulate endocytosis of ricin in Madin–Darby canine kidney (MDCK) cells (Llorente *et al.*, 1996) and sequestration of serotonin 5-HT_{1A} receptor in CHO cells (Della Rocca *et al.*, 1999). On the other hand, calmodulin antagonists inhibited transcytosis of IgA in MDCK cells (Enrich *et al.*, 1996) and sequestration of histamine H₁ receptors in human astrocytoma cells (Hishinuma *et al.*, 1998).

The Arp2/3 complex has been purified from *Acanthamoeba* (Machesky *et al.*, 1994), human platelets (Welch *et al.*, 1997b), human neutrophils (Machesky *et al.*, 1997), and *S. cerevisiae* (Winter *et al.*, 1997). It has been shown to consist of seven subunits in *Acanthamoeba* and human and of six subunits in yeast. Two of the subunits are the highly conserved actin-related proteins, Arp2 and Arp3. Components of the Arp2/3 complex have been localized to the cortical actin cytoskeleton in *Acanthamoeba* (Kelleher *et al.*, 1995), in human (Welch *et al.*, 1997a), in *S. cerevisiae* (Moreau *et al.*, 1996; Winter *et al.*, 1997), and in *Schizosaccharomyces pombe* (McCollum *et al.*, 1996). Activities demonstrated for the Arp2/3 complex include actin (Mullins *et al.*, 1997) and profilin binding (Ma-

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Table 1. Yeast strains

Strain	Relevant markers	Reference
B81	<i>Mata arp2-1::URA3 ade2 ura3 leu2 trp1 his3 lys2</i>	B. Winsor
CEN.PK2	<i>Mata/Mataα his3/his3 leu2/leu2 ura3/ura3 trp1/trp1</i>	K.D. Entian
DBY7443	<i>Mata cmd1-231 his3 leu2 ura3 lys2Δ trp1 bar1</i>	D. Botstein
DBY7445	<i>Mata cmd1-228 his3 leu2 ura3 lys2Δ trp1 bar1</i>	D. Botstein
DBY7446	<i>Mata cmd1-226 his3 leu2 ura3 lys2Δ trp1 bar1</i>	D. Botstein
DBY7449	<i>Mata cmd1-239 his3 leu2 ura3 lys2Δ trp1 bar1</i>	D. Botstein
EGY191	<i>Mata his3 leu2::pLEU2-lexAop2 ura3 trp1</i>	Estojak <i>et al.</i> , 1995
RH2584	<i>Mata cmd1-3 his4 leu2 ura3 bar1</i>	Kübler <i>et al.</i> , 1994
RH2616	<i>Mataα arc35-1 his4 leu2 ura3 bar1</i>	Riezman collection
RH2877	<i>Mata leu2 ura3 lys2 ade2 trp1 bar1</i>	Riezman collection
RH2962	<i>Mata/α arc35::URA3/ARC35 his4/his4 leu2/leu2 ura3/ura3 lys2/lys2 bar1/bar1</i>	Riezman collection
RH3429	<i>Mata arc35-1 his4 leu2 ura3 lys2 ade2 bar1</i>	This study
RH3431	<i>Mata/α arc35-1/ARC35 his4/HIS4 leu2/leu2 ura3/ura3 lys2/LYS2 ade2/ADE2 trp1/TRP1 bar1/bar1</i>	This study
RH4161	<i>Mata ARC35-GFP::kanMX4 his3 leu2 ura3 trp1</i>	This study
RH4164	<i>Mata arp2-1::URA3 ade2 ura3 leu2 trp1 his3 lys2 bar1::LYS2</i>	This study
Y90	<i>Mataα arp3-1::LEU2 ade2 ura3 leu2 trp1 his3 lys2</i>	B. Winsor

chesky *et al.*, 1994), nucleation of actin polymerization and induction of *Listeria* movement (Welch *et al.*, 1998), pointed end capping and branching of actin filaments (Mullins *et al.*, 1998), and actin patch movement and integrity (Winter *et al.*, 1997). Mutations in the *S. cerevisiae* Arp2 gene have been found to cause a defect in the internalization step of endocytosis (Moreau *et al.*, 1997). The above findings lead to the exciting possibility that the Arp2/3 complex may organize actin filaments at the sites of membrane dynamics (Machesky, 1997).

In this study, we demonstrate that mutations in the *ARC35* gene lead to defects both in the internalization step of endocytosis and in the organization of the actin cytoskeleton. Both the endocytic and actin defects can be suppressed by overexpression of calmodulin. Analysis of a series of temperature-sensitive (*ts*) *cmd1* mutant alleles for their ability to suppress the endocytic defect, the actin defect, and/or *ts* growth indicates that the endocytic and actin defects are tightly coupled. Two-hybrid and coimmunoprecipitation data indicate that Arc35p and Cmd1p interact. The involvement of Arc35p in endocytosis is likely to involve localization of Cmd1p to cortical sites for interaction with an essential endocytic target. Taken together, the data presented provide a novel function of the Arp2/3 complex in endocytosis via calmodulin.

MATERIALS AND METHODS

Yeast Media, Strains, and Genetic Techniques

Unless mentioned otherwise, strains without plasmids were grown in complete medium YPUADT (2% glucose, 2% peptone, 1% yeast extract, 40 μ g/ml uracil [Ura], 40 μ g/ml adenine, and 40 μ g/ml tryptophan [Trp], 2% agar for solid medium). Strains bearing plasmids were selected on SD minimal medium containing the required nutritional supplements (Dulic *et al.*, 1991). Sporulation, tetrad dissection, and scoring of genetic markers were performed as described (Sherman *et al.*, 1974). Recombinant lyticase was purified from *Escherichia coli* as described (Hicke *et al.*, 1997). Transformation of yeast cells was accomplished by the lithium acetate method (Gietz *et al.*, 1992).

All yeast strains used in this study are listed in Table 1. RH3431 was generated by crossing RH2877 and RH2616. RH3429 was generated from RH3431 by inducing sporulation on minimal medium, by tetrad dissection, and by scoring of adequate markers. For the construction of RH4161, CEN.PK2 was transformed with a green fluorescent protein (GFP)-*kanMX4* cassette for C-terminal tagging of *ARC35* (Wach *et al.*, 1997) amplified with the following oligos using pFA6a-GFP-*kanMX4* as a template: *ARC35*GFPUS (5'-CAA CAG GCA AGA AGA ACC TTC ACC GGT AGA AAG ATT GTC TAC GGT CGA CGG ATC CCC GGG) and *ARC35*GFPDS (5'-TAT AAC CCT TTT TAC GGA TTC TTA CGT ACT TAT TTA ATC CAT CGA TGA ATT CGA GCT CG). Homology to *ARC35* is shown in bold, and homology to pFA6a-GFP-*kanMX4* is shown in italic. The resulting strain was sporulated, the tetrads were dissected, and the adequate markers were selected. Strain RH4164 was constructed by transforming strain B81 with the *LYS2*-cassette for disrupting *BAR1* (Kübler and Riezman, 1993).

DNA Techniques and Plasmid Constructions

All DNA manipulations were performed according to standard techniques (Sambrook *et al.*, 1989) unless specified otherwise. Restriction enzymes, Klenow, T4 DNA polymerase, calf intestine phosphatase (CIP), and T4 DNA ligase were obtained from Boehringer Mannheim (Indianapolis, IN), New England Biolabs (Beverly, MA), United States Biochemical (Buckinghamshire, United Kingdom), or Stratagene Cloning Systems (La Jolla, CA). All DNA fragments were purified with the GeneClean II Kit (Bio 101, Vista, CA); all plasmids were purified with the Qiagen plasmid purification kit (Qiagen, Hilden, Germany), and transformation of *E. coli* was performed by electroporation (Dower *et al.*, 1988). All PCRs for cloning purposes were performed with a DNA polymerase with proof-reading activity (Pfu, Stratagene Cloning Systems) on a TRIO-thermoblock (Biometa GmbH, Goettingen, Germany). Oligonucleotides were synthesized by Microsynth GmbH (Balgach, Switzerland). All constructs were sequenced using a Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA) on an ABI 377 automated sequencer (Applied Biosystems).

