Visualization and Molecular Analysis of Actin Assembly in Living Cells

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Abstract. Actin filament assembly is critical for eukaryotic cell motility. Arp2/3 complex and capping protein (CP) regulate actin assembly in vitro. To understand how these proteins regulate the dynamics of actin filament assembly in a motile cell, we visualized their distribution in living fibroblasts using green flourescent protein (GFP) tagging. Both proteins were concentrated in motile regions at the cell periphery and at dynamic spots within the lamella. Actin assembly was required for the motility and dynamics of spots and for motility at the cell periphery. In permeabilized cells, rhodamine-actin assembled at the cell periphery and at spots, indicating that actin filament barbed ends were present at these locations. Inhibition of the Rho family GTPase rac1, and to a lesser extent cdc42 and RhoA, blocked motility at the cell periphery and the formation of spots. Increased expression of phosphatidylinositol 5-kinase promoted the movement of spots. Increased expression of LIM–kinase-1, which likely inactivates cofilin, decreased the frequency of moving spots and led to the formation of aggregates of GFP–CP. We conclude that spots, which appear as small projections on the surface by whole mount electron microscopy, represent sites of actin assembly where local and transient changes in the cortical actin cytoskeleton take place.

Key words: actin assembly • Arp2/3 complex • capping protein • cell motility • Rho family GTPase

Actin polymerization is a necessary event for many cellular movements. Classic photobleaching and photoactivation studies of motile cells showed that incorporation of actin monomer occurs predominantly near the leading edge (58, 59, 64) where actin filaments are short, branched, and have their fast-growing barbed ends oriented toward the membrane (33, 56). In addition, studies using pharmacologic inhibitors of actin polymerization (9, 19, 69) and studies of permeabilized cells (24, 47, 57) support the view that actin polymerization at free barbed ends creates a propulsive force that pushes forward the lamella of migrating cells (for review see ref. 39). The next challenges for understanding how actin dynamics contributes to cell motility is to observe the relationship in live cells and to define the molecular mechanism.

Two models for initiating actin assembly at the leading edge are elongation of existing actin filaments at newly created free barbed ends or creation of new actin filaments with free barbed ends that subsequently elongate. These models are not mutually exclusive. Both models require a mechanism for generating free barbed ends. In the latter model, a mechanism for the formation of new filaments having free barbed ends is required, because the rate of spontaneous actin oligomer formation is slow at the concentration of free actin monomer in cells. One likely candidate for a factor that promotes de novo assembly is the Arp2/3 complex (37, 45, 65, 66, 68), which nucleates the formation of actin filaments that elongate only at the barbed end (44). Arp2/3 complex remains bound at the pointed ends of filaments and also binds along the sides of filaments. Thus, Arp2/3 complex could create new actin filaments that could anchor via their pointed end to the existing filament network such that elongation at their barbed ends could propel the membrane forward (44). Arp2/3 complex, together with the *Listeria* surface protein ActA, promotes the assembly of actin filaments (67). In yeast, the Arp2/3 complex is essential for viability and necessary for the movement of cortical actin patches (41, 68).

In the model where assembly occurs on existing filaments, free barbed ends are proposed to be generated by severing filaments or by uncapping actin filament barbed ends. Support for actin filament severing comes from stud-

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ies of *Dictyostelium* stimulated by cAMP (16). On the other hand, capping protein (CP)[1] is readily removed from barbed ends in vitro by phosphatidylinositol 4,5-biphosphate (PI 4,5-P_2) (52), therefore, PI 4,5-P_2 in the membrane may induce localized uncapping of actin filaments close to the membrane. Capping protein is a potent barbed end capper as well, and much evidence suggests that capping protein functions to block barbed end growth and limit actin polymerization in vivo (14, 16, 26, 51).

Since Arp2/3 complex and capping protein affect actin assembly in vitro by different mechanisms and both are important for actin assembly in vivo, we reasoned that fluorescent probes of Arp2/3 complex and CP would reveal distinct features of actin assembly in motile cells. We prepared these probes using green fluorescent protein (GFP) tagging and analyzed their distributions in live cells under varying conditions that modulate cell motility. The distributions of the GFP-tagged proteins were identical to those of endogenous Arp2/3 complex and capping protein. Both GFP–Arp2/3 complex and GFP–CP were enriched at motile regions of the leading edge suggesting that both Arp2/3 complex and capping protein regulate actin dynamics at the leading edge. Unexpectedly, GFP–Arp2/3 complex and GFP–CP also were observed in dynamic structures at sites away from the cell periphery, in small spots scattered throughout the lamella. These localized sites of actin assembly may occur where transient changes in the cortical actin cytoskeleton are required for cellular events such as endocytosis, exocytosis, or signaling.

Materials and Methods

cDNA Constructs, Antibodies, and Reagents

The expression plasmid for GFP–CP was constructed from pEGFP-C1 (Clontech, Palo Alto, CA) and a cDNA encoding mouse CP-β2 (53). The expression plasmid for GFP–Arp3 was described (66). The expression plasmids for mutant forms of rac1 and cdc42 were as described (13). The plasmids for expression of LIM–kinase-1 and mutant LIM–kinase-1 were as described (2). Exoenzyme C3 transferase was purchased from Calbiochem-Novabiochem (La Jolla, CA). Activated RhoA was expressed in bacteria and purified as described (48). The expression plasmid for mouse phosphatidylinositol 5-kinase (PI 5-kinase) (GenBank/EMBL/DDBJ accession number AF048695) was constructed using pRK5myc (30) and a cDNA clone derived from American Type Culture Collection (no. 569886; Rockville, MD) and the NIH Image consortium (est no. ma36d03; National Institutes of Health, Bethesda, MD). The cloned PI 5-kinase is a variant of mouse type I alpha PI 5-kinase. Plasmid regions that had been amplified using PCR were sequenced to check for errors. Antibodies to Arp3, p34, and p21 of the Arp2/3 complex (65), CP-β2 (53), actin (mAb C4) (32), VASP (10), zyxin (36), mena (20), ezrin (3), and profilin (38) were as described. Anti-vinculin was purchased from Sigma (St. Louis, MO). Antibody to PI 4,5-P_2 was purchased from perSeptive Diagnostics (Framingham, MA) and was injected at 11 mg/ml, a concentration that had effects in other studies (21). Antibodies to myosin IIA and myosin IIB were gifts from R. Wysolmerski (St. Louis University, St. Louis, MO); anti–myosin V (17) and anti–myosin I (34) were as described. A peptide based on a polyphosphoinositide-binding site in gelsolin (residues 150–169) (28) was synthesized and injected at 10 mM. Rhodamine-labeled secondary antibodies were purchased from Chemicon (Temecula, CA). Rhodamine dextrans were purchased from Sigma.

Cell Lines

PtK1 fibroblasts were transfected using lipofectamine (GIBCO BRL, Gaithersburg, MD) according to the manufacturer's instructions and selected in media containing 1 mg/ml G418. Clones were screened by fluorescence microscopy and immunoblotting (29, 61). Cell lines were maintained in media containing 0.5 mg/ml G418.

Quantitative Immunoblotting

To determine the level of expression of the GFP-tagged proteins, whole cell extracts of each cell line, including the parental PtK1 cells, were prepared in SDS sample buffer. The extracts were subjected to electrophoresis and immunoblotting with antibodies to capping protein β2-subunit or anti-Arp3. The immunoblots were developed as described (18) and scanned to obtain a digital image. The intensity of the bands on the blot corresponding to the endogenous and GFP-tagged proteins were quantitated using NIH Image.

