Expression of Y53A-Actin in Dictyostelium Disrupts the Cytoskeleton and Inhibits Intracellular and Intercellular Chemotactic Signaling∗§

Shi Shu†, Xiong Liu†, Paul W. Kriebel‡, Myoung-Soon Hong§, Mathew P. Daniels§, Carole A. Parent‡, and Edward D. Korn†

From the †Laboratory of Cell Biology, NHLBI, the ‡Laboratory of Cellular and Molecular Biology, Center for Cancer Research, NCI, and the §Electron Microscopy Core Facility, NHLBI, National Institutes of Health, Bethesda, Maryland 20892

We showed previously that phosphorylation of Tyr\(^{53}\), or its mutation to Ala, inhibits actin polymerization in vitro with formation of aggregates of short filaments, and that expression of Y53A-actin in Dictyostelium blocks differentiation and development at the mound stage (Liu, X., Shu, S., Hong, M. S., Levine, R. L., and Korn, E. D. (2006) Proc. Natl. Acad. Sci. U.S.A. 103, 13694–13699; Liu, X., Shu, S., Hong, M. S., Yu, B., and Korn, E. D. (2010) J. Biol. Chem. 285, 9729–9739).

We now show that expression of Y53A-actin, which does not affect cell growth, phagocytosis, or pinocytosis, inhibits the formation of head-to-tail cell streams during cAMP-induced aggregation, although individual amoebae chemotax normally. We show that expression of Y53A-actin causes a 50% reduction of cell surface cAMP receptors, and inhibits cAMP-induced increases in adenyl cyclase A activity, phosphorylation of ERK2, and actin polymerization. Trafficking of vesicles containing adenyl cyclase A to the rear of the cell and secretion of the ACA vesicles are also inhibited. The actin cytoskeleton of cells expressing Y53A-actin is characterized by numerous short filaments, and bundled and aggregated filaments similar to the structures formed by copolymerization of purified Y53A-actin and wild-type actin in vitro. This disorganized actin cytoskeleton may be responsible for the inhibition of intracellular and intercellular cAMP signaling in cells expressing F-Y53A-actin.

Dictyostelium discoideum grows as single-cell amoebae on yeast, bacteria, or soluble medium with a generation time in liquid medium of about 8–12 h. Starvation initiates a 24-h developmental cycle, beginning with pulsed secretion of cAMP by a fraction of the amoebae creating cAMP gradients, which induce neighboring cells to polarize and chemotax toward these signaling/aggregation centers (1). The chemotaxing cells, in turn, are stimulated to synthesize and secrete cAMP, thereby relaying the cAMP signal to more distant cells in spatial and temporal waves formed by the pulsed secretion of cAMP and its degradation by extracellular phosphodiesterase (2).

Head-to-tail streams of chemotaxing cells migrate to the aggregation centers forming mounds composed of 100,000–200,000 cells, which differentiate into pre-stalk and pre-spore cells. The mounds transform, in several stages, into motile, multicellular slugs with the pre-stalk cells in their anterior and the pre-spore cells in their posterior. The slugs migrate until culmination is initiated with the pre-stalk cells forming a stalk rising from a basal disc anchored on the substratum, and the pre-spore cells moving up to the top of the stalk to form a fruiting body containing mature spores. Once favorable conditions arise, or when spores are placed in nutrient medium, the spores germinate and the life cycle starts again.

The actin cytoskeleton has critical roles in all stages of the life cycle. For example, actin, which is generally uniformly distributed around the cell cortex of vegetative (growing) cells, becomes enriched in the cleavage furrow of dividing cells where actomyosin II is required for cytokinesis of cells grown in suspension culture (3, 4), and facilitates cytokinesis of cells grown on a substrate (5, 6). Actin also has an essential role in phagocytosis, macropinocytosis, and formation of filopodia and pseudopodia in vegetative amoebae (7). The motility of polarized, chemotaxing amoebae is driven by waves of actin polymerization in the lamellipodia at the leading edge, which is correlated with the cAMP waves (8, 9), and actomyosin II-mediated contraction at the rear and sides inhibits formation of pseudopodia in those regions and facilitates forward movement of the cell body (10).

Actin Tyr\(^{53}\) becomes phosphorylated halfway (12 h) into the developmental cycle, reaching a maximum of ~50% phosphorylated actin in mature spores (~36 h), and rapid dephosphorylation precedes germination when spores are placed in growth medium (11–14). Tyr\(^{53}\) phosphorylation also occurs when vegetative cells are grown to high concentration or subjected to stress (15–18). The physiological function of Tyr\(^{53}\)-phosphorylated actin is not known.

