Functional Surfaces on the p35/ARPC2 Subunit of Arp2/3 Complex Required for Cell Growth, Actin Nucleation, and Endocytosis

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The Arp2/3 complex is comprised of seven evolutionarily conserved subunits and upon activation by WASp or another nucleation promoting factor nucleates the formation of actin filaments. These events are critical for driving a wide range of cellular processes, including motility, endocytosis, and intracellular trafficking. However, an in depth understanding of the Arp2/3 complex activation and nucleation mechanism is still lacking. Here, we used a mutagenesis approach in Saccharomyces cerevisiae to dissect the structural and functional roles of the p35/ARPC2 subunit. Using integrated alleles that target conserved and solvent-exposed residues, we identified surfaces on p35/ARPC2 required for cell growth, actin organization, and endocytosis. In parallel, we purified the mutant Arp2/3 complexes and compared their actin assembly activities both in the presence and in the absence of WASp. The majority of alleles with defects mapped to one face of p35/ARPC2, where there was a close correlation between loss of actin nucleation and endocytosis. A second site required for nucleation and endocytosis was identified near the contact surface between p35/ARPC2 and p19/ARPC4. A third site was identified at a more distal conserved surface, which was critical for endocytosis but not nucleation. These findings pinpoint the key surfaces on p35/ARPC2 required for Arp2/3 complex-mediated actin assembly and cellular function and provide a higher resolution view of Arp2/3 structure and mechanism.

The Arp2/3 complex nucleates branched actin filament networks essential for a wide range of cellular processes, including leading edge extension during cell motility, the internalization step of endocytosis, and intracellular trafficking of vesicles and organelles (reviewed in Ref. 1). The complex is composed of seven evolutionarily conserved subunits: p40/ARPC1, p35/ARPC2, p21/ARPC3, p19/ARPC4, and p15/ARPC5, and two actin-related proteins: Arp2 and Arp3 (2). Arp2 and Arp3 structurally resemble actin and are hypothesized to mimic an actin dimer, forming a seed that catalyzes filament growth (3). Alone the complex is a poor nucleator of actin polymerization. However, after the Arp2/3 complex is activated by an NPF (3) and then binds to the side of an existing (mother) actin filament, it efficiently assembles a new (daughter) filament at a characteristic 70-degree angle to produce a branched structure (reviewed in Ref. 4).

Much has been learned about the actin nucleation mechanism of the Arp2/3 complex, revealing it to be a highly intricate and dynamic machine. The crystal structure of bovine Arp2/3 complex revealed the positions of each subunit and provided key insights into their functional roles (3). Fluorescence resonance energy transfer and electron microscopy studies demonstrated that conformational rearrangements of the complex occur upon NPF activation (5, 6). Molecular modeling studies have made predictions about the locations of key functional surfaces on individual subunits in the complex, and electron microscopy studies have provided new information about the positions of subunits in the actin filament branch (7, 8). Despite these fundamental advances, our understanding of the activation and nucleation mechanism remains incomplete. Noticeably absent from the literature to date are any systematic mutational analyses on individual subunits to identify the specific protein surfaces required for Arp2/3 complex biochemical and cellular function.

The p35/ARPC2 subunit holds a central structural position in the Arp2/3 complex and mediates multiple physical interactions. It directly contacts the side of the mother actin filament and three other subunits in the complex (Arp2, Arp3, Arc19) and is required for maintaining the structural integrity of the entire complex (3, 9–11). p35/ARPC2 does not bind NPFs (11–13). Instead, it appears to serve as a structural/functional hub in the Arp2/3 complex, relaying signals and conformational changes to other subunits upon NPF activation and mother filament binding. Indeed, randomly generated alleles of p35/ARPC2 in Saccharomyces cerevisiae have been shown to cause either “impaired WASp response” or “leaky nucleation” (in the absence of any NPF) and have opposite conformational effects on Arp2/3 complex (inhibitory and activating, respectively) (6). However, the specific surfaces on p35/ARPC2 required for its functions have remained unexplored.

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The abbreviations used are: NPF, nucleation promoting factor; HA, hemagglutinin; ts, temperature-sensitive; 5-FOA, 5-fluoroorotic acid.
Here, we performed a systematic mutational analysis of conserved surface residues on p35/ARPC2 in S. cerevisiae. Our findings reveal the locations of three key sites required for both biochemical and cellular functions of the Arp2/3 complex. We also observe a strong correlation between the loss of actin nucleation activity in vitro and the loss of cellular actin organization, fluid phase endocytosis, and cell growth. These results pinpoint the key functional sites on p35/ARPC2, one of the most central subunits in the Arp2/3 complex, and offer new structural and functional insights into its biochemical and cellular mechanism.

