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) 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 (limbrin and capping proteins or fimbrin and Aplp), 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 centractin for the canine protein (Clark and Meyer, 1992). A gene encoding a protein with similarity to vertebrate centractin 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 centractin may be questionable. One novel aspect of Arp1 proteins is that these proteins have been found to be associated with the microtubule cytoskeleton. Canine Arp1 was localized to the centrosome in vivo and found to be part of a dynein-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* (Fyrborg 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* (Fyrborg and Fyrborg, 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-

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**Table I. Yeast Strains Used in This Study**

| Strains | Genotype | Source |
|---------|----------|--------|
| A364A   | MATa, ade1, ade2, his7, gal1, lys2, tyr1, ura1 | Y.G.S.C.* |
| FY1679  | MATa, his3-Δ200, leu2-Δ1, trpl-Δ63, ura3-52, GAL2 | B. Dujon* |
| I7012   | MATa, HIS3, LEU2, TRP1, ura3-52, GAL2 | Y.G.S.C. |
| 332     | MATa, ade1, ade2, cle1-10-1, his7, gal1, lys2, tyr1, ura1 | Y.G.S.C. |
| STX450-5B | MATa, ade1, ade2, cle1-11-1, his7, gal1, lys2, tyr1, ura1 | Y.G.S.C. |
| NY13    | MATa, gal2, ura3-52 | P. Novick |
| NY279   | MATa, act1-2, gal2, ura3-52 | P. Novick |
| NY273   | MATa, act1-3, gal2, ura3-52 | P. Novick |
| YB18    | MATa, leu2Δ, ura3-Δ5, his3-11,15, canR | Y.G.S.C.* |
| YPH499  | MATa, ade2-101, his3-Δ200, leu2-Δ1, lys2-801, trpl-Δ63, ura3-52 | P. Hieter |
| YPH500  | MATa, ade2-101, his3-Δ200, leu2-Δ1, lys2-801, trpl-Δ63, ura3-52 | P. Hieter |
| YPH501  | MATa, ade2-101, his3-Δ200, leu2-Δ1, lys2-801, trpl-Δ63, ura3-52 | P. Hieter |
| YMW1    | MATa, ARP2, ade2-1, trpl-Δ4, ura3-251,328,373, leu2-3,112 | This study |
| YMW3    | MATa, ARP2, LEU2, ade2-1, trpl-Δ4, ura3-251,328,373, leu2-3,112 | This study |
| YMW10   | MATa, ARP2, LEU2, ade2-1,101, his3-Δ200, leu2-Δ1, lys2-801, trpl-Δ63, ura3-52 | This study |
| YMW11   | MATa, ARP2, LEU2, ade2-1,101, his3-Δ200, leu2-Δ1, lys2-801, trpl-Δ63, ura3-52 | This study |
| YMW12   | MATa, ARP2, LEU2, ade2-1,101, his3-Δ200, leu2-Δ1, lys2-801, trpl-Δ63, ura3-52 | This study |
| YMW13   | MATa, ARP2, LEU2, ade2-1,101, his3-Δ200, leu2-Δ1, lys2-801, trpl-Δ63, ura3-52 | This study |
| YMW14   | MATa, ARP2, LEU2, ade2-1,101, his3-Δ200, leu2-Δ1, lys2-801, trpl-Δ63, ura3-52 | This study |
| YMW15   | MATa, ARP2, LEU2, ade2-1,101, his3-Δ200, leu2-Δ1, lys2-801, trpl-Δ63, ura3-52 | This study |
| YMW16   | MATa, ARP2, LEU2, ade2-1,101, his3-Δ200, leu2-Δ1, lys2-801, trpl-Δ63, ura3-52 | This study |
| YMW17   | MATa, ARP2, LEU2, ade2-1,101, his3-Δ200, leu2-Δ1, lys2-801, trpl-Δ63, ura3-52 | This study |
| YMW81   | MATa, ARP2, LEU2, ade2-1,101, his3-Δ200, leu2-Δ1, lys2-801, trpl-Δ63, ura3-52 | This study |
| YMW82   | MATa, ARP2, LEU2, ade2-1,101, his3-Δ200, leu2-Δ1, lys2-801, trpl-Δ63, ura3-52 | This study |
| YMW83   | MATa, ARP2, LEU2, ade2-1,101, his3-Δ200, leu2-Δ1, lys2-801, trpl-Δ63, ura3-52 | This study |

