Mammalian actin-related protein 2/3 complex localizes to regions of lamellipodial protrusion and is composed of evolutionarily conserved proteins

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Human neutrophils contain a complex of proteins similar to the actin-related protein 2/3 (Arp2/3) complex of Acanthamoeba. We have obtained peptide sequence information for each member of the putative seven-protein complex previously described for Acanthamoeba and human platelets. From the peptide sequences we have identified cDNA species encoding three novel proteins in this complex. We find that in addition to Arp2 and Arp3, this complex contains a relative of the human (Suppressor of Profilin) SOP2Hs protein and four previously unknown proteins. These proteins localize in the cytoplasm of fibroblasts that lack lamelipodia, but are enriched in lamellipodia on stimulation with serum or platelet-derived growth factor. We propose a conserved and dynamic role for this complex in the organization of the actin cytoskeleton.

INTRODUCTION

The actin-related proteins (Arps) have recently become a subject of much interest with regard to regulation of both the actin and microtubule cytoskeletons. Arps were first described from yeast but now have been found in amoebas and mammalian cells. Arp1, or dynactin, forms a short filament in association with four novel proteins and the p150/glued microtubule motor protein, which is important for microtubule organization and possibly membrane trafficking [1,2]. Similarly, Arp2 and Arp3 form a complex, with five other proteins, that binds to actin in vitro and is co-localized with the actin cytoskeleton by immunofluorescence in cells [3–5].

The Arp2/3 complex has been best characterized in Acanthamoeba. It is found largely in the supernatant after high-speed centrifugation of whole-cell lysates but binds to profilin and actin filaments in vitro [3,4]. Detailed immunofluorescence studies reveal that subunits in the Arp2/3 complex are concentrated in the leading edge of the amoeba, in microspikes containing bundled actin [4]. Biochemical studies suggest that the purified Acanthamoeba complex contains one each of seven proteins: Arp3, Arp2, p40, p35, p19, p18 and p16 [3,4]. Arp2 and Arp3 do not seem to polymerize like conventional actin or Arp1, but rather they form a heterodimer as a part of this larger complex. The sequences of p40, p35, p18 and p15 are unknown except for some partial peptide sequences [3], and R. D. Mullins, J. Vandekerckhove and T. D. Pollard, personal communication).

Human platelets and Saccharomyces cerevisiae also contain a similar complex, with Arp2, Arp3 and proteins with similar size and stoichiometry to those of proteins in the complex from amoeba [5,6]. However, with the exception of Arp2 and Arp3, no sequence data have yet been reported for any of the mammalian proteins. Interestingly, this complex is sufficient to reconstitute actin polymerization on the surface of the motile intracellular pathogen Listeria monocytogenes [5]. These pathogens recruit the host cell's cytoskeletal proteins and use actin polymerization to drive their own motility through the cytoplasm. Listeria motility currently serves as a model for actin dynamics at the leading edge of motile cells; the proteins of the host cell used in assembly of the Listeria actin tail are thought to be key players in cell motility [7]. In S. cerevisiae the Arp3 in Arp2/3 complex is required for the motility and integrity of the cortical actin patches [6]. It is important to understand the role of the Arp2/3 complex in mammalian cells, as it might regulate lamellipodial extension and actin-based motility.

We have purified the Arp2/3 complex from human neutrophils and obtained a peptide sequence from each of the seven proteins in the complex. We used this information to identify the cDNA species coding for three previously unknown proteins, p34, p21 and p16. We also found that our peptide sequences from a 40 kDa protein matched a predicted protein sequence of a human cDNA (Genbank accession number AA188179), which is related to, but not identical with, the SOP2Hs protein [8]. We generated polyclonal antisera to Arp3 and p34 to study tissue distribution and to follow these proteins through our purification. We also immunostained fibroblasts expressing Myc-tagged cDNA constructs encoding p34 and p21 and found that both proteins are highly enriched in lamellipodia.

MATERIALS AND METHODS

Chemicals

All chemicals and reagents were of reagent grade and were purchased from Sigma unless stated otherwise. Platelet-derived growth factor (PDGF)-ββ was purchased from Calbiochem (Nottingham, Notts., U.K.).