Rhodamine-Actin Incorporation in Permeabilized Cells

Incorporation of rhodamine-actin in saponin-permeabilized cells was performed as described (57). Rhodamine-actin (Cytoskeleton, Denver, CO) at 0.45 μM in 20 mM Hepes, pH 7.5, 138 mM KCl, 4 mM $MgCl_2$, 3 mM EGTA, 0.2 mg/ml saponin, 1 mM ATP, and 1% BSA was added to cells plated on coverslips. Latrunculin A (1 μM) or cytochalasin D (5 μM) were added to some samples. After 5 min in the presence of rhodamine-actin, the permeabilized cells on coverslips were fixed for 10 min in 3% paraformaldehyde in 127 mM NaCl, 5 mM KCl, 1.1 mM NaH_2PO_4, 0.4 mM KH_2PO_4, 2 mM $MgCl_2$, 5.5 mM glucose, 1 mM EGTA, and 20 mM Pipes, pH 7.3, rinsed in the same buffer containing 10 mM ethanolamine, and then mounted for microscopy.

Microscopy

Live cell imaging was performed using an intensifier silicon-intensifier target tube (ISIT) camera as described (62). Cells were maintained at 37°C in phenol red-free and $NaHCO_3$-free MEM-Earle's containing 10% fetal calf serum and 20 mM Hepes, pH 7.0. For immunofluorescence, cells on coverslips were fixed in 1% paraformaldehyde in IF buffer (127 mM NaCl, 5 mM KCl, 1.1 mM NaH_2PO_4, 0.4 mM KH_2PO_4, 2 mM $MgCl_2$, 5.5 mM glucose, 1 mM EGTA, and 20 mM Pipes, pH 7.3) for 10 min at 37°C, immersed in methanol at −20°C for 10 min, and then rehydrated in IF buffer containing 10 mM ethanolamine. Cells were blocked in 5% horse serum, 0.05% Tween-20 in IF buffer at pH 8.1. Antibodies were diluted in blocking solution. Coverslips were mounted in 1% *n*-propylgallate in 50 mM TrisCl, pH 8.5, with 50% glycerol. Images were collected with a model 300T-RC (Dage-MTI, Michigan City, IN) cooled charge-coupled device camera.

Analysis of Spot Movements

Spot velocities were calculated by marking the x and y coordinates of the position of each spot in several frames of the video recording. These data were tabulated using Microsoft Excel (Redmond, WA) and the total lifetime and distance covered by each spot was determined. Statistical analyses were performed with the tools available in the Microsoft Excel program.

Whole Mount Electron Microscopy

Cells observed by electron microscopy were first observed using fluorescence video microscopy as described above to record the dynamics of GFP–CP just before fixation for preparation of whole mounts. Cells were grown on coated electron microscopic grids and assembled in a flow chamber for video microscopy. A video recording was made of each cell before and during fixation in 0.25% glutaraldehyde, 0.1 M cacodylate, pH 7.4, 10 mM $CaCl_2$, and 10 mM $MgCl_2$ prewarmed to 37°C. The sample was kept at 37°C for 10–15 min and then the grid was transferred for 1 h at room temperature to 2% glutaraldehyde in the same buffer. Samples were postfixed with 0.1% OsO_4 for 10 min on ice, treated with 0.1% tannic acid for 20 min, and then stained, dehydrated, and subjected to critical point drying and carbon coating as described (4, 34). In some cases, samples were treated with 0.25% saponin before postfixation with OsO_4 and staining (49). The same cells visualized by video microscopy during fixation were identified on the grid and electron micrographs were prepared.

1. *Abbreviations used in this paper:* BDM, butanedione monoxime; CP, capping protein; GFP, green fluorescent protein; PI 3,4,5-P_3, phosphatidylinositol 3,4,5-triphosphate; PI 4,5-P_2, phosphatidylinositol 4,5-biphosphate; PI 5-kinase, phosphatidylinositol 5-kinase.

Results

Actin Assembly in Living Cells Revealed by GFP-tagged Forms of Arp2/3 Complex and Capping Protein

To observe sites of actin assembly in living cells, we selected stable PtK1 fibroblast lines expressing GFP-tagged Arp2/3 complex and capping protein and monitored their distributions over time using video microscopy. GFP–Arp3 and GFP–CP were found in two types of motile structures. First, both proteins were concentrated in motile regions of the cell periphery. The dynamics of GFP–Arp3 and GFP–CP at the cell periphery reflected movement of the edge of the lamella during extension and ruffling. Second, a surprising observation was the appearance of dynamic spots of GFP–Arp3 and GFP–CP within the lamella, but back from the cell edge. Collections of im-

