The Saccharomyces cerevisiae Actin-related Protein Arp2 Is Involved in the Actin Cytoskeleton

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Abstract. Arp2p is an essential yeast actin-related protein. Disruption of the corresponding ARP2 gene leads to a terminal phenotype characterized by the presence of a single large bud. Thus, Arp2p may be important for a late stage of the cell cycle (Schwob, E., and R.P. Martin, 1992. Nature (Lond.). 355:179–182). We have localized Arp2p by indirect immunofluorescence. Specific peptide antibodies revealed punctate staining under the plasma membrane, which partially colocalizes with actin. Temperature-sensitive arp2 mutations were created by PCR mutagenesis and selected by an ade2/SUP11 sectoring screen. One temperature-sensitive mutant that was characterized, arp2-H330L, was osmosensitive and had an altered actin cytoskeleton at a nonpermissive temperature, suggesting a role of Arp2p in the actin cytoskeleton. Random budding patterns were observed in both haploid and diploid arp2-H330L mutant cells. Endocytosis, as judged by Lucifer yellow uptake, was severely reduced in the mutant, at all temperatures. In addition, genetic interaction was observed between temperature-sensitive alleles arp2-H330L and cdc10-1. CDC10 is a gene encoding a neck filament-associated protein that is necessary for polarized growth and cytokinesis. Overall, the immunolocalization, mutant phenotypes, and genetic interaction suggest that the Arp2 protein is an essential component of the actin cytoskeleton that is involved in membrane growth and polarity, as well as in endocytosis.

The yeast actin cytoskeleton is essential for maintenance of cell shape, organization and polarized growth of the cell surface, morphogenesis, and cell division (Adams and Pringle, 1984; Kilmartin and Adams, 1984; Novick and Botstein, 1985; Drubin et al., 1991). Analysis of actin mutants revealed pleiotropic effects on yeast growth and development. Phenotypes such as alteration of the actin distribution, random budding pattern, delocalization of chitin, sensitivity to osmotic pressure, defective septation and nuclear segregation, reduced internalization in endocytosis, and accumulation of secretory vesicles have been demonstrated by analysis of temperature-sensitive (Ts)1 mutants (Novick and Botstein, 1985; Drubin et al., 1993; Kübler and Riezman, 1993). While actin has a demonstrated role in all of these processes, different functions may be mediated by interaction with one or several of numerous other cytoskeletal proteins. For example, among genetically redundant cytoskeletal proteins (fimbrin and capping proteins or fimbrin and Abplp), the lack of structural and functional homology has been taken as evidence that these proteins regulate the actin cytoskeleton by different mechanisms (Adams et al., 1993).

Whereas classical actins are highly conserved across eukaryotic phyla (e.g., Saccharomyces cerevisiae actin is 88% identical to rabbit skeletal α-actin), more divergent sequences that are homologous to actin have been identified in a number of organisms from yeast to humans (Schroer et al., 1994). Although the functions of actin and an increasing number of different actin-binding proteins and their interactions within the actin cytoskeleton are already well documented (Welch et al., 1994), the functions of these more recently discovered actin-related proteins (Arp) are just beginning to emerge (see reviews Herman, 1993; Frankel and Mooseker, 1996).

The best understood of the Arps is the divergent actin now known as Arp1, identified as actin-RPV (actin-related protein of vertebrates) for the human protein (Lees-Miller et al., 1992a) and centrinactin for the canine protein (Clark and Meyer, 1992). A gene encoding a protein with similarity to vertebrate centrinactin was isolated in S. cerevisiae as ACT3 (Clark and Meyer, 1994) or ACT7 (Muhua et al., 1994), although its identity as the closest homologue to centrinactin may be questionable. One novel aspect of Arp1 proteins is that these proteins are found to be associated with the microtubule cytoskeleton. Canine Arp1p was localized to the centrosome in vivo and found to be part of a dynactin-containing complex. The quasi-identical human Arp1 protein has been shown biochemically to be part of the dynactin complex, an activator of dynein-
driven vesicle movement on microtubules (Schafer et al., 1994). Characterization of the less closely related yeast Arp1p is consistent with its playing a similar role in S. cerevisiae, since deletion of the gene causes misorientation of the mitotic spindle and slight nuclear migration defects (Clark and Meyer, 1994; Muhua et al., 1994). The first gene coding for an Arp, ACT2, was isolated from S. cerevisiae in our laboratory (Schwob, 1988; Schwob and Martin, 1992; EMBL/GenBank/DDBJ accession number X61502). According to recent unifying classification and nomenclature based on sequence similarity and gene structure (Schroer et al., 1994), this gene will now be referred to as ARP2. The predicted 44-kD protein is 47% identical to S. cerevisiae actin and is essential for vegetative growth. Disruption of the ARP2 gene gave rise to a homogenous phenotype of cells with a large bud unable to complete formation of the first daughter cell. A possible role in cytokinesis was evoked (Schwob and Martin, 1992). Homologues of the ARP2 gene of S. cerevisiae have been identified in Acanthamoeba (Machesky et al., 1994), in Drosophila (Fyrberg et al., 1994), and in chicken (Michaille et al., 1995). Kelleher et al. (1995) have made a structural model based on actin which suggests that Arp2 contains a conserved profilin-binding site, but not the residues required to copolymerize with actin, and they have localized Arp2p in the Acanthamoeba cortex.

The family of ARP3 actin-related genes, first isolated as the ACT2 gene in Schizosaccharomyces pombe (Lees-Miller et al., 1992b), now includes Acanthamoeba (Machesky et al., 1994), Dictyostelium discoideum ACLA (Murgia et al., 1995), Drosophila (Fyrberg and Fyrberg, 1993), bovine homologues (Tanaka et al., 1992), and a protein fragment from Caenorhabditis elegans (EMBL/GenBank/DDBJ accession number M75768). Arp3 (p48) from Acanthamoeba was isolated in a complex bound to profilin, which also contained Arp2, actin, and several smaller proteins. Antibodies to this p48 stained the cortical cytoskeleton. In apparent contrast to these results, in D. discoi-

Table I. Yeast Strains Used in This Study

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†Institut Pasteur, Paris, France.
‡Yale University, New Haven, CT.
§Hans Knoll Institut, Jena, Germany.
¶Johns Hopkins University, Baltimore, MD.


