The Human Arp2/3 Complex Is Composed of Evolutionarily Conserved Subunits and Is Localized to Cellular Regions of Dynamic Actin Filament Assembly

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Abstract. The Arp2/3 protein complex has been implicated in the control of actin polymerization in cells. The human complex consists of seven subunits which include the actin related proteins Arp2 and Arp3, and five others referred to as p41-Arc, p34-Arc, p21-Arc, p20-Arc, and p16-Arc (Arp complex). We have determined the predicted amino acid sequence of all seven subunits. Each has homologues in diverse eukaryotes, implying that the structure and function of the complex has been conserved through evolution. Human Arp2 and Arp3 are very similar to family members from other species. p41-Arc is a new member of the Sop2 family of WD (tryptophan and aspartate) repeat–containing proteins and may be posttranslationally modified, suggesting that it may be involved in regulating the activity and/or localization of the complex. p34-Arc, p21-Arc, p20-Arc, and p16-Arc define novel protein families. We sought to evaluate the function of the Arp2/3 complex in cells by determining its intracellular distribution. Arp3, p34-Arc, and p21-Arc were localized to the lamellipodia of stationary and locomoting fibroblasts, as well to *Listeria monocytogenes* assembled actin tails. They were not detected in cellular bundles of actin filaments. Taken together with the ability of the Arp2/3 complex to induce actin polymerization, these observations suggest that the complex promotes actin assembly in lamellipodia and may participate in lamellipodial protrusion.

THE protrusion of the cell membrane is fundamental to cell shape change and locomotion. Actin polymerization plays a critical role in this process. The leading edge of motile cells is dominated by thin actin-rich structures called lamellipodia, which exhibit highly dynamic behavior characterized by rapid extension and retraction. In lamellipodia, actin filaments are oriented almost exclusively with their fast growing barbed ends facing the membrane (for review see Small, 1988). Actin polymerization adjacent to the membrane is coupled to protrusion, and may provide the driving force for this process (Hill and Kirschner, 1982; Mogilner and Oster, 1996).

Many aspects of the mechanism of lamellipodial protrusion are echoed in the intracellular motility of certain bacterial and viral pathogens (for review see Cossart, 1995; Theriot, 1995; Higley and Way, 1997). Of these, the most extensively studied is the bacterium *Listeria monocytogenes. L. monocytogenes* move in arcing trajectories within cell cytoplasm, forming cometlike tails composed of actin filaments oriented with their barbed ends towards the bacterium (Tilney et al., 1992). Actin polymerization at the bacterial surface is tightly coupled to propulsion (Sanger et al., 1992; Theriot et al., 1992), suggesting that it provides the motile force. Although a role for myosins in bacterial propulsion and lamellipodial protrusion cannot be definitively ruled out, the myosin inhibitor butanedione monoxime (BDM) which inhibits classic myosin-driven processes such as muscle contraction, does not affect either process (Cramer and Mitchison, 1995).

The central role of actin polymerization in both lamellipodial protrusion and *L. monocytogenes* propulsion suggests that the underlying mechanisms for promoting polymerization and generating force may be similar in both processes. Since *L. monocytogenes* motility can be reconstituted in cell extracts (Theriot et al., 1994; Laurent and Carlier, 1997; Welch et al., 1997) understanding the biochemical mechanisms that underlie this process has proven more tractable.

We have recently purified a multiprotein complex from human platelets that induces actin polymerization at the *L. monocytogenes* cell surface and mediates bacterial motility (Welch et al., 1997). This complex contains actin-related proteins in the Arp2 and Arp3 families and therefore was named the Arp2/3 complex. In addition to Arp2

and Arp3, the human complex consists of 41/40-, 34-, 21-, 20-, and 16-kD subunits, all present in approximately equal stoichiometry. These subunits are referred to as p41-Arc, p34-Arc, p21-Arc, p20-Arc, and p16-Arc (Arp complex). A similar Arp2/3 complex was first discovered by profilin affinity chromatography of cell extracts from *Acanthamoeba castellanii* (Machesky et al., 1994). In addition, Arp2 and Arp3 proteins appear to be found in all eukaryotes (for reviews see Frankel and Mooseeker, 1996; Mullins et al., 1996), suggesting that the Arp2/3 complex itself has been conserved through evolution. However, the molecular identities of all but one of the non-Arp subunits have remained undetermined. The exception is p41-Arc, which contains WD (tryptophan and aspartate) repeats and is similar to proteins in the Sop2 family (suppressor of profilin; Machesky et al., 1994; Balasubramanian et al., 1996; McCollum et al., 1996).

The mechanism by which the Arp2/3 complex promotes actin polymerization remains enigmatic. Structural modeling of Arp2 and Arp3 proteins has led to the prediction that the Arp2/3 complex serves as a seed for the nucleation of actin assembly (Kelleher et al., 1995). Thus far, only filament binding and bundling activity have been observed in vitro (Mullins et al., 1997). Despite the lack of detailed information regarding mechanism, several lines of evidence indicate that the Arp2/3 complex is important for actin function in cells. Subunits of the *A. castellanii* Arp2/3 complex colocalize with actin in ameba, particularly at sites of dynamic actin assembly at the cell cortex (Machesky et al., 1994; Kelleher et al., 1995; Mullins et al., 1997). The *Saccharomyces cerevisiae* Arp2 and *Schizosaccharomyces pombe* Arp3 proteins are also localized to cortical actin structures. Deletions of their corresponding genes are lethal, and temperature-sensitive mutations cause defects in actin function (Lees-Miller et al., 1992; Schwob and Martin, 1992; McCollum et al., 1996; Moreau et al., 1996).

In this study we have begun to explore the function of the Arp2/3 complex in animal cells. We have determined the complete amino acid sequence of all seven subunits of the human complex. Each protein has homologues in diverse eukaryotic organisms, indicating that the subunit composition, and most likely the function of the Arp2/3 complex, have been conserved through evolution. The ability of the complex to promote actin assembly and mediate *L. monocytogenes* motility suggests that it may play a corresponding role in cellular lamellipodia. In fact, multiple subunits of the complex are localized to *L. monocytogenes* assembled actin clouds and tails as well as to lamellipodia in stationary and motile fibroblast cells. These subunits are absent from less dynamic actin filament bundles in fibroblasts. Our observations further strengthen the analogy between *L. monocytogenes* motility and lamellipodial protrusion and suggest that the Arp2/3 complex plays a central role in both processes.

Materials and Methods

Protein and DNA Sequence Determination

To determine the amino acid sequence of the non-Arp polypeptide subunits of the Arp2/3 complex, the proteins were first subjected to SDS-PAGE, and then blotted to polyvinylidinedifluoride (PVDF)[1] membrane (Applied Biosystems, Inc., Foster City, CA). Proteins were identified by staining with Ponceau S (Hirano and Watanabe, 1990). The PVDF-immobilized proteins were reduced, S-carboxymethylated, and digested in situ with Achromobacter protease I and endoproteinase Asp-N (Iwamatsu, 1992). Digested peptides were chromatographed by reverse-phase HPLC using a Wakosil-II AR C18 300 Å column (2.0 × 150 mm; Wako Pure Chemical Industries, Ltd., Osaka, Japan). Amino acid sequencing was performed with a gas-phase sequencer (model PPSQ-10; Shimadzu Corp., Tokyo, Japan).