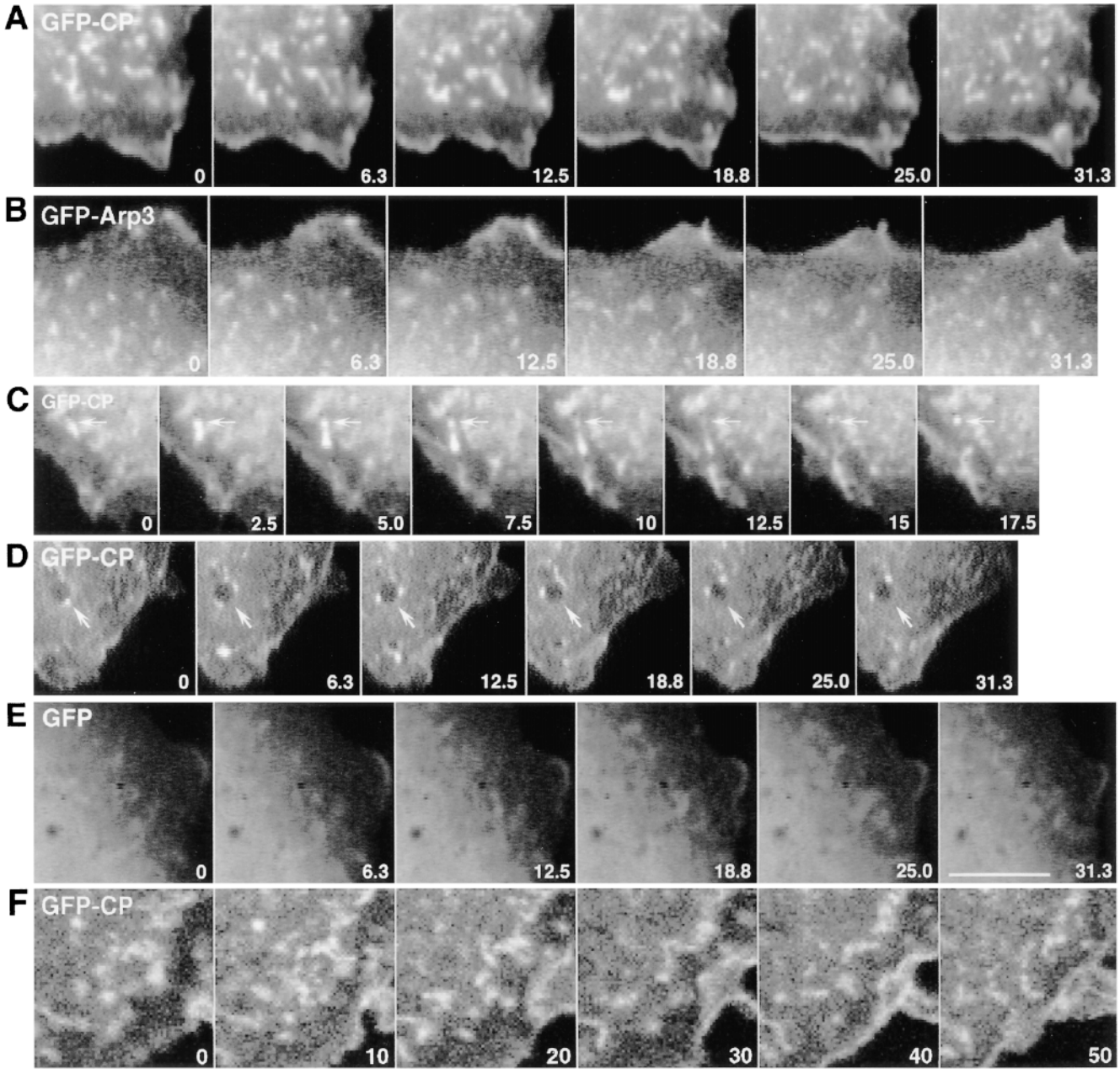

Figure 1. The distributions of GFP–CP (*A*, *C*, *D*, and *F*) and GFP–Arp3 (*B*) in living fibroblasts change dramatically over time. Each sequence of images (*A–F*) shows selected frames from a video sequence demonstrating that the distributions of GFP–CP and GFP–Arp3 change with time. Movies of the complete video sequences for *A–F* present these data more clearly and are available at www.cooperlab.wustl.edu or from the authors. Arrows in *C* and *D* indicate the initial position of a prominent motile spot formed in the lamella (*C*) or associated with a macropinosome (*D*). The distribution of GFP (*E*) is diffuse, as expected. Slight changes in the distribution of GFP over time reflect changes in cell thickness that accompany cellular movements. The sequence in *F* was obtained using a confocal microscope and shows that the accumulation of GFP–CP at the cell periphery is not due to increased cell thickness at the edge of the cell. Numbers in the lower corner of each image indicate elapsed time in seconds. Bar, 10 μm.

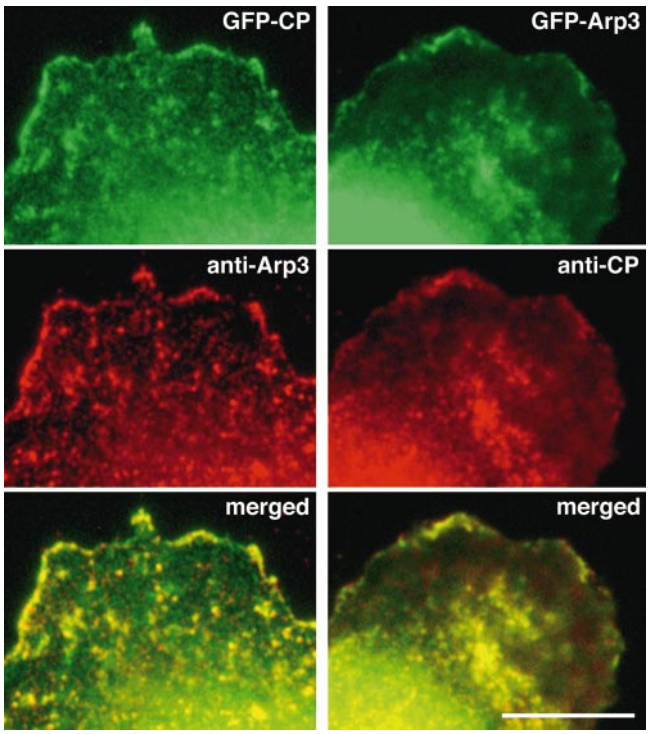

Figure 2. Arp2/3 complex and CP colocalize in punctate structures in the lamella and along the edge of cells. PtK1 cells expressing GFP–CP (*left*) or GFP–Arp3 (*right*) were fixed and labeled with antibodies to localize Arp2/3 complex (*left*) or CP (*right*), respectively. *Green*, GFP-tagged proteins; *red*, antibody labeling. *Bottom*, merged image of each red-green pair; *yellow*, regions of overlap of Arp2/3 complex and CP. Bar, 10 μm.

ages taken from the video sequences of individual cells illustrate the changes in the distributions of GFP–Arp3 (Fig. 1 *B*) and GFP–CP (Fig. 1, *A*, *C*, *D*, and *F*) in living cells over time. Movies (Movies 1A–1F; available at www.cooperlab.wustl.edu) present these data most clearly.

Spots of GFP–Arp3 and GFP–CP are components of the same structure because antibodies to subunits of the Arp2/3 complex (including Arp3, p21, and p34) labeled spots of GFP–CP and vice versa (Fig. 2). Capping protein and Arp2/3 complex were nearly coincident in punctate structures in the lamella and at the cell periphery.

The accumulation of GFP–Arp3 and GFP–CP at the cell periphery and in motile spots is not due to increased cell thickness at those locations. To control for changes in fluorescence intensity that result from changes in cell height, we compared the distributions of GFP–Arp3 and GFP–CP with those of GFP and rhodamine-dextran, two reagents that distributed uniformly in the cytoplasm. Ratio images of the fluorescence of GFP–CP or GFP–Arp3 to that of microinjected rhodamine-dextran (70 kD) showed that both proteins were specifically enriched in motile regions at the cell periphery and in the dynamic spots (data not shown). In addition, confocal microscopy of live cells expressing GFP–CP confirmed that CP was enriched in regions along the periphery and in motile spots (Fig. 1 *F*; Movie 1F available at www.cooperlab.wustl.edu). Time-lapse movies obtained using the confocal microscope focused at several planes above the substratum indicated that the moving spots were present on the upper cell surface.

The dynamic structures we observed are not artefacts of GFP tagging. The distributions of GFP–Arp3 and GFP–CP accurately represent the distributions of endogenous Arp2/3 complex and CP, respectively. Antibodies to two components of the Arp2/3 complex, p21 and p34, labeled regions at the cell periphery and spots within the lamella that colocalized with GFP–Arp3 at those locations (data not shown). The analogous experiment could not be done with GFP–CP due to the lack of specific antibodies, however, the distributions of GFP–CP tagged on the β2-subunit were identical to that of GFP–CP tagged on the α2-subunit. Thus, two different GFP-tagged probes for CP exhibited identical dynamics in cells. In addition, GFP–CP expressed in myotubes or cardiac myocytes in cell culture assembled at Z disks as does endogenous CP (8, 54). The distribution of sarcomeric actin in myotubes was not altered by expression of GFP–CP and myotubes contracted spontaneously, indicating that the contractile apparatus functioned (data not shown).

The distributions of GFP–Arp3 and GFP–CP in motile spots also are not artefacts of overexpression of the GFP-tagged proteins. GFP–Arp3 and GFP–CP β2-subunit represented 10 and 20%, respectively, of the total amount of Arp3 and CP β2-subunit expressed in the stably transfected cell lines as determined using quantitative immunoblots. Furthermore, the total amount of Arp3 and CP expressed in the cell lines was not increased. The moving spots also were not specific to PtK1 fibroblasts; other transfected cell lines in which motile spots were observed include mouse fibroblasts, rat mammary adenocarcinoma cells, and MDCK cells.

Properties of the Dynamic Spots

The majority of spots of GFP–Arp3 and GFP–CP appeared in the lamella, moved short distances, and then disappeared with an average life time of 11 s. The rate of movement of spots of GFP–Arp3 and GFP–CP were not significantly different; spots of CP and Arp3 moved at average rates of 0.17 μm/s (SEM 0.01, $n = 24$) and 0.14 μm/s (SEM 0.01, $n = 12$), respectively. The direction of spot movement appeared random relative to the cell edge and to the location and movement of other spots. In some cases a single spot diverged into two structures which moved apart from one another. In other cases spots coalesced into a single structure, which then continued to move. In rare instances, spots were trailed by comet-like tails similar to the actin structures assembled by moving *Listeria* monocytogenes (Fig. 1 *C*, *arrow*; Movie 1C available at www.cooperlab.wustl.edu).