**Materials and Methods**

**Plasmids, Strains, and Genetic Manipulations**

The plasmids and yeast strains used are listed in Tables I and II. All DNA manipulations were by standard techniques (Sambrook et al., 1989). Escherichia coli strain DH5α was used for the majority of bacterial manipulations. Bacteria were transformed by electroporation. Yeast cell cultures and genetic manipulations were essentially according to Guthrie and Fink (1991). Ura− strains were selected by culture on solid synthetic media containing 0.5 mg/ml 5-fluoroorotic acid. Yeast cells were transformed using LiAc, single-stranded carrier DNA, and DMso (Hill et al., 1991).

Null strains for *arp2* were created using pYWW202. This plasmid carries a 3,116-bp SacI-SphI genomic *arp2* fragment in which a 1,043-bp fragment between the unique SnaBI and NsiI sites was replaced with a Smal-PstI LEU2 fragment. The entire SacI-SphI fragment was used to transform different diploid *leu2Δ*/*leu2Δ* strains by one-step gene replacement (Rudstein, 1983). This yielded *Aarp2Δ*/*LEU2* strains YM10 (derived from Lacrout strains), YM3 (YB18 derived), and YM10 (YPH501 derived), respectively. The presence of one deleted allele at the *arp2* loci of these diploids was verified by Southern blot analysis (results not shown).

Schizosaccharomyces pombe strains (S. Elledge)*

Rescued haploid deleted strains constituted a "shuffle" system used to select mutants. These shuffle strains were obtained by transforming the diploid strain YM10 with plasmid pYCW204 carrying *URA3*, *ARP2*, and SUP11, and then sporulating and selecting Ura− colonies that could not lose the rescue plasmid when uracil was supplied. Spores YM11(a) and YM12(α) did not give red sectors when grown on limiting adenine, and died when inoculated onto 5FOA plates.

**PCR Mutagenesis and Mutant Isolation**

The *ARP2* gene was mutagenized by PCR amplification of a genomic fragment containing the entire coding sequence. Oligonucleotides 5′-CAG-ATTATAATTTAGTAGGACC-3′ and 5′-CATATGCTAGCG-GATAACTATCCTCT-3′ containing added external EcoRI and SphI sites were used as 5′ and 3′ primers to mutagenize the Dral-BsaBI genomic fragment (sites are underlined). 1 μg of each primer was added to a 100-μl

<table>
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<td>pGAL</td>
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| pAS1                                  | 2μ origin of replication (2μ); TRP1, ADH promoter, NLS-GAL4, HA tag, (S. Elledge)*  
| pYEW246                               | An Ncol-SphI fragment containing an intronless *ARP2* gene was placed under the control of the ADH promoter by ligating into the Ncol and SmaI sites of pAS1 (This study) |
| p423GALL                              | pBluescript, 2μ; HIS3, GALL promoter (Mumberg et al., 1994) |
| p424GAL1                              | pBluescript, 2μ; TRP1, GALL1 promoter (Mumberg et al., 1994) |
| pYEW247                               | p424GAL1 (Mumberg et al., 1994) plus HAN*ARP2* under the GALL1 promoter, by inserting the EcoRI-SalI fragment of pYEW246 containing 5′ HA tagged *ARP2*, into the EcoRI-SalI sites of p424GAL1 (This study) |
| pYEW248                               | pUN20 plus 3HAC*ARP2*, constructed by cloning a PCR-generated copy of the *ARP2* gene (site DRAI-TAG), having an added NotI site preceding the TAG codon and added external EcoRI and BgIII restriction sites, into the EcoRI-BamHI sites of pUN20 (This study) |
| pYEW250                               | p423GAL1 plus 3HAC*ARP2* under the GALL promoter, by inserting 3HAC*ARP2* contained in the SnaBI-SalI fragment of pYEW248 into the SnaBI-SalI sites of p423GALL (This study)  
| pYEW251                               | The same as pYEW250, but lacking the NotI fragment containing the 3HA epitope tag (This study) |

*Not a natural genomic site.

†Howard Hughes Medical Institute, Houston, TX.
reaction containing 200 ng of pBON34 DNA template as source of the Arp2-coding sequence, 3 U Taq polymerase, 200 mM dNTPs, 50 mM KCl, 10 mM Tris-HCl (pH 8.8), 1 mM DTT, and 1.5 mM MgCl2. The reaction was cycled 30 times in a thermal cycler at 95°C for 1 min melting, 42°C for 1 min annealing, and 70°C for a 2-min extension. The reaction mix was digested with EcoRI and SpHl, and the 1,550-bp amplified product purified from an agarose gel was then ligated into EcoRI and SpHl cut pYC2007 vector. DNA prepared from a culture of all the transformants constituted the pool of potential mutants.

To isolate arp2ts mutants, the ade2-101mahi supU111 sectoring system was used. The pYC2007CR plasmids were transformed into shuffle strain YMW11 and selected on minimal selective plates lacking histidine with limiting adenine (2 μg/ml) at 37°C. Colonies that remained white at 25°C but formed sectors at 25°C were retained as potential Ts mutants. As a secondary screen, these colonies were tested for thermosensitivity at 37°C in the presence of SFOA to counterselect the pYC2004 rescue plasmid. Candidate plasmids were isolated and transformed into fresh YMW11 cells, which were then restested for thermostolerance of both ade sectoring and SFOA resistance.

**Growth and Viability of Mutants**

Cultures of wild-type and mutant strains were first grown to early log phase at 25°C in rich medium (liquid YPD). The culture was diluted to A00 = 0.2, divided, and incubated at 25°C and 37°C. Aliquots of the cultures were removed every hour. Cell density, viability, and osmosensitivity were determined at each time point. To monitor cell death, 100 μl of a 10-3 dilution in sterile water was plated on YPD and incubated at 25°C for 2 d before counting colonies. To analyze osmosensitivity, a drop of each culture was spotted on solid YPD media containing increasing concentrations of NaC1, KCl, or sorbitol. Plates were incubated at 25°C or 37°C for 2 d then photographed.

**Preparation of Antibodies**

Comparison of the amino acid sequence of Arp2p with the three-dimensional structure of rabbit skeletal actin (Kabsch et al., 1990) predicts that peptide 40-RAERASVATPLKD-54 localizes in subdomain 2 of Arp2p on a probable external loop structure. This peptide was synthesized and conjugated to ovalbumin by Neosystem (Strasbourg, France). Two rabbits were injected at 2-3-wk intervals with 100 μg of peptide in conjugated form. Sera were collected before the first injection and 2 wk after the third and subsequent injections, and then titrated by ELISA against fixed peptide and ovalbumin-conjugated peptide. Individual antisera with the highest titers against the peptide were affinity-purified against the peptide antigen bound to an Epoxy-activated Sepharose 6B column (Pharmacia Fine Chemicals, Piscataway, NJ) according to the manufacturer’s instructions. Fractions containing antibody were pooled, concentrated by filtration in microcentrators (Millipore Corp., Bedford, MA), aliquoted, and stored at −20°C in 20% glycerol.