EXPERIMENTAL PROCEDURES

Yeast Strains and Plasmid Constructions—Standard methods were used for the growth and transformation of yeast strains (14). To generate plasmids carrying arc35 alleles, PCR-based site-directed mutagenesis was performed on pBG175 (pRS315-ARC35) and pBG959 (pRS305-ARC35) and verified by restriction digest (each allele contained a unique silent restriction site) and DNA sequencing. For testing the lethality of each allele, mutant pBG175 plasmids were transformed into the BGY589 strain (MATa, leu2, his3, lys2, ura3, arc35::KANMX, pRS316-ARC35::URA3) and selected on Leu− medium. The cells were then streaked on media containing 5-FOA to counter-select against the wild type ARC35::URA3 plasmid. We also integrated mutant arc35 alleles at the LEU2 locus by digesting wild type and mutant pBG959 plasmids with XcmI and transforming strains BGY589 and BGY694 (MATa, leu2, ura3, his3, ARPC2-TEV-3::HA::HISMX, arc35::KANMX, pRS316-ARC35::URA3). Transformants were selected on Leu− medium and then plated on medium containing 5-FOA to counter-select against the wild type ARC35::URA3 plasmid. Integrations were confirmed by PCR amplification of the ARC35 open reading frame and restriction digest as above. To compare cell growth, serial dilutions of wild type and mutant cultures were plated on YEPD and grown at 25 °C or 37 °C. Purifying the lethal Arp2/3 complex containing arc35–105 and arc35–106 mutations required construction of new yeast strains with multiple tags. First, a 9× Myc tag was integrated at the C terminus of the ARC35 gene in BGY694 (MATa, ade2, his3, leu2, trp1, ura3, ARC18-TEV-3::HA::HIS3) using pML11 as a PCR template (15). Next, the resulting strain was crossed to BGY325 (MATa, leu2, ura3, his3, trp1, abp1Δ::LEU2) and sporulated, and tetrad were dissected to obtain the haploid strain BGY1229 (MATα, his3, leu2, trp1, ura3, ARC35-9xmyc). This strain was used to integrate a second copy of the ARC35 gene (wild type or mutant) tagged with TEV-3×HA. For this purpose, we introduced an Nhe1 site at the C terminus of ARC35 on pBG959 by site-directed mutagenesis. Next, a TEV-3×HA cassette was amplified by PCR from pBG265 and cloned into the Nhe1 site. The resulting integration plasmid (pBG960) encodes the wild type Arc35 protein with a C-terminal alainineserine linker and in-frame TEV-3×HA tag. pBG960 was altered by site-directed mutagenesis to introduce the arc35–105 and arc35–106 alleles. To integrate the alleles at the LEU2 locus, wild type and mutant plasmids were digested with XcmI and transformed into BGY1229.

Antibodies and Immunoblotting—Rabbit polyclonal antibodies against yeast tubulin were a kind gift from Frank Solomon (Massachusetts Institute of Technology). Chicken polyclonal antibodies were raised against a fragment of p35/Arc35 (residues 1–287) (Aves; Tigand, OR). HA (HA.11) antibodies conjugated to either horseradish peroxidase or IRDye700DX were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) or Rockland Immunoochemicals (Gilbertsville, PA). Horseradish peroxidase was visualized using ECL (Pierce). All other antibodies were visualized using the Odyssey system (Li-Cor).

Purification of Proteins—The yeast strains BGY848 (containing integrated ARPC2-TEV-3×HA) and BGY1229 (containing integrated wild type or mutant copies of ARC35-TEV-3×HA) were grown to A600 = 1–2, washed, and resuspended in a 2:1 w/v ratio of cell pellet to water. The cells were frozen in liquid nitrogen and lysed by cryo-mechanical shearing (16). 6 g of frozen yeast powder was thawed with the addition of 2.5 ml of 2.5× HEK buffer (50 mM HEPES, pH 7.5, 2.5 mM EDTA, 125 mM KCl) and a protease inhibitor mixture: 1 mM phenylmethylsulfonyl fluoride and 0.5 μg/ml each of antipain, chymostatin, aprotinin, pepstatin A, and leupeptin. Thawed lysates were centrifuged for 20 min at 80,000 rpm at 4 °C in a TLA100.3 rotor (Beckman Coulter; Fullerton, CA), and the supernatants were harvested. Clarified extracts were incubated for 2 h at 4 °C with 50 μl of CL4B beads (GE Healthcare) prebound to 50 μg of α-HA antibodies (HA.11; Covance, Philadelphia, PA). The beads were washed three times with 1 ml of ice-cold HEK buffer (20 mM HEPES, pH 7.5, 1 mM EDTA, 50 mM KCl), then two times with 1 ml of ice-cold HEK500 buffer (20 mM HEPES, pH 7.5, 1 mM EDTA, 500 mM KCl), and finally twice with 1 ml of ice-cold HEK buffer. The beads were resuspended in 50 μl of HEK buffer to generate a 100-μl bead slurry and incubated for 2 h at 25 °C with 20 units of TEV (Invitrogen). The 50-μl supernatant was harvested, and then the beads were incubated with an additional 50 μl of HEK buffer. After 10 min, the 50 μl of supernatant was harvested and combined with the first 50 μl that was removed. Aliquots of this mixture were flash frozen in liquid nitrogen and stored at −80 °C. Rabbit skeletal muscle was purified (17) and labeled with pyrenylidoacetamide (18) as described. Full-length yeast WASp/Las17 was purified as described (19).