In previous papers (14, 19), we reported some of the biochemical and biophysical differences between phosphorylated and unphosphorylated actin. Briefly, the phosphate group on Tyr\(^{53}\) of phosphorylated actin hydrogen bonds with amino acids in and near the DNase I-binding loop of actin (residues 40–50) partially stabilizing the conformation...
Inhibition of cAMP Signaling by Y53A-Actin Mutant

of the D-loop,2 which significantly inhibits the rate of actin polymerization, increases actin critical concentration, and leads to the formation of fragmented filaments. More recently, to determine whether these differences are due to the addition of phosphate or to the loss of Tyr, we studied the biochemical and biophysical properties of purified Y53F-, Y53A-, and Y53E-actin mutants expressed in and purified from Dictostelium (20). In brief, we found no difference between Y53F-actin and endogenous wild-type actin, but the Y53A- and Y53E-actins differed substantially from WT-actin: the affinity for DNase I was reduced, the rate of nucleotide exchange was increased, filament elongation was inhibited, the critical concentration was increased, and polymerized actin was in the form of small oligomers and imperfect filaments. We concluded that Tyr or Phe at position 53, but not Ala or Glu, maintains the functional conformations of the D-loop that are required not only for interaction with DNase I, but for the interactions between actin subunits in F-actin (21, 22).

To facilitate their purification and separation from endogenous actin, the expressed mutant actins in the previous experiments had an N-terminal FLAG tag, with a tobacco etch virus protease cleavage site between the FLAG tag and the actin N terminus for removal of the FLAG tag after purification of the actins. We noted that the amoebae expressing the F-TEVCS-Y53A-actin (to the extent of 30% of total actin) developed more slowly and did not develop beyond the mound stage. We now report in detail the effects of expression of the Y53A-actin mutant on cell growth, phagocytosis, pinocytosis, cell streaming in a cAMP gradient, and morphological and biochemical development. These results may be informative on the role of Tyr53-phosphorylated actin in development and the reaction to stress.

EXPERIMENTAL PROCEDURES

Plasmids and Cell Lines—Dictostelium actin cDNA (a generous gift of G. Gerisch, Max Planck-Institute für Biochemie, Martinsried, Germany) was mutated at position Tyr53 to Ala by PCR. A FLAG sequence (DYKDDDDK) was added to the N terminus of the mutant actin (F-Y/A), and also to WT-actin (F-Y/Y) as a control for possible effects of the FLAG tag. Actin cDNAs were cloned onto a pTIKL vector, which has an actin 15 terminus of the mutant actin (F-Y/A), and also to WT-actin (F-Y/Y) as a control for possible effects of the FLAG tag. Actin cDNAs were cloned onto a pTIKL vector, which has an actin 15 promoter and G-418 resistance (23). Expression plasmids were introduced into Dictostelium AX3 cells using a gene pulser (Bio-Rad) (24).

For coexpression of actin and ACA in aca− cells, the actin and promoter sequences amplified by PCR from pTIKL F-Y/Y-actin and pTIKL F-Y/A-actin were subcloned into pCV5 using restriction enzymes XbaI and BglII and the sequences were confirmed. Then equal amounts of pCV5 ACA-YFP (25) and pCV5 actin were mixed and transfected into aca− cells (25) by electroporation.

2 The abbreviations used are: D-loop, DNase I-binding loop; ACA, adenyllylcyclase A; CAR, cAMP receptor; pERK2, phospho-ERK2; PB, phosphate buffer; WT, endogenous wild-type actin; F-Y/A, expressed FLAG-tagged actin with Y53A mutation; F-Y/Y, expressed FLAG-tagged wild-type actin; F-TEVCS, FLAG-tobacco etch virus protease cleavage site; TORC, target of rapamycin complex 2; TRITC, tetramethylrhodamine isothiocyanate.

Cell Culture and Differentiation—Dictostelium AX3 cells were grown at 21 °C in HL5 medium (number LG0101, Formedium) containing 60 μg/ml each of penicillin and streptomycin (26), and cells transformed with cDNAs (with and without an N-terminal FLAG tag) were selected and maintained in the same medium containing 16 μg/ml of G418 (number 61-234-RG, Cellgro). Cells were differentiated to the chemotaxis-competent stage as described (27, 28). Briefly, log-phase cells (~2 × 10^6 cells/ml) were harvested by low-speed centrifugation, washed, and re-suspended in developmental buffer (5 mM Na_2HPO_4, 5 mM KH_2PO_4, pH 6.2, 2 mM MgSO_4, 0.2 mM CaCl_2) at 2 × 10^6 cells/ml (25), and developed in suspension at 100 rpm for 5–6 h with pulses of 50 μl of 4 μM cAMP into 5 ml of cells every 6 min. Differentiated cells were processed according to the assay to be performed.