Spots also were observed moving along the periphery of macropinosomes, which appeared as dark, round structures (Fig. 1 *D*, *arrow*; Movie 1D available at www.cooperlab.wustl.edu); these organelles were identified as macropinosomes based on their uptake of rhodamine-dextran from the media (data not shown). Compared with the short-lived spots moving in the lamella, spots associated with macropinosomes persisted throughout the entire observation period (>1 min) and moved along the organelle mem-

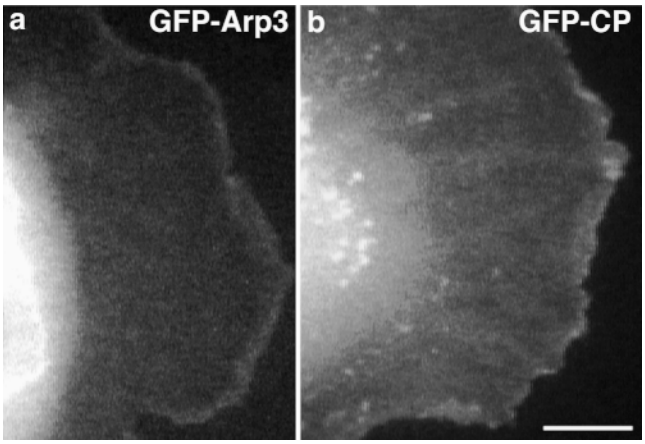

Figure 3. Spots were reduced in number and spot motility was suppressed in migrating cells. Confluent monolayers of PtK1 fibroblasts expressing GFP–Arp3 (*A*) or GFP–CP (*B*) were "wounded" to obtain a clear area on the coverslip. Cells migrating into the cleared region were monitored 2–4 h after scratching. Both Arp2/3 and CP were enriched in regions of motile activity at the cell edge. Few motile spots were observed and they were generally restricted to a location at the rear of the extended lamella. Movies of the complete video sequence for each panel are available at www.cooperlab.wustl.edu or from the authors. Bar, 10 μm.

brane with frequent stops and changes in direction. The rate of movement of spots on macropinosomes was similar to that of the short-lived spots moving in the lamella.

Motile spots were most frequently observed in cells of preconfluent cultures in which PtK1 fibroblasts were well spread, but were not actively migrating. In contrast, motile spots were less abundant in cells of confluent cultures induced to migrate into a cleared zone scratched in the monolayer (Fig. 3; Movies 3A and 3B available at www.cooperlab.wustl.edu). The migrating cells extended a broad flat lamella that was nearly devoid of spots; GFP–Arp3 and GFP–CP were enriched at the leading edge of the lamella. Thus, the appearance and movement of spots was suppressed in actively migrating cells.

Actin Assembly Drives Motility at the Cell Periphery and at Spots

To confirm that GFP–Arp3 and GFP–CP identify sites of actin assembly, we observed the incorporation of rhodamine-labeled actin in saponin-permeabilized cells. Rhodamine-actin assembled at sites along the cell periphery and at spots of GFP–CP (Fig. 4). The incorporation of rhodamine-actin was inhibited by latrunculin A and cytochalasin D. In addition, treatment of intact cells with 1 μM cytochalasin D and 200 nM latrunculin B (which sequesters actin monomers [11, 55]) caused spots to stop moving within 2 min, prevented the formation of new spots, and blocked motility at the cell periphery (Fig. 5; Movies 5A and 5B available at www.cooperlab.wustl.edu). Two agents that alter actin dynamics, swinholide A (which binds actin dimers and severs filaments [6]) and jasplakinolide (which stabilizes F-actin [5]), also inhibited motility of spots (Table I). The effects of all these reagents are consistent with the hypothesis that actin polymerization is required for spot motility and for motility at the cell periphery. If actin polymerization is necessary for spot motility, then filamentous actin should also be a component of spots. Actin, as detected using anti-actin (Fig. 6, *A* and *B*) or rhodamine-phalloidin (Fig. 6 *C*), colocalized with GFP–Arp3 and GFP–CP in spots and at the cell periphery. Other proteins that regulate actin assembly or bind actin filaments, including profilin, vinculin, WASP, VASP, mena, zyxin, ezrin, myosins IIA and IIB, myosin I, and myosin V were not detected in spots using immunofluorescence analysis; these proteins were detected at locations expected for each protein based on previous studies.

Moving Spots Correspond with Fin-like Projections of the Plasma Membrane

Those motile spots in the lamella not associated with macropinosomes were observed in whole mount electron micrographs to correspond with structures on the membrane surface that protruded from the cell (Fig. 7). The structures most often appeared as small fin-like projections that tapered along their length. The structures differed from microvilli, which appeared as finger-like projections with uniform thickness along their length. No vesicles were observed in the vicinity of the projections. The increased staining of the structures obtained after brief saponin-treatment (49) suggests that the structures are dense, consistent with the presence of actin filaments.

Regulation of Actin Assembly and Cell Motility by Small GTPases and Polyphosphoinositides

Small GTPases have been implicated in regulating actin

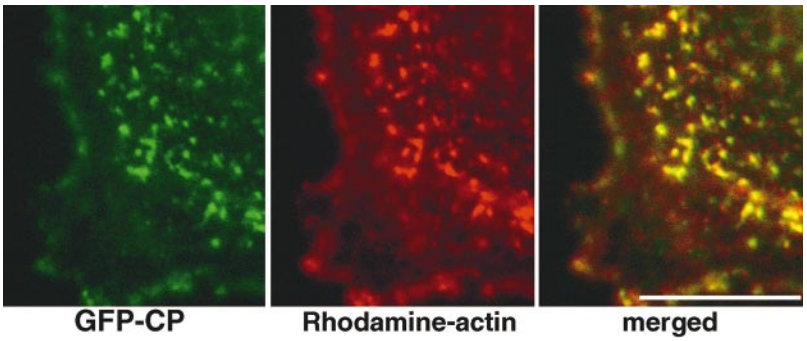

Figure 4. Actin assembly occurs at spots and at the cell periphery. To identify sites of actin assembly, cells were permeabilized with saponin in the presence of rhodamine-labeled G-actin, which indicates sites of actin assembly. Rhodamine-actin (*red*) was incorporated along the edge of the cell and at GFP–CP spots (*green*). *Yellow*, merged image shows regions of overlap of the GFP–CP and rhodamine-actin. Bar, 10 μm.

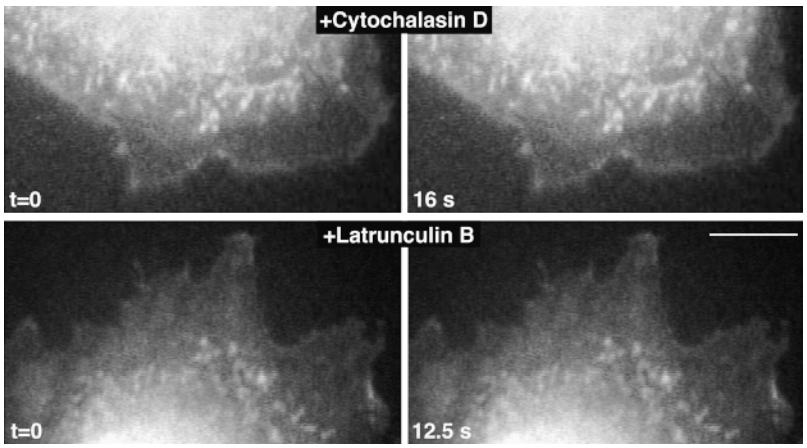

Figure 5. Actin polymerization is required for formation and movement of spots and for movements at the cell periphery. Fibroblasts expressing GFP–CP were filmed within 2 min after treatment with 1 μM cytochalasin D or with 200 nM latrunculin B. Two images separated by 16 or 12.5 s in each video sequence are shown. The distribution of GFP–CP did not change during this interval. Movies of the complete video sequence, including views of the cell before and after treatment with cytochalasin D or latrunculin B are available at www.cooperlab.wustl.edu or from the authors. Bar, 10 μm.

filament dynamics in response to extracellular signals (for review see refs. 22 and 23). To evaluate the role of small GTPases in spot motility and motility at the cell periphery, we microinjected or expressed mutant forms of RhoA, rac1, and cdc42 (Fig. 8; Movies 8A–8F available at www.cooperlab.wustl.edu). Expression of a dominant-negative form of rac1 (N17rac1) resulted in the loss of spots, a loss of motility at the periphery of the cell, and a concomitant diffuse distribution for both GFP–CP and GFP–Arp3 (12 out of 13 cells) (Fig. 8 *B*; Movie 8B available at www.