To identify cDNA clones encoding the individual subunits of the human Arp2/3 complex, the peptide sequences were used to search the EMBL/GenBank/DDBJ database of expressed sequence tags (dbEST). The following cDNA clones were identified in the database and were obtained from Genome Systems, Inc. (St. Louis, MO) (clone Id numbers are indicated): Arp3, 328293; Arp2, 587272; p41-Arc, 527050; p34-Arc, 547253; p21-Arc, 326562; p20-Arc, 187446; and p16-Arc, 300395. In addition, a cDNA-encoding p34-Arc was isolated from a λ phage Uni ZAP HeLa cell cDNA expression library (Stratagene, La Jolla, CA) by screening with anti-p34 antibody using standard procedures. The DNA sequence of each clone was determined by sequencing both strands (performed by the Biomolecular Resource Center Sequencing Facility at the University of California, San Francisco, CA).

Protein Expression, Antibody Production, and Immunoblotting

Polyclonal anti-peptide antibodies that recognize p34-Arc were generated by immunizing rabbits (Berkeley Antibody Co., Richmond, CA) with the synthetic peptide (C)KVFMQEFKEGRRASHTAPQVLFSHRE, corresponding to amino acids 179–204 of the protein. An amino-terminal cysteine was added for sulfhydryl coupling. Peptide conjugation and antibody affinity purification were carried out as previously described (Sawin et al., 1992). Antibodies were concentrated by dialysis into 10 mM MOPS, pH 6.8, 150 mM NaCl, 35% glycerol.

To generate polyclonal antibodies that recognize p21-Arc (formerly referred to as the 23-kD subunit; Welch et al., 1997), the corresponding gene was amplified by PCR and cloned into pGEX-3X (Smith and Johnson, 1988) to create a glutathione S-transferase (GST) fusion protein. Purified GST fusion protein was used to immunize rabbits (Berkeley Antibody Co.). An affinity matrix for purification of anti-p21 antibodies was created by cleaving p21-Arc from the GST-p21 fusion protein using Factor Xa (New England Biolabs Inc., Beverly, MA) and covalently coupling it to affigel-10 (Bio Rad Laboratories, Hercules, CA). Antibody affinity purification was performed using standard procedures (Harlow and Lane, 1988). Antibodies were concentrated by dialysis into 10 mM MOPS, pH 6.8, 150 mM NaCl, 35% glycerol. Immunoblotting was carried out using affinity-purified anti-Arp3 (0.4 μg/ml), anti-p34 (0.04 μg/ml), and anti-p21 (0.6 μg/ml) antibodies using the ECL system (Amersham Corp., Arlington Heights, IL).

Cell Culture and Immunofluorescence

Swiss 3T3 fibroblasts were grown in DME-H16 medium with 10% FBS, penicillin, and streptomycin. To create a confluent layer of cells for the wounding experiment, cells were plated at a density of 3×10^4 cells per ml onto 22-mm diam coverslips and allowed to grow to confluence. A wound was created using a plastic pipette tip. HeLa cells were grown in DME-H16 medium with 10% FBS, penicillin, and streptomycin. Cells were plated onto glass coverslips 1–2 d before immunofluorescence experiments. Infection of HeLa cells with *L. monocytogenes* strain 10403S was carried out as previously described (Theriot et al., 1994). Locomoting fibroblasts were prepared from heart explants from 7-d-old chick embryos as described in Cramer et al., 1997. Cells were plated onto glass coverslips treated with 1 mg/ml poly-D-lysine (Sigma Chemical Co., St. Louis, MO), and 125 μg/ml matrigel (Collaborative Research, Inc., Waltham, MA) and were grown in DME-H16 medium with 10% FBS and 10% heat-inactivated chicken serum, penicillin, and streptomycin. Immunofluorescence experiments were carried out within 20 h of plating, which was shown by Cramer et al. (1997) to be optimal for observing rapidly locomoting cells.

For immunofluorescence experiments, cells were extracted for 30 s in

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1. *Abbreviations used in this paper*: CBS, cytoskeleton buffer with sucrose; dbEST, database of expressed sequence tags; EGFP and GFP, green fluorescent protein; GST, glutathione S-transferase; PH, pleckstrin homology.

cytoskeleton buffer with 0.32 M sucrose (CBS) (Small, 1981; Symons and Mitchison, 1991) also containing 0.1% Triton X-100 and 1 μg/ml phalloidin. Cells were fixed in CBS plus 4% formaldehyde for 20 min followed by −20°C methanol for 3 min (methanol fixation was necessary to observe staining with the anti-p34 and anti-p21 antibodies, indicating that they recognize only denatured protein). Indirect immunofluorescence was performed using affinity-purified rabbit anti-Arp3 (4 μg/ml), anti-p34 (0.4 μg/ml), anti-p21 (6 μg/ml) and mouse anti-actin (5 μg/ml; Boehringer Mannheim Biochemicals, Indianapolis, IN) primary antibodies, and Texas red–conjugated goat anti–rabbit and FITC-conjugated goat anti–mouse secondary antibodies (Jackson Immunoresearch Laboratories, Inc., West Grove, PA). Fluorescent phalloidin staining of filamentous actin was carried out with 1 μg/ml FITC-conjugated phalloidin (Sigma Chemical Co.). Photomicrographs were taken using a cooled CCD camera (Princeton Scientific Instruments, Inc., Princeton, NJ) on a Nikon Optiphot-2 microscope. Images were handled digitally using Adobe Photoshop (Adobe Systems Inc., Mountain View, CA).

Construction of Arp3-Green Fluorescent Protein Fusion and Cell Transfection

The gene encoding Arp3 was isolated by PCR and was cloned into pEGFP-N1 (Clontech, Palo Alto, CA) to create a fusion of Arp3 with green fluorescent protein (EGFP) attached to its carboxy terminus (pArp3-EGFP). (The DNA sequence of the Arp3 gene isolated by PCR was identical to the sequence of the Arp3 cDNA clone.) pArp3-EGFP was introduced into HeLa cells using calcium phosphate–mediated transfection as described previously (Heald et al., 1993). To visualize Arp3-EGPF in *L. monocytogenes*–infected cells, HeLa cells were infected 1 d after transfection. For fluorescence experiments, cells were extracted in CBS with 0.1% Triton X-100 and 1 μg/ml phalloidin for 30 s, and then fixed in CBS with 3.2% formaldehyde for 10 min. To visualize polymerized actin, cells were stained with 1 μg/ml 5-carboxytetramethyl rhodamine phalloidin (Sigma Chemical Co.) for 2 min. Cells were photographed as described above except that a blocking filter was used to eliminate bleed through from the rhodamine to the fluorescein channel.

Results

The Sequence of Human Arp2 and Arp3

To further understand the role of the Arp2/3 complex in the control of actin assembly in cells, we determined the predicted amino acid sequences of all of the subunits of the human protein complex. Using peptide sequence information (Welch et al., 1997) to search the EMBL/GenBank/DDBJ dbEST we obtained and sequenced full-length human cDNA clones encoding Arp2 and Arp3 (these sequence data are available from EMBL/GenBank/DDBJ under accession numbers AF006082 for Arp2 and AF-006083 for Arp3). The Arp2 cDNA encodes a predicted protein of 394 amino acids with a molecular mass of 45 kD (43 kD as determined by SDS-PAGE; Welch et al., 1997). Human Arp2 is 99% identical to chicken Arp2 (these data are available from EMBL/GenBank/DDBJ under accession number X73971), and is also highly similar to Arp2 proteins from *Drosophila melanogaster* (81% identical; accession number X78486), *Caenorhabditis elegans* (76% identical; accession number Z71181), *A. castellanii* (72% identical; accession number U29609), and *S. cerevisiae* (67% identical; accession number X61502). Human Arp3 is 418 amino acids in length and has a predicted molecular mass of 47 kD (50 kD as determined by SDS-PAGE; Welch et al., 1997). It is identical in sequence to bovine Arp3 (accession number D12816), and shows extensive similarity to Arp3 proteins from *D. melanogaster* (80% identical; accession number X71789), *A. castellanii*

(70% identical; accession number U29610), *Dictyostelium discoideum* (69% identical; accession number Z46418), *Neurospora crassa* (59% identical; accession number U79737), *S. pombe* (59% identical; accession number M81068), and *S. cerevisiae* (58% identical; accession number Z49565).

p41-Arc Is a Member of the Sop2 Family of WD Repeat Containing Proteins

We also searched the dbEST database using peptide sequences derived from the five non-Arp subunits of the Arp2/3 complex. Full-length cDNA clones encoding each subunit were identified, and their DNA sequences were determined. The predicted polypeptides encoded by these clones are similar in sequence to proteins from a wide variety of eukaryotes.