All plasmids used in this study are listed in Table 2. Plasmid pASZ12::*ARC35* was constructed by removing the *PstI/XbaI* fragment spanning the complete *ARC35* gene and 125 bp upstream, 2000 bp downstream sequence approximately, respectively, from a genomic library in plasmid YCplac111 (Gietz and Sugino, 1988) followed by Klenow and T4 DNA polymerase treatment and liga-

Table 2. Plasmids

Plasmid	Yeast ori	Marker	Insert	Reference
pASZ12	2 μ	ADE2		A. Stotz and P. Linder, 1990
pASZ12::ARC35	2 μ	ADE2	ARC35	This study
pCMD1	2 μ	ADE2	CMD1	M.I. Geli
pASZ12::cmd1-3	2 μ	ADE2	cmd1-3	This study
pASZ12::cmd1-226	2 μ	ADE2	cmd1-226	This study
pASZ12::cmd1-228	2 μ	ADE2	cmd1-228	This study
pASZ12::cmd1-239	2 μ	ADE2	cmd1-239	This study
pASZ12::cmd1-231	2 μ	ADE2	cmd1-231	This study
pASZ12::cmd1-247	2 μ	ADE2	cmd1-247	This study
pYOC547	CEN	TRP1	GAL1::cmd1-247	Y. Ohya and D. Botstein, 1994b
pSH18-34	2 μ	URA3	8 lexA op. lacZ	Gyuris <i>et al.</i> , 1993
pRFHM-1	2 μ	HIS3	lexAbicoid	Gyuris <i>et al.</i> , 1993
pEG202::ARC35	2 μ	HIS3	lexAARC35	R. Lombardi and H. Riezman, 1997
pEG202::arc35-1	2 μ	HIS3	lexAarc35-1	This study
pJG4-5::CMD1	2 μ	TRP1	B42CMD1	R. Lombardi and H. Riezman, 1997
pJG4-5::cmd1-3	2 μ	TRP1	B42cmd1-3	This study
pJG4-5::cmd1-226	2 μ	TRP1	B42cmd1-226	This study
pJG4-5::cmd1-228	2 μ	TRP1	B42cmd1-228	This study
pJG4-5::cmd1-239	2 μ	TRP1	B42cmd1-239	This study
pJG4-5::cmd1-231	2 μ	TRP1	B42cmd1-231	This study
pJG4-5::cmd1-247	2 μ	TRP1	B42cmd1-247	This study

tion into pASZ12 (Stotz and Linder, 1990), cut open at *Sma*I, and treated with CIP. The *arc35-1* allele was amplified from genomic DNA from the strain RH3429 with the primers ARC352HYB-1 (5'-GGC CGA CTC GAG CTA CAC TTA CAA CCA) and ARC352HYB-2 (5'-GGC CGA CTC GAG CGT ACT TAT TTA ATC), both of which include an *Xho*I site for in-frame ligation to the lexA DNA binding domain of pEG202 (Ruden *et al.*, 1991), cut with *Xho*I, and treated with CIP. The *cmd1* mutant alleles for cloning into pASZ12 were amplified from genomic DNA of the strains RH2584, DBY7443, DBY7449, DBY7446, and DBY7445 or from plasmid pYOC547 with the primers CMDASZ-1 (5'-ACG CGT CGA CAT GTA TTT ATA TTT TCG TGT A) and CMDASZ-2 (5'-ACG CGT CGA CAG AAT GGT AAG GGT AAG ATA G) for RH2584 or primers CMDASZ-3 (5'-CGC GGA TCC ATG TAT TTA TAT TTT CGT GTA) and CMDASZ-4 (5'-TGA CAT GCA TGC AGA ATG GTA AGG GTA AGA TAG) for all other alleles. Primers CMDASZ-1 and CMDASZ-2 include a *Sal*I restriction site for cloning into pASZ12 (Stotz and Linder, 1990), cut open at *Sal*I, and treated with CIP. Primers CMDASZ-3 and CMDASZ-4 bear a *Bam*HI or *Sph*I site for cloning into pASZ12 (Stotz and Linder, 1990), cut open with *Bam*HI and *Sph*I, and treated with CIP. The *cmd1* mutant alleles for the construction of the pJG4-5 series were amplified as described using the primers CMD2H-1 (5'-CCG CTC GAG TCC TCC AAT CTT ACC GAA GAA) and CMD2H-2 (5'-CCG CTC GAG CTA TTT AGA TAA CAA AGC AGC). Both primers bear an *Xho*I restriction site for in-frame cloning to the acid blob domain B42 of pJG4-5 (Gyuris *et al.*, 1993), cut with *Xho*I, and treated with CIP.

Protein Techniques and Antibodies

SDS-PAGE for protein separation was performed as described (Laemmli, 1970) using a Minigel System (Hoefer Scientific Instruments, San Francisco, CA). Acrylamide gels (16.5%) were used to separate Cmd1p, Arc35p, and Act1p. Low-range SDS-PAGE molecular weight standards (Bio-Rad Laboratories, Hercules, CA) were used for determination of apparent molecular weight. Total yeast protein extractions were performed as described (Horvath and Riezman, 1994). Western blotting was performed as described (Horvath and Riezman, 1994), except for the use of 10% (wt/vol) milk and the addition of 0.1% (vol/vol) Nonidet P-40 in the solutions for blocking

and subsequent incubations. Incubation with the primary antibodies, α -Cmd1p and α -Arc35p-N at a dilution of 1:1000 and α -actin (Roche Diagnostics, Mannheim, Germany) at 10 μ g/ml, was followed by incubation with the secondary antibody conjugated to horseradish peroxidase (Sigma, St. Louis, MO). Membranes (Schleicher and Schuell, Dassel, Germany) were developed using an ECL immunoblotting detection reagent kit (Amersham, Arlington Heights, IL).

The antibody against Cmd1p has been described (Geli *et al.*, 1998). The α -Arc35pN antibody was raised in rabbits against the Arc35p peptide LHLQPQNLLIQTK synthesized by Neosystem Laboratoire (Strasbourg, France). Arc35p antibody specificity was assessed on immunoblots by comparing extracts from cells expressing ARC35 and ARC35 on a 2- μ plasmid (see Figure 8A).

Microscopy

The procedure used for immunolocalization of calmodulin was adapted from Sohrmann *et al.*, 1996. Fixation was performed during a period of 90 min by adding formaldehyde directly to the culture to a final concentration of 3.7%. Incubation with the primary antibody or the secondary antibody coupled to CY3 was performed for 2 h or 30 min, respectively, at room temperature.

The calmodulin antibody was depleted of antibodies against chitin by passage over a chitin affinity column for use in immunofluorescence experiments. Briefly, 15 ml packed chitin particles were washed with 100 ml CMF-PBS (137 mM NaCl, 2.7 mM KCl, 1.5 mM Na₂HPO₄, 8.1 mM KH₂PO₄) followed by 100 ml CMF-PBS + 0.4 M NaCl, 100 ml 0.1 M HCl, and 400 ml CMF-PBS. The calmodulin antibody was loaded onto the column followed by washing with CMF-PBS. A 15-ml fraction corresponding to the void volume of the column was collected. Flow-through fractions in CMF-PBS were collected and used for immunolocalization of calmodulin in a dilution of 1:100 in PEMBAL. For the background control, the purified calmodulin antibody was preabsorbed with 60 μ g recombinant GST-calmodulin from *E. coli*.

Labeling of actin with rhodamine-phalloidin was performed as described (Bénédetti *et al.*, 1994). To preserve the GFP signal, sorbitol was added to a final concentration of 1 M, and the pH was adjusted to 7.5 during the pre-fixation and fixation processes.

For localizing GFP chimeras, strains were grown in SD minimal medium supplied with required nutrients. Cells were collected by centrifugation, resuspended in $1 \times$ PBS (137 mM NaCl, 2.7 mM KCl, 3.19 mM Na_2HPO_4 , 2.67 mM KH_2PO_4), and mounted onto poly-L-lysine-treated slides. Cells were observed using a Zeiss Axiophot microscope with a 100 \times objective. Images were taken with a Zeiss camera coupled to a video system (MWG Biotech, Ebersburg, Germany). Exposure times and other processing parameters were adjusted to the signal strength of the sample.

Latrunculin A Sensitivity

Halo assays were used for determining latrunculin A sensitivity (Ayscough *et al.*, 1997). Relative apparent sensitivities were calculated according to Reneke *et al.* (1988).

α -Factor Uptake Assay

^{35}S α -factor uptake assays were performed as described (Dulic *et al.*, 1991). The samples were processed as described above. The internalization rates were calculated as the percentage of counts internalized per minute between 2.5 and 15 min. All uptake assays were performed at least twice.

Two-Hybrid Techniques

The interaction trap two-hybrid system was used (Gyuris *et al.*, 1993). The assay was performed as described (Geli *et al.*, 1998). The X-Gal plates (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) were supplied with the indicated concentration of calcium in the form of CaCl_2 . Pictures were taken after 1 d of growth at 37°C. Expression of all *lexA*-Arc35p fusion proteins was confirmed by immunoblotting using the α -Arc35p-N antibody. Expression of the B42-Cmd1p, B42-Cmd1-226p, B42-Cmd1-228p, B42-Cmd1-239p, B42-Cmd1-231p, and B42-Cmd1-247p fusion proteins was confirmed on total protein extractions of cultures grown in SD/Gal/Raff-Ura-His-Trp by immunoblotting using the calmodulin antibody. All *lexA* fusions and B42 fusions were tested for their ability to activate transcription of the *lacZ* gene without the prey or bait, respectively. None of them was found to induce transcription significantly.