Table I. Summary of the Effects of Pharmacologic and Biochemical Reagents on Dynamics of GFP–CP and GFP–Arp3

Reagent tested	Concentration and duration	Effects	
		On spots	On the cell periphery
Inhibitors of actin polymerization			
Cytochalasin D	1 μM, 2 min	Motility inhibited	Motility inhibited
Latrunculin B	200 nM, 2 min	Motility inhibited	Motility inhibited
Swinholide A	50 nM, 5 min	Motility inhibited	Motility inhibited
Jasplakinolide	1.4 μM, 15 min	Motility inhibited	Motility inhibited
Microtubule reagents			
Nocodazole	1 μM, 30 min	No effect	No effect
Taxol	10 nM, overnight	No effect	No effect
Myosin inhibitors			
Butanedione monoxime	10 mM, 15–60 min	No effect	n.d.*
KT5926	100 nM, 15–60 min	No effect	No effect
Kinase and phosphatase inhibitors			
Okadaic acid	100 nM, 30 min	No effect	No effect
Genistein	25 μM, 30 min	No effect	No effect
Expression/modification of small G proteins			
N17Rac	2 h after cDNA injected	CP and Arp3 diffuse	Motility inhibited
V12Rac	2 h after cDNA injected	No effect	No effect
N17cdc42	2 h after cDNA injected	CP and Arp3 diffuse	Motility inhibited
V12cdc42	2 h after cDNA injected	No effect	No effect
V14RhoA	15–60 min after injection at 300 μg/ml	No effect	No effect
Exoenzyme C3 transferase	10–50 min after injection at 200 μg/ml	CP and Arp3 diffuse	Motility inhibited
Growth factors and mitogens			
Phorbol myristic acid	2 μM, 30 min	No effect	Motility increased
EGF	500 ng/ml, 60 min	No effect	No effect
Reagents that alter phosphoinositide metabolism			
Wortmannin	100 nM, 60 min	No effect	No effect
Anti-PIP2	15–60 min after injection at 11 mg/ml	No effect	No effect
PIP2-binding peptide	15–60 min after injection at 10 mM	No effect	No effect
PI 5-kinase	2 h after cDNA injected	Increased movement	n.d.
Effects of cofilin regulators			
LIMK-1	2 h after cDNA injected	Inhibited movement	Inhibited
Mutant LIMK-1	2 h after cDNA injected	No effect	No effect

**n.d.*, not determined. Changes in morphology at the cell periphery made it difficult to observe motility at the edge of the cell.

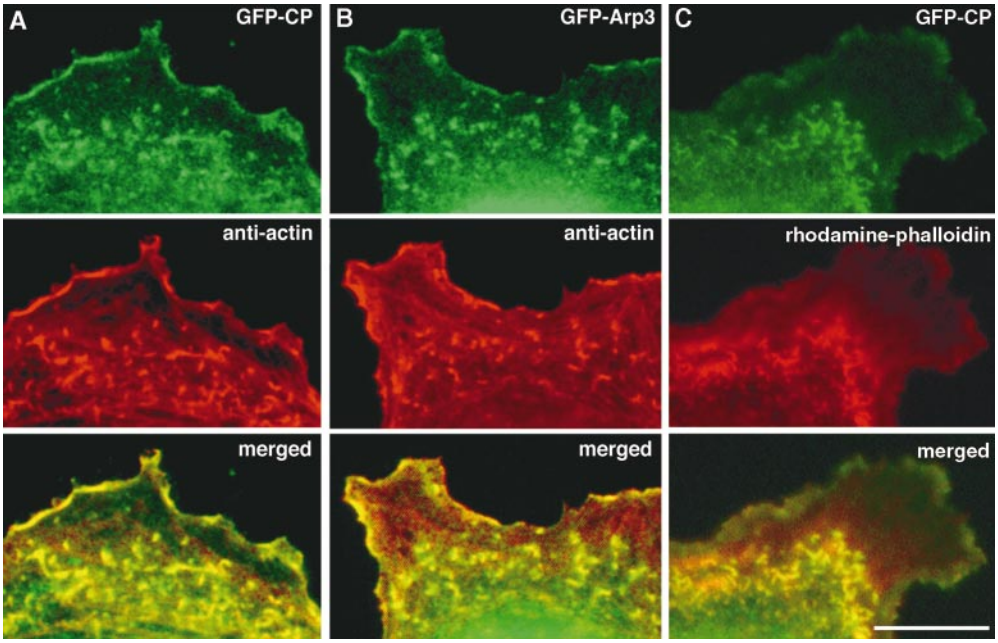

Figure 6. Actin colocalizes with Arp2/3 complex and CP at the cell periphery and on spots in the lamella. (*A* and *B*) Fibroblasts expressing GFP–CP (*A*, *green*) or GFP–Arp3 (*B*, *green*) were fixed and labeled with anti-actin mAb C4 (*red*). *Yellow*, merged images show the colocalization of actin with Arp2/3 complex or CP. (*C*) Actin filaments, detected with rhodamine-phalloidin are components of spots. Cells expressing GFP–CP (*green*) were fixed and stained with rhodamine-phalloidin (*red*). *Yellow*, merged image shows the colocalization of F-actin with CP. Bar, 10 μm.

cooperlab.wustl.edu). Expression of a dominant-negative form of cdc42 (N17cdc42) (Fig. 8 *D*; Movie 8D available at www.cooperlab.wustl.edu) or injection of *Clostridium botulinum* C3 toxin (Fig. 8 *F*; Movie 8F available at www.cooperlab.wustl.edu) resulted in a similar diffuse distribution for GFP–Arp3 in most cells, however, the effect was not as complete as with N17rac1 with a few spots remaining in some cells. Expression of activated forms of rac1 (v12rac) (Fig. 8 *A*; Movie 8A available at www.cooperlab.wustl.edu) or cdc42 (V12cdc42) (Fig. 8 *C*; Movie 8C available at www.cooperlab.wustl.edu) or microinjection of activated RhoA (v14RhoA) (Fig. 8 *E*; Movie 8E available at www.cooperlab.wustl.edu) did not alter the distributions of the GFP-tagged proteins or motility of spots. These results suggest that the small GTPases, RhoA, cdc42, and especially rac1, participate in the formation and movement of spots and in motility at the cell periphery.

Polyphosphoinositides have been implicated as mediators of actin assembly (27), and as effectors of Rho GTPases (24, 60). To investigate the role of polyphosphoinositides in spot motility, we overexpressed type 1 alpha PI 5-kinase. PI 5-kinase catalyzes the synthesis of PI 4,5-P_2, and phosphatidylinositol 3,4,5-trisphosphate (PI 3,4,5-P_3), which may increase actin polymerization by effects on actin-binding proteins (27). In cells expressing PI 5-kinase, long-lived spots of GFP–CP and GFP–Arp3 having an average lifetime of 18.5 s moved through the cytoplasm at an average rate of 0.33 μm/s (SEM 0.05, $n = 12$), twofold greater than spots in the lamella of control cells (Fig. 9; Movie 9 available at www.cooperlab.wustl.edu). Wortmannin, an inhibitor of phosphatidylinositol 3-kinases, did not affect the motility of spots in the lamella when tested at concentrations (100 nM for up to 30 min) that alter 3-phosphoinositide levels in other systems. Together, these data suggest that 5-phosphoinositides, but not 3-phosphoinositides, participate in the assembly of F-actin in cells.