Actin Assembly Assays—Actin assembly assays were performed as described (20). Monomeric rabbit skeletal muscle actin was prepared by gel filtration using a Sephacryl S-200 column (GE Biosciences) equilibrated in G-buffer (10 mM Tris, pH 8.0, 0.2 mM ATP, 0.2 mM CaCl2, and 0.2 mM dithioretil). Actin assembly was measured in 60-μl reactions. Gel-filtered monomeric actin (final concentration, 2 or 4 μM; 5% pyrene-labeled) was converted to Mg-ATP-actin for 2 min immediately following Mg-ATP addition. Reactions were stopped with 2 μg/ml vinblastine or nocodazole, and 1 μg/ml pyrene-labeled actin was added to the reactions 2 μg/ml vinblastine or nocodazole. All samples were mixed and flash frozen in liquid nitrogen.
before use in the reaction as described (20) and then mixed with 10–15 μl of HEK buffer or proteins in HEK buffer and added immediately to 3 μl of initiation mix (40 mM MgCl₂, 10 mM ATP, 1 M KCl) to initiate assembly. Pyrene fluorescence was monitored over time at 25 °C in a fluorescence spectrophotometer (Photon Technology International, Lawrenceville, NJ) at excitation 365 nm and emission 407 nm. To compare wild type and mutant Arp2/3 complexes, rates of actin assembly were calculated from the slopes of the assembly curves at 25–50% polymerization. The barbed end concentration in each reaction was calculated as described previously (21). We also compared the lag time (time to 50% actin assembly) for wild type and mutant Arp2/3 complexes. The results were indistinguishable from the slope measurements above, i.e. the mutant defects were the same by either criteria.

Microscopy—To stain cells for filamentous actin, the cells were grown to log phase at 25 °C in 5-ml YEPD liquid cultures and then incubated for an additional 2 h at 25 or 37 °C. The cells were fixed in formaldehyde and stained with Alexa 488-conjugated phalloidin (Invitrogen). The cell images were captured using a Zeiss Axioskop mot plus microscope (Zeiss, Thornwood, NY) outfitted with a Hamamatsu IEEE1394 digital camera (Hamamatsu Photonics, Bridgewater, NJ), and the images were processed using Openlab software (Improvision, Lexington, MA). Fluid phase endocytosis in wild type and mutant strains was assayed by Lucifer yellow dye uptake, as described (22). Lipid endocytic uptake in wild type and arc35–101 strains was compared in assays as described (23) using FM1–43FX (Invitrogen).

RESULTS

Genetic Analysis of arc35 Alleles—To dissect p35/ARPC2 function, we performed a scanning mutagenesis of its conserved surface residues. We aligned p35/ARPC2 sequences from S. cerevisiae (S.c.) and B. taurus (B.t.) to identify conserved residues and then mapped their positions on the crystal structure of bovine Arp2/3 complex (3). The p35/ARPC2 subunit is tan, and each subunit is labeled.

![Figure 1. Scanning mutagenesis of conserved surface residues on the p35/ARPC2 subunit of Arp2/3 complex. A. Alignment of p35/ARPC2 sequences from S. cerevisiae (S.c.) and B. taurus (B.t.). Mutant arc35 allele numbers are color-coded according to cell growth phenotypes: pseudo wild type, green; mild ts, yellow; strong ts, orange; lethal, red. Black dots denote conserved residues that are surface exposed on the crystal structure of bovine Arp2/3 complex (3). B. Positions of residues mutated in each arc35 allele mapped on the bovine Arp2/3 complex structure (3). The p35/ARPC2 subunit is tan, and each subunit is labeled.](image-url)
ARC35 plasmid. This analysis revealed that 12 of the 14 alleles supported viability in the absence of wild type ARC35 (not shown); only arc35–105 and arc35–106 failed to complement growth (Fig. 2A). These alleles introduce alanine substitutions at three and four conserved residues, respectively (Fig. 1A), and interestingly, the locations of arc35–105 and arc35–106 are proximal on the surface of p35/ARPC2 (Fig. 1B).

We dissected in greater detail the two essential surfaces on p35/ARPC2, mutated in arc35–105 and arc35–106, by generating six new alleles that disrupt smaller subsets of those residues: arc35–115, arc35–116, arc35–117, arc35–118, arc35–119, and arc35–120 (Fig. 2B). Our dissection of arc35–106 revealed that a single highly conserved residue is essential for cell viability (Arg215 in S. cerevisiae, Arg189 in Bos taurus), as shown by the lethality of arc35–120 (Fig. 2A). In contrast, our dissection of arc35–105 revealed a more complex pattern of contribution to cell growth. Mutation of the Lys-Val pair caused ts growth (arc35–115 in Fig. 2C). Mutation of the conserved Gln alone caused no obvious change in cell growth (arc35–116 in Fig. 2C) but when combined with the KV/AA mutation caused lethality.

To analyze in more depth the cell growth phenotypes of the initial 12 viable arc35 alleles, each allele was integrated into the genome of an arc35Δ strain, such that the mutant copies are expressed under the control of the ARC35 promoter. This strain also has an integrated TEV-3×HA tag at the C terminus of ARP2 to facilitate purification of the mutant Arp2/3 complexes (6). Wild type and mutant cells were serially diluted, plated, and compared for growth at 25 °C and 37 °C (Fig. 2C). Four strains showed strong temperature-sensitive (ts) growth at 37 °C (arc35–104, arc35–108, arc35–109, and arc35–112), whereas two others exhibited minor ts growth (arc5–102 and arc35–111). One of the strong ts strains, arc35–112, also was partially impaired for growth at 25 °C, suggesting that among the 12 viable alleles its cellular defects may be the most severe.