Native Protein Extracts and Coimmunoprecipitations

Cells (2×10^9) were grown to a density of 1×10^7 cells/ml in YPUADT. Cells were collected by centrifugation, resuspended in 100 ml 0.1 M Tris- SO_4 , pH 9.4, containing 28 mM 2-mercaptoethanol, and incubated at 24°C for 30 min. Cells were collected by centrifugation, resuspended in 20 ml spheroplasting buffer (2% yeast extract, 1% peptone, 0.7 M sorbitol, 10 mM Tris-HCl, pH 8.0, 0.6 U lyticase), and incubated for 90 min at 24°C. Spheroplasts were collected, washed in 20 ml washing buffer (0.7 M sorbitol, 0.1 M NaCl, 10 mM Tris-HCl, pH 8.0, 5 mM MgCl_2), resuspended in 1 ml lysis buffer (20 mM MES, pH 6.7, 0.1 M NaCl, 5 mM MgCl_2 , 0.5 mM PMSF, 1 $\mu\text{g}/\text{ml}$ each leupeptin, pepstatin, antipain) supplemented with the indicated concentration of CaCl_2 , and dounce-lysed on ice with 40 strokes. Addition of a nonionic detergent such as Nonidet-P-40 to the lysis buffer did not affect the results. The lysate was spun to remove cellular debris. The lysate (500 μl) was added to the antibodies preabsorbed on protein A-Sepharose beads and incubated for 2 h at 4°C. The beads were subjected to three washes. The pellet was resuspended in 300 μl 125 mM Tris-HCl 6.8, 2% SDS, 0.1 M DTT, 30% glycerol, 5% β -mercaptoethanol, and bromophenol blue, boiled for 5 min, centrifuged for 5 min, and analyzed by SDS-PAGE. The supernatant from the antibody incubation was precipitated with trichloroacetic acid and resuspended in 300 μl as described for the pellet. Fifty microliters of each pellet and supernatant were loaded onto the gel.

RESULTS

Arc35p Is Required for Endocytosis and Organization of the Actin Cytoskeleton

The *END9* gene has been identified in a screen for endocytosis-deficient mutants (Munn and Riezman, 1994). *END9* was found to be identical to *ARC35*, which encodes the 35-kDa subunit of the Arp2/3 complex (Winter *et al.*, 1997). Genetic analysis has revealed that the *ARC35* gene is essential for cell viability. Dissection of a diploid strain in which one allele of *ARC35* was replaced with the *URA3* marker (RH2962) resulted in a 2:2 (viable/dead) spore segregation. Eighteen tetrads gave two viable spores, and two tetrads gave only one viable spore. All of the viable spores were *ura3*, indicating that deletion of *ARC35* in our strain background is lethal (our unpublished results). A ts strain carrying the *arc35-1* mutation is viable at 24°C but undergoes growth arrest after temperature shift to 37°C (see Figure 4B, sector A). Sequencing of the genomic copy of *arc35-1* revealed two mutations. Nucleotide 1023 was changed from C to T, resulting in a silent mutation in Val₃₄₁, and nucleotide 1024 was changed from T to G, resulting in replacement of Tyr₃₄₂ by Asp.

The *arc35-1* mutant was shown to be impaired in the internalization of radiolabeled α -factor after a preincubation of 15 min at 37°C (Munn and Riezman, 1994). We found that the mutant was already significantly slowed down for endocytosis at the permissive temperature of 24°C when compared with an isogenic wild-type (Wt) strain (Figure 1A, Wt, 24°C and *arc35-1*, 24°C). While initial internalization of α -factor still occurred in the mutant upon a 15-min preincubation at 37°C (Munn and Riezman, 1994), a preincubation of 20 min abolished the internalization of α -factor almost completely (Figure 1A, *arc35-1*, 37°C). Under the same conditions, the isogenic Wt strain internalized α -factor with identical kinetics as at 24°C (Figure 1A, Wt, 37°C).

Because actin is required for endocytosis (Kübler and Riezman, 1993) and mutations in the Arp2/3 complex lead to actin defects (Moreau *et al.*, 1996; Winter *et al.*, 1997, 1999), we examined the organization of the actin cytoskeleton in an *arc35-1* strain. Wt and *arc35-1* cells were stained for filamentous actin (F-actin) at 24°C and after a preincubation of 3 h at 37°C (Figure 2). Microscopic inspection of the *arc35-1* cultures at both 24°C (Figure 2E) and 37°C (Figure 2G) revealed that the mutant cells were considerably larger and more elongated than the Wt cells (Figure 2, A and C). Wt cells showed the expected actin staining at both temperatures (Figure 2, B and D), with F-actin localizing predominantly to the sites of active cell growth (Adams and Pringle, 1984). The *arc35-1* mutant displayed mild defects in the organization of the actin cytoskeleton at both 24°C (Figure 2F) and 37°C (Figure 2H). The most obvious defect was the presence of highly staining actin cables that followed the cell cortex in both mother and daughter cells. Actin patches were often mislocalized to the bud neck and were apparently clustered into aggregates as described for an *arc35 Δ* strain (Winter *et al.*, 1999). The actin defects for other mutants in the Arp2/3 complex contrast with that of *arc35-1* in that no increased staining of the actin cables was observed (Moreau *et al.*, 1996; Winter *et al.*, 1997, 1999; our own observation). This finding emphasizes the importance of the Arp2/3 complex in organization of the actin cytoskeleton and points to possibly different or multiple functions of the subunits in the process.

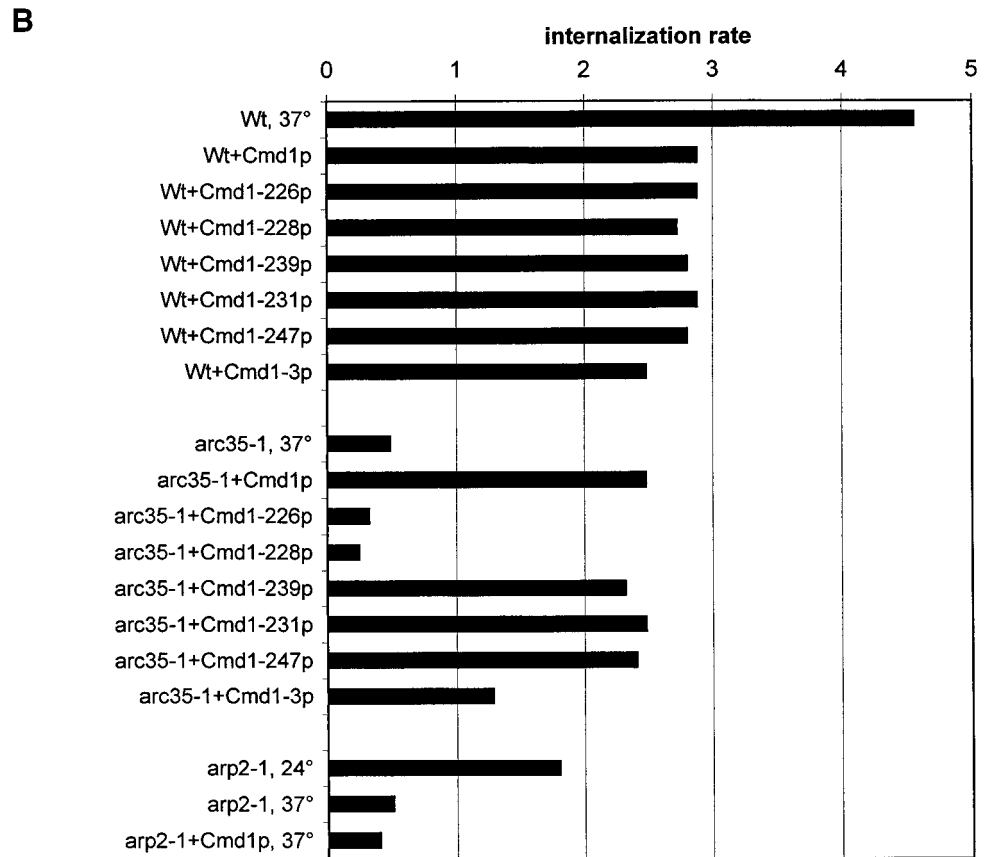
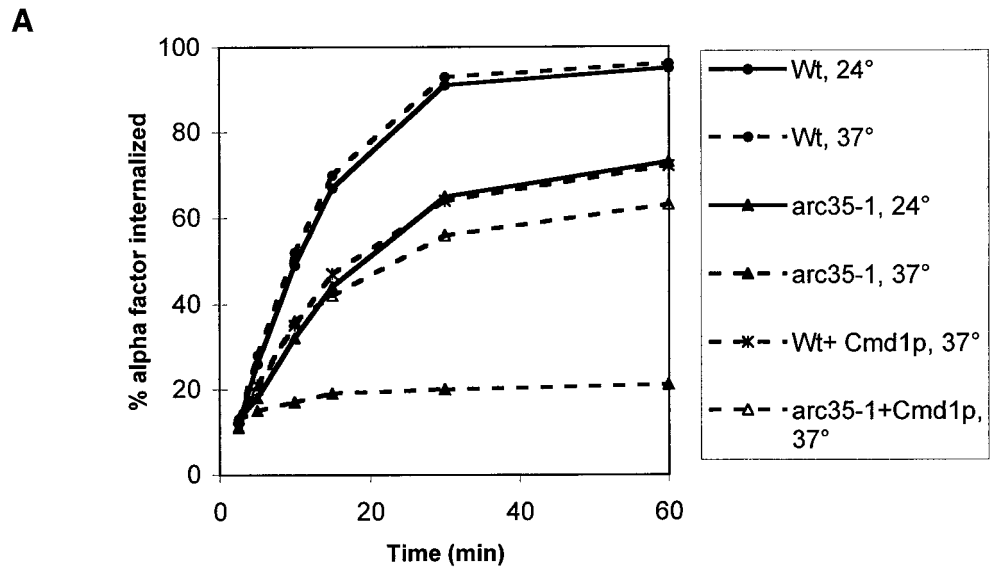


Figure 1. Overexpression of calmodulin and distinct calmodulin mutant alleles restores endocytosis in *arc35-1*. (A) Wt (RH2877) and *arc35-1* (RH3429) strains without plasmid or bearing plasmid pCMD1 (+Cmd1p) were assayed for their ability to internalize radioactive α -factor at 24 and 37°C after a preincubation of 20 min at the indicated temperature. (B) Wt (RH2877), *arc35-1* (RH3429), and *arp2-1* (RH4164) strains without plasmid or overexpressing the indicated mutant calmodulin protein from pASZ12 were assayed for the internalization of radioactive α -factor at 24 or 37°C after a preincubation of 20 min. The results are presented as the percentage of radioactive α -factor internalized per minute during the linear range of the uptake reaction.