Role of Other Proteins in Spot Motility

Several recent studies implicate cofilin, which promotes actin filament depolymerization, as a regulator of actin dynamics in vivo. Phosphorylation of cofilin at Ser-3 inactivates cofilin (1, 43) via the activity of LIM–kinase-1 (LIMK-1) (2, 70). To investigate a role for cofilin in motility of spots and the cell periphery, we expressed LIMK-1 in cells expressing GFP–CP. Expression of LIMK-1 resulted in the loss of motility at the cell periphery and a decrease in the motility of GFP–CP spots in 11 out of 14 injected cells (Fig. 10 *A*; Movie 10A available at www.cooperlab.wustl.edu). In a majority of cells expressing LIMK-1, aggregates of GFP–CP accumulated. In contrast, expression of a mutant form of LIMK-1 that lacks a portion of the catalytic kinase domain had little effect on the motility of spots or movements at the cell periphery (Fig. 10 *B*; Movie 10B available at www.cooperlab.wustl.edu). These data suggest that cofilin participates in the actin filament dynamics required for movement of spots and for motility at the cell periphery.

To determine whether or not myosin motor activity was necessary for spot motility, we treated cells with butanedione monoxime (BDM), which inhibits the ATPase activity of myosins (12) and with KT5926, which inhibits myosin light chain kinase (46). Spots continued to move in cells treated for 15 min in 10 mM BDM, a concentration known to inhibit postmitotic cell spreading and whole cell motility in fibroblasts (12). KT5926 at 160 nM for up to 60 min also did not effect spot motility. These results, together with the absence of several myosins from spots by immunofluorescence analysis, suggest that myosins are not involved in spot motility. Whether or not BDM or KT5926 inhibit all unconventional myosins is not known, thus, these results do not rule out the participation of all myosins.

A summary of the effects on spot motility and motility at the cell periphery of all agents tested is presented in Table I. Each agent was used at concentrations and durations

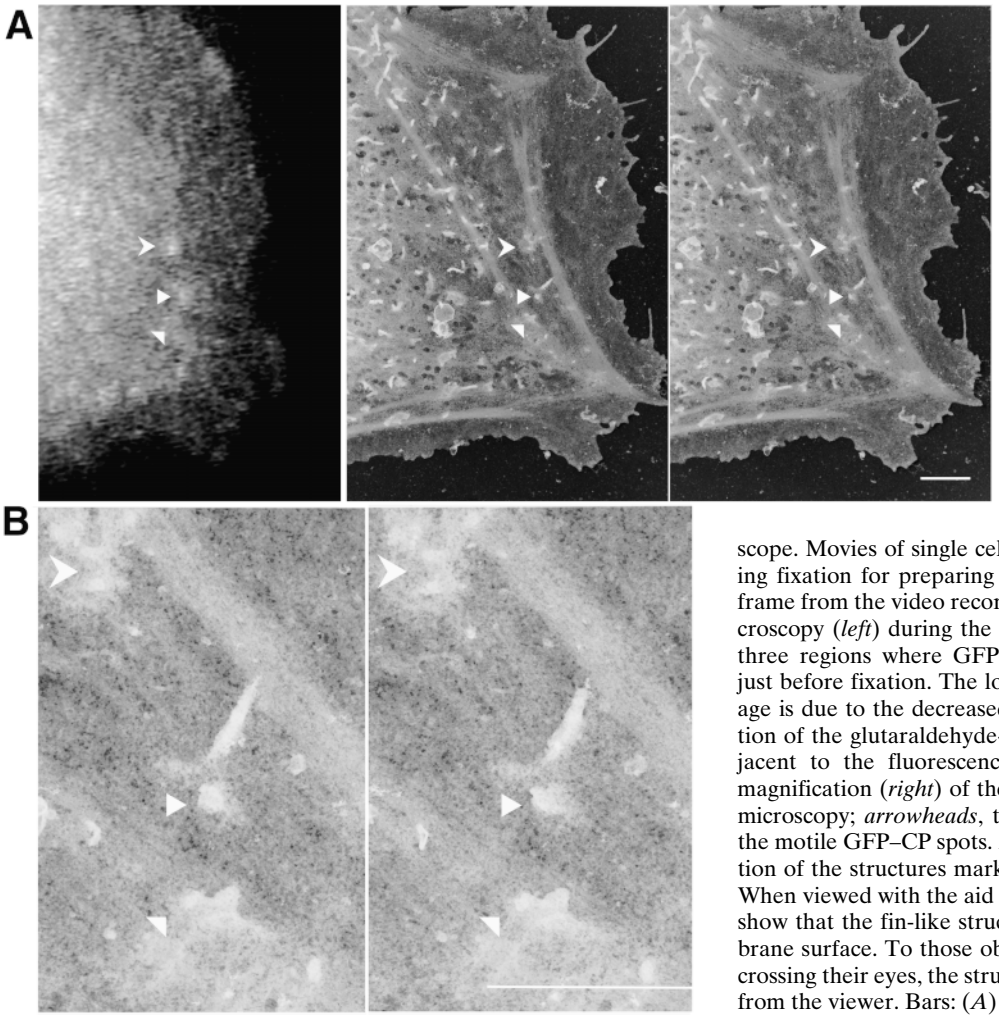

Figure 7. Motile spots in the lamella are small, fin-like projections that taper toward their ends. Cells expressing GFP–CP were grown on electron microscopic grids, placed in a flow chamber on the fluorescence microscope to document the movement of spots and the lamella, and then prepared as whole mounts for viewing in the electron microscope. Movies of single cells were obtained before and during fixation for preparing the whole mounts. *A* shows one frame from the video recording taken using fluorescence microscopy (*left*) during the addition of fixative. *Arrowheads*, three regions where GFP–CP spots were actively moving just before fixation. The low quality of the fluorescence image is due to the decreased signal to noise ratio upon addition of the glutaraldehyde-containing fixation solution. Adjacent to the fluorescence view is a stereo pair at low magnification (*right*) of the same cell viewed using electron microscopy; *arrowheads*, three structures corresponding to the motile GFP–CP spots. A stereo pair at higher magnification of the structures marked by arrowheads is shown in *B*. When viewed with the aid of stereo viewers, the stereo pairs show that the fin-like structures project out from the membrane surface. To those observers who view stereo pairs by crossing their eyes, the structures will appear to project away from the viewer. Bars: (*A*) 2 μm; (*B*) 1.5 μm.

known to be effective in other studies. Agents that did not affect spot motility or motility at the cell periphery included the protein phosphatase inhibitor, okadaic acid, the tyrosine kinase inhibitor, genistein, and two agents that disrupt microtubule function, taxol and nocodazole. Depletion of serum from the culture medium for 24 h did not suppress motility of spots or the cell periphery in PtK1 cells and addition of the growth factors EGF and PDGF to serum-starved cells did not enhance the motility. Phorbol 12-myristic acetate (PMA) stimulated cell spreading and the accumulation of GFP–CP at the cell periphery, but motile spots continued to be observed in the lamella for up to 60 min after addition of PMA.