The positions of the mutated surfaces on p35/ARPC2 are shown on the crystal structure of Arp2/3 complex with color-coded growth defects (Fig. 1B). Importantly, we verified that the defects caused by the arc35 alleles were not due to reduced expression levels of p35/ARPC2 (supplemental Fig. S2).

Biochemical Analysis of Nonlethal arc35 Arp2/3 Complexes—To investigate the biochemical basis of the arc35 mutant phenotypes, we purified the corresponding mutant Arp2/3 complexes (Fig. 3A) and compared their actin nucleation activities (10 nM Arp2/3 complex) in the presence of yeast WASp/Las17 (10 nM) and 2 μM actin (Fig. 3B). Mutant activities varied from close to wild type to severely impaired. Arp2/3 complexes containing arc35–101, arc35–102, arc35–103, arc35–107, arc35–111, arc35–113, and arc35–114 mutations were relatively normal, with activities ranging from 60 to 97% of wild type (Fig. 3B, yellow and green bars), whereas Arp2/3 complexes containing arc35–104, arc35–108, arc35–109, and arc35–112 mutations were severely defective, with activities ranging from 13–55% of wild type (Fig. 3B, orange bars). The defects in severe mutants (arc35–104, arc35–108, arc35–109, and arc35–112) occurred over a range of Arp2/3 complex concentrations (Fig. 3, C and D). We also analyzed one pseudo wild type allele, arc35–110; this mutant showed only a slightly reduced activity compared with wild type at each concentration. Allele effects graphed in Fig. 3 (B and D) are color-coded by their growth phenotypes (Fig. 2C), which indicates a close correlation between loss of nucleation activity and severity of cell growth phenotype. We considered the possibility that the mutants showed reduced actin assembly activity because they were impaired in binding WASp. To address this point, we compared the actin assembly activities of wild type, arc35–104, and arc35–108 Arp2/3 complexes over a range of WASp concentrations (Fig. 3E). The data showed that increasing WASp concentrations have the same relative effect on actin assembly for mutant and wild type Arp2/3 complexes, suggesting that the defects are not due to impaired WASp binding.

We also compared wild type and mutant Arp2/3 complexes for their actin assembly activities in the absence of WASp. Although the Arp2/3 complex is a poor nucleator in the absence of an NPF, it retains some activity. This is especially...
relevant for yeast Arp2/3 complex, which is inherently more "leaky" for nucleation than bovine Arp2/3 complex (6, 24).

Nucleation activity was plotted as a function of Arp2/3 complex concentration, 0–20 nM (Fig. 4, A and B), and the slopes of the line fits were further compared (Fig. 4C). These analyses revealed that all of the mutants with nucleation defects in the presence of WASp (Fig. 3, B and D) also showed nucleation defects in the absence of WASp. In a few cases, the mutant defects were even more pronounced in the absence of WASp. For example, arc35–104, arc35–108, and arc35–109 mutants had activity levels ranging from 30 to 50% of wild type in the presence of WASp (Fig. 3, B and D), compared with only 2–13% of wild type in the absence of WASp (Fig. 4C). Surprisingly, one mutant (arc35–107) showed higher nucleation activity than wild type in the absence of WASp, suggesting that it may be "leaky," similar to the previously described arc35–5 allele (6).

Actin Nucleation Defects of Lethal arc35 Alleles—To isolate Arp2/3 complexes carrying lethal arc35–105 and arc35–106 mutations and analyze their defects, it was necessary to develop a modified purification strategy. A 9×Myc tag was integrated at the C terminus of the endogenous ARC35 gene. Then a second ARC35 gene (mutant or wild type) with a C-terminal TEV-3×HA tag was integrated at the LEU2 locus. The resulting strains express two separate p35/ARPC2 proteins, the wild type Arc35-9×Myc and wild type or mutant Arc35-TEV-3×HA. The wild type and mutant TEV-3×HA-tagged Arp2/3 complexes were isolated on HA antibody-coated beads and released by TEV protease digestion. Immunoblots confirmed the
absence of wild type Arc35-9×Myc protein in the preparations (not shown). Then the actin nucleation activities of the Arp2/3 complexes were compared at a range of concentrations in the presence of WASp (Fig. 5, A and B). Strong nucleation defects were observed for arc35–106, with reduced activity levels similar to the most impaired nonlethal allele, arc35–112 (Fig. 5C). In contrast, arc35–105 showed a less severe reduction in nucleation activity, comparable with the activity levels of ts alleles (arc35–104, arc35–108, and arc35–109) (Fig. 5C).