Based on its high degree of sequence similarity, the p41-Arc subunit of the platelet Arp2/3 complex is a new member of the Sop2 family of proteins. As is shown in Fig. 1 *A*, p41-Arc is 67% identical to the previously identified human Sop2 protein Sop2Hs (Balasubramanian et al., 1996), making them similar but distinct members of the same family. p41-Arc is also similar to Sop2 proteins from *D. melanogaster* (49% identical; accession number L39625), *S. pombe* (37% identical; accession number Y08998), and *S. cerevisiae* (34% identical; accession number Z36103). Previous evidence has suggested that Sop2 family proteins encode the 41-kD subunit of the Arp2/3 complex. The canonical member of this family, *S. pombe* Sop2p, associates with Arp3 in a protein complex, and mutations in the *sop2* and *arp3* genes interact genetically. Moreover, Sop2 proteins contain sequences that are similar to peptide sequences from the 40-kD subunit of the *A. castellanii* Arp2/3 complex (Balasubramanian et al., 1996). Like previously identified Sop2 family proteins, human p41-Arc contains WD repeat sequences (also known as β-transducin repeats). We identified seven potential WD repeats in p41-Arc (Fig. 1 *A*) that correspond to the consensus sequence described by Neer et al. (1994) with one to five mismatches. These repeats are highly conserved in both sequence and position within p41-Arc and Sop2Hs.

The 41- and 40-kD subunits of the platelet Arp2/3 complex (Fig. 2, *far left lane*), which differ slightly in their mobility by SDS-PAGE, are different versions of the p41-Arc polypeptide. Digestion of p41 and p40 with Achromobacter protease I and endoproteinase Asp-N produced identical peptide maps by reverse phase HPLC (not shown). This suggests that p41 and p40 may differ in their state of post-translational modification. Furthermore, this indicates that the platelet Arp2/3 complex contains seven polypeptides, identical to the number found in the *A. castellanii* complex. Both p41 and p40 are the only subunits of the platelet Arp2/3 complex that are present in substoichiometric quantities, while the other six subunits are represented in slightly larger and roughly equal amounts (Welch et al., 1997). However, when summed together, the quantity of the p41 and p40 is stoichiometric with that of the other subunits. This indicates that the seven polypeptide subunits of the complex are present in a 1:1:1:1:1:1:1 stoichiometry, similar to that found in the *A. castellanii* Arp2/3 complex (Machesky et al., 1994; Mullins et al., 1997).
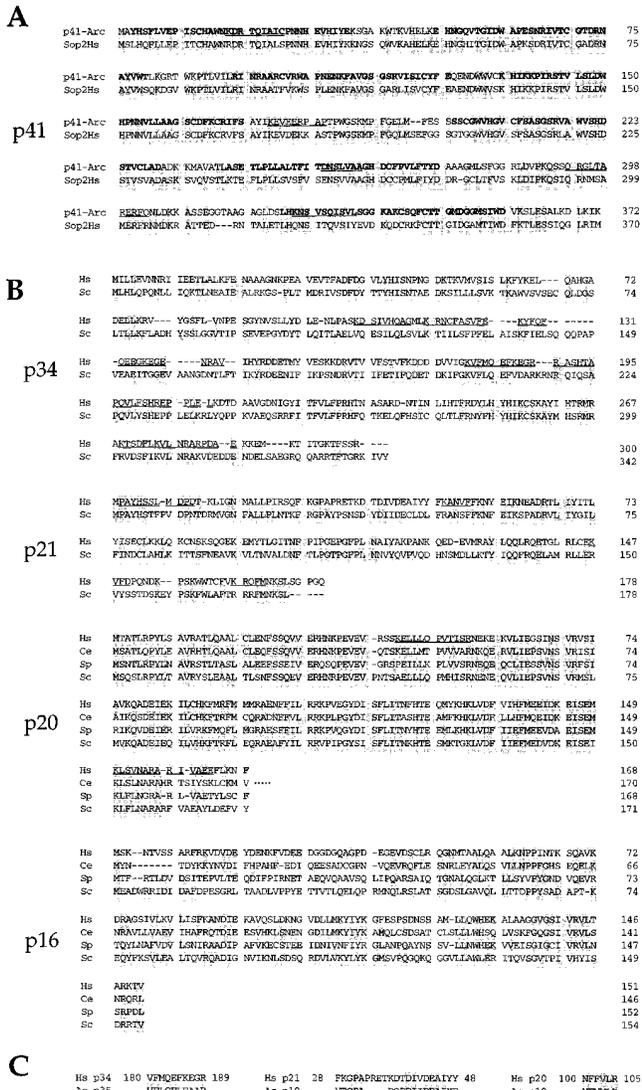
A

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         p41-Arc  MAYHSFLVEP ISCHAWNEDR TQIAICPNNN EVHIYEKSGA KWTKVHELKE ENKQVTGIDW APESNRIVTC GTDRN  75
         Sop2Hs   MSLHQFLLEP ITCHAWNRDR TQIALSPNNH EVHIYKENGS QWVKAHELKE HNGHITGIDW APKSDRIVTC GADEN  75

         p41-Arc  AYVWTLKGRT WKPTLVILKI HRAARCVRWA PNENKFAVGS GSHRVISICYF EQENDAWVCK HIKKPIRSTV LSLLDW  150
         Sop2Hs   AYVWSQKDGV WKPTLVILKI NRAAITVKWS PLENKFAVGS GARLISVCYF EAENDAWVGK HIKKPIRSTV LSLLDW  150

p41      p41-Arc  HPNNVLLAAG SCDFKCRIFS AYIKEVEERP APTPWGSKMP FGELM---FS SSSCGWVHGV CPSASGSRVA WVSHD  223
         Sop2Hs   HPNNVLLAAG SCDFKCRVFS AYIKEVDEKK ASTPWGSKMP FGQLMSEFGG SGTGGWVHGV SPSASGSRLA WVSHD  225

         p41-Arc  STVCLADADK KMAVATLASE FLFLLALTFI TENSLVAAGH DCFFVLFTID AAAGMLSFGG RLDVFKQSSQ RGLTA  298
         Sop2Hs   SIVSVADASK SVQVSTLKTE FLFLLSVSFV SENSVVAAGH DCCHMLFIYD DR-GCLTFVS KLDDIFKQSIQ RNMSA  299

         p41-Arc  RERFPQNLDKK ASSEGGTAAG AGLDSLHKNS VSQLEVLSGG KAKCSQFCTT GMDQGMSTWD VKSLESALKD LKIK  372
         Sop2Hs   MERFPNNMDKR ATPED---RN TALETLHQNS ITQVSIYEVD KQDCRKFCTT GIDGAMTIWD FKTLESSIQG LRIM  370
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B

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         Hs  MILLEVMNRI IEETLALKFE NAAAGNKPEA VEVTFADFDG VLYHISNPNG DKTKVMVSIS LKFYKEL--- QAHGA  72
         Sc  MLHLQFQNLL IQKTLNEAIE ALRKGS-PLT MDRIVSDFDY TTYHISNTAE DKSILLLSVK TKAWVSVSEC QLDGS  74