**Proteins, Electrophoresis and Blotting**

Protein extracts were prepared by agitating cell suspensions with glass beads on a mechanical agitator for 5 × 30 s. After boiling for 1 min in the presence of SDS-PAGE loading buffer, proteins were separated by SDS-PAGE, and then stained with Coomassie blue or immunoblotted onto re-inforced nitrocellulose membrane (Schleicher & Schuell, Inc., Keene, NH). Immunoblots were incubated with antibody diluted in PBS and revealed using the ECL detection system (Amerham, Arlington Heights, IL.).

**Immunofluorescence, Phalloidin and Calcofluor Staining**

Yeast cells were grown to early log phase in YPD or supplemented YNB synthetic media, fixed, and processed for immunofluorescence as described by Frang Bennett et al. (1991). A (3.7%) final concentration of formaldehyde 3 was added directly to cultures for 1 h (except for the 12CA5 epitope where fixation was for 20 min). Cells were washed and digested with zymolyase 100T to obtain spheroplasts. The spheroplasts were washed with PBS/sorbitol, then attached to polylysine treated multwell slides and treated with cold methanol/acetone. After incubation for 2 h with primary antibody, five washes with PBS, incubation 1 h with FITC or rhodamine-conjugated secondary antibodies (Sigma Chemical Co., St. Louis, MO), and five washes with PBS, preparations were analyzed using an Optiphot microscope (Nikon, Inc., Melville, NY) equipped with fluorescence optics. Affinity-purified polyclonal rabbit antiactin antibody was a generous gift from D. Drubin (University of California, Berkeley, CA), and YOL1/34 antieutobulin mAb was bought from Serotec. Primary antibodies for Arp2p/Act1p double-labeling were affinity-purified polyclonal goat antiactin antibody, a generous gift from J. Cooper (Washington University, St. Louis, MO), and affinity-purified polyclonal rabbit anti-Arp2p antibody (described above). Mouse monoclonal hemagglutinin (HA) antibody (clone 12CA5) was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN).

To reveal actin in whole cells, cells fixed for 1 h were incubated with 1.5 μM rhodamine-phalloidin for 2 h and washed extensively before mounting.

Chitin labeling of bud scars was observed after incubation of fixed cells in a 200 μg/ml solution of calcofluor (Fluorescent Brightener 28; Sigma Chemical Co.) for 5 min, three to five washes in water, and resuspension in PBS.

**Vacuole and Endocytosis Analysis**

Accumulation of the naturally fluorescent ade2 fluorophore was observed to analyze vacuolar morphology and inheritance (Weisman et al., 1987). Yeast cells were grown overnight at 25°C and then diluted to early logarithmic phase. After preincubation for 30 min at 25°C or 37°C, LY-CH was added to the culture to a final concentration of 16 mg/ml. LY-CH accumulation in unfixed whole cells was analyzed after a 1-h incubation.

**Results**

**Preparation and Specificity of Anti-Arp2p Antibodies**

To obtain specific antibodies against Arp2p, which is 47% identical to actin, we decided to raise antipeptide antibodies against areas of the protein predicted to be surface exposed by comparison with the three-dimensional structure of rabbit skeletal muscle actin (Kabsch et al., 1990). A synthetic peptide corresponding to Arp2p residues 40-54, which corresponds to residues 41-52 in actin (plus an insertion of three amino acids), but is divergent from actin, was synthesized and used to raise antibodies in rabbits. Crude sera revealed multiple bands in yeast crude extracts (data not shown). Antipeptide/40-54 antibodies from antisera that gave the highest titers in ELISA tests were purified using a peptide column. Affinity-purified antibody revealed a single 44-kD polypeptide band in wild-type yeast extract by Western blot (Fig. 1 A, lane J). This same band could be competed out by previous incubation of the antibody with the peptide antigen (Fig. 1 A, lane 2). By classical SDS-PAGE, we were not able to completely separate Arp2p and Act1p (Fig. 1 A, lanes 3 and 4). In view of this problem, we constructed a hybrid Arp2p-fused COOH-terminal to the DNA-binding domain of the transcriptional activator Gal4p and expressed it in a Δarp2 strain. The chromosomal deletion was rescued by this fusion plasmid (transformed strain YMW17). Extracts from this strain were revealed by antipeptide/40-54 antibodies (Fig. 1 B, lane J) and by antiactin antibodies (Fig. 1 B, lane 6). The antipeptide antibodies revealed the Arp2-Gal4 fusion and some smaller sized bands that could be degradation prod-
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Figure 1. Identification of Arp2p on Western blots. (A) Immuno-detection of Arp2p in a crude extract of wild-type YPH501 cells with rabbit antipeptide/40-54 antibodies (lane 3). With antiactin antibodies (lanes 4-6). (B) Proteins were extracted from glucose-grown cells almost as well as wild-type, and with mouse antiactin mAbs (lane 4). (C) Proteins were extracted from glucose-grown cells almost as well as wild-type, and with mouse antiactin mAbs (lane 4). Detection of Act1p and Arp2p by sequential incubation with antiactin and antipeptide/40-54 antibodies (lanes 3 and 4) are sequential revelations of the same blot strip. Note that Act1p migrates with slightly lower mobility (45 kD; Water et al., 1980) than expected for its molecular mass (42 kD) and in superposition with or very close to Arp2p, which is larger (44 kD). Since Act1p and Arp2p are revealed here with two different types of antibodies with very different specific affinities, and they are used at different concentrations, relative cellular concentrations cannot be estimated from the intensity of the bands. (B) Immunodetection of chromosome-encoded Arp2p in crude extracts of wild-type YPH499 cells (lanes 1 and 4), of YMW11 shuffle strain rescued by a functional Arp2p-Gal4DBD fusion (lanes 3 and 6) with antipeptide/40-54 antibodies (lanes 1 and 2) and with mouse antiactin mAbs (lanes 3 and 4) and with antiactin antibodies (lanes 4-6). (C) Proteins were extracted from glucose and galactose grown yeast cells transformed with pYCW245 that has the ARP2 gene under the control of an inducible hybrid GAL10-CYC1 promoter. Equal amounts of the different extracts (50-60 µg) were electrophoresed on SDS-PAGE, and then blotted and revealed with the antipeptide/40-54 antibodies. WT, wild type.