Cell Growth, Phagocytosis, and Pinocytosis—To measure cell growth, 50 ml of cells (2 × 10^6 cells/ml) were cultured in a 250-ml flask on a rotary shaker at 145 rpm and 21 °C, and cells were counted daily using a cellometer (AutoT4 cell count, Nexcelom Bioscience). Phagocytosis and pinocytosis assays were performed as described (29) with some modifications (30). For phagocytosis, cells were collected, washed, and re-suspended at 5 × 10^6/ml in Sorenson’s buffer (17 mM KH_2PO_4, 2 mM Na_2HPO_4, pH 6.1), and fluorescein isothiocyanate-conjugated, 1-μm polystyrene-latex beads (Polysciences) were added at a ratio of 300 beads per cell. For pinocytosis, cells were harvested, re-suspended in HL5 medium at 5 × 10^6/ml, and TRITC-dextran (Sigma) was added to a final concentration of 2 mg/ml. In both the phagocytosis and pinocytosis assays, 1-ml samples were transferred to a 1.5-ml tube containing 100 μl of 0.4% trypan blue (to quench background fluorescence), the tubes were vortexed and centrifuged at 2,800 rpm for 3 min, and the pellets were washed and re-suspended in 1 ml of Sorenson's buffer. Fluorescence was measured with excitation and emission wavelengths of 485 and 520 nm, respectively, for the phagocytosis assay, and 544 and 574 nm, respectively, for the pinocytosis assay, using an LS55 Luminescence spectrometer (PerkinElmer). All assays were repeated at least three times.

Fluorescence Microscopy—Indirect immunofluorescence microscopy was performed as described (31). For localization of total actin and expressed FLAG-actins, cells were fixed with 1% formaldehyde, 0.1% glutaraldehyde, and 0.01% Triton X-100 in 5 mM sodium phosphate buffer, pH 6.2, at room temperature for 15 min, washed and incubated for 60 min at 37 °C with 100-fold diluted rabbit anti-actin (number A-2066, Sigma) and 500-fold diluted anti-FLAG M2 antibody (number F-3165, Sigma) in PB supplemented with 1% bovine serum albumin and 0.2% saponin. Secondary antibodies, FITC-conjugated goat anti-mouse IgG and Texas Red-goat anti-rabbit IgG (Molecular Probes) were diluted 750-fold. Images were acquired with an LSM-510 laser scanning fluorescence microscope (Carl Zeiss). For immunolocalization during chemotaxis and streaming, cells were first differentiated for 5–6 h, and then extensively washed with PB, placed on a chambered coverslip, and fixed when they had polarized or formed streams.

Cell Streaming and Development—To examine self-streaming, cells were harvested, re-suspended at 5 × 10^6/ml in HL5
medium, and $1.5 \times 10^7$ cells were plated on 60-mm Petri dishes and allowed to adhere for 30 min (30). The medium was then removed by aspiration, and the cells were carefully washed twice with starvation buffer (20 mM MES, pH 6.8, 0.2 mM CaCl$_2$, 2 mM MgSO$_4$), and 2 ml of the same buffer was carefully applied to the plates. Aggregation and streaming were visualized 6–8 h after plating. Images of self-streaming cells were taken every minute with a Discovery V12 stereo microscope (Carl Zeiss) equipped with a PlanApos ×1.0 objective and an AxioCam camera automated by AxioVision 4 software.

Development was observed 24 or 72 h after cells were spotted on 1.5% agarose plates in starvation buffer. Results were recorded with the same stereo microscope used to visualize self-streaming.

**Chemotaxis Assays**—The micropipette assay of cAMP-induced Dictyostelium chemotaxis was performed as described (28). Cells were starved for 5–7 h to induce differentiation to the chemotaxis stage and then re-suspended in PB on a chambered coverslip. A chemotactrant gradient was generated with a microinjector (Eppendorf) attached to a micropipette filled with 1 μM cAMP. Chemotactic migration was continuously recorded at intervals of 10 s using an Axiovert 200 inverted microscope and Axiovision software (Carl Zeiss), and processed with Metamorph software.