In Vivo Actin Organization Defects of arc35 Alleles—Next, we examined the correlation between the loss of nucleation activity in vitro and the loss of actin organization in vivo. In S. cerevisiae, the Arp2/3 complex localizes to cortical actin patches, which are sites of endocytic internalization (reviewed in Ref. 25). Mutant alleles of different subunits in the Arp2/3 complex (and NPFs) have been shown to cause depolarization of actin patches and impaired endocytosis, accompanied by diminished cable staining (6, 26–30). However, in few instances have the underlying biochemical defects of those mutants been determined. Our analysis of the arc35 strains revealed a close correlation between reduction in nucleation activity and severity of defects in cellular actin organization (Fig. 6). In the wild type ARC35 strain, the cells have polarized patch organization at 25 °C but show a slight depolarization of actin patches at 37 °C. This is due to the integrated Arp2-TEV-3×HA tag, which causes a minor impairment of ARP2 function. Three mutants that were pseudo wild type for growth (arc35–101, arc35–107, and arc35–113) and one mutant that was mildly ts (arc35–111) had normal actin organization at 25 °C but a higher percentage of cells with depolarized actin patches at 37 °C compared with wild type cells (Table 1). Two other mutants that were pseudo wild type for growth (arc35–101 and arc35–110) and one
mutant that was mildly ts (arc35–102) showed an abnormal accumulation of actin patches in the mother compartment at both 25 and 37 °C. Three mutants that were more severely ts for growth (arc35–104, arc35–108, and arc35–109) had more severe defects in patch polarity at both temperatures. Quantification of this phenotype revealed a 1.8-fold increase in number of patches in the mother compartment for arc35–108 relative to wild type cells. The allele with the most dramatic growth defects (arc35–112) accumulated large clumps of actin in the bud at both 25 (66%) and 37 °C (46%), similar to the actin clump

![Cellular actin organization in arc35 mutants.](image)

**TABLE 1**
Summary of genetic and biochemical effects of ARC35 alleles

| Allele     | Mutations   | Cell growth 25 °C | Actin nucleation activity | Cells with polarized actin patches 25 °C | Endocytosis |
|------------|-------------|-------------------|---------------------------|------------------------------------------|-------------|
| arc35      |             | + + + +           |                           |                                          |             |
| arc35–101  | D50A, K51A  | + + + +           | 100 ± 3.1 a               | 85 ± 3.1 a                               |             |
| arc35–102  | K79A        | + + + +           | 75 ± 7.8 a               | 28 ± 12                                  |             |
| arc35–103  | R173A, D174A, E175A | + + + + | 50 ± 5.7 a               | 47 ± 11                                  | 29          |
| arc35–104  | D185A, R186A | + + + +           | 92 ± 15.4 a              | 74 ± 19                                  | 79          |
| arc35–105  | K205A, V206A, Q209A | + + + + | 45 ± 8 a               | 15 ± 6                                   | 6           |
| arc35–106  | D213A, R215A, K216A, R217A | + + + + | ND                       | 40 ± 2.7 b                 | NA          |
| arc35–107  | H231A, E232A | + + + +           | 97 ± 23.2 a              | 85 ± 28                                  | 69          |
| arc35–108  | L235A, E236A, K238A | + + + + | 37 ± 4.5 a             | 8 ± 0                                    | 8           |
| arc35–109  | F259A, R261A | + + + +           | 2 ± 3.3 ± 5.9 b         | 2 ± 1                                     | 8           |
| arc35–110  | L228K       | + + + +           | 77 ± 11.1 b             | 18 ± 2                                    | 27          |
| arc35–111  | K61A        | + + + +           | 70 ± 7.0 b               | 81 ± 14                                  | 73          |
| arc35–112  | K307A, K310A, R311A | + + + + | 19 ± 3.9 b             | 4 (clumps)                               | 10          |
| arc35–113  | R297A, R299A, R301A | + + + + | 74 ± 1.7 a             | 75 ± 18                                  | 69          |
| arc35–114  | S223A, Q226A | + + + +           | ND                       | ND                                       | ND          |

* This WASp-dependent actin nucleation activity was taken from Fig. 3B.

* This WASp-dependent actin nucleation activity was taken from Fig. 5C.
Structure and Function of Arp2/3 Complex

Despite its pivotal role as an actin nucleation factor in animal, fungal, and plant cells, our understanding of Arp2/3 complex mechanism and function remains incomplete. For instance, we still lack a detailed map of the functional surfaces of each subunit and how they contribute to Arp2/3 complex biochemical and cellular activities. A major step in resolving Arp2/3 complex inner workings was solution of its crystal structure in its inactive state (3), which provided an atomic resolution view of each subunit and the necessary blueprint for performing more refined structure-function analyses. Here, we have used this crystal structure to guide a systematic mutagenesis of the p35/ARPC2 subunit and define the conserved sites on its surface required for in vitro actin nucleation and in vivo functions in endocytosis, actin organization, and cell growth (summarized in Fig. 1B and Table 1). Until now, mutational analyses on this subunit have been limited to full gene deletions and/or randomly generated ts mutants (10, 26, 27). From these genetic data, it remained uncertain whether the essential requirement for p35/ARPC2 stemmed primarily from its role in maintaining structural integrity of the Arp2/3 complex (10). However, our data show that loss of specific functional surfaces on p35/ARPC2 can cause lethality without affecting the structural integrity of the complex, demonstrating that in addition to its requirement as part of the structural backbone of the complex, p35/ARPC2 has essential activities that facilitate actin nucleation and drive endocytosis in vivo.