Independent of the expressed 44-kD polypeptide revealed by affinity-purified polyclonal antibodies against a unique 15-amino acid long constituent peptide (as described above), and secondly, by replacing the normal genomically encoded wild-type protein with HA-epitope tagged versions that could be revealed by commercially available mAbs. Affinity-purified rabbit antipeptide antibodies and FITC-labeled goat anti-rabbit IgG were used to visualize Arp2p in wild-type diploid FY1679 cells (Fig. 2 A, a-f, upper panels). Punctate spots under the plasma membrane were visible in both the mother cell and in buds. In logarithmically growing cells, a polarized distribution of spots was observed with label concentrated at the site of bud emergence (Fig. 2 A, b), in buds until nuclear migration (Fig. 2 A, c-e), and at the neck between the mother and daughter cells just before cytokinesis (Fig. 2 A, f) resembling cortical actin patches. Cytoskeletal cable filaments were not visibly labeled. No labeling of the nucleus or mitochondria was detected (Fig. 2 A, a-f, compare Arp2p and DAPI stainings in upper and lower panels, respectively). Control staining without primary antibodies (Fig. 2 A, g) showed no significant label.

Alleles with an HA epitope inserted after the initiator ATG or a triple HA epitope immediately upstream of the TAG termination codon (HAN'arp2 or 3HAC'arp2) were constructed as described in Table II (plasmids YEW247 and YEW248) and tested for their capacity to replace a wild-type rescue plasmid in a cell with a genomic arp2 null allele. The HAN' and 3HAC'Arp2 alleles both rescued ∆arp2 cells, but grew slowly and gave rise to cell populations where most of the cells were morphologically abnormal even in the presence of a wild-type allele. Immunocytochemical analyses of these “dominantly sick” cells were abandoned because the relevance of HAN' and 3HAC'Arp2 localization to normal Arp2p localization is difficult to judge in these cells. To try to overcome this problem, the 3HAC'arp2 allele (which allowed slightly better growth than the HAN'arp2 allele), was cloned under the control of a truncated GAL1 promoter (source plasmid GALL). This plasmid (pYEW250) was found to rescue ∆arp2 strains when grown on either glucose or galactose, pYEW250 rescued glucose-grown cells almost as well as wild-type, and 50-60% of the cells in the population showed normal morphology. Swollen cells, misshapen large buds, and mother cells that rebudded before cytokinesis of a first daughter cell were observed among the morphologically abnormal cells, but any one individual phenotype was seen in only ~10-15% of the population. The expression of the tagged protein was verified by Western blotting and immunode-
Immunolocalization of Arp2p. (A) Wild-type diploid cells (FY1679) were decorated with antipeptide/40-54 antibody plus FITC-conjugated secondary antibody (top panels) and with DAPI (bottom panels). Representative cells at different stages of the mitotic cell cycle are shown in a–f. Control cells decorated only with the FITC-conjugated secondary antibody are shown in g. (B) Δarp2::LEU2 diploid cells rescued by pYEW250 bearing the 3HAC'ARP2 allele were decorated with clone 12CA5 antibodies and TRITC-conjugated secondary antibodies, and were stained with DAPI (a–c). Control cells in (d–f) are Δarp2::LEU2 diploid cells rescued by pYEW251 containing the NotI-modified ARP2 gene without the HA epitope. a and d, Nomarski; b and e, TRITC anti-HA; c and f, DAPI. Bar, 5 μm.

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under the plasma membrane, and these spots localized to bud sites and small buds, but not to cytoplasmic structures (Fig. 2 B, b). Cells expressing an ARP2 gene with the COOH-terminal Not1 site, but no HA-encoding sequence (pYEW251), showed the same morphologies as cells expressing the tagged version. This control also gave rather high background cytoplasmic fluorescence, but no significant punctate staining (Fig. 2 B, c). Taken together, these results suggest that Arp2p is located under the plasma membrane in areas of cell-surface growth. It was then of interest to know whether Arp2p colocalizes with cortical actin.

Colocalization of the Arp2 and Act1 Proteins

Wild-type yeast cells have a characteristic actin organization that changes during the cell cycle. Cortical actin structures are located predominantly at sites of surface growth and actin cables in the mother cell are parallel to the mother–bud axis. At the beginning of the cell cycle, patches of actin are found at the site of bud emergence. During bud growth, cortical patches are found enriched in the bud and actin cables extend towards the daughter cell. Before cytokinesis, a ring of actin is located around the bud neck. The image obtained with the antipeptide antibodies was similar to cortical actin staining. The photos in Fig. 3, a–c, show wild-type cells that were doubly labeled using goat antiactin (b) and rabbit anti-Arp2p (c) primary antibodies with TRITC anti–goat- and FITC anti–rabbit-conjugated secondary antibodies. Both antibodies label sites of membrane growth. Most punctate dots seen with anti-Arp2p antibodies appeared to colocalize with actin in patches, e.g., most but not all individual spots could be superimposed. In budding cells, anti-Act1p antibodies labeled normal cytoplasmic cables. Cytoplasmic cables were not detected with the anti-Arp2p antibody. The colocalization of Arp2p and Act1p is thus partial and restricted to cortical actin dots. Control cells are depicted in Fig. 3, d–i; Fig. 3, d–f, which shows cells labeled using only rabbit anti-Arp2p primary antibody; and Fig. 3, g–i, which show cells labeled using only anti-Act1p antibody, while all control cells were incubated with both secondary antibodies. This shows that there is no overlap of the rhodamine and fluorescein channels. These results suggest that Arp2p is part of or very closely associated with cortical actin patches.

Isolation of Arp2 Conditional Mutants

Earlier work on the ARP2 gene in strain FL100 showed that sporesses carrying either an arp2::URA3 gene disruption or Δarp2::URA3 deletion were not viable; they give rise after germination to one cell with a single large bud (Schwob and Martin, 1992). To verify that Δarp2 is lethal in different laboratory strains and to create more convenient multiply auxotrophic strains (for use in red ade2 sectoring screens and screens requiring counterselection on URA3 by 5FOA), we constructed a LEU2 deletion allele (see Materials and Methods) and integrated it into other laboratory strain backgrounds. In both YB18 and YPH499 backgrounds, the Δarp2::LEU2 allele conferred inviability on haploid meiotic progeny within one division cycle, as had been previously described. We consistently observed a single budded cell as the terminal phenotype in different strain backgrounds.