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deum an Arp3p was reported to be localized in the mitochondria (Murgia et al., 1995). Although little functional information is available, it is now clear that in addition to these defined families of ARPs, several other less closely related Arps coexist in a cell, with Drosophila and yeast having the most known examples (Schroer et al., 1994; Fyrberg et al., 1994; Harata et al., 1994).

Our aim is functional analysis of Arp2p. We report here, using a specific peptide antibody, a cortical localization of the protein and its colocalization with actin patches. We also characterize one of several thermosensitive mutants isolated. This mutant has a single amino acid change, His330 to Leu. Pleiotropic phenotypes implicate Arp2p in the 10-nm neck filaments that are necessary for bud site selection, polarized growth, and cytokinesis (Byers and Goetsch, 1976; Haarer and Pringle, 1987; Ford and Pringle, 1991; Kim et al., 1991; Flescher et al., 1993).

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). The ARP2 gene was mutagenized by PCR amplification of a genomic fragment containing the entire coding sequence. Alloligosucleotides 5′-CAGGATATCAATTTTAGGACC-3′ and 5′-CATATCGCATGCGGATAACTATCCTC-3′ containing added external EcoRI and SphI sites were used as 5′ and 3′ primers to mutagenize the DraII-BsaBI genomic fragment (sites are underlined). 1 μg of each primer was added to a 100-μl PCR reaction containing 1.5 μM of each primer, 200 μM each dNTP, 200 μM of each primer, and 1× PCR buffer. The reaction was incubated for 50 cycles of 30 seconds at 94°C, 30 seconds at 55°C, and 60 seconds at 72°C.

Table II. Plasmids Used in This Study

| Plasmids | Characteristics |
|----------|----------------|
| pUC18, ARP2; a 2.8-kb EcoRI*-Sphl fragment containing the complete wild-type ACT2/ARP2 gene plus part of the PRP9 gene upstream and part of the RPK1/MP51 gene downstream was inserted into the EcoRI-Sphl sites of pUC18 (Schwob, 1988) |
| pYIW202 | pUC18, arp2Δ::LEU2, constructed by deleting the SnaBI-Nsil fragment from ARP2 and replacing it with a PstI-Smal LEU2 fragment (This study) |
| pFL34 | pUC19, URA3 (Bonneaud et al., 1991) |
| pBON34 | pFL34 containing the ACT2/ARP2 gene as a 2.7-kb EcoRI*-KpnI fragment (Schwob, 1988) |
| pFL34-B8 | pFL34 containing the arp2-H330L gene as an EcoRI*-DraII fragment (This study) |
| pUN20 | pUC18, CEN11, TRP1, SUP11 (Elledge and Davis, 1988) |
| pUN90 | pUC18, CEN11, HIS3 (Elledge and Davis, 1988) |
| pUN60 | pUC18, CEN11, URA3, SUP11 (Elledge and Davis, 1988) |
| pYCW204 | pUN60, ARP2, constructed by inserting a 2.8-kb EcoRI*-Sphl fragment containing ARP2 into the EcoRI-Sphl sites of pUN60 (This study) |
| pYCW207 | pUN90, ARP2, constructed by inserting a 2.8-kb EcoRI*-Sphl fragment containing ARP2 into the same sites of pUN90 (This study) |
| pGAL | CEN11, URA3, TRP1, GAL10-CYC1 promoter (Blum et al., 1989) |
| pYCW245 | CEN11, TRP1, GAL10-CYC1 promoter, ARP2; the ARP2 gene was placed under the control of the GAL10-CYC1 promoter by introducing the SnaBI-SspI fragment into the Smal site of a slightly modified version of the pGAL vector (Camasses, A., unpublished data, and this study) |
| pAS1 | 2μ origin of replication (2μ); TRP1, ADH promoter, NLS-GAL4, HA tag. (S. Elledge) |
| pYEWMU26 | An NcoI-SspI fragment containing an intronless ARP2 gene was placed under the control of the ADH promoter by ligating into the NcoI and Smal sites of pAS1 (This study) |
| p423GALL | pBluescript, 2μ; HIS3, GALL promoter (Mumberg et al., 1994) |
| p424GAL1 | pBluescript, 2μ; TRP1, GAL1 promoter (Mumberg et al., 1994) |
| pYEWM247 | p424GAL1 (Mumberg et al., 1994) plus HAN*ARP2 under the GAL1 promoter, constructed by inserting the EcoRI-SalI fragment of pYEWM246 containing 5′ HA tagged ARP2, into the EcoRI-SalI sites of p424GAL1 (This study) |
| pYEWM248 | pUN20 plus JHAC*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) |
| pYEWM250 | p423GALL plus JHAC*ARP2 under the GALL promoter, constructed by inserting JHAC*ARP2 contained in the SnaBI-SalI fragment of pYEWM248 into the SnaBI-SalI sites of p423GALL (This study) |