         Hs  DEELKRV--- YGSFL-VNPE SGYNVSLLYD LE-NLPASKD SIVHQAGMLK RNCFASVES- ---KYFQE--  131
         Sc  LTLLKFLADH YSSLGGVTIP SEVEPGVDYT LQITLAELVQ ESILQLSVLK TIILSFPFEL AISKFIELSQ QQPAP  149

p34      Hs  -CEEGKEGE- ----NRAV-- IHYRDDETMY VESKKDRVTV VFSTVFKDDG DVVIGKVFMQ EFKEGR---E AGHTA  195
         Sc  VEAEITGGEV AANGDMTLFY IKYRDEENIF IKFPSNDRVTI IFETIPQDET DKIFGKVFLQ EFVDARKRNR QIQSA  224

         Hs  PQVLFSHREP -PLE-LXDTD AAVGDNIGYI TFVLFPRHTN ASARD-NTIN LIHTERDVLH YHIECSKAYI HTRMR  267
         Sc  PQVLYSHEPP LECKRLYQPP KVAEQSRRFI TFVLFPRHFQ TKELQFHSIC QLTLFRNYPH YHIECSKAYM HSRMR  299

         Hs  AKTSDFLKVL NRARPDA--E KKEM----KT ITGKTFSSR- ---  300
         Sc  PRVDSFIKVL NRAKVDEDDE NDELSAEGRQ QARRTFTGRK IVY  342
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         Hs  MPAYHSSL-M DKDT-KLIGN MALLPIRSQF KGPAPRETKD TDIVDEAIYY FKANVFKNY EIKNEADRTL LYITL  73
         Sc  MPAVHSTFPV DPMTDRMVGN FALLPLNTKF RGPAYPSNSD YDIIDECLDL FRANSFPKNF EIKSPADRVL TYGIL  75

p21      Hs  YISECLKKLQ RCNSKSQGEK EMYTLGITNP PIPCELPGPL NAIYAKPANK QED-EVMRAY LQQLRQETGL RLCEE  147
         Sc  FINDXLAHLK ITTSPRNEAVK VLTNVALDNF MLPGTPGFPL NNVYQVPVQD HNSMDLLKTY IQQFRQELAM RLLER  150

         Hs  VEDPQNDK-- PSKWWTCFVK RQFMNKSLSG PGQ  178
         Sc  VVSSTDSKEY PSKFWLAFTR RRFMNKSL-- ---  178
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         Hs  MTATVLRPYLS AVRATLQAAL CLENFSSQVV ERHNKPEVEV- -RSSRELLLQ PVTISRENEKE KVLIEGSINS VRVSI  74
         Ce  MSATLQPYLE AVRHTLQAAL CLEQFSSQVV ERHNKPEVEV -QTSKELLMT PVVVARNKQE RVLIEFSVNS VRISI  74
         Sp  MSNTLRFYIN AVRSTLTASL ALEEFSSEIV ERQSQPEVEV -GRSPEILLK PLVVSRNEQE QCLIESSVNS VRFSI  74
         Sc  MSQSLRFYLT AVRYSLEAAL TLSNFSSQEV ERHNRPEVEV PNTSAELLLQ FMHISRNENE QVLIEPSVNS VRMSL  75

p20      Hs  AVKQADEIEK ILCHKFMRFM MKRAENFPIL RRKPVEGYDI SFLITWFHTE QMYKHKLVDF VIHFMEEIDK EISEM  149
         Ce  AIKQSDEIEK ILCHKFTRFM CQRADNEFVL RRKPLPGYDI SFLKTASHTE AMFKHKLVDF LLHFMQEIDK EISEM  149
         Sp  RIKQVDEIER ILVRKFMQFL MQRAESFEIL RRKPVQGYDI SFLITWFHTE EMLKHKLVDF IIEFMEEVDA EISEM  149
         Sc  MVKQADEIEQ ILVHKFTRFL EQRAEAFYIL RRVPIPGYSI SFLITNKHTE SMKTGKLVDF IIEFMEDVDK RISEI  150

         Hs  KLSVNARA-R I-VAEEFLKM F  168
         Ce  KLSLNARAHR TSIYSKLCMM V  170
         Sp  KLFLNSRA-R L-VAETYLSC F  168
         Sc  KLFLNARARF VAEAYLDEFV Y  171
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         Hs  MSK--NTVSS ARFRKVDVDE YDENKFVDEB DGGDGQAGPD -EGEVDSCLR QGNMTAALQA ALKNPPIVTK SQAVK  72
         Ce  MYN------- TDYKKYNVDI FHPANF-EDI QEESAIXGRN -VQEVRQFLE SNRLEYAIQS VILNPFFQHS EQRLK  66
         Sp  MTF--RTLDV DSITEPVLTE QDIFPIRNET AEQVQAAVSG LIPQARSAIQ TGNALQGLKT LLSYVFYGND VQEVR  73
         Sc  MEADWRRIDI DAFDPESGRL TAADLVPPYE TTVTLQELQP RMNQLRSLAT SGDSLGAVQL LTTDPFYSAD APT-K  74

p16      Hs  DRAGGSIVLKV LISFKANDTE KAVQSLDKNG VDLLMKYIYK GFESPSDNSS AM-LLQWHEK ALAAGGVGSI VRVLT  146
         Ce  NRAVLLVAEV IHAFRQTDIE ESVHKLSNEN GDILMKYIYK AMQLCSDSAT CLSLLLWHSQ LVKHFGQGSI VRVLS  141
         Sp  TQYLNAFVDV LSNIRAADIP AFVKECSTEB IDNIVNFIYR GLANPQAYNS SV-LLMWHEK VVEISGIQCI VRVLN  147
         Sc  EQYFKSVLEA LTQVRQADIG NVIKNLSDSQ RDVLVKYLYK GMSVPQGQKQ GGVLLANLKR ITQVSGVTPI VHYIS  149

         Hs  ARKTV  151
         Ce  NRQRL  146
         Sp  SRPDL  152
         Sc  DRRTV  154
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C

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Hs p34  180 VFMQEFKEGR 189     Hs p21  28 FKGPAPRETKDTDIVDEAIYY 48      Hs p20  100 NFFVLR 105
Ac p35      VFLQELSAAR             Ac p18     YTGFA----DQPDIIDEAIXF            Ac p19      NFVVLR
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Figure 1. Predicted amino acid sequences of p41-Arc, p34-Arc, p21-Arc, p20-Arc, and p16-Arc aligned to their homologues from diverse organisms. (*A*) The sequence of p41-Arc is similar to Sop2Hs (these data are available from EMBL/GenBank/DDBJ under accession number Y08999). Bold type indicates the location of the WD repeat sequences and underlined type indicates the location of the peptide sequences used to determine the identity of p41. (*B*) The sequence of p34-Arc is similar to EMBL/GenBank/DDBJ accession number Z71650 from *Sc*. The sequence of p21-Arc is similar to accession number U19103 from *Sc*. The sequence of p20-Arc is similar to accession numbers U21324 from *Ce*, Z81317 from *Sp*, and Z28013 from *Sc*. The sequence of p16-Arc is similar to accession numbers U88314 from *Ce*, Z69795 from *Sp*, and Z38060 from *Sc*. Underlined type indicates the location of the peptide sequences used to determine the identity of the proteins. (*C*) Sequences from within p34, p21, and p20 are similar to peptide sequences from the 35-, 18-, and 19-kD subunits of the *Ac* Arp2/3 complex (Machesky et al., 1994), respectively. *Hs*, *Homo sapiens*, *Ce*, *C. elegans*, *Ac*, *A. castellanii*, *Sc*, *S. cerevisiae*, *Sp*, *S. pombe.* The sequence data for the subunits of the human Arp2/3 complex are available from EMBL/GenBank/DDBJ under accession numbers AF006084 for p41-Arc, AF006085 for p34-Arc, AF006086 for p21-Arc, AF006087 for p20-Arc, and AF006088 for p16-Arc.