To obtain chemotactic metrics, 200 or more cells were analyzed. Images were binarized using the ImageJ software by subtracting the background using a rolling ball algorithm and then thresholding. Tracking cell centers-of-mass was carried out using custom MATLAB code (The Mathworks, Natick, MA). Velocities were determined by dividing cell displacements by the time interval, and velocities were only counted when there was a net displacement of at least 20 μm over a 5-min period. The instantaneous chemotactic index is defined as the cosine of the angle between the vector from a cell center in the current frame to its center in the next frame and the vector from the cell center to the pipette tip.

**Mixed cell experiments** were performed as previously described (25). Briefly, differentiated F-Y/Y or F-Y/A cells ($2 \times 10^7$ cells/ml) were mixed at a 9:1 ratio with GFP-labeled aca$^-$ cells, and the mixtures were diluted 1:10 in PB, pH 6.2, and spotted on a chambered slide. A micropipette containing 10 μM CAMP was placed into the chamber. As the cells migrated toward the micropipette tip, fluorescence and bright field images were captured every 10 s using an Axiovert 200 inverted microscope (Carl Zeiss) and Axiovision software (Carl Zeiss).

3 W. S. Rasband, National Institutes of Health, Bethesda, MD.
Electrophoresis and Immunoblotting—For actin, SDS-PAGE was performed by standard procedures (32) using Tris glycin gels (Invitrogen). Gels were stained with Coomassie Blue or immunoblotted, and then quantified with the Odyssey infrared imaging system (Li-COR Biosciences). Dilution of primary antibodies was 1,000-fold for rabbit anti-actin (number A-2066, Sigma) and 4,000-fold for anti-FLAG M2 (number F-3165, Sigma). Secondary antibodies, goat IRDye800, anti-rabbit IgG (Rockland Immunochemicals), and Alexa Fluor 680 goat anti-mouse IgG (Molecular Probes), were diluted 1:7,000.

For ACA, cAR1, and ERK2, cell lysates were subjected to 10% Criterion gel system and transferred to Immuno-Blot PVDF membrane (Bio-Rad). The membrane was blotted with anti-cAR1 (33) (1:2000), anti-ACA (34) (1:2000), and anti-ERK2 (35) (generous gift of Dr. Jeffrey Segall, Albert Einstein College of Medicine) polyclonal antibodies. Detection was performed by chemiluminescence using a donkey anti-rabbit horseradish peroxidase-coupled antibody (1:5000; GE Healthcare, Piscataway, NJ) and GE Healthcare ECL Western blotting detection reagents (36).

cAMP Binding Assay—Binding of [3H]cAMP to the cell surface was assayed using the (NH4)2SO4 stabilization method (37, 38) with slight modifications. Vegetative and differentiated cells were collected, washed, and re-suspended in 10 mM PB, pH 6.5, with 10 mM DTT to a density of 1 \times 10^6/ml. To aliquots of 100 μl on ice, 100 μl of 100 nm [3H]cAMP (PerkinElmer Life Sciences Inc.) was added, and, after 1 min, 1.2 ml of saturated (4 M) (NH4)2SO4 and 100 μl of 10 mg/ml of bovine serum albumin, in that order. After 6 min on ice, the cells were centrifuged at 4 °C for 2 min at 14,000 rpm. The supernatant was carefully and completely removed, the pellet was dissolved in 200 μl of 1% SDS in 10 mM Tris-HCl, pH 7.5, and the radioactivity measured in a Beckman scintillation counter. To determine nonspecific binding of [3H]cAMP, control cells were first incubated with 10 μl of 1 mM unlabeled cAMP for 5 min on ice.

**TABLE 1**

Activation of myosin ATPase and *in vitro* motility activities of WT-actin and FLAG-actin

| Actin | Myosin ATPase | In vitro motility |
|-------|---------------|------------------|
|       | \(V_{max}\) | \(K_m\) | Filament number | Velocity \(\mu m/s\) |
| WT    | 12.7 | 70 | 223 | 4.10 ± 0.57 |
| F-Y/Y  | 40  | 3.42 | 4 | 3.42 ± 0.78 |
| F-Y/A  | 57  | 3.33 | 50 | 4.01 ± 0.50 |
| F-Y/Y + WT | 11.6 | 127 | 72 | 3.98 ± 0.50 |
| F-Y/A + WT | 15.6 | 151 | 72 | 3.98 ± 0.50 |

**FIGURE 2.** Polymerized F-Y/A-actin forms small aggregates and broken filaments. Actins were polymerized overnight in 100 mM KCl, 2 mM MgCl2, and 5 mM Tris buffer, negatively stained with 0.5% uranyl acetate on formvar/carbon-coated grids, and imaged with a JEM electron microscope. F-Y/A-actin forms very few filaments, and copolymers of F-Y/A-actin and WT-actin form short filaments.