Abbreviations used: Arp, actin-related protein; PDGF, platelet-derived growth factor.

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Protein purification

Human neutrophil lysates were prepared as follows: 10^6 neutrophils were suspended in 10 ml of PBS [0.15 M NaCl/10 mM NaHPO_4/10 mM KCl (pH 7.3)] containing 5 mM glucose for 5 min. Cells were then stimulated with 1 µg/ml PMA for 3 min, aliquoted three times for 5 s at maximum power with a MSE Soniprep 150 sonicator. Break buffer contained 10 mM KCl (pH 7.3) containing 5 mM glucose for 5 min, diluted 10-fold in ice-cold PBS and centrifuged at 200 g for 5 min. The pellets were resuspended in 10 ml of break buffer (see below) and sonicated three times for 5 s at maximum power with a MSE Soniprep 150 sonicator. Break buffer contained 10 mM KCl, 3 mM NaCl, 4 mM MgCl_2, 10 mM Pipes, protease inhibitors (1 µg/ml leupeptin, 1 µg/ml pepstatin, 1 µg/ml 7-aminomethylestramidoheptan-2-one and 0.5 mM PMSF) and phosphatase inhibitors (0.1 µM microcystin, 5 mM sodium pyrophosphate and 25 mM NaF). After centrifugation for 10 min at 100000 g, the supernatant cytosol (approx. 1400 mg of protein) was chromatographed successively as follows. (1) Starting material was loaded on Fast Flow S-Sepharose (3 cm x 20 cm, 2 ml/min, 12 ml fractions collected, eluted with a 500 ml linear gradient of 0–1 M NaCl). (2) Fractions 12–18 from the Fast Flow S-Sepharose were pooled and eluted in heparin–agarose gradient of 0–1 M NaCl. (3) Fractions 18–26 from the Fast Flow S-Sepharose were pooled and loaded on heparin–agarose column were pooled and loaded on Superose 12 (48 cm x 2 cm, 0.5 ml/min, 1 ml fractions collected). (4) Fractions 45–55 from the Superose 12 column were pooled and loaded on a Mono S column (1 ml, 0.5 ml/min, 1 ml fractions collected, eluted with a 20 ml gradient of 0–1 M NaCl). (5) Fractions 10–18 from the Mono S column were pooled and loaded on Superose 12 (26 cm x 1 cm, 0.5 ml/min, 0.25 ml fractions collected). All columns were run with break buffer. Gel-filtration markers for both of the Superose 12 columns were apoferritin (440 kDa), alcohol dehydrogenase (160 kDa) and BSA (67 kDa). The 20 ml sucrose gradient was poured in layers consisting of 5%, 7.5%, 10%, 12.5%, 15%, 17.5% and 20% (w/v) sucrose in break buffer and incubated at 37 °C for 45 min. The peak of the Arp2/3 complex (0.5 ml of fraction 42 of the final Superose 12 column) was loaded on top of the 5% sucrose solution and centrifuged at 50000 g for 10 h; 0.5 ml aliquots were removed and run on SDS/PAGE [12.5% (w/v) gel] for Coomassie Blue staining and Western blotting. For all columns and the sucrose gradient, fractions were run on SDS/polyacrylamide gels, transferred to nitrocellulose and probed with antisera to p34 and Arp3 to determine the position of the Arp2/3 complex. Fractions containing p34 and Arp3 were pooled and loaded on the next column.

Antiserum and Western blotting

Swiss 3T3 fibroblasts were injected with keyhole limpet haemocyanin-coupled peptides from Arp3 (residues 348–361, CKLSEELSGGRLPK; residues 37–50, CIKESAKVGDQ-KQRR) and p34 (residues 57–70, CVSSLKFKYKELQAH; residues 285–298, CEKKEMKTGKTF) [9]. In each peptide the N-terminal cysteine residue was added to facilitate coupling to the keyhole limpet haemocyanin. Arp3 antisera was specifically raised against human sequences corresponding to the predicted loop structures from the Acanthamoeba Arp3 three-dimensional structure model proposed by Kelleher et al. [10]. Antiserum was tested against Western blot strips of whole cell lysates from human neutrophils prepared as described above.