Arc35-GFP Colocalizes with Filamentous Actin

Both Arp2 (Moreau *et al.*, 1996) and Arp3 (Winter *et al.*, 1997) have been localized to actin patches. Because Arc35p belongs to the same complex and exhibits an actin defect, we

determined the localization of Arc35p. Because our α -Arc35p antibodies failed to recognize the protein in fixed cells (our unpublished results), we integrated an Arc35-GFP chimera at the *ARC35* locus. The chimeric protein was fully

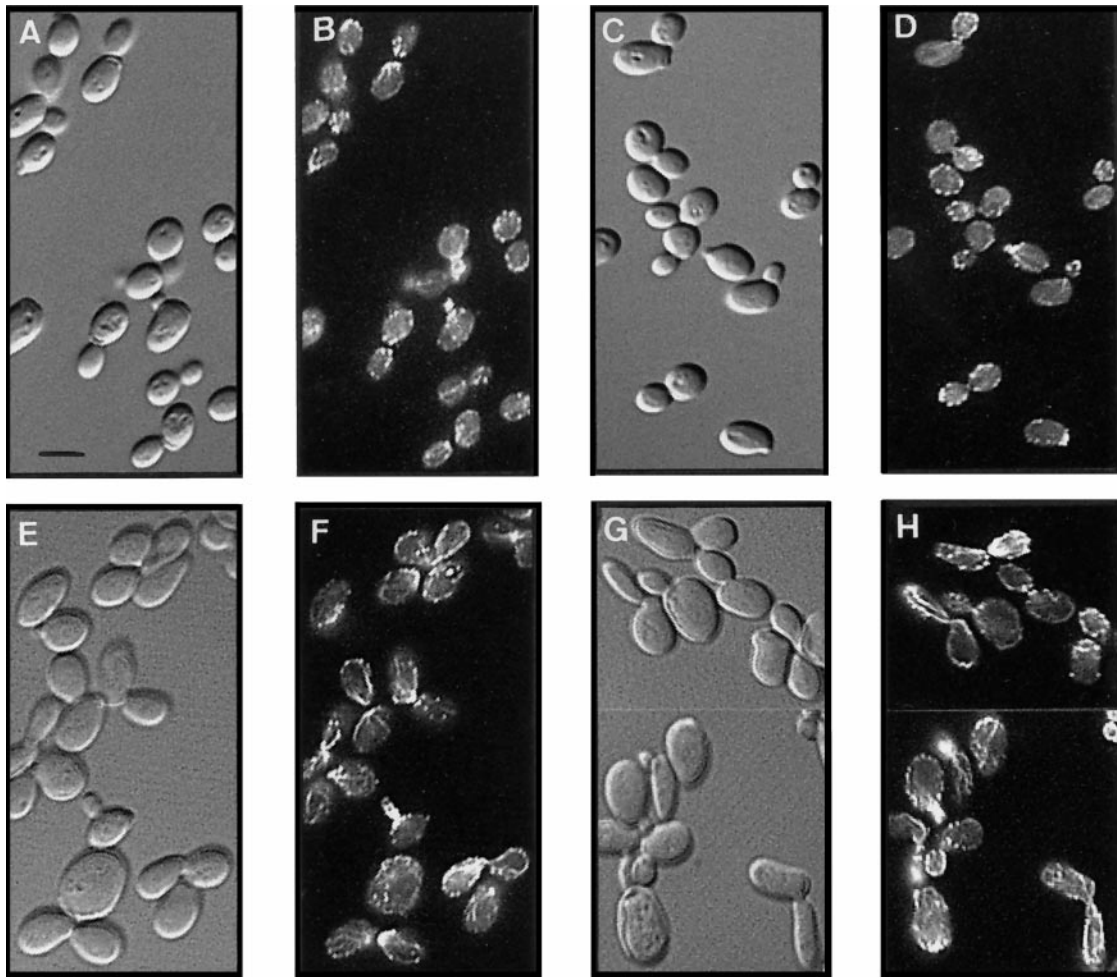


Figure 2. Defective actin organization in *arc35-1* cells. Wt (RH2877) and *arc35-1* (RH3429) strains were preincubated for 3 h at either 24 or 37°C, fixed, stained with rhodamine-phalloidin, and visualized either by Nomarski optics or by epifluorescence. Shown are the Wt strain at 24°C (A, B) and 37°C (C, D) and the *arc35-1* strain at 24°C (E, F) and 37°C (G, H), respectively. Bar, 5 μ m.

functional based on cell morphology, F-actin stainings (Figure 3A), and endocytosis assays (our unpublished results). Arc35-GFP localized predominantly to sites of active growth in a Wt background (Figure 3A) with some cortical staining visible in the mother cell. Arc35-GFP fluorescence was not observed on cable-like structures. To test for colocalization of actin and Arc35p, an Arc35-GFP-expressing strain was stained to visualize F-actin (Figure 3B). Although integrity of the actin cytoskeleton was affected by the use of sorbitol during fixation to preserve Arc35-GFP, the signal from Arc35-GFP was found to overlap nicely with the staining of F-actin for all visible actin patches. Because the cables were lost when using this special fixation protocol, it was impossible to determine localization of Arc35-GFP to those structures.

Overexpression of Calmodulin Restores Growth of *arc35-1* at 37°C

Because the *arc35-1* mutant exhibited defects in actin organization, in endocytosis, and in nuclear division (Schaerer-

Brodbeck and Riezman, 2000), analogous to *cmd1* mutants, we wanted to test for a potential genetic interaction between Arc35p and Cmd1p. We found that overexpression of Cmd1p rescued the growth defect of *arc35-1* at 37°C (Figure 4B, sector B). Growth of the Wt control strain was not affected by overexpression of Cmd1p at any temperature (Figure 4A). No suppression of the ts growth of various *cmd1* mutants was observed upon overexpression of Arc35p (our unpublished results).

Intragenic complementation studies of ts *cmd1* mutants have revealed at least four distinct, essential cellular functions for calmodulin (Ohya and Botstein, 1994). For a summary of the ts *cmd1* mutant phenotypes, see Table 3. Class A mutants (e.g., *cmd1-226*) are defective in the organization of the actin cytoskeleton. Class B mutants (*cmd1-228*) mislocalize calmodulin. Class C mutants (*cmd1-239*) are defective for establishment/elongation of the nuclear spindle, and class D mutants (*cmd1-231*) do not form a bud. In addition, calmodulin performs at least two different functions in endocytosis (Geli *et al.*, 1998).