**FIGURE 3.** Expressed FLAG-tagged actins and endogenous actin colocalize in vegetative (A) and chemotaxing (B) *Dictyostelium*. Cells were fixed and total actin was stained with anti-actin antibody (red), and FLAG-tagged actin was stained with anti-FLAG antibody (green). Note that the expression of FLAG-actin (green) relative to total actin (red) was not the same for all cells in each experiment.

**cAMP Stimulation of ERK2 Phosphorylation and TORC2 Activity**—ERK2 phosphorylation was assayed as described (39, 40). Briefly, differentiated cells were re-suspended at a density of \(1 \times 10^7/ml\) in 5 mM PB, pH 6.5, containing 2 mM caffeine, and shaken at 200 rpm for 30 min at room temperature. Cells were then washed twice with PB to remove the caffeine, and re-suspended at a concentration of \(1 \times 10^7/ml\) in ice-cold PB containing 2 mM MgSO4. Aliquots of 2 ml were placed in small plastic cups that were rotated at 200 rpm for 1.5 min, and the cells were stimulated by adding 20 μl of cAMP to final concentrations of 100 μM and 10 μM. Aliquots of 100 μl were removed at the indicated times, cells were lysed by addition of 5 times concentrated SDS-PAGE sample buffer, and, after SDS-PAGE, the gels were blotted with 1,000-fold diluted polyclonal anti-

**FIGURE 4.** Inhibition of cAMP Signaling by Y53A-Actin Mutant

Inhibition of cAMP Signaling by Y53A-Actin Mutant

**FIGURE 5.** Inhibition of cAMP Signaling by Y53A-Actin Mutant

...
Expression of F-Y/A-actin disrupts the actin cytoskeleton. Representative electron micrographs of the actin cytoskeleton of polarized WT cells, F-Y/Y cells, and F-Y/A cells were prepared as described under “Experimental Procedures.” The boxes in the low magnification images (top row) show the regions between the tail and nucleus of the cells that are seen at higher magnifications in the second and third rows. The cytoskeletons of WT cells consist of a largely homogeneous array of long actin filaments. In contrast, the cytoskeletons of F-Y/A cells contain fewer long filaments and many of the filaments are bundled, leaving large open spaces. In addition, the F-Y/A cytoskeletons display many filament ends, suggesting the presence of short filaments (arrowheads in bottom row indicate examples), and short filament bundles (arrows indicate examples). The actin cytoskeletons of F-Y/Y cells are slightly less homogeneous than those of the WT cells but resemble them more closely than they do the cytoskeletons of the F-Y/A cells. Bars are 2 μm (top row) and 0.2 μm (middle and bottom rows).

Inhibition of cAMP Signaling by Y53A-Actin Mutant

cAMP stimulation of TORC2 activity was determined by assaying phosphorylation of PKBR1 as described (41). Cells were prepared as described for ERK2 phosphorylation, stimulated with 1 μM cAMP, and SDS-PAGE gels were blotted with phospho-PKC (pan) rabbit antibody (number 9101, Cell Signaling Technology) diluted 1:1000. All experiments were performed at least three times.

Instant Actin Polymerization and Adenylyl Cyclase Activity Assays—The time course of actin polymerization was determined as described (42, 43). Differentiated cells were harvested, washed with PB, and re-suspended (3 × 10^7 cells/ml) in 10 mM PB containing 2 mM MgSO_4_ and 3 mM caffeine. Cells were stimulated with 1 μM cAMP, fixed, and stained in buffer containing 3.7% formaldehyde, 0.15% Triton X-100, 250 mM TRITC-phalloidin, 20 mM KCl, 10 mM Pipes, 5 mM EGTA, and 2 mM MgCl_2, pH 6.8. F-actin was quantified by measuring TRITC-phalloidin fluorescence with a LS55 Luminescence spectrometer.

Adenylyl cyclase activity was assayed as described (36, 44). Briefly, differentiated cells were treated with 2 mM caffeine in PB for 30 min, then washed twice with PB plus 2 mM MgSO_4, re-suspended at 8 × 10^7 cells/ml, and shaken on ice for 10 min. Adenylyl cyclase activity was assayed at room temperature before and after the addition of 10 μM cAMP.