p34-Arc, p21-Arc, p20-Arc, and p16-Arc Define Novel Protein Families

The four other subunits of the human Arp2/3 complex—p34-Arc, p21-Arc, p20-Arc, and p16-Arc (formerly the 34-, 23-, 22-, and 19-kD subunits as determined by SDS-PAGE; Welch et al., 1997)—are similar to proteins from diverse eukaryotic organisms. However, they do not share similarity with any proteins of known function. These therefore define four novel protein families, which are shown in Fig. 1 *B*. The p34-Arc family is comprised of the human and *S. cerevisiae* proteins (34% identical). Furthermore, a region in p34-Arc is similar in sequence to a peptide from 35-kD subunit of the *A. castellanii* complex (Fig. 1 *C*). A partial sequence of human p34-Arc had previously been determined and its gene maps to chromosome 13 in the BRCA2 region (Couch et al., 1996). Like p34-Arc, p21-Arc has a single homologue in *S. cerevisiae* (45% identical; Fig. 1 *B*) and contains a region similar to a peptide in the 18-kD subunit of the *A. castellanii* complex (Fig. 1 *C*). The p20-Arc family has members in multiple organisms (Fig. 1 *B*) including *C. elegans* (73% identical in its amino-terminal 168 amino acids), *S. pombe* (68% identical), and *S. cerevisiae* (67% identical). In addition, it contains a sequence that is similar to the 19-kD subunit of the *A. castellanii* complex (Fig. 1 *C*). Finally, p16-Arc resembles proteins in *C. elegans* (34% identical), *S. pombe* (26% identical), and *S. cerevisiae* (21% identical). The *S. cerevisiae* proteins that share sequence similarity with human p34-Arc, p21-Arc, p20-Arc, and p16-Arc have recently been shown to be components of the yeast Arp2/3 complex (Winter et al., 1997).

Although the sequences of these subunits have not provided clues to their potential function in the Arp2/3 complex, we have used the sequence information as well as the cloned genes to generate reagents to probe the function of the complex in cells. We have raised polyclonal antibodies to a GST-p21 fusion protein and to a peptide from p34-Arc. Affinity-purified antibodies recognize p21-Arc and p34-Arc in the pure Arp2/3 complex (not shown), and specifically recognize 21- and 34-kD proteins in whole cell extracts made from a variety of mammalian and avian cell types (Fig. 2). In addition, we have taken advantage of the previously described anti-Arp3 antibody (Welch et al., 1997), which specifically recognizes Arp3 in the same cell
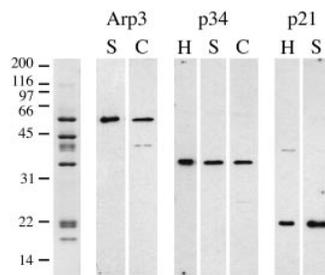
Figure 2. Immunoblots of whole cell extracts probed with anti-Arp3, anti-p34, and anti-p21 antibodies. The *far left* lane, which shows pure Arp2/3 complex run on a 12% SDS-PAGE gel and stained with Coomassie blue, illustrates the protein composition of the complex. The other lanes are immunoblots of: Swiss 3T3 (*S*) and chick heart fibroblast (*C*) extracts probed with anti-Arp3 (*Arp3*); HeLa (*H*), Swiss 3T3 (*S*), and chick heart fibroblast (*C*) extracts probed with anti-p34 (*p34*); HeLa (*H*) and Swiss 3T3 (*S*) extracts probed with anti-p21 (*p21*).

extracts (Fig. 2). Together, these reagents provide a means of examining the function of the Arp2/3 complex by determining its intracellular distribution.

Arp3, p34-Arc, and p21-Arc Are Localized to L. monocytogenes Assembled Actin Tails

The Arp2/3 complex promotes the assembly of actin filaments at the surface of *L. monocytogenes.* Moreover, the Arp3 subunit of the complex is localized to the actin tails assembled by moving intracellular bacteria and to actin clouds surrounding stationary bacteria (Welch et al., 1997). To confirm that other subunits of the Arp2/3 complex exhibit the same localization pattern, we carried out immunofluorescence experiments on *L. monocytogenes–*infected HeLa cells using anti-p34 and anti-p21 antibodies. As is shown in Fig. 3, *A* and *C*, both p34-Arc and p21-Arc are localized to *L. monocytogenes* assembled actin clouds, and throughout tails with a distribution that mirrors that of Arp3 (Welch et al., 1997; and see below) and polymer-

ized actin (Fig. 3, *B* and *D*). Punctate cytoplasmic staining also was evident, and was reduced by briefly pre-extracting cells with detergent in the presence of phalloidin to stabilize actin structures (see Materials and Methods).

To further confirm the observed localization pattern, we created a tagged Arp3 with green fluorescent protein (GFP) at its carboxy terminus. We chose to create this particular fusion because *S. cerevisiae* Arp3 tagged at its carboxy terminus can complement a null mutation in the *ARP3* gene (Winter et al., 1997). HeLa cells were transfected with pArp3-EGFP plasmid DNA, and 1 d later were infected with *L. monocytogenes.* Arp3-GFP fluorescence was observed in actin clouds and tails with a distribution indistinguishable from that seen by immunofluorescence (Fig. 3 *E*; actin localization in *F*). GFP alone did not localize to actin clouds or tails (not shown). As was seen in the immunofluorescence experiments, cytoplasmic Arp3-GFP fluorescence was always apparent, even in cells expressing lower levels of the fusion protein. A brief pre-extraction of the cells before fixation significantly reduced this cytoplasmic background.
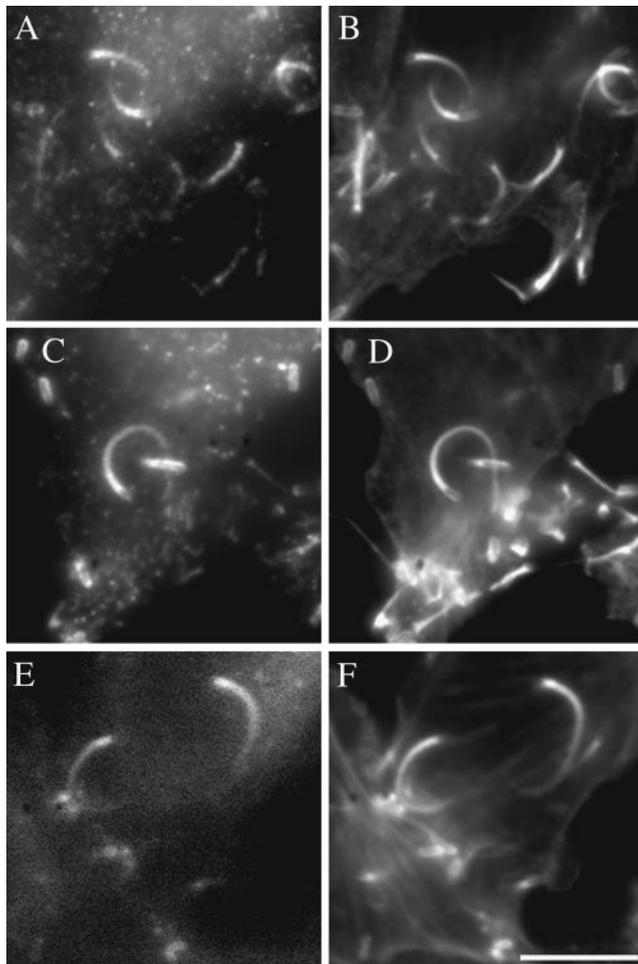
The Arp2/3 Complex Is Localized to Lamellipodia in Stationary Cells