To try to understand the role(s) of Arp2p, we created conditional alleles of the ARP2 gene. Using "standard" conditions of PCR amplification, the entire coding sequence was mutagenized. Δarp2::LEU2 haploid strains carrying an ARP2 rescue plasmid were constructed (see Materials and Methods). These strains allowed us to test mutagenized plasmids in plasmid shuffle experiments. An ade2 SUP11 colored colony sectoring screen was then used to identify Ts mutant plasmids. Plasmids that did confer sectoring ability on the test strain at 37°C, but did not at 25°C, i.e., which could not replace pYC204 in strain YM11 at 37°C (but could at 25°C), were then tested further. Test strain YM11 carrying the mutagenized plasmid was tested for thermostensitivity on 5FOA. Nucleotide sequencing of these plasmid-borne mutant ARP2 genes identified eight different Ts alleles. Mutations were distributed in all four presumed subdomains of Arp2p (Moreau, V., unpublished observation).

One of these contained a single mutation situated in an insertion in Arp2p relative to Act1p. This region may form an external loop in subdomain 3. This mutant was chosen for further characterization. Histidine 330 was changed to leucine, resulting in one less charged residue. The arp2-H330L allele was cloned into the integrative plasmid pFL34. The resulting plasmid was cut with PstI and transformed into wild-type strains YPH499 and YPH500 to integrate at the ARP2 locus. Ura− colonies were transferred to 5FOA at 25°C and Ts clones were sought among the Ura− colonies. The integrity of the arp2-H330L locus was verified by Southern blot hybridization. Haploid strains were named YMW81 (a) and YMW82 (a), and the diploid strain that was obtained by mating, YMW83.

Growth and Viability of the arp2-H330L Mutant

Growth and viability of the arp2-H330L mutant were examined. Growth of wild-type and mutant strains was monitored in YPD liquid medium at 25°C and at 37°C by measuring absorbance at 600 nm (Fig. 4 A). At 25°C, the YMW83 strain grew as well as the wild-type strain. Growth of the mutant strain slowed down after 2 h at 37°C and stopped by 4 h. YMW83 cells were fixed at different time points and examined by phase microscopy. At the permissive temperature, mutant cells appeared normal. After 4 h at 37°C, arp2-H330L cells did not show a uniform terminal morphology. Unbudded cells had lost the ellipsoidal shape of normal yeast cells. Some cells appeared swollen, the vacuoles occupying nearly the entire cytoplasm, and cell debris was apparent in the medium. The ratio of budded to un budded cells was increased by 10–15% relative to the wild type.

Cell viability was followed for the YMW83 and YPH501 strains at 25°C and after shift of a permissively grown culture to 37°C (Fig. 4 B). The number of viable cells per unit volume started to decrease between 1 and 2 h after shift to 37°C. After 4 h, 10–20% of the initial number of cells were recoverable at 25°C. The drop in the number of viable cells is consistent with our observation of lysed cells after prolonged incubation at restrictive temperature (result not shown). This delayed death by cell lysis is also a character-
Double labeling of Arp2p and Actlp. Wild-type diploid cells (YPH501 strain) were incubated with a mixture of rabbit polyclonal anti-Arp2p peptide and goat polyclonal antiactin primary antibodies (a−c), or with only anti-Arp2p antibodies (d−f), or with only antiactin antibodies (g−i), followed in all cases by incubation with a mixture of FITC anti-rabbit and TRITC anti-goat secondary antibodies. a, d, and g, Nomarski; b, e, and h, TRITC filter for Actlp; c, f, and i, FITC filter for Arp2p. Bar, 5 μm.

Figure 3

Characteristic phenotype of the act1-1 and act1-2 actin mutants (Novick and Botstein, 1985).

Sensitivity to Osmotic Pressure

The swollen cells and delayed lysis phenotype of the arp2-H330L mutant are suggestive of a defect in osmotic stability. We examined the effect of increased osmotic pressure on growth of the arp2-H330L mutant at temperatures of 25°, 30°, 34°, and 37°C. Several concentrations and forms of added osmotic support (KCl, NaCl, and sorbitol) were tested. Actin mutants act1-2 and act1-3, described as osmosensitive (Novick and Botstein, 1985), and their corresponding parent were tested in parallel. Results were similar whether salt or sorbitol was added to change the osmolarity. A representative sample of growth tests is presented in Fig. 5. Growth inhibition of arp2-H330L at 37°C was partially relieved on 1 M sorbitol medium, while the act1-2 mutant strain failed to grow significantly at 37°C regardless of the concentration of osmotic support. Similarly, 0.5 M NaCl or 0.5 M KCl gave some protection to the arp2-H330L mutant (data not shown). Although the arp2-H330L mutant failed to grow on 1 M NaCl at 37°C or at the normally permissive temperature of 25°C, it did grow at intermediate temperatures of 30°C and 34°C on 1 M NaCl. Thus, the arp2-H330L mutant is osmosensitive, albeit less so than the act1-2 mutant strain, which failed to grow on 1 M NaCl and grew only poorly on 0.5 M NaCl at all temperatures. We suppose that the act1-3 strain we used represents either a partial revertant of the original isolate or differences because of its genetic background, since it is less thermosensitive than the act1-3 strain originally described. It shows slightly better growth on 1.0 M sorbitol, but remains osmosensitive. Overall, slightly increased osmotic support protected the arp2-H330L mutant cells, whereas high osmolarity induced cell lysis. This inability to respond normally to changes in osmolarity is reminiscent of a number of cytoskeletal mutants.

The arp2-H330L Mutation Affects Polarization of Actin Distribution

A possible alteration of the actin cytoskeleton due to the arp2-H330L mutation was investigated by examining the actin distribution in wild-type and arp2-H330L mutant cells at 25°C and 37°C. To visualize actin distribution, we
used rhodamine-phalloidin to label whole cells and antiactin antibodies with rhodamine-conjugated secondary antibodies to label spheroplasts. At 25°C, no remarkable difference between the mutant and the wild-type strains was seen (Fig. 6A, a–f). Mutant cells showed a grossly abnormal actin distribution after 2 h at the restrictive temperature, while wild-type cells recovered from the depolarization effect of temperature shift. The difference in polarized distribution of actin patches was evident (Fig. 6A, g–l). About 80% of small buds in mutant cells were not intensely stained, compared to less than 15% in wild-type cells after 2 h at 37°C. In many cells, cortical actin patches were distributed over the entire cell surface of the mother and emerging daughter cells with little or no concentration at the site of bud emergence or in small buds (as shown by arrows in Fig. 6A, h). At this time, cables reappear but are less prominent than when revealed after constant growth at 25°C.