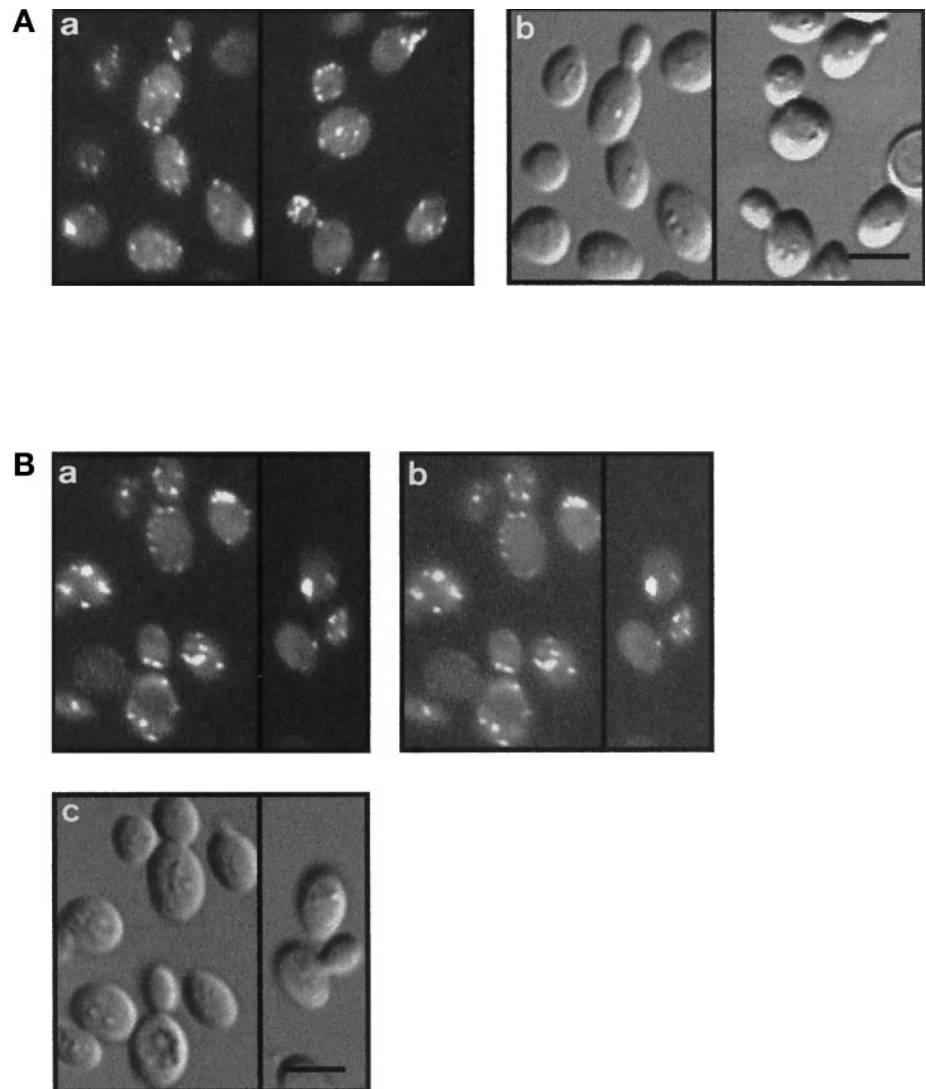


Figure 3. Arc35-GFP colocalizes with filamentous actin. (A) A Wt strain expressing Arc35-GFP (RH4161) was visualized with epifluorescence (a) or Nomarski optics (b). Bar, 5 μ m. (B) A Wt strain expressing Arc35-GFP (RH4161) was grown at 24°C, stained for F-actin with rhodamine-phalloidin, and visualized either with Nomarski optics (c) or with epifluorescence filters suitable to distinguish between GFP and rhodamine signals. The F-actin staining is shown in a, and the signal of Arc35-GFP is shown in b. Bar, 5 μ m.

One function, affected by the *cmd1-247* mutation and referred to as the *Cmd1-247* pathway, acts through the type I myosins. The other function, impaired by the *cmd1-228* mutation and referred to as the *Cmd1-228* pathway, operates via an unknown target. The *cmd1-226* mutation affects both endocytic functions of calmodulin. To examine genetically which of the functions of Cmd1p Arc35p interacts with, Wt and *arc35-1* mutant cells were transformed with a series of high-copy plasmids expressing the ts *cmd1* alleles described above. All of the resulting strains were tested for growth at 37°C. Overexpression of the mutant alleles did not affect growth of the Wt control strain at either 24 or 37°C (Figure 4A). The ts growth of *arc35-1* (Figure 4B, sector A) could be suppressed by overexpression of *Cmd1-226p* (panel C), *Cmd1-231p* (panel F), or *Cmd1-247p* (panel G). Overexpression of neither *Cmd1-228p* (panel D) nor *Cmd1-239p* (panel E) could restore growth at 37°C to *arc35-1*. The lack of suppression by the B and C class calmodulin mutants (for a summary of the

data, see Table 4) indicates that Arc35p may share two functions with calmodulin, calmodulin localization (*Cmd1-228* pathway) and nuclear division (*Cmd1-239* pathway). At least one of the functions of Arc35p may be calcium-dependent because overexpression of *Cmd1-3p*, which is defective for calcium binding in vitro (Geiser *et al.*, 1991), did not compensate for loss of Arc35p activity (Figure 4B, panel H). Moreover, a genetic interaction between *ARC35* and *CMD1* was confirmed in crosses. An *arc35-1* mutant was crossed to *cmd1-3*, *cmd1-226*, *cmd1-228*, *cmd1-239*, *cmd1-231*, or *cmd1-247* strains. The resulting diploids were sporulated, and tetrads were dissected. No double mutants between *arc35-1* and *cmd1-3*, *cmd1-228*, or *cmd1-239* were obtained, indicating a synthetic lethal interaction (our unpublished results). This correlates well with the suppression of ts growth data.

Interestingly, the ts growth of an *arp2-1* strain (Figure 4C) or an *arp3-1* strain (our unpublished results) was not suppressed by overexpression of calmodulin or selected mutant

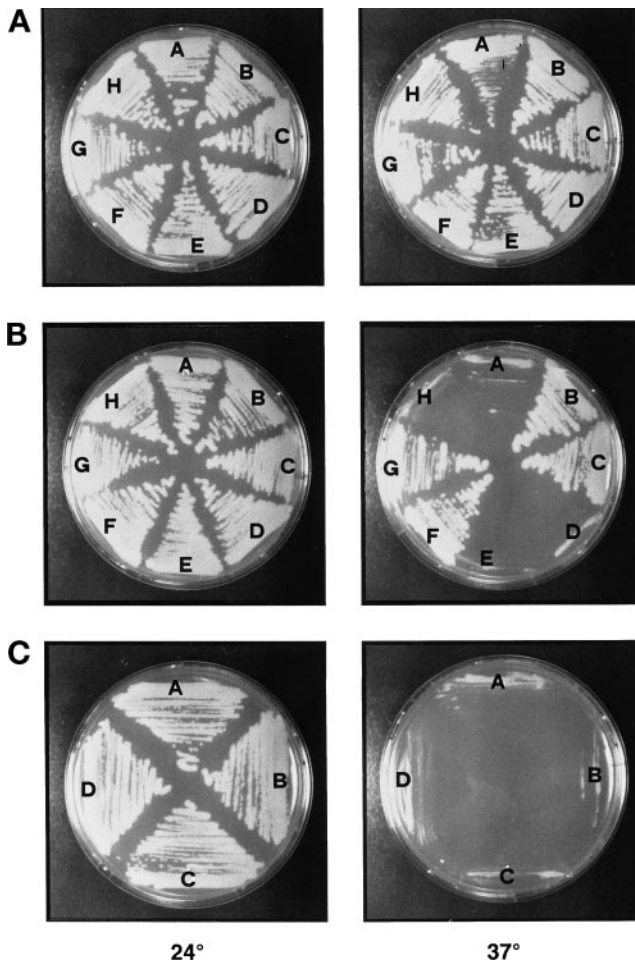


Figure 4. Suppression of *arc35-1* ts growth by calmodulin. Wt (RH2877) (A), *arc35-1* (RH3429) (B), and *arp2-1* (RH4164) (C) strains were transformed with the following plasmids: (A) pASZ12, (B) pCMD1, (C) pASZ12::*cmd1-226* (actin), (D) pASZ12::*cmd1-228* (calmodulin mislocalized), (E) pASZ12::*cmd1-239* (nuclear defect), (F) pASZ12::*cmd1-231* (bud emergence), (G) pASZ12::*cmd1-247* (endocytosis), (H) pASZ12::*cmd1-3* (calcium binding). The strains were grown on glucose-selective medium for 3 d at 24°C and for 2 d at 37°C.

calmodulin proteins. This suggests that the genetic interaction observed between the Arp2/3 complex and Cmd1p could be unique to Arc35p.

Overexpression of Cmd1p and Distinct Cmd1 Mutant Proteins Restores Endocytosis in *arc35-1*

We wanted to determine whether overexpression of Cmd1p suppresses the endocytic defect of *arc35-1*. A Wt and *arc35-1* strain expressing Cmd1p were assayed for receptor-mediated endocytosis after a preincubation of 20 min at 37°C (Figure 1A). Overexpression of Cmd1p reduced the internalization kinetics slightly in a Wt background. In the *arc35-1* background, the endocytic rate was increased to ~80% of Wt levels upon overexpression of Cmd1p if compared with the mutant strain without plasmid.

Table 3. Summary of the calmodulin mutant phenotypes

Mutant allele Class	Phenotype at 37°C	Endocytosis at 37°C
<i>cmd1-226</i>	A Delocalized actin	–
<i>cmd1-228</i>	B Delocalized calmodulin	–
<i>cmd1-239</i>	C Defective mitotic spindle formation	+
<i>cmd1-231</i>	D No bud emergence	+
<i>cmd1-247</i>		–
<i>cmd1-3</i>	No Ca ²⁺ binding	+

The phenotypes for one representative allele of each calmodulin mutant class are shown. These are based on results of Geiser *et al.* (1991), Ohya and Botstein (1994), and Geli *et al.* (1998).

The collection of ts *cmd1* alleles was assayed for their ability to restore endocytosis in *arc35-1* at 37°C upon overexpression (Figure 1B). None of the mutant alleles was found to decrease the internalization rate more than Cmd1p upon overexpression in a Wt background. This allowed a direct comparison of the internalization rates observed for the different *arc35-1* strains. Overexpression of Cmd1-239p (nuclear defect), Cmd1-231p (bud emergence), or Cmd1-247p (myosin endocytic function) restored endocytosis in *arc35-1* to almost Wt level as observed for overexpression of Cmd1p. Overexpression of Cmd1-226p or Cmd1-228p did not improve the internalization kinetics. Overexpression of Cmd1-3p restored endocytosis to 50% of the Wt level. The data (for a summary, see Table 4) suggest that Arc35p shares specifically one endocytic function with Cmd1p, namely the *Cmd1-228* but not the *Cmd1-247* pathway.

An involvement of other subunits of the Arp2/3 complex in endocytosis has been demonstrated previously (Moreau *et al.*, 1997). We found that the ts *arp2-1* strain, which fails to internalize uracil permease (Moreau *et al.*, 1997), showed a reduced rate of α -factor internalization already at the permissive temperature (Figure 1B, *arp2-1*, 24°C). The internalization rate decreased further upon a 20 min preincubation at 37°C (*arp2-1*, 37°C). Overexpression of calmodulin did not improve the internalization kinetics. This points to different defects affecting endocytosis in mutants of the Arp2/3 complex and is in good correlation with the findings for the actin defects of the different mutants.