The heterodimer comprised of p35/ARPC2 and p19/ARPC4 not only forms the central core of the Arp2/3 complex but is sufficient to bind the sides of actin filaments with similar affinity to the intact Arp2/3 complex (11). Recent homology modeling and electron microscopy studies have implicated one broad surface of p35/ARPC2 in filament binding (7, 8). Our unbiased mapping approach, introducing mutations at 31 of the 33 conserved surface residues on p35/ARPC2, identified this same surface as being crucial for cell growth and actin nucleation. This establishes the functional importance of this site and supports the hypothesis that filament side binding is instrumental in branched nucleation. Finally, we identified two additional functional sites on p35/ARPC2 that had not been predicted by homology modeling. Thus, in total there appear to be three distinct, conserved surfaces on p35/ARPC2 that are important for actin nucleation and/or cellular functions (Fig. 1B). Each surface is discussed below.

Surface I: Predicted Mother Filament-binding Site—A new high resolution three-dimensional reconstruction of the actin filament branch based on electron tomography suggests that all seven subunits of the Arp2/3 complex may interact with the mother filament (7). The p35/ARPC2-p19/ARPC4 heterodimer occupies a central position in this structure and is predicted to be crucial for filament side binding and branch stabilization, which is supported by several functional studies (9–11, defects in arc35Δ and arc35-1 alleles (10, 31) and in yeast WASp mutants (las17Δ/bee1Δ) (32). arc35–112 mutants also had fewer budded cells compared with wild type cells at both temperatures, consistent with their severe growth defect.

Endocytic Defects Caused by the arc35 Alleles—Finally, we examined arc35 mutants for defects in fluid phase endocytosis, by monitoring cells for uptake of Lucifer yellow dye in the vacuole (Fig. 7A) and quantifying this effect (Fig. 7B). Five mutants (arc35–101, arc35–104, arc35–108, arc35–109, and arc35–112) showed severe defects in endocytosis; two mutants (arc35–102 and arc35–110) showed partial defects; four mutants (arc35–103, arc35–107, arc35–111, and arc35–113) showed no defect or only a modest defect in endocytosis. For all but one mutant, there was a fairly close correlation between the loss of endocytosis function in vivo and the loss of actin nucleation activity in vitro. The exception was arc35–101, which despite being pseudo wild type for growth and minimally impaired in actin organization and exhibiting normal levels of actin nucleation in vitro was severely impaired in endocytosis. To further investigate the endocytic defect of arc35–101, we monitored the uptake of the lipid-binding dye FM1–43 in this mutant. arc35–101 cells showed a reduced rate of FM1–43 delivery to/accumulation in the vacuole, roughly two times slower than wild type cells (supplemental Fig. S3). This suggests that arc35–101 may have only a mild defect in endocytosis, which is consistent with its very mild defects in actin organization and actin nucleation activity.

DISCUSSION

Despite its pivotal role as an actin nucleation factor in animal, fungal, and plant cells, our understanding of Arp2/3 complex mechanism and function remains incomplete. For instance, we still lack a detailed map of the functional surfaces of each subunit and how they contribute to Arp2/3 complex biochemical and cellular activities. A major step in resolving Arp2/3 complex inner workings was solution of its crystal structure in its inactive state (3), which provided an atomic resolution view of each subunit and the necessary blueprint for performing more refined structure-function analyses. Here, we have used this crystal structure to guide a systematic mutagenesis of the p35/ARPC2 subunit and define the conserved sites on its surface required for in vitro actin nucleation and in vivo functions in endocytosis, actin organization, and cell growth (summarized in Fig. 1B and Table 1). Until now, mutational analyses on this subunit have been limited to full gene deletions and/or randomly generated ts mutants (10, 26, 27). From these genetic data, it remained uncertain whether the essential requirement for p35/ARPC2 stemmed primarily from its role in maintaining structural integrity of the Arp2/3 complex (10). However, our data show that loss of specific functional surfaces on p35/ARPC2 can cause lethality without affecting the structural integrity of the complex, demonstrating that in addition to its requirement as part of the structural backbone of the complex, p35/ARPC2 has essential activities that facilitate actin nucleation and drive endocytosis in vivo.

The heterodimer comprised of p35/ARPC2 and p19/ARPC4 not only forms the central core of the Arp2/3 complex but is sufficient to bind the sides of actin filaments with similar affinity to the intact Arp2/3 complex (11). Recent homology modeling and electron microscopy studies have implicated one broad surface of p35/ARPC2 in filament binding (7, 8). Our unbiased mapping approach, introducing mutations at 31 of the 33 conserved surface residues on p35/ARPC2, identified this same surface as being crucial for cell growth and actin nucleation. This establishes the functional importance of this site and supports the hypothesis that filament side binding is instrumental in branched nucleation. Finally, we identified two additional functional sites on p35/ARPC2 that had not been predicted by homology modeling. Thus, in total there appear to be three distinct, conserved surfaces on p35/ARPC2 that are important for actin nucleation and/or cellular functions (Fig. 1B). Each surface is discussed below.

Surface I: Predicted Mother Filament-binding Site—A new high resolution three-dimensional reconstruction of the actin filament branch based on electron tomography suggests that all seven subunits of the Arp2/3 complex may interact with the mother filament (7). The p35/ARPC2-p19/ARPC4 heterodimer occupies a central position in this structure and is predicted to be crucial for filament side binding and branch stabilization, which is supported by several functional studies (9–11, defects in arc35Δ and arc35-1 alleles (10, 31) and in yeast WASp mutants (las17Δ/bee1Δ) (32). arc35–112 mutants also had fewer budded cells compared with wild type cells at both temperatures, consistent with their severe growth defect.
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This new structural model is in good agreement with previous homology modeling studies (8) and shows that the largest conserved surface region on p35/ARPC2 (Fig. 1B, side view) binds to actin. Our analysis provides experimental validation of the functional importance of this site, demonstrating that surface I is crucial for Arp2/3 complex-mediated actin nucleation and cellular functions.