After 4 h at 37°C, ~15% of the mutant cells had misshapen buds. The tips of these “beak-shaped” buds contained brightly staining spots of actin (data not shown). Thus, cells with an apparent lack of polarization and cells with hyperpolarized actin were present in the same culture, although we cannot eliminate the possibility that this hyperpolarized phenotype may represent dying cells. In addition, actin bars without distinct orientation were occasionally observed at 37°C. Mutant cells 3 h after shift-up were spheroplasted and stained with antiactin antibody (Fig. 6B) to visualize cable structures more clearly at 37°C. Cytoplasmic cables are present but are fainter and appear more tangled at 37°C compared to 25°C. These effects of the arp2-H330L mutation on actin distribution are consistent with a role for Arp2p in the polarity of actin filament organization and/or cellular polarity requisite for bud growth. In contrast, staining of microtubules with antitubulin antibodies revealed essentially normal mitotic spindles with extending cytoplasmic microtubules at both 25°C and 37°C (results not shown).

4',6-diamidino-2-phenylindole (DAPI) staining of mutant cells after shift to 37°C also showed what appeared to
Figure 6. Actin organization in the arp2-H330L mutant strain. (A) Cultures of the YPH501 (wild type) and YMW83 (arp2ts) strains were grown at 25°C to early log phase and then half the culture was shifted to 37°C. Whole cells were labeled with rhodamine-phalloidin and DAPI. Photos were taken after 2 h at 25°C (a–c) and 37°C (g–i) for the mutant strain and after 2 h at 25°C (d–f) and 37°C (j–l) for the wild-type strain. Arrows (h) indicate unstained small buds seen in Nomarski; triangles (i) indicate condensed mitochondria. a, d, g, and j, Nomarski; b, e, h, and k, phalloidin; c, f, i, and l, DAPI. (B) Cultures of YMW83 strain was grown at 25°C to A600 0.2, and half of each culture was shifted to 37°C for 3 h. Mutant cells are shown at 25°C (a and b) and 37°C (c and d). Cells were decorated with actin antibodies (b and d) and stained with DAPI (a and c). Bar, 5 μm.
be occasional condensed or clumped mitochondrial genomes (see triangles in Fig. 6 A, i). This contrasted with the string-like appearance of normal mitochondria, which follow cytoplasmic actin cables. This phenotype has been described for certain act1 mutants by Drubin et al. (1993). The number and position of nuclei, as revealed by DAPI staining, were comparable to those observed in wild-type cells in both experiments. Overall, nuclear division appeared normal. Very occasionally, a mother cell was observed to have two separate nuclei before bud emergence.

Altered Budding Patterns in the arp2 Mutant

Budding in arp2 mutant strains was examined in both haploid and diploid cells. S. cerevisiae reproduces mitotically by asymmetric cell growth initiated at a nonrandom site on the plasma membrane. Bud sites are selected in an axial pattern in haploid a and a cells and in a bipolar pattern in a/α diploid cells. Chitin synthase is an integral membrane protein implicated in the structural changes occurring during bud formation. Deposited chitin can be stained with the fluorescent dye calcofluor. Wild-type yeast cells deposit a ring of chitin at the neck of the emerging bud that remains after cell division on the mother cell as a chitin rich bud scar (Pringle, 1991). Calcofluor staining revealed that the majority of both haploid and diploid mutant cells with three or more bud scars showed random budding patterns. Some cells showed a diffuse chitin distribution over the entire cell surface and some cells, especially haploids, had abnormal random patches of fluorescence. In fact, no patches of chitin were seen on cells with small mishapen buds, indicating that these cells had stopped growth before completing the first division. However, large unbudded haploid cells showed many randomly distributed patches (result not shown). Fig. 7 illustrates bud scars in wild-type and mutant diploid cells. Whereas the wild-type budding pattern was clearly bipolar (Fig. 7, a and b), most mutant cells had lost this polarity. In Fig. 7, c and d, the budding pattern of the older cell is completely random although shifted to nonpermissive temperature for only one generation, while the younger cell appears to have lost the polarity of bud site selection after having first divided with a bipolar pattern. It thus appears that polarity of budding is drastically reduced, even at 25°C.

Analysis of Vacuoles and Endocytosis

Vacuolar morphology and inheritance were analyzed using the endogenous fluorophore accumulated in ade2 mutant cells. When ade2 cells are grown in limiting adenine, they accumulate a polymer with a red fluorescent component in the vacuole (Weisman et al., 1987). This stable fluorophore allows one to follow the portion of the vacuole that migrates from the mother to the daughter cell. We examined vacuolar morphology in wild-type and mutant strains (YPH499 and YMW81) at 25°C and at 37°C. Although some mutant vacuoles appeared slightly larger than wild-type vacuoles, no major differences in vacuolar inheritance were detected between the two strains either at 25°C (data not shown) or after shift to 37°C (Fig. 8 A). The majority of mother cells had one major vacuole. The fluorescence in the mutant cells was slightly more intense than that in the wild-type cells. This might be explained by a slower division rate of mutant cells at 37°C, with consequently less dilution of the accumulated dye during inheritance of vacuoles by daughter cells.

To study endocytosis in the mutant strain, we investigated its ability to take up and deliver LY-CH to the vacuole. LY-CH is a marker for fluid-phase endocytosis (Riezman, 1985). Compared to wild-type cells, mutants cells revealed a strong defect in the accumulation of the fluorescent dye (Fig. 8 B). In wild-type cells, vacuoles were brightly stained; in contrast, arp2-H330L cells showed very little uptake of the dye into the cell at any temperature. Mutant cells exhibited very weak fluorescence in the vacuole (recognizable by Nomarski optics) relative to the rest of the cell. These results suggest that the arp2-H330L mutation affects endocytosis even at temperatures permissive for growth, but it appears that what little dye is taken up finds its way to the vacuole. Further experiments to quantitate early steps of endocytosis are currently in progress.