The Same Calmodulin Mutant Proteins that Restore Endocytosis Suppress the Actin Defect of *arc35-1*

It has been shown that defects in the organization of the actin cytoskeleton result frequently in increased or decreased sensitivity to the actin depolymerizing drug latA (Ayscough *et al.*, 1997). We found that the *arc35-1* mutation leads to almost twofold sensitivity to latA at 24°C if compared with a Wt control (Figure 5B; Wt+pASZ12, *arc35-1*+pASZ12). Overexpression of Cmd1p in *arc35-1* resulted in a restoration of Wt sensitivity to latA (Figure 5B). In support of the idea that latA sensitivity is a measure of integrity of the actin cytoskeleton, the actin cytoskeleton of an *arc35-1* strain expressing Cmd1p was found to be properly organized at 37°C (Figure 5A, panel c; compare with *arc35-1* at the same temperature in panel a).

In contrast to overexpression of Wt Cmd1p (Figure 5A, panel d), overexpression of most other ts *cmd1* alleles in

Table 4. Summary of the suppression of the defects observed in *arc31-1* by different ts calmodulin mutant alleles

	Growth	Actin	Endocytosis	Interacts with Arc35p
<i>arc35-1</i> , 24°	+	–	+	
<i>arc35-1</i> , 37°	–	–	–	
<i>arc35-1</i> + pASZ12, 37°	–	–	–	
<i>arc35-1</i> + Cmd1p, 37°	+	+	+	+
<i>arc35-1</i> + Cmd1-226p, 37°	+	–	–	–
<i>arc35-1</i> + Cmd1-228p, 37°	–	–	–	–
<i>arc35-1</i> + Cmd1-239p, 37°	–	+	+	–
<i>arc35-1</i> + Cmd1-231p, 37°	+	+	+	+
<i>arc35-1</i> + Cmd1-247p, 37°	+	+	+	+
<i>arc35-1</i> + Cmd1-3p, 37°	–	+/-	+/-	–

arc35-1 did not restore Wt morphology (Schaerer-Brodbeck and Riezman, 2000). We have shown previously that overexpression of Cmd1-228p did not restore proper actin localization in *arc35-1* cells, whereas high doses of Cmd1-239p did (Schaerer-Brodbeck and Riezman, 2000); however, it may be misleading to draw any conclusions concerning restoration of the actin integrity in mutant cells with an abnormal morphology. Therefore, we determined sensitivity to latA of *arc35-1* strains overexpressing the collection of ts *cmd1* alleles as a measure for integrity of the actin cytoskel-

eton. Overexpression of any of the ts alleles, except for Cmd1-3p, did not alter the sensitivity of a Wt strain to latA (Figure 5B). Overexpression of Cmd1-3p increased the sensitivity of the Wt strain to latA 1.3-fold. Overexpression of Cmd1-239p, Cmd1-231p, or Cmd1-247p in *arc35-1* restored latA sensitivity to Wt values (Figure 5B). Overexpression of Cmd1-228p did not decrease the sensitivity of *arc35-1* to latA, whereas overexpression of Cmd1-226p increased the sensitivity of *arc35-1* to latA. Overexpression of Cmd1-3p (calcium binding) resulted in some reduction of sensitivity

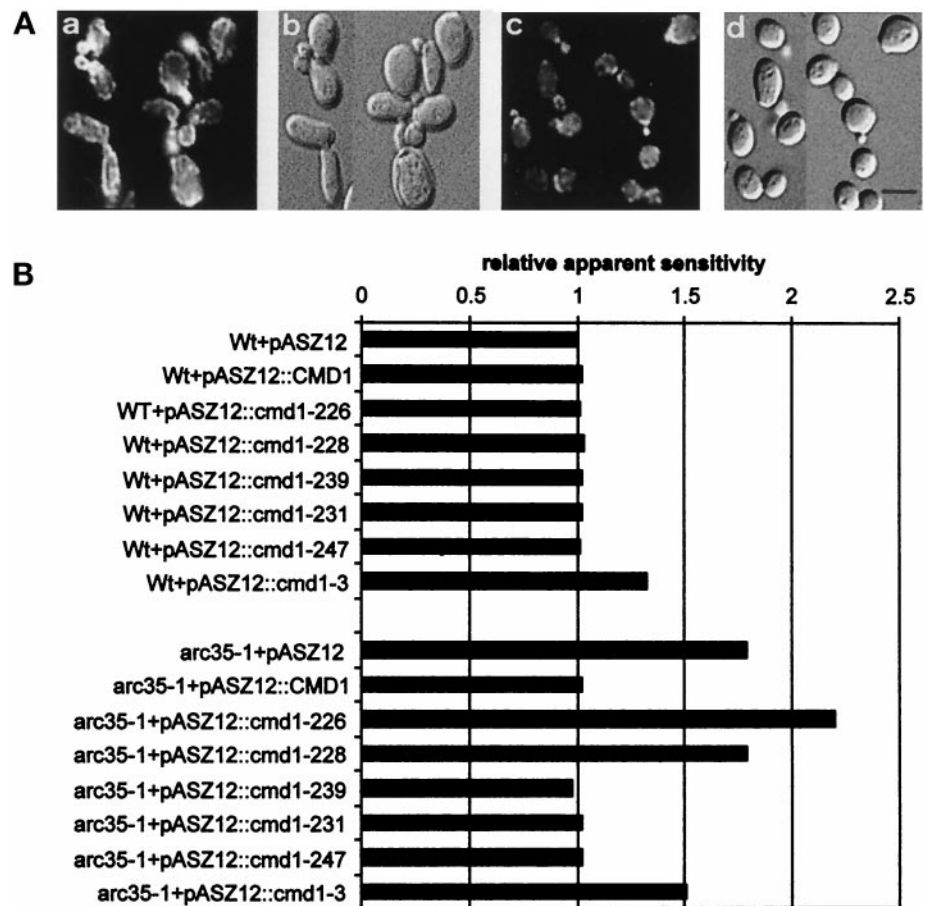


Figure 5. Sensitivity of *arc35-1* expressing various calmodulin mutant proteins to latrunculin A. (A) An *arc35-1* strain (RH3429) without plasmid (a, b) or overexpressing Cmd1p from pCMD1 (c, d) was preincubated for 3 h, stained with rhodamine-phalloidin, and visualized with epifluorescence. Bar, 5 μ m. (B) Wt (RH2877) and *arc35-1* (RH3429) strains bearing the empty control plasmid (pASZ12) or overexpressing the indicated calmodulin mutant protein from pASZ12 were used for determination of the apparent relative sensitivity to latrunculin A.