The actin-based motility of *L. monocytogenes* may be mechanistically similar to actin-driven cellular movements such as the protrusion of lamellipodia. To evaluate whether the Arp2/3 complex may be involved in actin polymerization–driven membrane protrusion, we determined its intracellular distribution. We observed the localization of Arp2/3 complex subunits in a variety of tissue culture lines by immunofluorescence. Mouse Swiss 3T3 fibroblast cells gave the most consistent results, although a similar staining pattern was evident in all cell lines tested (including human HeLa epithelial-like cells and mouse MC7 3T3 fibroblasts). As is shown in Fig. 4, Arp3 (*A*), p34-Arc (*C*), and p21-Arc (*E*) were distributed throughout the thin actin-rich lamellipodia at the edges of Swiss 3T3 cells (actin staining in the same cells is shown in Fig. 3 *B*, *D*, and *F*). Staining was not observed along the entire margin of the cell, but was confined to lamellipodia (Figs. 4 and 5). Strikingly, other actin-containing structures such as stress fibers and focal adhesions were not stained. The punctate cytoplasmic staining pattern was especially prominent in thicker regions of the cell in the vicinity of the nucleus (Fig. 5).

The Arp2/3 Complex Is Localized to the Leading Lamellipodia of Motile Cells

Stationary Swiss 3T3 fibroblasts produce actin-rich lamellipodia that actively protrude and retract but are not involved in directional motility. These were randomly dispersed over the cell surface as is illustrated by the distribution of the p21 subunit of the Arp2/3 complex and actin in these cells (Fig. 5, *A* and *B*). To ascertain whether the Arp2/3 complex may be involved in membrane protrusion during cell migration, we observed the localization of the complex in motile fibroblasts. We generated a population of moving Swiss 3T3 fibroblasts by wounding a confluent layer of cells. Cells at the margin of the wound responded immediately by generating membrane ruffles at
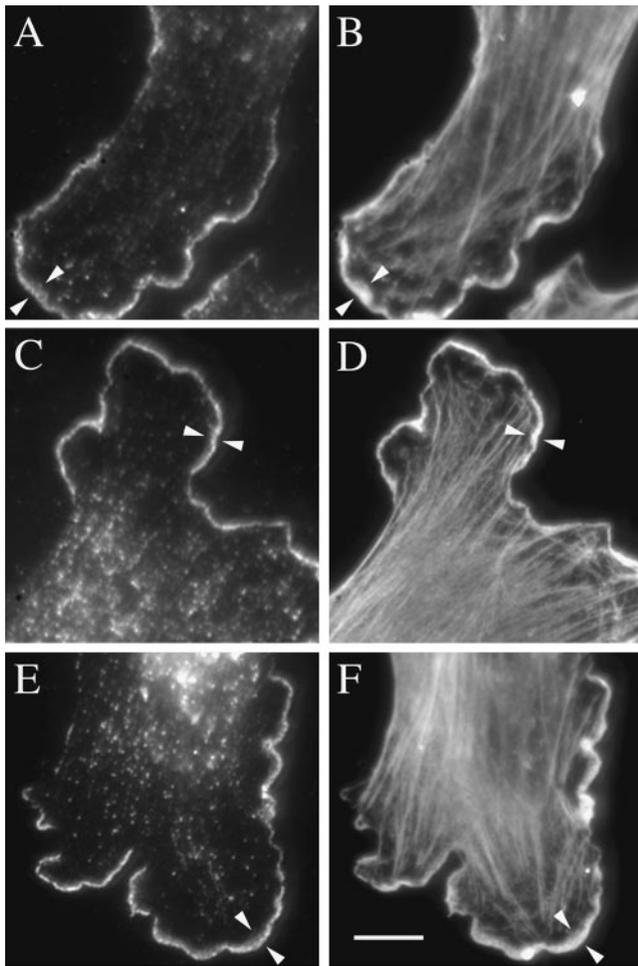

Figure 3. Localization of Arp2/3 complex subunits in *L. monocytogenes*–infected HeLa cells. (*A*) Distribution of p34-Arc and (*B*) of actin in the same cell, visualized by immunofluorescence. (*C*) Distribution of p21-Arc and (*D*) of actin in the same cell, visualized by immunofluorescence. (*E*) Distribution of Arp3-EGFP and (*F*) distribution of actin in the same cell, visualized by staining with rhodamine-phalloidin. Bar, 10 μm.

Figure 4. Localization of Arp2/3 complex subunits in Swiss 3T3 fibroblasts by indirect immunofluorescence. (*A*) Distribution of Arp3, and (*B*) of actin in the same cell. (*C*) Distribution of p34-Arc, and (*D*) of actin in the same cell. (*E*) Distribution of p21-Arc and (*F*) of actin in the same cell. The arrowheads indicate the position of lamellipodia. Bar, 10 μm.

their edges facing the wound, indicating that they had begun to reorient their actin cytoskeleton. Within 30 min, they began to move outwards towards the center of the wound. p21-Arc was localized to the broad actin-rich lamellipodia at the leading edges of these moving cells (Fig. 5, *C* and *E*, and actin localization in *D* and *F*).

We also generated a population of motile primary cultured fibroblasts and looked at the distribution of Arp2/3 complex subunits in these cells by immunofluorescence. To prepare moving fibroblasts, tissue explants from the hearts of 7-d-old embryonic chicks were plated onto coverslips. Cells migrate outward from explants within 12 h, and continue to move rapidly for up to 20 h (Cramer et al., 1997). These cells assumed a morphology typical of locomoting cells, with a cone- or fan-shaped cell body and broad lamellipodia at their leading edges (Fig. 6). Both Arp3 (Fig. 6 *A*) and p34-Arc (Fig. 6 *C*) were localized throughout lamellipodia. Neither protein was found in actin bundles, fully consistent with their distribution in Swiss 3T3 fibroblasts.

Discussion

The Arp2/3 Complex Consists of Evolutionarily Conserved Subunits

The seven subunits of the human Arp2/3 complex define seven protein families that are present in diverse eukaryotic organisms. Six share sequence similarity with subunits of the *A. castellanii* complex (sequence information for *A. castellanii* p14 is not yet available). Moreover, every subunit has a homologue *S. cerevisiae*, and the yeast equivalents of human p34-Arc, p21-Arc, p20-Arc, and p16-Arc have recently been shown to be components of the Arp2/3 complex (Winter et al., 1997). This indicates that the structure of the Arp2/3 complex has been conserved through evolution and suggests a corresponding conservation of its activity in all eukaryotic cells. The complex appears to be essential for cell viability and actin cytoskeletal function, as deletion of the genes encoding Arp2, Arp3, and Sop2p is lethal in yeast, and less severe mutations cause defects in actin organization and disrupt actin-dependent processes (Lees-Miller et al., 1992; Schwob and Martin, 1992; Moreau et al., 1996; McCollum et al., 1996; Balasubramanian et al., 1996; Winter et al., 1997).