Protein Purification—Dictyostelium FLAG-tagged actins were purified by FLAG affinity chromatography on an anti-FLAG antibody-conjugated resin (Sigma) eluted with 0.1 mg/ml of FLAG-peptide (EZ Biolab, Inc.) as described (45). Endogenous WT-actin was purified from the concentrated flow-through of the FLAG affinity column as described (20). Purified actins were dialyzed against G-buffer (4 mM Tris, 0.1 mM CaCl_2, 0.2 mM ATP, 1 mM DTT, pH 7.5) overnight, centrifuged at 100,000 rpm for 2 h at 4 °C in a Beckman TL-100 centrifuge, and used immediately.

Dictyostelium myosin II and regulatory light chain kinase were purified as described (23). The myosin II regulatory light chain was phosphorylated by addition of the kinase (20 μg/ml), and incubating overnight at 4 °C. Dictyostelium myosin II S1 was expressed and purified as described (46). Myosin and kinase concentrations were determined by the Bradford method with BSA as standard. Actin concentration was determined by absorbance at 290 nm using an extinction coefficient of 0.62 cm^2/mg.

Polymerization and Critical Concentration—Acts at the indicated concentrations in G-buffer were polymerized at 22 °C by adding KCl and MgCl_2 to final concentrations of 100 and 2 mM, respectively. Polymerization was measured by right angle light scattering in an LS55 Luminescence spectrometer (PerkinElmer) with both excitation and emission wavelengths of 360 nm and a slit width of 4 nm. Critical concentrations were determined by incubating various concentrations of actin at room temperature overnight in G-buffer plus 100 mM KCl and 2 mM MgCl_2. The extent of polymerization was measured by light scattering.

Actin-activated Myosin ATPase Activity and In Vitro Motility Assays—steady-state ATPase activity of actin-activated Dictyostelium myosin S1 was assayed as described (46). Briefly, 150 nM S1 was incubated with the indicated concentrations of polymerized acts in 20 mM imidazole, pH 7.5, 4 mM MgCl_2, 25 mM KCl, and 2 mM [γ-32P]ATP, and production of 32P was determined after incubation for 12 min at 32 °C as described (47). In vitro motility activity was assayed at 30 °C according to Ito et al. (48) with Dictyostelium myosin II in 25 mM Hepes, pH 7.4, 2 mM...
ATP, 4 mM MgCl₂ and 10 mM DTT. Actin, 5 μM, was polymerized at room temperature for 3–5 h by addition of MgCl₂ to 2 mM and rhodamine-phalloidin to 5 μM, and kept on ice until use.

Electron Microscopy—Negatively stained images of actin filaments were obtained as described by Liu et al. (14). Actin was polymerized overnight in the presence of 100 mM KCl, 2 mM MgCl₂, and 5 mM Tris, pH 7.5. One drop of the filament suspension (0.1 mg/ml) was placed on a formvar/carbon-coated 400-mesh copper grid and excess fluid was removed by filter paper. The grid was negatively stained with one drop of 0.5% aqueous uranyl acetate for 30 s, and excess fluid absorbed on filter paper. The images were observed with a JEM-1200EX II transmission electron microscope (JEOL). Platinum-carbon replicas of detergent-extracted cytoskeletons of polarized amoebae migrating on glass coverslips were prepared essentially as described (49). The only significant modification was in the extraction buffer in which the concentration of EGTA was increased to 2 mM and MgCl₂ to 5 mM. In brief, the living cells were extracted for 4 min with 1% Triton X-100 in a “cytoplasmic” buffer containing 2 μM phalloidin. The cytoskeletons were fixed with glutaraldehyde and further stabilized with tannic acid and uranyl acetate before ethanol dehydration and critical point drying. Platinum-carbon replicas of the dried cytoskeleton were viewed with a JEM-1200EX electron microscope equipped with an AMT XR-60 digital camera.

RESULTS

Initially, we expressed Y/A-actin without a FLAG tag to avoid any phenotypic changes that a FLAG tag might cause independent of the Y53A mutation. The expression level was determined by mass spectrometric analysis of the relative amounts of the endogenous wild-type and mutant peptide Asp⁵¹–Lys⁶¹ produced by Lys-C proteolysis of the actin band on an SDS-PAGE gel (14). For undetermined reasons, Y/A-actin was consistently expressed at levels of only 1% or less of the total actin, which was too low to determine whether the mutation affected the cell phenotype.