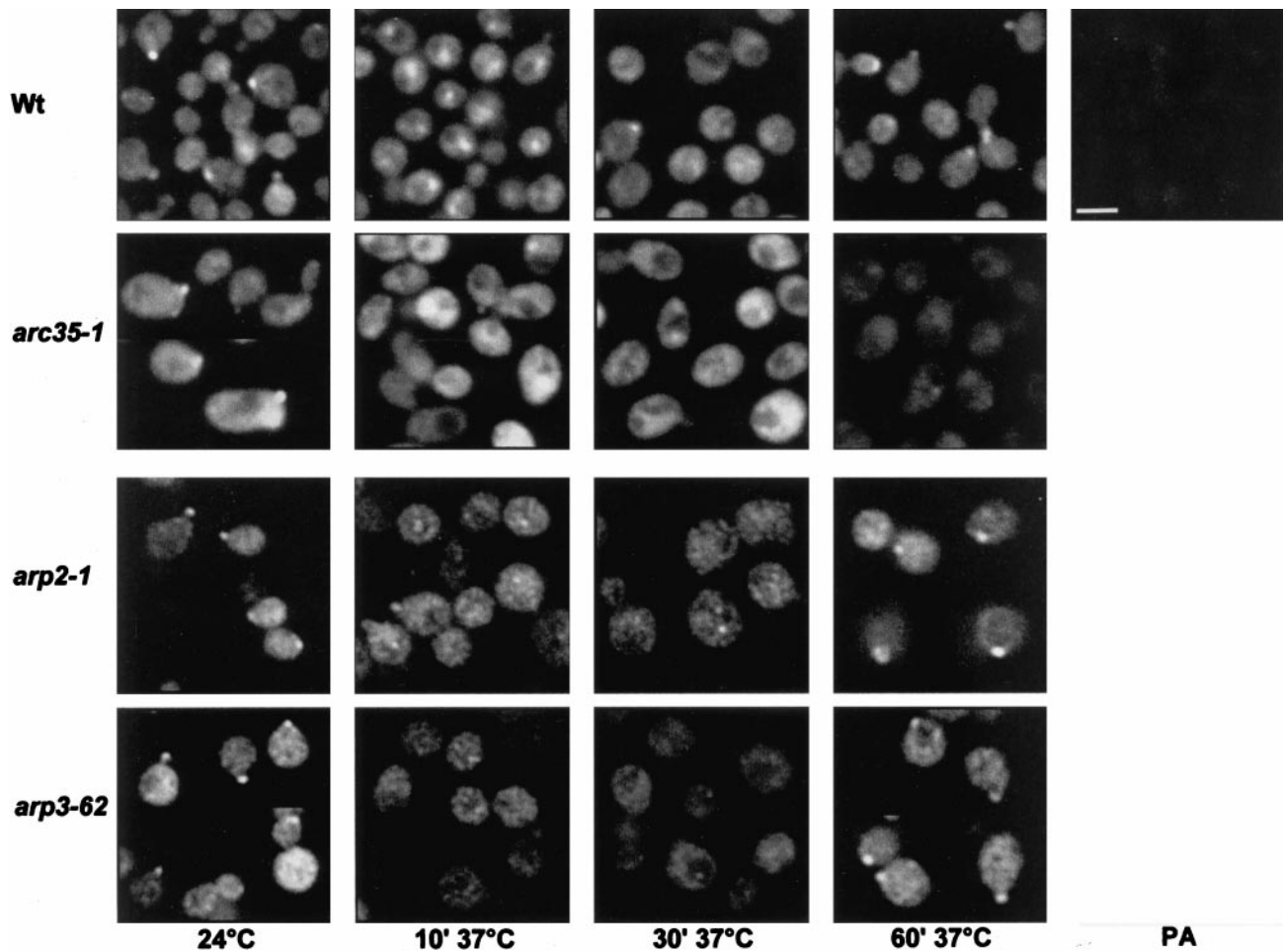


Figure 6. Arc35p is required to localize calmodulin to the cortical sites. Wt (RH2877), *arc35-1* (RH3429), *arp2-1* (RH4164), and *arp3-1* (Y90) strains were grown at 24°C and then shifted to 37°C for 10, 30, or 60 min before fixation and staining for calmodulin by indirect immunofluorescence. The staining obtained using preabsorbed α -calmodulin antibody is also shown (PA). Bar, 5 μ m.

of *arc35-1* to *latA*, although the suppression was not complete. It is striking that calmodulin mutant proteins that cannot suppress the endocytic defect of *arc35-1* cannot suppress the increased sensitivity to *latA* either.

Arc35p Is Required to Localize Calmodulin to Sites of Active Growth

The pathway affected by *cmd1-228* is required to localize calmodulin to the sites of active growth. Because the endocytic defect in an *arc35-1* mutant is most likely linked to the *Cmd1-228* function, we determined the intracellular localization of Cmd1p in an *arc35-1* mutant at the restrictive temperature. Wt and *arc35-1* strains were processed for immunofluorescence with the α -Cmd1p antibody after various time intervals at 37°C (Figure 6). The Wt strain showed localization of calmodulin to the sites of active growth at 24°C as described previously (Ayscough *et al.*, 1997). After the shift to 37°C, calmodulin underwent a transient relocalization to the cytoplasm. Recovery of cortical calmodulin localization began 30 min after the shift and was completed

after 1 h at 37°C. To confirm the specificity of the observed signal, the Wt strain was stained with calmodulin antibody that had been preabsorbed with recombinant calmodulin (PA). The fluorescent images revealed that the signal observed was due to calmodulin.

In *arc35-1* mutant cells, Cmd1p was mainly localized to the sites of active growth, although increased cytoplasmic staining was observed (Figure 6, *arc35-1*, 24°C). Staining of calmodulin at sites of active growth disappeared completely upon shift for 10 min to 37°C as in Wt cells. In contrast to Wt cells, a relocalization of calmodulin to sites of active growth was not seen in the *arc35-1* mutant at 37°C. In contrast, no mislocalization of Arc35-GFP in any of the *ts cmd1* mutants was observed (our unpublished results), indicating that Arc35p localization does not depend on Cmd1p.

It should be considered that the observed mislocalization of Cmd1p in *arc35-1* cells on shift to 37°C could be caused by misorganization of the actin cytoskeleton. To address this question, Cmd1p localization was analyzed in *arp2* and *arp3* mutants, which fail to organize the actin cytoskeleton prop-

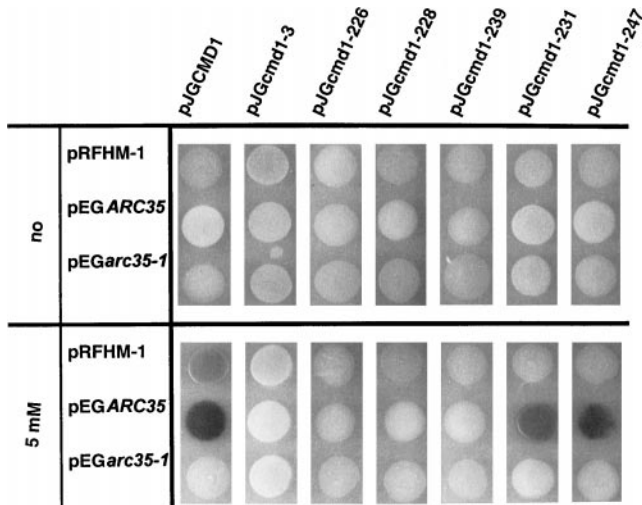


Figure 7. Two-hybrid interaction between Arc35p and Cmd1p. ARC35 and *arc35-1* were cloned in frame to the *lexA* DNA binding domain of pEG202. *CMD1* and the collection of *ts cmd1* alleles were cloned in frame behind the B42 transactivation domain of pJG4-5. β -Galactosidase activity indicative of interaction was monitored on X-Gal plates at 37°C supplemented with no calcium or 5 mM calcium.

erly at 37°C (Moreau *et al.*, 1996; our own observation). At 24°C, both mutants localized Cmd1p to the sites of active growth (Figure 6). A short shift to 37°C caused mislocalization of Cmd1p to the cytoplasm as observed for Wt and *arc35-1* cells. In contrast to the *arc35-1* mutant, recovery of cortical calmodulin was observed in *arp2* and *arp3* mutants within 1 h at 37°C. Although the kinetics of recovery was slightly decreased when compared with Wt, disruption of the actin cytoskeleton by the *arp2-1* and *arp3-1* mutations did not affect the long-term localization of Cmd1p. It is therefore unlikely that the mislocalization of Cmd1p observed in *arc35-1* cells is caused by the failure to organize the actin cytoskeleton properly. These data suggest that Arc35p is required to localize calmodulin to the sites of active growth.

Arc35p Interacts Specifically with Cmd1p

The data presented suggest a role for Arc35p as a regulator of calmodulin localization and probably function. We therefore investigated a possible physical interaction between Arc35p and Cmd1p with the two-hybrid system and coimmunoprecipitations. For the analysis with the yeast two-hybrid system, *ARC35* and *arc35-1* were used as baits (pEG series), and *CMD1* and the collection of *ts cmd1* mutant were used as prey (pJG series). β -Galactosidase activity indicative of interaction was monitored at 37°C on X-Gal-enriched plates containing no calcium or 5 mM calcium. Strong β -galactosidase activity was detected in the strain bearing the *ARC35* bait and *CMD1* prey on plates with 5 mM calcium (Figure 7).

The observation that only some *ts cmd1* alleles are capable of suppressing the *ts* growth and/or the endocytic defect of the *arc35-1* strain prompted us to determine the interaction profile of Arc35p with the same calmodulin mutants (Figure

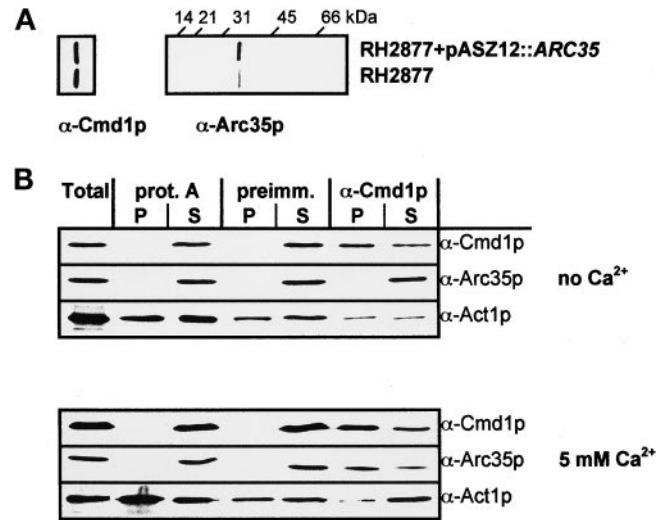


Figure 8. Coimmunoprecipitation of Arc35p and Cmd1p. (A) To assess the specificity of the Arc35p antibody, Wt cells expressing either endogenous Arc35p or overexpressing Arc35p (RH2877+pASZ12::ARC35) were grown in selective medium and harvested, and proteins were extracted. The extracts were immunoblotted for Arc35p and Cmd1p as a loading control. (B) A Wt strain (RH2877) was grown to logarithmic phase and harvested. Total and native protein extracts were prepared. Protein A-Sepharose (prot. A), protein A-Sepharose bound to preimmune serum (preimm.), or protein A-Sepharose bound to α -Cmd1p serum (α -Cmd1p) was used for immunoprecipitation of native cell extracts in the presence of no calcium or 5 mM calcium. Immunoprecipitates (P) and supernatants (S) were immunoblotted for Arc35p, Cmd1p, or Act1p with α -Arc35p-N, α -Cmd1p, or α -actin antibodies.