Sequence, Structure, and Function—the Role of the Arp2 and Arp3 Subunits

The Arp2/3 complex induces actin polymerization at the cell surface of *L. monocytogenes* (Welch et al., 1997), suggesting that it performs a similar function in cells. It is likely to promote actin assembly by filament nucleation. Structural modeling of Arp2 and Arp3 proteins (Kelleher et al., 1995) has suggested that these subunits may serve as a template for the nucleation of polymerization, similar to the function performed by γ-tubulin in the initiation of microtubule assembly (Moritz et al., 1995; Zheng et al., 1995). Although structural considerations led Kelleher et al. (1995) to propose that the Arp subunits interact with the pointed end of the filament, interaction with the barbed end remains a possibility. Of the Arp2/3 complex subunits, the sequences of Arp2 and Arp3 proteins are among the most highly conserved between species, consistent with their proposed role in nucleating actin polymerization.

Sequence, Structure, and Function—the Role of p41-Arc

The other five subunits of the Arp2/3 complex may be important for stabilizing its structure, regulating its biochemical activity, and/or determining its intracellular localization. The sequence and biochemical properties of one of these subunits, p41-Arc, suggest that it plays an important regulatory role. This subunit is a member of the Sop2 family of proteins and is most similar to another human protein, Sop2Hs. The similarity between these proteins suggests that they may both function as the p41 subunit of the human Arp2/3 complex. Multiple versions of the p41 subunit may adapt the functions of the complex to different cell types or developmental stages. Platelets appear to use only p41-Arc since the peptides derived from the 41- and 40-kD polypeptides were all identical to it in sequence,
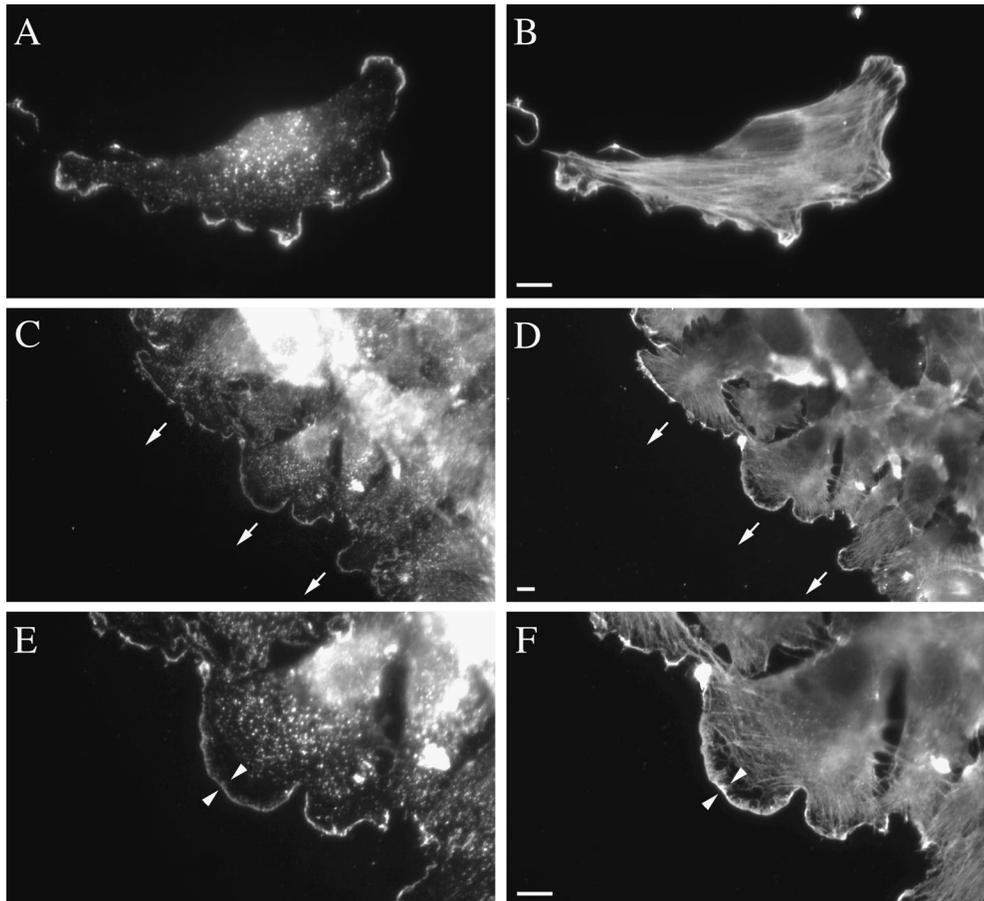
Figure 5. A comparison of the localization of Arp2/3 complex subunits in stationary (*A* and *B*) and moving (*C–F*) Swiss 3T3 fibroblasts by indirect immunofluorescence. (*A*) Higher magnification view of the distribution of p21-Arc, and (*B*) of actin in a stationary fibroblast. (*C*) Lower magnification view of the distribution of p21-Arc and (*D*) of actin in fibroblasts at the edge of a wound 2 h after wounding a monolayer. Cells move in the direction of the arrows, which point towards the center of the wound. (*E*) Higher magnification view of p21-Arc and (*F*) of actin in a cell from *C* and *D*, respectively. The arrowheads indicate the position of the lamellipodium. Bars, 10 μm.

and clearly different from the corresponding regions in Sop2Hs.

p41-Arc, like Sop2Hs, contains seven putative WD repeats that are evenly dispersed over the entire sequence of the protein. The presence of WD repeats may provide some insight into the potential functions of this subunit. These repeats, which were first identified in the G_β subunit of heterotrimeric GTP-binding proteins, have since been found in between four to ten copies in proteins with a wide variety of functions (for review see Neer et al., 1994). The repeat unit is involved in the formation of a characteristic three-dimensional structure known as a β-propeller, each repeat constituting one blade of the propeller (Wall et al., 1995; Lambright et al., 1996; for review see Neer and Smith, 1996). Because many WD repeat-containing proteins participate in the formation of multiprotein complexes, one possibility is that p41-Arc is involved in assembling and maintaining the structure of the Arp2/3 complex.

Another intriguing possibility is that p41-Arc mediates the interaction of the complex with other molecules that regulate its localization or activity. One known ligand of WD repeats is the pleckstrin homology (PH) domain (for reviews see Lemmon et al., 1996; Shaw, 1996), which is found in a variety of cell signaling and cytoskeletal proteins that interact with cell membranes. In addition to binding WD repeat sequences, PH domains can simultaneously bind phosphatidyl inositol 4,5-bisphosphate, and thus may serve as adapters that link WD repeat–containing proteins to the cell membrane. We have found that the Arp2/3 complex is localized to the cell edges in lamellipodia. An interaction between p41-Arc in the Arp2/3 complex and a PH domain–containing protein may mediate the recruitment of the complex to the cell membrane and may also serve to activate the actin polymerization promoting capacity of the complex. A protein that recruits the Arp2/3 complex to cell membranes would be functionally similar to the *L. monocytogenes* ActA protein, which is required for actin polymerization by bacteria in vivo (Domann et al., 1992; Kocks et al., 1992) and for the activity of the Arp2/3 complex at the bacterial surface (Welch et al., 1997).

Posttranslational modification of p41-Arc may also regulate the activity and intracellular distribution of the complex. We have determined that the 41- and 40-kD subunits of the platelet Arp2/3 complex are the same polypeptide, suggesting that they may differ in their state of posttranslational modification. However, the nature of this putative modification and its effect on the Arp2/3 complex remain to be determined.