Therefore, knowing from previous results that F-TEVCS-actins are expressed at high levels (20), we expressed N-terminal F-Y/A-actin, and also expressed F-Y/Y-actin as a control for possible effects of the FLAG tag on the properties of the mutant actins. Both constructs were expressed well, accounting for about 30–50% of the total actin in vegetative amoebae (Fig. 1A). The presence of the FLAG tag also provided a tool for separation of the expressed actins from endogenous actin (WT-actin), and their purification (Fig. 1B). We first determined the properties of the purified FLAG-tagged actins to determine whether they differed significantly from the previously characterized expressed actins without a FLAG tag (20).

Biochemical and Biophysical Characterization of F-Y/Y-Actin and F-Y/A-Actin—Purified endogenous WT-actin and F-Y/Y-actin polymerized at essentially the same rate, indicating that the FLAG tag had no effect on polymerization (Fig. 1C). However, F-Y/A-actin polymerized substantially more slowly with no significant polymerization at 40 min, by which time polymerization of WT-actin and F-Y/Y-actin was complete (Fig. 1C). Polymerization of all of the actins reached steady state by 24 h allowing determination of their critical concentrations. The critical concentrations of both WT-actin and F-Y/Y-actin were ~0.2 μM (Fig. 1D), i.e. the N-terminal FLAG tag had no detectable effect on critical concentration, but the critical concentration of F-Y/A-actin was substantially higher, ~1.2 μM (Fig. 1D). Interestingly, F-Y/A-actin copolymerized with WT-actin; the amount of
WT-actin and F-Y/Y-actin and 75% WT-actin (similar to the ratios of the copolymers of 25% F-Y/A-actin and 75% WT-actin, as reflected by the higher 

Thus, the FLAG tag appears to reduce the affinity of actin for myosin, as shown previously for their greater activity in the motility assay than in the ATPase assay, as was shown previously for the mutant and wild-type actins without a FLAG tag (20). However, as in the myosin ATPase assays, the FLAG-tagged actins had a reduced affinity for myosin in the in vitro motility assays resulting in fewer filaments binding to the myosin (Table 1).

Summarizing the in vitro data, other than the somewhat reduced affinity for myosin, the N-terminal FLAG tag had little or no effect on the biochemical and biophysical properties of purified F-Y/A-actin, which were essentially the same as the properties of Y/A-actin without the FLAG tag (20). It is not surprising that an N-terminal FLAG tag reduced the affinity of actin for myosin given that N-terminal residues of actin comprise some of the myosin-binding sites (50–54). An important new observation is that copolymers of F-Y/A-actin and WT-actin formed abnormal filaments and small aggregates. With this background information, we now describe the phenotype of vegetative and developing cells expressing either F-Y/Y-actin or F-Y/A-actin.

Inhibition of cAMP Signaling by Y53A-Actin Mutant

Expression of F-Y53A-Actin Disrupts the Actin Cytoskeleton—Consistent with the copolymerization of the purified F-Y/Y-actin and F-Y/A-actin with WT-actin in vitro, we found that expressed F-Y/Y-actin and F-Y/A-actin colocalized with endogenous actin in both vegetative and chemotaxing cells (Fig. 3). However, whereas the cytoskeletons of WT-cells (Fig. 4) consisted of a mostly homogeneous array of long actin filaments, the cytoskeletons of F-Y/A-cells contained significantly fewer long filaments, and displayed many filament ends, indicative of short filaments, and many bundles and aggregates of both long and short filaments. Also, there were many more large empty spaces in the cytoskeleton of F-Y/A cells than in the WT cells. The actin cytoskeletons of F-Y/Y cells were somewhat less homogeneous than those of WT cells, but much more closely resembled the actin cytoskeletons of WT cells than the cytoskeletons of F-Y/A cells.

Actin Y53A Mutation Does Not Affect Cell Growth, Phagocytosis, or Pinocytosis—Despite the abnormal cytoskeleton of F-Y/A cells, neither of the FLAG-tagged actins affected growth of amoebae in suspension culture (Fig. 5A) or phagocytosis (Fig. 5B), however, pinocytosis was about 40% inhibited in cells expressing either F-Y/Y-actin or F-Y/A-actin (Fig. 5C). This inhibition can be attributed entirely to the FLAG tag, with no effect of the Y53A mutation, as pinocytosis by F-Y/A cells was essentially indistinguishable from pinocytosis by F-Y/Y cells (Fig. 5C). Having shown that expression of F-Y/A-actin, and the resultant abnormal cytoskeleton, had no effect on growth (cytokinesis), phagocytosis, or pinocytosis attributable to the muta-