*Not a natural genomic site.
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The Escherichia coli strain DH5a 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 0.9 μg/ml 5-fluoroorotic acid. Yeast cells were transformed using LiAc, single-stranded carrier DNA, and DNAO (Hi1 et al., 1991).

Null strains for ARP2 were created by pYIW202. This plasmid carries a 3,116-bp SacI-Sphl genomic ARP2 fragment in which a 1,043-bp fragment between the unique SbaI and NsiI sites was replaced with a Smal-PstI LEU2 fragment. The entire SacI-Sphl fragment was used to transform different diploid leu2/leu2 strains by one-step gene replacement (Rudnstein, 1983). This yielded Arp2::LEU2 strains YMW1 (derived from Lacroute strains), YMW3 (YB18 derived), and YMW10 (YPH501 derived), respectively. The presence of one deleted allele at the ARP2 locus of these diploids was verified by Southern blot analysis (results not shown).

Rescued haploid deleted strains constituted a "shuffle" system used to select mutants. These shuffle strains were obtained by transforming the diploid strain YMW10 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 YMW11(a) and YMW12(a) 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. Alloligosucleotides 5′-CAGGATATCAATTTTAGGACC-3′ and 5′-CATATCGCATGCGGATAACTATCCTC-3′ containing added external EcoRI and SphI sites were used as 5′ and 3′ primers to mutagenize the DraII-BsaBI genomic fragment (sites are underlined). 1 μg of each primer was added to a 100-μl PCR reaction containing 1.5 μM of each primer, 200 μM each dNTP, 200 μM of each primer, and 1× PCR buffer. The reaction was incubated for 50 cycles of 30 seconds at 94°C, 30 seconds at 55°C, and 60 seconds at 72°C.
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 re-
caction was cycled 30 times in a thermal cycler at 95°C for 2 min melting, 42°C
for 1 min annealing, and 70°C for a 2-min extension. The reaction mix
was digested with EcoRI and SphI, and the 1,550-bp amplified product puri-
ified from an agarose gel was then ligated into EcoRI and SphI cut
pYCW207 vector. DNA prepared from a culture of all the transformants
constituted the pool of potential mutants.