Sequence, Structure, and Function—the Role of p34-Arc, p21-Arc, p20-Arc, and p16-Arc

The four other subunits of the Arp2/3 complex (p34-Arc, p21-Arc, p20-Arc, and p16-Arc) define four novel protein families. The extent of sequence conservation between
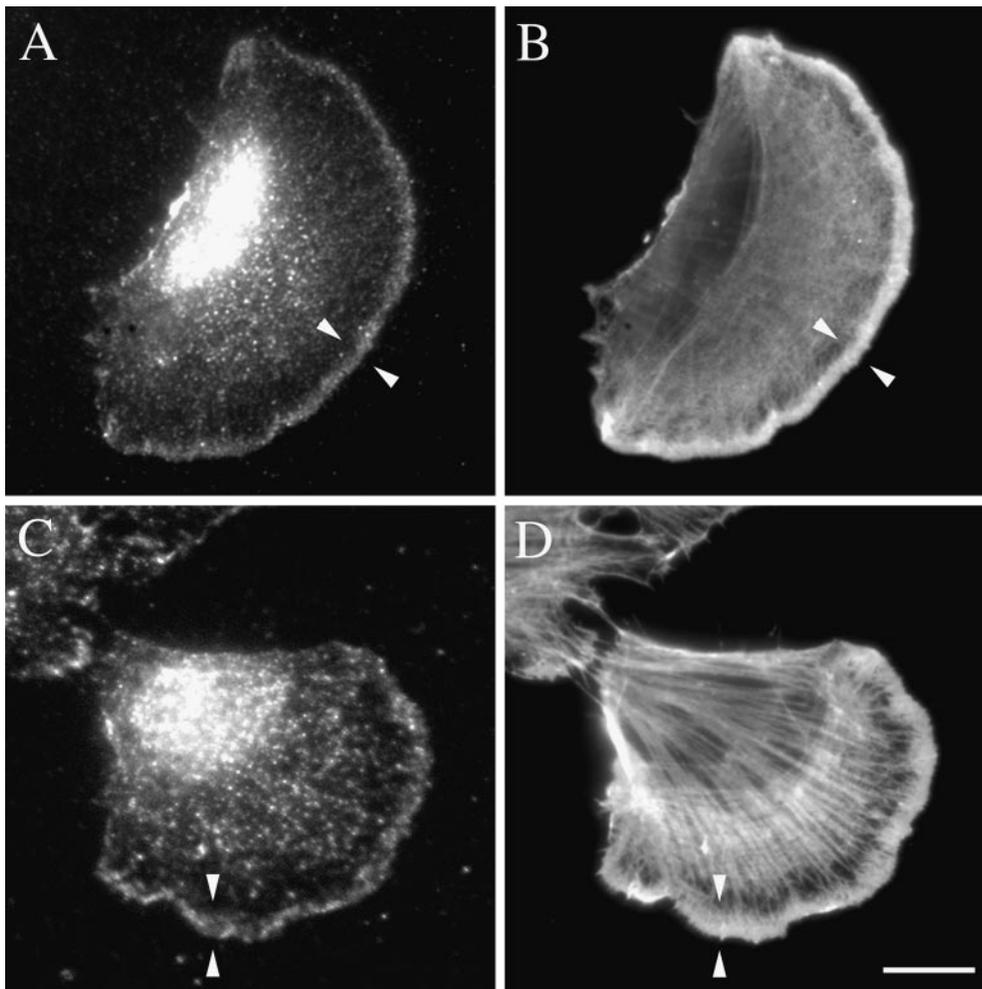
Figure 6. Localization of Arp2/3 complex subunits in primary culture fibroblasts from embryonic chick hearts by indirect immunofluorescence. (*A*) Distribution of Arp3 and (*B*) of actin in the same cell. (*C*) Distribution of p34-Arc and (*D*) of actin in the same cell. The arrowheads indicate the position of the lamellipodia. Bar, 10 μm.

family members from different species indicates that the structure of these subunits has been conserved through evolution. However, the sequences of these proteins do not indicate what their role in the structure or regulation of the complex may be.

Two of these subunits of the *A. castellanii* complex—the 35-, and either the 19-, 18-, or 14-kD subunits—were found by chemical cross-linking to be intimately associated with actin (Mullins et al., 1997). The Arp3 subunit and actin could be also cross-linked. It was suggested that these subunits mediate the ability of the *A. castellanii* complex to bind the sides of actin filaments and to bundle actin filaments into parallel arrays in vitro. However, these subunits do not exhibit any similarity to known actin-binding proteins, implying that they may possess novel actin-binding motifs.

Localization of the Arp2/3 Complex in Vertebrate Cells

The localization of subunits of the Arp2/3 complex in yeast and *A. castellanii* is consistent with its proposed role in promoting actin polymerization in cells. The *S. cerevisiae* Arp2 and Arp3 proteins are found in the motile cortical actin patches, but not in cytoplasmic cables that consist of bundled actin filaments (Moreau et al., 1996; Winter et al., 1997). *S. pombe* Arp3 shares a similar distribution, and

in addition is absent from the contractile ring (McCollum et al., 1996). In ameba, subunits of the complex colocalize with actin at the cell cortex (Machesky et al., 1994; Kelleher et al., 1995). Staining is enriched in actin fibrils that may represent bundles of actin filaments, in agreement with the bundling activity exhibited by the *A. castellanii* complex in vitro (Mullins et al., 1997).

We have now determined the localization of multiple subunits of the Arp2/3 complex in vertebrate cells. The Arp3, p34-Arc, and p21-Arc subunits are found in the lamellipodia of motile and stationary fibroblasts. They are also localized to *L. monocytogenes* assembled actin clouds and tails in infected cells. In contrast, Arp3, p34-Arc, and p21-Arc were not detected in cellular bundles of actin, including stress fibers in tissue culture fibroblasts and graded polarity bundles (Cramer et al., 1997) in primary cultures of chick embryo fibroblasts. Moreover, they have not been observed in filopodia, which also contain bundled actin (DePace, A., M. Welch, and T. Mitchison, unpublished results).

Actin filaments in lamellipodia and those in actin bundles differ in their spatial organization and dynamic properties. Filaments in lamellipodia are arranged as an orthogonal meshwork and are oriented at an angle of ~45°, relative to the direction of protrusion (Small et al., 1995). They exhibit rapid polymerization dynamics, turning over

with a $t_{1/2}$ of <1 min in some cell types (Theriot and Mitchison, 1991). This rapid turnover is similar to that seen for filaments in *L. monocytogenes* tails (Theriot et al., 1992). Conversely, filaments in stress fibers and other cellular bundles are arranged in closely spaced parallel arrays. They exhibit much slower polymerization dynamics than filaments in lamellipodia (Theriot and Mitchison, 1991). We do not detect the Arp2/3 complex in actin bundles, suggesting that the filament side-binding and bundling activity exhibited by the complex in vitro (Mullins et al., 1997) may not be a primary determinant of its function in vertebrate cells. Instead, we find that the Arp2/3 complex is present with the rapidly polymerizing and depolymerizing populations of actin filaments in lamellipodia and *L. monocytogenes* tails, further strengthening the analogy between bacterial motility and lamellipodial protrusion. Together with the biochemical activity of the Arp2/3 complex, these results suggest a model in which the complex promotes actin polymerization in the lamellipodium and participates in lamellipodial protrusion. The ability of the complex to bind filaments might serve to retain the complex in the lamellipodium, making it readily available for additional rounds of polymerization.

Before we can put together a comprehensive model of actin polymerization dynamics in lamellipodia, several outstanding questions need to be answered. For example, it is not clear whether actin polymerization occurs throughout the lamellipodium, matching the distribution of Arp2/3 complex, or only at the leading edge. In addition, it is not known whether the mechanism of new actin filament polymerization involves the elongation of existing filaments or continuous nucleation of new filaments. Finally, the mechanisms that regulate the spatial and temporal control of actin polymerization have yet to be determined. Further characterization of the interactions of the Arp2/3 complex with actin filaments and with factors that regulate its activity and localization should help shed light on the answers to these outstanding problems.

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