7). Strong β -galactosidase activity was detected in the strains bearing the *ARC35* bait and either the *cmd1-231* or *cmd1-247* prey. Strains bearing the *ARC35* bait and the *cmd1-226*, *cmd1-228*, or *cmd1-239* preys did not show detectable β -galactosidase activity. These data (for a summary, see Table 4) support the hypothesis that Arc35p exerts its functions via the pathways affected by *cmd1-226/228* and *cmd1-239* alleles. No interaction was monitored in a strain expressing the *ARC35* bait and the *cmd1-3* prey, indicating Ca^{2+} -dependent binding. β -galactosidase activity was not observed with the *cmd1* preys when the *ARC35* bait was replaced with *arc35-1* as a bait, showing that functional Arc35p is required for this interaction. The strain bearing the *Drosophila* bicoid protein as a control for the specificity of the assay did not show any significant interaction.

To confirm the interaction seen in the two-hybrid interaction trap, an extract of Wt cells was used for immunoprecipitations with the α -calmodulin antiserum in the presence or absence of 5 mM calcium. The immunoprecipitates (P) and supernatants (S) were assayed for Arc35p and Cmd1p content by immunoblotting. Arc35p was recovered significantly in the immunoprecipitates using Cmd1p antibody (α -Cmd1p) for immunoprecipitation in the presence of 5 mM calcium (Figure 8). No recovery was detected without calcium. At 5 mM calcium, no Arc35p was recovered in the immunoprecipitates when protein A-Sepharose (prot. A) or preimmune serum (preimm.) was used for immunoprecipitation, confirming the specificity of the interaction. Because

the interaction between Arc35p and Cmd1p could be mediated by filamentous actin, the immunoprecipitates and supernatants were assayed for the actin content as well (Figure 8). Actin was found to precipitate nonspecifically in all control reactions where no Arc35p could be recovered in the immunopellets. It is therefore unlikely that filamentous actin mediates the observed interaction. Unlike experiments with the two-hybrid system, a strain expressing Cmd1-3p could not be used for an independent confirmation of the calcium dependence of the observed interaction because Cmd1-3p is not recognized by our antibody (our unpublished results).

DISCUSSION

The Arp2/3 complex is involved in the organization and integrity of the cortical actin cytoskeleton (Zigmond, 1998; for review, see Machesky and Gould, 1999). Analysis of an *arc35-1* (ts) mutant, defective in a relatively uncharacterized component of the complex, also revealed defects in the organization of the actin cytoskeleton. The mutant displayed exaggerated actin cables along the mother–daughter cell axis, and F-actin aggregates localized predominantly in the bud neck. Although accumulation of F-actin aggregates has been demonstrated for *arp2* (Moreau *et al.*, 1996) and *arp3* (Winter *et al.*, 1997) mutants, an increase in actin cable staining has not been seen in *arp2/3* mutants. The phenotype of *arc35-1* suggests a role for the Arp2/3 complex, not only in actin polymerization (Welch *et al.*, 1998) but also in dynamics of actin cables.

Suppression of *arc35-1* by Calmodulin

We have shown that overexpression of calmodulin can suppress the ts growth, the endocytic defect, and the actin defect of the *arc35-1* mutant. This suppression was specific to *arc35-1* and was not observed for *arp2-1* or *arp3-1*. The unusual actin defect of *arc35-1* compared with other mutants in the Arp2/3 complex and the unique genetic interaction observed between Arc35p and Cmd1p suggest that Arc35p may regulate some aspects of F-actin dynamics via calmodulin and that its function may be different from other subunits of the complex; however, we cannot rule out that the differences we see between mutants in the Arp2/3 complex are due to the specific alleles rather than individual functions of the subunits.

We observed that overexpression of *cmd1* alleles that do not affect endocytosis restored normal actin staining and endocytosis in the *arc35-1* mutant. Overexpression of Cmd1-3p, which does not bind calcium (Geiser *et al.*, 1991), could only partially suppress the endocytic and actin defect of *arc35-1*, although a ts *cmd1-3* strain internalizes α -factor with Wt kinetics (Kübler *et al.*, 1994). On the other hand, overexpression of two *cmd1* alleles that affect endocytosis, *cmd1-226* and *cmd1-228*, could not restore the actin and endocytic defects, whereas overexpression of another allele, *cmd1-247*, could. For a summary of the suppression data, see Table 4. These data are consistent with the previous hypothesis (Geli *et al.*, 1998) that calmodulin plays at least two roles in endocytosis. One role is to regulate the type I myosin, and this function is affected by the *cmd1-247* and *cmd1-226* mutations. The target of the second function, which is affected by the *cmd1-226* and *cmd1-228* mutations, is still unknown. Our data would suggest that Arc35p is required for actin

organization and endocytosis and that it is associated with the *Cmd1-228* function of calmodulin. Thus, it is likely that the endocytic function defined by the *arc35-1* mutation is independent of the type I myosins.

One striking observation is that both the *cmd1-228* and *arc35-1* mutants mislocalize cortical calmodulin after shift to the restrictive temperature. For other mutants in the Arp2/3 complex, recovery of cortical calmodulin after a shift to the restrictive temperature was observed, albeit with slower kinetics than in the Wt control strain. Because both *arc35-1* and *cmd1-228* mislocalize calmodulin and are defective for receptor-mediated endocytosis as well, it is tempting to speculate that the endocytic function of Arc35p is to localize cortical calmodulin. The endocytic defect could be a direct consequence of the mislocalization of calmodulin in the *arc35-1* strain. One line of evidence argues against this simple model. In Wt cells, after short times of temperature shift to 37°C (up to 30 min), calmodulin is transiently localized to the cytoplasm. At the same time, endocytosis occurs with normal kinetics. We therefore postulate that Arc35p is not only required for localizing calmodulin cortically, but could be required for interaction of calmodulin with the target of the *Cmd1-228* function. Prolonged incubation at 37°C results in localization of Cmd1p back to the cortical sites in Wt cells. It is possible that during this time at 37°C in which wild-type cells recover calmodulin localization, the target of the *Cmd1-228* function remains in place; however, in *arc35-1* cells, calmodulin is never relocalized cortically, perhaps because of lack of interaction with Arc35-1p. As a consequence, the target of the *Cmd1-228* function may become mislocalized or inactivated, which might be the cause of the endocytic defect in both the *cmd1-228* and *arc35-1* mutants. This idea is supported by the observation that the endocytic defect of an *arc35-1* strain is not immediate after a shift to restrictive temperature but requires 15–20 min preincubation.

Overexpression of some calmodulin mutant proteins suppressed the ts growth phenotype of *arc35-1* cells, whereas others, such as Cmd1-228p and Cmd1-239p, could not. In addition, overexpression of Cmd1-226p in *arc35-1* restored growth at 37°C without restoring endocytosis or actin organization. This indicates that the actin/endocytic defect of *arc35-1* cells is not the only determinant of ts growth and suggests that Arc35p regulates another essential function through calmodulin. We have evidence that Arc35p is involved in spindle elongation during mitosis via a function affected by the *cmd1-239* allele (Schaerer-Brodbeck and Riezman, 2000).

Arc35p Interacts with Calmodulin in a Calcium-dependent Manner

We have characterized Arc35p as a regulator of Cmd1p localization and activity. Two-hybrid data showed that Arc35p interacts optimally with Cmd1p under conditions of 5 mM extracellular calcium. Consistent with the calcium stimulation is the fact that Arc35p–Cmd1-3p interaction was not detectable even with 5 mM extracellular calcium. This suggests that the calcium-binding domains of calmodulin loaded with calcium strengthen the interaction with Arc35p.

Results using calmodulin mutant proteins in the two-hybrid assays showed that the interaction we detected is specific and most likely significant for function. For a summary of the interaction data, see Table 4. Calmodulin mutant

proteins that could suppress the ts growth defect of *arc35-1* (Cmd1-231p, Cmd1-247p) bound Arc35p strongly in the assay, whereas ones that could not suppress the ts growth defect or the endocytic defect (Cmd1-226p, Cmd1-228p, Cmd1-239p) did not. Coimmunoprecipitation assays were in entire agreement with the two-hybrid studies and indicate that Arc35p and Cmd1p can be found together in a protein complex. In addition, our data suggest that the interaction observed may not be mediated through filamentous actin. This is the first suggestion of complex formation between these two important players in regulation of the dynamics of the actin cytoskeleton. We cannot determine from these experiments whether the interaction of the two proteins is direct or through other proteins.

The internalization step of endocytosis in yeast has been shown to require calmodulin, but apparently the calcium binding capacity of calmodulin is not essential for this calmodulin function because *cmd1-3* mutant cells do not show a strong endocytic defect (Kübler *et al.*, 1994). On the other hand, we show here that calcium is needed to detect an interaction between Cmd1p and Arc35p, two proteins required for endocytosis. One possibility to explain this is if the interaction between the two proteins would be dynamic and calcium ions would favor the state in which the two proteins interact. This would allow detection of a stable complex *in vitro*. *In vivo* a transient interaction without calcium could be sufficient for endocytic function.

In summary, the data presented show that Arc35p is required for endocytosis and provide evidence that its endocytic function is mediated through actin and calmodulin. This major point is supported by multiple genetic interactions as well as a physical association between Arc35p and Cmd1p. Calmodulin has two functions in endocytosis. One of these is associated with the type I myosin and is not affected in the *arc35-1* mutant. The other calmodulin function, characterized by the *cmd1-228* allele, is affected in the *arc35-1* mutant. For the moment, it seems that this novel calmodulin-dependent function of the Arp2/3 complex is mediated by the Arc35p subunit; however, our studies do not address the question of whether this function of Arc35p requires association with other subunits of the complex.

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