To isolate arp2 mutants, the ade2-101/ade2 SUP111 sectoring system
was used. The pYCW207/CR 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 37°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 5FOA to counterselect the pYCW204 rescue plasmid.
Candidate plasmids were isolated and transformed into fresh YMW11
cells, which were then restested for thermosensitivity of both ade sectoring
and 5FOA 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
A600 = 0.2, divided, and incubated at 25°C and 37°C. Aliquots of the cul-
tures 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 concentra-
tions of NaCl, 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-dimen-
sional structure of rabbit skeletal actin (Kabsch et al., 1990) predicts that
peptide 40-RAEERASVATPLKD1-54 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 pep-
tide and ovalbumin-conjugated peptide. Individual antisera with the high-
est titers against the peptide were affinity-purified against the peptide an-
tigen 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 re-
vealed using the ECL detection system (Amersham, 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 de-
scribed by Pringle et al. (1991). A (3.7%) final concentration of formamide-
hyde 3 was added directly to cultures for 1 h (except for the 12CAS
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 polylsine 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 waches with PBS, preparations were analyzed using an
Optiphot microscope (Nikon, Inc., Melville, NY) equipped with fluo-
rescence optics. Affinity-purified polyclonal rabbit antiactin antibody was
a generous gift from D. Drubin (University of California, Berkeley, CA),
and YOL134/antiactin mAb was bought from SeroTec. Primary antibodies for Arp2p/Act1p double-labeling were affinity-purified pol-
yclonal goat antiactin antibody, a generous gift from J. Cooper (Washing-
ton 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 Mann-
heim 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).
Wild-type YPH501 and mutant YM883 diploid strains were grown to sta-
tionary phase in YPD with limiting adenine concentration to allow accu-
mulation of the ade2 fluorophore. Cells were diluted into fresh medium
prewarmed to 25°C or 37°C and grown until the cell density doubled. The
red fluorescence of vacuoles was visualized using the G filter set on a Ni-
kon microscope.

Lucifer yellow carbohydrazine (LY-CH from Fluka) uptake experi-
ments were performed as described by Dulic et al. (1991). The strains
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 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 antibod-
ies against areas of the protein predicted to be surface ex-
posed by comparison with the three-dimensional structure
of rabbit skeletal muscle actin (Kabsch et al., 1990). A syn-
thetic peptide corresponding to Arp2p residues 40-54,
which corresponds to residues 41-52 in actin (plus an inser-
tion of three amino acids), but is divergent from actin, was
synthesized and used to raise antibodies in rabbits. Crude
several revealed multiple bands in yeast crude extracts (data
not shown). Antipeptide/40-54 antibodies from antisera
gave the highest titers in ELISA tests were purified
using a peptide column. Affinity-purified antibody re-
vealed 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 anti-
body with the peptide antigen (Fig. 1 A, lane 2). By classi-
cal 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 transcrip-
tional activator Gal4p and expressed it in a Δarp2 strain.
The chromosomal deletion was rescued by this fusion plas-
mid (transformed strain YMW17). Extracts from this strain
were revealed by antipeptide/40-54 antibodies (Fig. 1 B, lane J) and by antibactin antibodies (Fig. 1 B, lane 6). The
antipeptide antibodies revealed the Arp2-Gal4p fusion and some smaller sized bands that could be degradation prod-
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...ter 6 and 9 h of induction. Cated that Arp2p was induced by a factor of at least 10 af-
Figure 2. 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.

Tection with anti-HA and anti-Arp2p antibodies (data not shown). Since the higher expression levels of galactose growth resulted in extremely poor growth, only glucose-grown cells were used for immunofluorescence studies. Transformant Δarp2/Δarp2 diploid yeast cells expressing HA epitope-tagged Arp2p (pYEW250) were decorated with 12CA5 antibodies. Background labeling was high, but some punctate staining was revealed in areas of cell growth, an image similar to that observed with the antipeptide antibodies. In particular, punctate spots were observed un-
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 antia actin (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 spores 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 (Schwandt 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 mutated. *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 not confer sectoring ability on the test strain at 37°C, but did at 25°C, i.e., which could not replace pYCW204 in strain YMW11 at 37°C (but could at 25°C), were then tested further. Test strain YMW11 carrying the mutagenized plasmid was tested for thermosensitivity 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-
The 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 1.0 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. 6 A, 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. 6 A, 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. 6 A, 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. 6 B) 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).
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/a diploid cells. Chitin synthase is an integral membrane protein implicated in the structural changes occurring during bud formation. Deposition of 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 (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