Our data also define more precisely the important residues on surface I. Of the initial 14 alleles we generated in p35/ARPC2, 8 were compromised for cell growth (two lethal and six temperature-sensitive) (Fig. 1A). Five of the eight alleles (arc35–104, arc35–105, arc35–106, arc35–108, and arc35–109), including both lethals, are clustered on the postulated actin binding surface (Fig. 1B, side view). These five alleles target 13 of the conserved residues predicted to be involved in actin binding by modeling studies (8), and we found that seven of these residues make important contributions to actin nucleation and cellular function (supplemental Fig. S4). The substructure that is most crucial for in vivo functions is targeted by the two lethal alleles, arc35–105 and arc35–106. The residues mutated in these alleles are adjacent on the surface of p35/ARPC2, suggesting that they may comprise a single active site essential for in vivo function. Three residues were mutated in arc35–105, with two residues (Lys307 and Val309) appearing to be the most important for function. The next most most important part of surface I is disrupted by several nearby ts alleles (arc35–104, arc35–108, and arc35–109). Four residues were mutated in arc35–106, and we were able to identify one highly conserved residue (Arg215) as being essential for cell viability. Because this residue is conserved in all species examined to date, its targeted disruption in other organisms will provide a new tool for study.

One of the most interesting structural features of p35/ARPC2 is its long α helix that extends toward Arp2 (Fig. 1B, front view). Although sequence homology in this region is low, the predicted helical structure is conserved across distant species, suggesting that this element has an important function. The only conserved solvent-exposed residues in the helix were targeted by two of our alleles (arc35–112 and arc35–113). arc35–112 had severe defects in cell growth, endocytosis, and actin nucleation, whereas arc35–113 was pseudo-wild type in all assays. The residues mutated in arc35–112 (Lys310, Lys311, and Arg311) contact p19/ARPC4. These mutations could alter the structure of the p35/ARPC2-p19/ARPC4 interaction region and thereby affect mother filament binding. Alternatively, these mutations could interrupt important conformational signals normally transmitted from one subunit to the other. Indeed, the conspicuous length and position of the long helix in p35/ARPC2 is suggestive of a lever arm that might transduce signals from NPF and/or F-actin binding to other subunits in the complex (e.g. Arp2). Another interesting feature of this helix is an 11-amino acid S. cerevisiae-specific insertion that occurs in a section of the helix that is disordered in the Arp2/3 complex crystal structure (3). The functional significance of this insertion has remained unclear. We generated an internal deletion of the sequence and found the allele to be lethal (not shown). This was somewhat unexpected, because the insertion is absent in other organisms. This suggests that the insertion may compensate for other yeast-specific structural differences in Arp2/3 complex and/or provide an interaction site for a cellular factor that is essential for regulating Arp2/3 complex in yeast but not other species.

Surface III: Essential for Endocytosis but Not Actin Nucleation—A third surface on p35/ARPC2 was defined by two alleles (arc35–101 and arc35–102) with severe defects in endocytosis but not actin nucleation (Fig. 1B, back view). This surface, which is distal to intersubunit contacts and the predicted mother filament-binding site (8), is highly conserved, pointing to its functional importance. Our data demonstrate that it is important in vivo for efficient endocytic delivery to the vacuole, as indicated by mutant defects in uptake of Lucifer yellow and FM1–43 dyes (Fig. 7 and supplemental Fig. S3). What is not yet clear is whether surface III performs a related or separate function in endocytosis from surfaces I and II. For instance, surface III could function in a later, post-internalization step of endocytosis. In support of this model, there is mounting evidence that Arp2/3 complex is important not only for the early internalization step of endocytosis, but also in endosome movement, vesicle budding from the trans-Golgi network, and vacuolar

3 M. Welch, personal communication.
4 D. Sept and M. Welch, personal communication.
fusion events (35–37). These phases of membrane trafficking may require a distinct set of Arp2/3–interacting proteins, which could involve interactions with surface III on p35/ARPC2.

Summary—We have defined three important functional sites on the p35/ARPC2 subunit of Arp2/3 complex. In doing so, we have (a) demonstrated that the putative mother filament-binding site is essential for cell growth and actin nucleation (surface I), (b) shown that the contacts between p35/ARPC2 and p19/ARPC4 are important for actin nucleation and endocytosis (surface II), and (c) identified a novel third site (surface III) required for efficient endocytosis and actin nucleation, which does not overlap with functional sites predicted by earlier modeling studies. This represents the first detailed mutational analysis of any subunit in the Arp2/3 complex. It is our hope that the details provided within this study will enhance models of Arp2/3 complex structure and mechanism and help guide future studies aimed at disrupting specific interactions and activities of the Arp2/3 complex.