All Western blots were probed with the ECL enhanced chemiluminescence system (Amersham, Little Chalfont, Bucks, U.K.) in accordance with the manufacturer’s instructions. Quantification was done in the program NIH Image with a scanner to digitize dried Coomassie-stained gels or luminescence images on X-ray film (Kodak; Scotlab, Coatbridge, Strathclyde, Scotland, U.K.) from Western blots.

Cell culture and immunofluorescence

Swiss 3T3 fibroblasts were grown in Dulbecco’s modified Eagle’s medium containing 5% (v/v) fetal calf serum as described previously [11]. Tissue culture plastic was purchased from Nunc (Roskilde, Denmark). For serum starvation, cells were placed overnight in Dulbecco’s modified Eagle’s medium without serum. Briefly, 0.1 mg/ml DNA in PBS was injected and cells were incubated for 2 h at 37 °C, fixed in 4% (w/v) paraformaldehyde in PBS and blocked with 50 mM NH_4Cl before immunocytochemistry.

Identification of Arp complex proteins and cDNA species, and preparation of Myc-tagged constructs

Peptide sequencing was performed as previously described [12]. Peptide sequences were screened against the non-redundant and dBEST databases with the program BLAST [13]. The 57 kDa protein was identified as coronin by identity with the published sequence [14] and Genbank accession number X89109. Arp2 and Arp3 were identified by similarity to the chicken and bovine predicted protein sequences respectively [15,16]. Strains of Escherichia coli containing the full-length human cDNA species coding for the other proteins (p34, p21 and p16) were purchased from the American Type Culture Collection (ATCC) for all matches. ATCC catalogue numbers for each clone are as follows:
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Figure 2 Purification of the Arp2/3 complex: columns and sucrose gradient

Quantification of Western blots of Arp3 (●) and p34 (○) in column fractions from each stage in the purification of the Arp2/3 complex. At the left side of the graph, the amount of immunoreactive material as assayed by scanning densitometry of Western blots is given in arbitrary units against the volume of the column gradient. At the right side of (A), (B) and (D) the NaCl concentration (M) for the elution gradient is shown against the volume of the column gradient. The broken line on these graphs represents the salt gradient. The solid line on each graph represents the total protein across the column or in (F) across the sucrose gradient. The shaded bar marks the fractions that we pooled to load on the next column. The peak fraction from the final Superose-12 column was loaded on the sucrose gradient. For the gel-filtration columns, (C) and (E), the chromatographic position of the 443 and 150 kDa molecular mass markers is shown. For the sucrose gradient, (F), the percentage of sucrose (w/w) is shown on the right against the fraction number (1 ml fractions); *, coronin as measured by immunoblotting. (A) Fast flow S-Sepharose; (B) heparin–agarose; (C) first Superose-12 column; (D) Mono-S; (E) second Superose-12 column; (F) sucrose gradient.

RESULTS

Purification of the Arp2/3 complex

Human neutrophils are highly motile cells, rich in actin cytoskeletal components and morphologically similar to Acanthamoeba. Therefore we chose to isolate the mammalian Arp2/3 complex from human neutrophils by conventional column chromatography and a final sucrose gradient. Coomassie-stained gel bands from the peak fraction containing the Arp2/3 complex for each step in the purification are shown in Figure 1. The starting