Discussion

The dynamic distributions of GFP–Arp3 and GFP–CP revealed the nonsteady-state behavior of actin assembly in living cells. Actin assembled at the cell periphery to extend the lamella and create ruffles. Actin assembly also occurred throughout the lamella at small spots that likely represent dynamic regions of the cortical actin cytoskeleton. The GFP-tagged proteins are valuable new tools for observing where actin assembles and investigating how actin assembly is controlled in living cells. Other methods for observing actin assembly in living cells, such as photobleaching of rhodamine-actin (63) and photoactivation of "caged" actin (58), two techniques that require microinjection of modified actin, did not reveal these novel sites of actin assembly. The motile spots, which were observed in several different types of cells, may prove to be as useful as *Listeria* monocytogenes for understanding the mechanism for actin assembly in vivo.

Mechanism for Actin-based Motility at Spots and at the Leading Edge

Movement of spots and movement at the cell periphery have many properties in common, including the participation of Arp2/3 complex and CP, a requirement for actin polymerization, regulation by Rho family GTPases and the participation of the actin-binding protein, cofilin. We propose that the biochemical mechanisms underlying extension of lamella at the cell periphery and the formation and movement of spots are related. One possible mechanism for the actin-based motility observed in spots and at the cell periphery is shown in Fig. 11.

We propose that Arp2/3 complex is activated near the

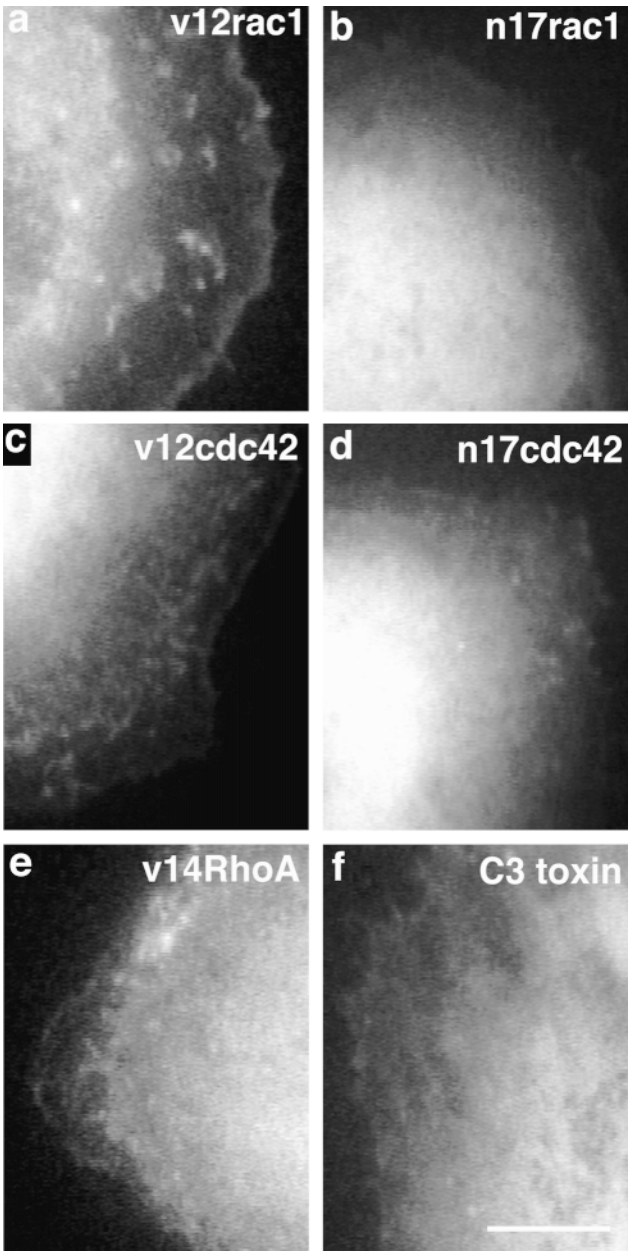

Figure 8. Small GTPases regulate spot formation and motility at the cell periphery. GFP–Arp3 fibroblasts were microinjected with plasmids for expression of constitutively active (*v12*) or dominant-negative forms (*n17*) of rac1 and cdc42. Other cells were microinjected with an active form of RhoA (*v14 RhoA*) protein (0.5 mg/ml) or with C3 toxin (160–200 μg/ml). GFP–Arp3 was diffusely distributed in cells containing N17rac1, N17cdc42, or C3 toxin. In contrast, motile spots were observed in cells containing V12rac1, V12 cdc42, or V14RhoA. Movies of the complete video sequence for each panel are available at www.cooperlab.wustl.edu or from the authors. Bar, 10 μm.

membrane in response to an unidentified signal. The mechanism for the localized activation or recruitment of Arp2/3 complex at sites of assembly is unknown, but small G proteins may participate since the distribution of Arp2/3 complex was diffuse when Rho family GTPases were inhibited. Arp2/3 complex is a good candidate for initiating actin assembly because it nucleates actin assembly in vitro to create free barbed ends (44). Nucleation activity of the Arp2/3 complex is activated by ActA (67), the *Listeria* protein required for actin assembly at the bacterial surface. An unknown factor may activate Arp2/3 complex similarly to ActA to promote de novo actin assembly at spots and at the leading edge. Actin polymerization would then proceed at the new free barbed ends until blocked by capping protein. The short, newly assembled actin filaments would subsequently disassemble via the action of cofilin, which promotes actin filament disassembly (7, 31, 40, 50). The formation of stable aggregates of GFP–CP (this paper) and actin (2) in cells expressing LIMK-1, which inactivates cofilin, is consistent with the idea that cofilin functions to disassemble actin formed at spots, but a role for cofilin in initiating actin assembly has not been ruled out.

At first glance, the presence of both Arp2/3 complex, which promotes assembly, together with CP, which limits assembly, at sites of actin assembly at the leading edge may seem paradoxical. However, the actin filaments in an extending lamella or in the cortex are generally short (33, 56), thus, the concomitant appearance of Arp2/3 and CP at the leading edge may represent actin nucleation followed by actin assembly and subsequent capping within a region below the level of resolution of the light microscope. In addition, some studies (24, 52), but not all (16), suggest that PIP_2-mediated uncapping of barbed ends at the leading edge is one mechanism for creating free barbed ends to initiate new actin assembly, thus, CP and PIP_2 at the membrane, together with Arp2/3 complex, may promote actin assembly.

Although the mechanism for motility at the periphery and for spot movement may be related, lamellipodia, filopodia, or ruffles do not appear to form via the coalescence or stabilization of new actin filaments formed at spots. Neither the location nor the direction of moving spots correlated with motility at the cell periphery. Indeed, the loss of spots in migrating cells, together with the concomitant increase in GFP–Arp3 at the cell periphery, suggests that Arp2/3 complex and CP are recruited to the cell periphery for actin assembly that drives the lamella forward at the expense of motile spots.