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REFERENCES

1. Goley, E. D., and Welch, M. D. (2006) Nat. Rev. Mol. Cell. Biol. 7, 713–726
2. Machelsky, L. M., Atkinson, S. J., Ampe, C., Vandekerckhove, J., and Pollard, T. D. (1994) J. Cell Biol. 127, 107–115
3. Robinson, R. C., Turbedsky, K., Kaiser, D. A., Marchand, J. B., Higgs, H. N., Choe, S., and Pollard, T. D. (2001) Science 294, 1679–1684
4. Welch, M. D., and Mullins, R. D. (2002) Annu. Rev. Cell Dev. Biol. 18, 247–288
5. Goley, E. D., Rodenbusch, S. E., Martin, A. C., and Welch, M. D. (2004) Mol. Cell 16, 269–279
6. Rodal, A. A., Sokolova, O., Robins, D. B., Daugherty, K. M., Hippenmeyer, S., Riezman, H., Grigorieff, N., and Goode, B. L. (2005) Nat. Struct. Mol. Biol. 12, 26–31
7. Rouiller, I., Xu, X. P., Amann, K. J., Egile, C., Nickell, S., Nicastro, D., Li, R., Pollard, T. D., Volkman, N., and Hanein, D. (2008) J. Cell Biol. 180, 887–895
8. Beltzner, C. C., and Pollard, T. D. (2004) J. Mol. Biol. 336, 551–565
9. Mullins, R. D., Stafford, W. F., and Pollard, T. D. (1997) J. Cell Biol. 136, 331–343
10. Winter, D. C., Choe, E. Y., and Li, R. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 7288–7293
11. Gournier, H., Goley, E. D., Niederstrasser, H., Trinh, T., and Welch, M. D. (2001) Mol. Cell 8, 1041–1052
12. Zalevsky, I., Lempert, L., Kranitz, H., and Mullins, R. D. (2001) Curr. Biol. 11, 1903–1913
13. Weaver, A. M., Heuser, J. E., Karginov, A. V., Lee, W. L., Parsons, J. T., and Cooper, J. A. (2002) Curr. Biol. 12, 1270–1278
14. Guthrie, C., and Fink, R. (1991) Methods Enzymol. 194, 1–933
15. Longtine, M. S., McKenzie, A., 3rd, Demarini, D. J., Shah, N. G., Wach, A., Brach, A., Philippen, P., and Pringle, J. R. (1998) Yeast 14, 953–961
16. Goode, B. L. (2002) Methods Enzymol. 351, 433–441
17. Pollard, T. D. (1984) J. Cell Biol. 99, 769–777
18. Higgs, H. N., Blanchon, L., and Pollard, T. D. (1999) Biochemistry 38, 15212–15222
19. Rodal, A. A., Manning, A. L., Goode, B. L., and Drubin, D. G. (2003) Curr. Biol. 13, 1000–1008
20. Moseley, J. B., and Goode, B. L. (2005) J. Biol. Chem. 280, 28023–28033
21. Pollard, T. D. (1986) J. Cell Biol. 103, 2747–2754
22. Dulin, Y., Egerton, M., Elguindi, L., Raths, S., Singer, B., and Riezman, H. (1991) Methods Enzymol. 194, 697–710
23. Baggett, J. J., Shaw, J. D., Sciambi, C. J., Watson, H. A., and Wendland, B. (2003) Current Protocols in Cell Biology, Chapter 4, Unit 4.13, Wiley, Hoboken, NJ
24. Wen, K. K., and Rubenstein, P. A. (2005) J. Biol. Chem. 280, 24168–24174
25. Moseley, J. B., and Goode, B. L. (2006) Microbiol. Mol. Biol. Rev. 70, 605–645
26. Schaerer-Brodbeck, C., and Riezman, H. (2000) Mol. Biol. Cell 11, 1113–1127
27. Schaerer-Brodbeck, C., and Riezman, H. (2000) J. Cell Sci. 113, 521–532
28. Moreau, V., Galan, J. M., Devilliers, G., Haguenauer-Tsapis, R., and Winser, B. (1997) Mol. Biol. Cell 8, 1361–1375
29. Madania, A., Dumoulin, P., Grava, S., Kitamoto, H., Schaerer-Brodbeck, C., Soulard, A., Moreau, V., and Winser, B. (1999) Mol. Biol. Cell 10, 3521–3538
30. Winter, D., Podtelejnikov, A. V., Mann, M., and Li, R. (1997) Curr. Biol. 7, 519–529
31. Munn, A. L., and Riezman, H. (1994) J. Cell Biol. 127, 373–386
32. Li, R. (1997) J. Cell Biol. 136, 649–658
33. Baill, M., Ichetovkin, I., Grant, W., Zebda, N., Machelsky, L. M., Segall, J. E., and Condeelis, J. (2001) Curr. Biol. 11, 620–625
34. Beltzner, C. C., and Pollard, T. D. (2008) J. Biol. Chem. 283, 7135–7144
35. Etzen, G., Wang, L., Thorngren, N., and Wickner, W. (2002) J. Cell Biol. 158, 669–679
36. Chang, F. S., Stefan, C. J., and Blumer, K. J. (2003) Curr. Biol. 13, 455–463
37. Carreno, S., Engqvist-Goldstein, A. E., Zhang, C. X., McDonald, K. L., and Drubin, D. G. (2004) J. Cell Biol. 165, 781–788

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