p34, ATCC 705661; p21, ATCC 535809; p16, ATCC 333225. Plasmid DNA prepared from each clone was sequenced by fluorescent automatic sequencing in accordance with the manufacturer’s instructions (ABI; Perkin-Elmer, Warrington, U.K.). Alignments of the predicted protein sequences were performed with the program MACAW [17–19]. Expression constructs for mammalian cells were prepared with the BamH1 and EcoR1 cloning sites in the vector pRK5-myc [20]. DNA was prepared by CsCl banding and injected into the nucleus of Swiss 3T3 fibroblasts as described previously [11].
material (Figure 1A, lane 1) was a lysate of 10^{8} human neutrophils prepared as described in the Materials and methods section. The Arp2/3 complex (as assayed with antiserum against Arp3 and p34) was fully purified on a Fast Flow S-Sepharose column and eluted between 250 and 350 mM NaCl (Figure 1A, lane 2, and Figure 2A). The peak of the Arp2/3 complex was loaded on heparin–agarose; it was eluted between 450 and 650 mM NaCl (Figure 1A, lane 3, and Figure 2B). The peak fractions were then pooled and loaded on a Superose 12 gel filtration column, where they migrated at approx. 300 kDa (Figure 1A, lane 4, and Figure 2C). The Arp2/3 complex peak was loaded on a Mono S column, from which it eluted at approx. 175–375 mM NaCl (Figure 1A, lane 5, and Figure 2D). Finally, a further gel-filtration step yielded visible Arp2/3 complex on Coomassie gels, running at approx. 300 kDa, with coronin being the most abundant co-purifying protein (Figure 1A, lane 6, and Figure 2E). To purify the Arp2/3 complex further, we sedimented the peak fraction from the gel filtration column on a 5–20% (w/v) sucrose gradient for 10 h and obtained essentially pure Arp2/3 complex with a substoichiometric amount of coronin (Figure 1B). The coronin clearly sedimented at a different region on the sucrose gradient (Figure 2F, □), and the tail of this peak appears in Figure 1 (lane 7). The Arp2/3 complex peaked at approx. 10% (w/v) sucrose, whereas the coronin peak was at approx. 7.5% sucrose (Figure 2F). Quantification of Coomassie Blue-stained gels from the Arp2/3 complex peak of the final gel filtration-column and the sucrose gradient revealed a 1:1 stoichiometry of association of the Arp2/3 complex components, with some variability in the lower subunits. Standardizing the amount of Arp3 as 1, we obtained ratios of the other components as follows: Arp2, 1.0 ± 0.1 (S.D.); p40 (combined upper and lower bands), 1.1 ± 0.09; p34, 1.0 ± 0.1; p21, 0.8 ± 0.3; p20, 0.8 ± 0.3; p16, 0.8 ± 0.3 from an average of two gel filtration columns and two sucrose gradients run in separate preparations. Yields from all steps of an average purification are shown in Table 1.

Identification of the proteins in the Arp2/3 complex

From the previously published mammalian sequence of Arp3 [15] and from the predicted protein sequence from the cDNA encoding p34, specific polyclonal antisera were raised in rabbits (Figure 3). Antiserum against both p34 and Arp3 was used to mark the position of the Arp2/3 complex during our purification. We found by Western blotting that Arp3 and p34 were present in all human tissues tested (muscle, kidney, brain, smooth muscle, skeletal muscle and pancreas; results not shown), as well as in Swiss 3T3 mouse fibroblasts (results not shown). The Arp3 distribution results agree with previous studies on bovine Arp3 [15].

We sequenced peptides from each of the major bands that co-purified on the final gel-filtration column. Peptide sequences and the identities of some of these proteins are shown in Figure 4(A). We used the program BLAST [13] to show that the 57 kDa co-purifying protein was identical with human coronin (Genbank accession number X89109) [14]. The 47 kDa protein matched bovine Arp3 [15] as well as other Arp3 sequences in the database and the 41 kDa protein matched nearly identically with chicken Arp2 [16]. The peptide sequences from p40 nearly matched SOP2Hs (Figure 4B) [8], and identically matched the open reading frame encoded by the human cDNA Genbank accession number AA188179 (results not shown). The peptide sequences that we obtained from p34 were nearly identical with a partly sequenced open reading frame in the region of the BRCA2 gene.

Table 1 Purification of the human neutrophil Arp2/3 complex

<table>
<thead>
<tr>
<th>Sample Description</th>
<th>Total volume (ml)</th>
<th>Total protein (mg)</th>
<th>Specific activity (a.u.)</th>
<th>Yield of Arp2/3 complex (nmol)</th>
<th>Estimated purity (%)</th>
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<td>22</td>
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<tr>
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<tr>
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<td>4.5</td>
<td>58</td>
</tr>
<tr>
<td>Sucrose gradient*</td>
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<td>0.25</td>
<td>2.57</td>
<td>3.9</td>
<td>88</td>
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</table>

* A 0.5 ml sample from the gel-filtration column was loaded on the sucrose gradient, so yields have been adjusted (multiplied by a factor of 4) to reflect this.