Genetic Interaction between arp2-H330L and the cdc10-1 Mutations

On the basis of the large-budded terminal phenotype of Δarp2 cells, a role for the Arp2 protein in cytokinesis has been postulated (Schwob and Martin, 1992). This prompted us to investigate possible interactions with 10-nm filament proteins. 10-nm filaments are found in the neck between mother and daughter cells and are known to be involved in cytokinesis (Byers and Goetsch, 1976; Haarer and Pringle, 1987). We searched for possible genetic interactions by...
crossing the YMW82 strain with strains harboring Ts mutations affecting neck filament–associated proteins cdc10-1, cdc11-1, and cdc12-1. We first looked for unlinked noncomplementation of these Ts alleles in diploid cells. No significant difference in growth rates in rich medium was seen between restrictive and permissive temperature for any of the heterozygous diploids. However, microscopic analysis of cell morphology revealed a possible interaction between the cdc10-1 and the arp2-H330L mutations (Fig. 9, a–d). At 37°C, these doubly heterozygous diploid cells showed a phenotype similar to the cdc10-1 phenotype. That is, the cdc10-1/arp2-H330L doubly heterozygous population showed at least 20% of cells with abnormally elongated buds, whereas <1% of wild-type diploids and single heterozygous diploids had abnormally elongated buds. No particular phenotype was observed for cdc11-1 or cdc12-1 doubly heterozygous diploid cells. This effect observed in heterozygous diploids prompted us to look for possible synthetic effects of these mutations in haploid cells.

The diploids of the three pairwise crosses cited above were sporulated, and viable progeny were analyzed for the temperature threshold of growth and morphology. A synthetic effect was observed between the cdc10-1 and arp2-H330L mutations. This ranged from increased thermostability (death at 30–34°C) to deduced lethality (Table III). Of the 18 doubly mutant spores, in 17 tetrads analyzed, 11 cdc10-1 arp2-H330L spores were dead and 7 showed increased thermostability. Cultures of all cdc10-1 arp2-H330L double-mutant spores showed some cells with aberrant morphology at 25°C. The double mutants also had a temperature threshold for growth that was lower than that of the single parental mutants. At 25°C, ~35% of a cdc10-1 mutant culture showed abnormal cells, while >90% of doubly mutant cells were abnormal. These genetic data are indicative of an interaction between the CDC10 and ARP2 gene products.

**Discussion**

Arp2p is a protein that is essential for growth, but its function was unknown when these studies were undertaken. Revealing the cellular localization of a protein can indicate with which cellular structures it is associated. Examination of defects in conditional mutants should point more directly, if not to precise function, at least to the processes in which a protein is involved. Here we have combined these approaches. Obtaining specific antibodies to Arp2p, a protein that is close in size and 47% identical to (74% similarity with) a protein as abundant as actin was a major objective of this work. The inability to predict the outcome when raising antipeptide antibodies prompted us to resort to a tagging strategy as well. One of the two peptide antibodies raised was shown to be specific for Arp2p on Western blots and revealed cytological localization similar to the HA-tagged protein. Arp2p-specific antibody showed punctate staining under the plasma membrane at sites of cell-surface growth with little cytoplasmic background. Staining of cytoplasmic cable filaments was not visible. This constitutes a first indication that Arp2p may interact with, or be part of, the cortical actin cytoskeleton. We cannot exclude the possibility of a weak interaction with actin cables, but this was not obvious from our labeling. A less abundant protein than actin (which appears to be the case for Arp2p) might have different stoichiometry in cables than in patches and be in such relatively low abundance in cables that the antipeptide/40-54 antibody does not visibly decorate it. Moreover, the peptide antibody recognizes only a 15-amino acid stretch of the protein, and it is possible that a particular peptide region may not always be accessible in situ. This is especially pertinent in view of the fact that two different filamentous actin structures are visible in cytological staining of the actin cytoskeleton (Adams and Pringle, 1984). Since antibodies raised against a second peptide situated in a different probable external loop of the protein were not clearly specific to Arp2p (re-
Unlinked noncomplementation of the *arp2-H330L* and *cdc10-1* mutations in diploid cells. Diploid strains were grown to log phase in YPD liquid medium at 25°C and shifted to 37°C for 2 h before examining their morphology with Nomarski optics. Diploid strains resulting from the mating of (a) YMW82 (*arp2-H330L*) and 17012 (*cdc10-1*), (b) YMW82 and A364A (*CDC10*), (c) YPH500 (*ARP2*<sup>+</sup>), and (d) YMW82 and 332 (*cdc11-1*). Bar, 10 μm.

Results of simultaneous decoration with Arp2 and Act1 antibodies lend credence to the partial colocalization suggested by single-antibody labeling. As has been amply demonstrated for actin, Arp2p localizes in polar fashion throughout the cell cycle to sites of bud emergence, in small and medium-sized buds, and to the neck region in both mother and daughter cells before cytokinesis. However, since not all individual dots of fluorescence coincide, we cannot exclude the possibility that Arp2p is located in cortical structures other than actin patches. Determination of the precise time at which Arp2p first appears at the site of bud emergence and when it appears and disappears from the neck might help to settle this question. More high resolution localization using confocal or immuno-EM could also resolve this issue. Certain cytoskeletal components such as Abp1p, Cap (Caplp, Cap2p), and cofolin (Cof1p) have been localized in patches, while tropomyosin has been localized only to cables and fimbrin (Sac6p) is clearly found in both (see review by Welch et al., 1994). The reasons for localization to patches or cables are not known. Moreover, it is surprising at first glance that tropomyosin mutants (*Atpml*) have severely delocalized actin patches while tropomyosin itself is localized only in cables. These findings, however, are consistent with the model of Mulholland et al. (1994), proposing that cortical patches are directly linked to cables. The submembrane localization of yeast Arp2p is in agreement with the recent observation of an *Acanthamoeba* Arp2p homologue localized in the cortical cytoskeleton. Immunolocalization of Arp2p is also similar to filamentous actin in fixed amoebas (Kelleher et al., 1995). This Arp2p homologue was identified in a complex isolated by its affinity for profilin. Arp2p is in tight interaction with Arp3p and other proteins in this complex (Machesky et al., 1994). Actin also associates with this complex, but is more easily dissociated in vitro. Kelleher et al. (1995) propose that an Arp2p/Arp3p heterodimer present in the profilin-binding complex might serve as a pointed-end nucleus for actin polymerization. At the present time, little evidence other than the existence of *S. cerevisiae PFY1, ARP2*, and *ARP3* genes is available to know whether this type of complex is found in yeast. In our hands, the overexpression of profilin was unable to suppress the *arp2-H330L* mutation (result not shown), whereas it has been shown that overexpression of profilin alleviated the toxicity due to actin overexpression (Magdolen et al., 1993).