Regulation of Actin Dynamics

The Rho family GTPases regulate the assembly different actin structures (22), however, few studies have investigated directly the effects of the Rho family GTPases on actin dynamics in vivo. It is likely that the Rho GTPases act cooperatively to regulate actin dynamics in vivo. In our studies using PtK1 cells, rho, rac1, and cdc42 were involved in initiating actin assembly at the cell edge and at sites within the cortical actin network. Rho, rac, and cdc42 may act by recruiting components necessary for actin assembly because spots disappeared and GFP–Arp3 became diffuse upon inhibition of these GTPases. In contrast, actin assembly in cell-free extracts was found to require specifically cdc42 (35, 42, 72). A specific role for rho in actin assembly in cell-free extracts has not consistently been tested in the different cell-free systems. The involvement

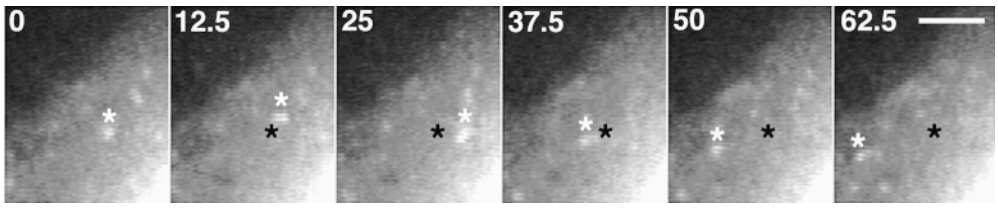

Figure 9. Expression of PI 5-kinase promotes the movement of spots. Nuclei were injected with DNA for expression of PI 5-kinase and cells were observed 2–3 h after injection. A white asterisk follows one moving spot and a black asterisk in each frame marks the initial position of the spot. Numbers indicate elapsed time in seconds. The complete video sequence for these data are available at www.cooperlab.wustl.edu or from the authors. Bar, 10 μm.

of multiple G proteins for actin assembly in vivo as detected here using GFP-tagged proteins suggests that some components of the system for actin assembly in cells may be lost or inactivated in cell-free extracts.

5-Phosphoinositides also may regulate the actin dynamics observed with GFP–Arp3 and GFP–CP where movement of spots through the cytoplasm was stimulated in cells expressing PI 5-kinase. It is not clear how phosphoinositides promote the movement of spots or regulate actin assembly. Polyphosphoinositides, particularly PI 4,5-P_2, may promote actin polymerization at membranes by directly stimulating actin nucleation activity of Arp2/3 complex or by uncapping and preventing capping of actin filament barbed ends (25, 52). The effects of phosphoinositides on the actin nucleating activity of Arp2/3 complex have not been tested. Alternatively, polyphosphoinositides may promote actin assembly by activating or localizing small G proteins and their guanine nucleotide exchange factors at the membrane, which in turn may recruit nucleation factors such as Arp2/3 complex. Support for this idea is that RhoGDI, an inhibitor of guanine nucleotide exchange activity, blocks phosphoinositide-stimulated actin assembly in cell free extracts (35). In addition, polyphosphoinositides possess GEF-like activity (71), and could act directly to maintain a sufficient supply of activated small G protein at the membrane.

The function of the novel sites of assembly is unknown. The lack of correlation of motile spots with various markers for endocytosis (Schafer, D.A., unpublished results) and the absence of membrane vesicles in the vicinity of the motile spots does not support a role for the transient actin structures in processes that involve movement of components across the membrane. In addition, spots are not precursors of the extending lamella. An alternate possibility is that the transient actin filament structures formed at spots serve as scaffolding upon which proteins involved in signal transduction are recruited or assembled. We have localized CD2AP, a protein having multiple SH3 domains (15), with GFP-CP spots (Schafer, D.A., unpublished results). CD2AP has been implicated as a molecular scaffold required for organization of surface receptors and for polarizing the cytoskeleton during T cell activation (15). At spots and at the cell periphery, CD2AP may bind components required for actin assembly resulting in localized bursts of actin assembly. On the other hand, the actin cytoskeleton in living cells may be constitutively poised to assemble actin at the edge of the lamella and at locations within the lamella. In cells like PtK1 fibroblasts that are

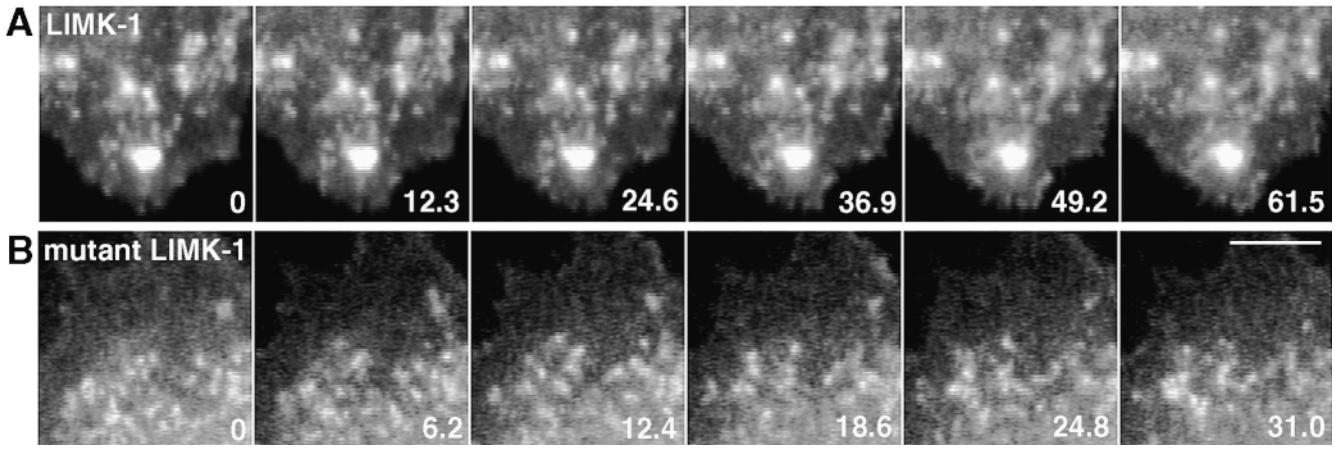

Figure 10. Expression of LIMK-1, which inactivates cofilin, results in decreased motility of spots and of the cell periphery. Nuclei were injected with DNA for expression of LIMK-1 (*A*) or a mutant form of LIMK-1 lacking a portion of the catalytic kinase domain (*B*) and cells were observed 2–3 h after injection. Expression of LIMK-1 caused spots to stop moving and, in some cases, to the formation of aggregates of GFP–CP. In contrast, expression of an inactive mutant form of LIMK-1 did not affect spot movement or motility at the cell periphery. Numbers indicate elapsed time in seconds. Movies that show the complete video sequences for these data are available at www.cooperlab.wustl.edu or from the authors. Bar, 10 μm.

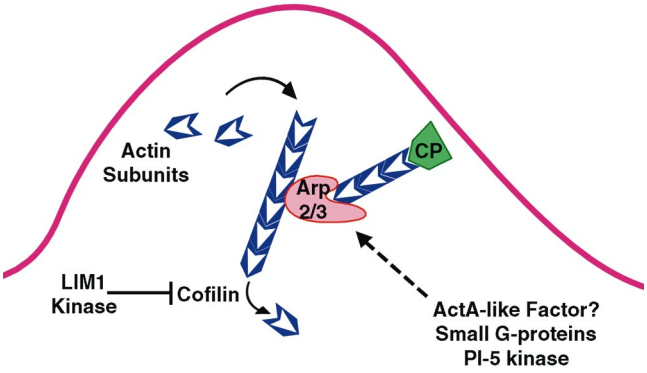

Figure 11. Model for signal-induced actin assembly at motile spots and at the leading edge. When Arp2/3 complex is activated, free barbed ends are created near the membrane, which assemble actin (*blue*). The mechanism for the local activation or recruitment of Arp2/3 complex at sites of assembly is unknown. Small G proteins, phosphoinositides, and an endogenous ActA-like factor may participate in the recruitment or activation of Arp2/3 complex at the membrane. Actin polymerization then proceeds at the new free barbed ends until blocked by CP. The short-lived actin filaments would subsequently disassemble via the action of cofilin. LIM–kinase-1, which inactivates cofilin and is also stimulated by GTP-rac, may regulate the disassembly of actin filaments. Spatial regulation of the processes of actin assembly and disassembly could result in the growth of filaments in the extending lamella and their subsequent disassembly at sites further from the leading edge.

not actively migrating, the moving spots within lamella and the meanderings at the cell periphery may occur when the activation energy for de novo actin assembly is reached focally at a stochastic rate.

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