Figure 3 Characterization of polyclonal antisera used to assay column fractions for Arp3 and p34

The positions of Molecular mass standards are indicated at the left. Lane 1, Coomassie Blue-stained SDS/PAGE lane of 10 µg of the supernatant from a high-speed spin of human neutrophil lysates prepared as described above; lane 2, Western blot strip from the same gel as in lane 1 probed with antiserum to p34; lane 3, Western blot strip from the same gel as in lane 1 probed with antiserum to Arp3.
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Figure 4 Identification of the proteins of the human Arp2/3 complex

(A) Identity (as determined from the peptide sequence), apparent molecular mass (MW; position on SDS gel), protease used to create peptide fragments, assigned peptide number (Pep. No.) and peptide sequence for each of the major bands found in the final step of the purification. Bold letters are shown where the peptide does not match the predicted protein sequence from one of our cDNA species. (B) Comparison of SOP2Hs and p40 (human neutrophil peptides). (C) Human p34 and Acanthamoeba p35 (amoeba peptides). (D) Human p21 and Acanthamoeba p18 (amoeba peptides). (E) Human p16

Predicted Amino Acid Sequences

B) SOP2Hs and p40 (human neutrophil peptides)

Genbank accession SOP2Hs: Y08999

C) Human p34 and Acanthamoeba p35 (amoeba peptides)

Genbank accession: U05023

D) Human p21 and Acanthamoeba p18 (amoeba peptides)

Genbank accession: N66229: est: yj68c05

E) Human p16

Genbank accession: R98163

Localization of the Arp2/3 complex in cells

Previous data suggested that the amoebic Arp2/3 complex co-localizes with dynamic actin structures in the cortex of cells. We explored this localization further by the expression of epitope-tagged cDNA constructs for p21 and p34 in Swiss 3T3 fibroblasts, which are particularly well characterized with respect to dynamic and stable actin structures and amenable to microinjection and subsequent localization of specific actin cytoskeletal structures [22]. Components of the Arp2/3 complex (p21 and p34) co-localized with polymerizing actin in lamellipodia and membrane ruffles but did not co-localize with actin in stress fibres. Subconfluent cells growing in the presence of serum were dynamic and extended lamellipodia rich in polymerizing actin. Cells microinjected with epitope-tagged p21 cDNA in the expression vector pRK5-myc showed an enrichment of p21 in the lamellipodia in addition to diffuse staining in the cytoplasm (Figure 6A), but no co-localization with stress fibres, as revealed by counterstaining with rhodamine phallolidin (Figure 6B). Serum-starved cells generally lose many of their actin-containing structures and do not extend lamellipodia. Under these conditions, p21 (or p34; results not shown) was diffuse in the cytoplasm (Figure 6C, and rhodamine phallolidin counterstain in Figure 6D). Treatment of serum-deprived cells with PDGF-activated membrane ruffling and lamellipodia extension [23] caused p21 (or p34; results not shown) to be concentrated in peripheral regions (Figure 6E) that co-stained brightly with polymerizing actin (Figure 6F). The expression of epitope-tagged p21 (or p34) did not seem to affect the morphology of cells in any condition that we tested.

DISCUSSION

The Arp2/3 complex has been highly conserved among S. cerevisiae [6], amoebas [3,4] and mammals [5] and is likely to have an important role in actin dynamics during cell motility. To begin to understand the function of this complex in mammalian cells, we have purified it to near homogeneity from human neutrophil extracts and used the purified proteins to obtain full sequences for three novel proteins in the complex. We could not detect any Arp3 or p34 (by Western blotting) that did not co-localize with the predicted open reading frame shown in Figure 4C (3). The Acanthamoeba peptides for p35 in the Arp2/3 complex matched the p34 human sequence as shown in Figure 4C (3), and R. D. Mullins, J. Vandekerckhove and T. D. Pollard, personal communication). The peptide sequences that we obtained for p21 (Genbank accession number N66229), p20 (Genbank accession number R85133) and p16 (Genbank accession number R98163) matched human cDNA species in the dBEST as indicated. The predicted amino acid sequence of the open reading frame in the cDNA encoding p21 also showed similarity to the amoeba p18 peptides (Figure 4D) [3]. The predicted amino acid sequence of the open reading frame in the cDNA encoding p16 is shown in Figure 4E). No amoeba peptide sequences are available for this protein. For the three new components identified, homologues in yeast and C. elegans are aligned diagrammatically, with identities shaded, in Figure 5. p34 also has a homologous sequence in Drosophila melanogaster, which corresponds to Genbank accession number AA246886 (results not shown).
purify with the Arp2/3 complex. This suggests that at least these two proteins are in a stable association in the Arp2/3 complex in neutrophils, and that a pool of unassembled components is not maintained. By Western blotting and inspection of Coomassie-stained gels, coronin partly co-chromatographed with the Arp2/3 complex on all columns but did not migrate as a tightly associated member of the complex. We do not think that coronin is an integral part of the Arp2/3 complex, because in Acanthamoeba [3] and in human platelets [5] coronin was not associated with the Arp2/3 complex.