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Figure 7. Chitin deposition in the arp2-H330L mutant. Diploid wild-type (a and b) and mutant (c and d) cells were grown in YPD medium at 25°C, and then shifted to 37°C for 2 h before staining with calcofluor to label chitin rings. Bar, 5 μm.
Figure 8. (A) Vacuolar morphology. Vacuoles were visualized by the accumulation of the ade2 fluorophore. YPH499 (a and b) and YMW81 (c and d) strains were grown at 25°C to stationary phase in YPD without added adenine, diluted into fresh YPD + adenine at 37°C, and grown for one-cell doubling. Exposure times shown are the same for wild-type and mutant cells. a and c, Nomarski; b and d, fluorescence. (B) The arp2 mutant is defective for endocytosis. Accumulation of LY was examined at 25°C in wild-type YPH501 (a and b) and mutant YMW83 (c and d) cells. All cells were incubated, washed, and mounted as described in Materials and Methods. Cells were visualized using Nomarski (a and c) and fluorescence (b and d) optics. Bar, 5 \mu m.

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 non-complementation 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 thermosensitivity (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 thermosensitivity. 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 either 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 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-
Figure 9. 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**+) and 17012, 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 (Cap1p, Cap2p), and coflin (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). 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

| Tetrad | Spore genotype | Wild type | Ts | Dead |
|--------|----------------|-----------|----|------|
| 5 PD   | **ARP2 cdc 10-1** | 10        |    |      |
|        | **arp2 CDC10**   | 10        |    |      |
| 6 NPD  | **ARP2 CDC10**   | 12        | 5  | 7    |
|        | **arp2 cdc 10-1**| 6         | 2  | 4    |
| 6 TT   | **ARP2 CDC10**   | 6         | 2  | 4    |
|        | **arp2 cdc 10-1**| 6         | 2  | 4    |

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 tetatype (TT) tetrads 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 budded phenotype, was also seen for mutants defective in Rho proteins. Rho proteins regulate cytoskeletal dynamics. Cells depleted of the Ras-related proteins Rho3p and Rho4p (Matsui and Toh-e, 1992) initiated bud emergence and then lost cell polarity. Once budding was initiated, the cells became sensitive to osmotic pressure. Matsui and Toh-e (1992) suggested a role for the *RHO3* and *RHO4* gene products in an essential stage of bud growth. These gene products may be required for the maintenance of cell polarity, which may correspond to the maintenance and development of the bud site complex. Rho1p, like Rho3p and Rho4p, is necessary for bud enlargement, and Rho1p has been localized to growing areas of the cell surface, i.e., Rho1p has the same distribution as actin cortical patches (Yamochi et al., 1994) and Arp2p. Thus, both the osmo-sensitivity phenotype observed in mutants and specific localization of the wild-type protein are characteristic of the regulatory and structural proteins that are involved in the budding process.

Normally, haploid cells bud in an axial pattern. During axial budding, cells bud near the previous bud scar. Diploid cells bud in a bipolar pattern. During bipolar budding, cells bud alternately from each end (Flescher et al., 1993; Chant and Pringle, 1991). In the *arp2-H330L* mutant, random budding patterns were observed in both haploid and diploid cells, implicating Arp2p in budding. This phenotype was evident at temperatures permissive for growth of the *arp2-H330L* mutant, whereas disorganization of the actin cytoskeleton filamentous structures was seen only at more severe temperatures. One should keep in mind, however, that random budding patterns were observed in all of the *act1* Ts mutants that were examined in a comparative study (Drubin et al., 1993). A large number of genes necessary for bud site selection and formation have been defined (Chant and Herskowitz, 1991; Drubin, 1991). However, to establish whether Arp2p might be directly involved in bud site selection (like *RSLI/BUD1, BUD2-5*), assembly of the bud site, the establishment of polarity or bud development (like *CDC42, CDC24, CDC43, BEM1, BEM2, RHO1-4*), or in bud structure and orientation as a component of the cytoskeleton (like *ACT1, ABP1, PFY1, CAP1,2, TPM1, MYO2, SLA2/END4*, etc.) (Drubin, 1991; Amatruda et al., 1992; Chant, 1994; Chant and Pringle, 1991; Holtzman et al., 1993), it will be important to know with which of the proteins already implicated in these particular functions Arp2p interacts.

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 *cdc10-10* 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 *cdc10-1* 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 *Aarp2::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 *Aarp2-H330L* protein thus has physiological effects that are different from the depletion 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
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 suggest 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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