### Table III. Synthetic Interaction between *arp2-H330L* and *cdc10-1* Mutations

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<td><em>ARP2 cdc 10-1</em>&lt;sup&gt;+&lt;/sup&gt;</td>
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YMW82 and 17012 were mated and the resulting diploid strain was sporulated. The haploid segregants were dissected, and the viability of each was determined. Numbers of parental ditype (PD), nonparental ditype (NPD), and tetrasporate (TT) tetrad are indicated.
Furthermore, it may be pertinent that an essential role for profilin in cytokinesis has been observed in S. pombe (Balasubramanian et al., 1994).

To try to understand the function of Arp2p, we isolated *arp2* conditional mutants. The *arp2-H330L* mutation was generated by PCR mutagenesis and isolated in a screen for thermosensitive capacity to rescue a null allele. We originally characterized the *arp2-H330L* allele on the chance that it might be altered in a function specific to Arp2p (the H330L mutation is situated in a loop divergent from Act1p). However, the pleiotropic phenotypes revealed by temperature shift experiments using this *arp2* Ts strain suggest that Arp2p functions in many of the same processes as Act1p. The observations that *arp2* mutants display a random budding pattern, osmosensitivity, an abnormal actin distribution, and apparently defective endocytosis are all consistent with a proposed role for Arp2p in the actin cytoskeleton. It is possible that Arp2p may play a role in the regulation of actin in vivo, but we have no direct evidence for this hypothesis.

We have distinguished at least two morphological terminal phenotypes in the Ts *arp2-H330L* strain that may be related to cellular polarity. In cultures shifted to the restrictive temperature, there is an increase in the number of swollen unbudded cells and abnormally budded cells. Polarity of bud growth, as judged by the concentration and localization of cortical patches and chitin scars, was lost or altered in the budded cells. We also observed some instances of hyperpolarized growth (as revealed by the actin distribution) in misshapen buds in some cells after long incubation at 37°C. These abnormal buds were unable to continue growth. However, since this phenotype was predominant only 4 or more h after shift up (when most cells were dead or dying), its physiological significance is not certain. Loss of cellular polarity, leading to a terminal budding pattern, osmosensitivity, an abnormal actin distribution, and apparently defective endocytosis are all consistent with a proposed role for Arp2p in the actin cytoskeleton. It is possible that Arp2p may play a role in the regulation of actin in vivo, but we have no direct evidence for this hypothesis.

The genes *CDC3*, *CDC10*, *CDC11*, *CDC12*, and *SPA2* encode proteins that localize to the bud site before bud emergence and at the site of cytokinesis (Haarer and Pringle, 1987; Ford and Pringle, 1991; Kim et al., 1991; Snyder et al., 1991; Flescher et al., 1993). The products of the four *CDC* genes are constituents of the ring of neck filaments described by Byers and Goetsch (1976), but the nature of the main protein that forms the 10-nm filaments is still unknown. A model for a relationship between bud site selection and cytokinesis has been proposed by Snyder et al. (1991). They suggested that a tag remaining from cytokinesis marks the site of new bud emergence and surface growth. The genetic interaction of *cde10-10* and *spa2* reported by Flescher et al. (1993) supports the idea that a component involved in cytokinesis is also important in bud site selection. The genetic interaction between *cde10-1* and *arp2-H330L* could be taken as an indication of Arp2p playing a role in cytokinesis. This result is compatible with the potential role of Arp2p in cytokinesis evoked by the lethal uniform large bud phenotype of *Δarp2*:URA3 cells (Schwob and Martin, 1992). Furthermore, the fact that Cdc10p is also implicated in chitin deposition and bud growth (Ford and Pringle, 1991), which are both affected by the *arp2-H330L* mutation, may be pertinent to the observed genetic interactions. At the present time, we have no data as to whether the *arp2-H330L* mutation could disrupt the normal interaction of these two proteins. Additionally, we saw no increase in the proportion of large budded cells at a restrictive temperature in the mutant, contrary to what was observed in spores carrying null alleles. Although it seems less likely, the large bud of *Δarp2* cells after germination might reflect a growing cell that has used up the available supply of Arp2p that is necessary for normal growth and therefore stopped growing before reaching cytokinesis. If this is true, then *Δarp2* cells do not show a specific block in cytokinesis. The mutant Arp2p-H330L protein thus has physiological effects that are different from the deletion of the normal protein in germinating spores.

Finally, the *arp2-H330L* mutant is defective in endocytosis, as judged by the uptake of LY-CH, a widely used marker for fluid-phase endocytosis. Mutants affected in other cytoskeletal proteins such as actin and fimbrin are defective for LY-CH uptake. These mutants have been

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shown to be defective in the internalization step of receptor-mediated endocytosis (Kübler and Riezman, 1993). The participation of Act1p and other constituent proteins of the actin cytoskeleton in internalization of LY-CH and specific membrane receptors suggests an involvement of the actin cytoskeleton in the endocytic pathway. In addition, a disorganized actin cytoskeleton has been observed in mutants that were isolated in screens for mutants defective in endocytosis (Raths et al., 1993; Bénédicti et al., 1994; Munn et al., 1995). The severe defect in LY-CH uptake in the arp2-H330L mutant, even at 25°C, which is permissive for growth, and the observed cytolocalization under the plasma membrane at sites of actin cortical structures are both in good agreement with Arp2p playing a role in endocytosis. In the screen which gave rise to the arp2-H330L allele we have isolated other mutant alleles of ARP2. The study of these mutants should provide a better understanding of the apparent multiple functions of Arp2p.

Taken together, we believe that the results presented here provide strong evidence that Arp2p is an essential component of the actin cortical cytoskeleton. Alternatively, the phenotypes we have described here might be explained by interaction between Arp2p and actin, and one (Cdc10p) or several components of the actin cytoskeleton necessary for polarized budding, cytoskeletal dynamics, endocytosis, and perhaps cytokinesis. While direct interaction between actin and Arp2p has not yet been shown, the actin-like phenotype revealed by arp2p mutants would justify screening for interaction between multiple mutant alleles of the two genes. To understand at which of the possible functional levels Arp2p might act, one must determine whether Arp2p plays a role in bud site selection, assembly of the bud site complex leading to polarity, or bud enlargement. Arp2p might be important for the coordination of budding with the cell division cycle (Lew and Reed, 1994) if the ARP2/CDC10 interaction reflects a role in cytokinesis. Investigating interactions between arp2p and mutations affecting these functions could help us understand the specific event for which Arp2p is required.

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