The Arp2/3 complex, as marked by p21 and p34, is dynamically associated with peripheral regions of the actin cytoskeleton in Swiss 3T3 fibroblasts. The co-localization with dynamic actin structures agrees with previous studies in Acanthamoeba [3,4], S. cerevisiae [6] and motile Listeria monocytogenes [5]. Arp3 has also been previously localized to the lamellae of HeLa cells [5]. Interactions of the Arp2/3 complex with actin must be spatially regulated because the larger actin bundles in the cell do not seem to contain this complex and because the distribution of the complex seems to change when the cells are stimulated to polymerize actin in the peripheral lamella. The mechanism of this regulation is currently unknown.

The sequences of p34, p21 and p16 yielded scant information about possible functions of these proteins but did reveal a high degree of conservation among various phyla. cDNA species encoding all of the novel Arp2/3 complex proteins have been identified in S. cerevisiae (Figure 5), and Arp2 and Arp3 have been previously cloned and sequenced from S. cerevisiae [24,25]. The complex has been biochemically purified from both S. cerevisiae and Acanthamoeba, where it seems to be composed of the same subunits, with the exception of the S. cerevisiae 40 kDa subunit [3,6]. There are a few discrepancies between our peptide sequences and the predicted amino acid sequences from our cDNA species (shown in bold in Figure 3A). At present we do not know whether this is due to variability between isoforms or errors in the sequencing.

SOP2Hs and SOP2 are WD-repeat-containing proteins [8], which are thought to be regulatory proteins for protein–protein interactions [26]. Thus the 40 kDa subunit might have a role in regulating the interactions of this complex with other proteins.
The absence of the 40 kDa subunit from the *S. cerevisiae* Arp2/3 complex suggests that it is not required for the structural integrity of the complex [6]. It is interesting that SOP2 was found as a suppressor of a profilin mutation in *Saccharomyces pombe* that interacts with Arp3p [8]. It is possible that SOP2Hs and p40 have similar roles in the Arp2/3 complex. They could compete for interactions or they might have cell-type or developmental specificity. Another interesting feature of the SOP2Hs-like protein is that it often appears as a doublet on SDS/polyacrylamide gels (e.g. Figure 1B) [5]. Although we have not specifically sequenced each of these bands on the gel, M. D. Welch and T. J. Mitchison (personal communication) have done this for the human platelet p40 protein and found them to be identical with each other and with our p40.

There are currently two models of how the Arp2/3 complex might regulate actin dynamics. The *Acanthamoeba* Arp2/3 complex cross-links actin filaments *in vitro* and localizes to microspikes in amoebas. Thus it is possible that the complex serves to regulate actin filament cross-linking, which could selectively stabilize bundles of actin filaments to assemble into microspikes in lamellipodia. This could serve to make actin bundles that are precursors of larger actin cables in the cell or to hold the lamella out as the cytoplasm streams forward into it. Our results suggest that the Arp2/3 complex is not associated with large actin filament bundles, but is recruited to dynamic polymerizing actin in lamellipodia. The Arp2/3 complex might nucleate the formation of new actin filaments in the cell [5]. This could serve to determine the position and direction of new lamellipodial extension. Distinguishing between these models will require functional data on the contribution of this complex to actin dynamics in live cells.

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