polymer formed by a mixture of 1.5 μM F-Y/A-actin and 4.5 μM WT-actin was greater than the sum of the polymer formed when the two actins were polymerized separately (Fig. 1E). Negative staining electron microscopy revealed no difference between filaments of F-Y/Y-actin and endogenous WT-actin, but polymerized F-Y/A-actin formed only small aggregates and a few unstable filaments (Fig. 2). Importantly, copolymerization of a 1:3 mixture of F-Y/A-actin and WT-actin (their approximate ratios in cells expressing F-Y/Y-actin and F-Y/A-actin) formed a mixture of short, irregular filaments and some small aggregates (Fig. 2).

The FLAG tag impaired the ability of the expressed actins to activate the MgATPase activity of myosin S1 (compare F-Y/Y to WT, Fig. 1F), and the Y53A mutation was additionally inhibitory (compare F-Y/A-actin to F-Y/Y, Fig. 1F). However, the activation of myosin ATPase by copolymers of 25% F-Y/A-actin or F-Y/Y-actin and 75% WT-actin (similar to the ratios of the FLAG-actins to WT-actin in the transformed cells) were essentially indistinguishable, although still lower than the activity of WT-actin (Fig. 1G); the two copolymers had the same V_max as 100% WT-actin but about twice the K_m of WT-actin (Table 1). Thus, the FLAG tag appears to reduce the affinity of actin for myosin, as reflected by the higher K_m values of the copolymers, but the additional inhibition by the Y/A mutation is reversed when F-Y/A-actin is copolymerized with WT-actin (Fig. 1G and Table 1).

In contrast to their different activations of myosin ATPase activity, the velocities of F-Y/A-actin and F-Y/Y-actin in the in vitro motility assay were very similar to WT-actin (Table 1). The presence of phalloidin in the in vitro motility assay probably converted the aggregates and fragmented filaments of polymerized Y/A-actin into more “normal” filaments, accounting for their greater activity in the motility assay than in the ATPase assay, as was shown previously for the mutant and wild-type actins without a FLAG tag (20). However, as in the myosin ATPase assays, the FLAG-tagged actins had a reduced affinity for myosin in the in vitro motility assays resulting in fewer filaments binding to the myosin (Table 1).
Inhibition of cAMP Signaling by Y53A-Actin Mutant

![Image of cAMP streaming assay](https://example.com/inhibition_camp.png)

**FIGURE 7.** Expression of F-Y/A-actin inhibits cAMP-induced streaming in the needle assay. Vegetative cells were starved for 5–7 h, re-suspended in phosphate buffer and placed on a chambered coverslip, and a cAMP gradient was established by injection of 1 μM cAMP through a micropipette. Cells were imaged every 10 s for —1 h. WT and F-Y/Y cells formed chemotaxing streams aggregating at the needle source of cAMP by 30 min, whereas F-Y/A cells chemotaxed to the needle as individual cells (middle row) that were rounder and less polarized than WT and F-Y/Y cells (bottom row). Bar, 100 μm.

**TABLE 2**

Quantification of chemotaxis of individual WT, F-Y/Y, and F-Y/A cells

| Cells       | Cell number | Velocity (μm/min) | Chemotactic index |
|-------------|-------------|-------------------|-------------------|
| WT          | 247         | 11.4 ± 2.6        | 0.80 ± 0.04       |
| F-Y/Y       | 271         | 9.8 ± 0.4         | 0.86 ± 0.04       |
| F-Y/A       | 469         | 8.5 ± 0.7         | 0.76 ± 0.13       |

To gain more insight into the difference in cell streaming of F-Y/Y cells and F-Y/A cells, we performed needle assays on cells that had been exogenously pulsed with cAMP for 5 h. This allowed us not only to synchronize the cells at the chemotactic-competent stage, but also to visualize the migration of the cells in real time. We found (Fig. 7 and supplemental Movie 2, A–C) that F-Y/Y cells formed streams similar, but not identical, to the streams formed by WT cells (Fig. 7, middle row). In contrast, as we had observed with the self-streaming assay, F-Y/A cells exhibited severe streaming defects migrating mainly as individual cells to the point source of cAMP where they accumulated (Fig. 7, middle row). At higher magnification (Fig. 7, bottom row), we observed that the Y/A cells were generally rounder than the highly polarized WT cells and Y/Y cells. Although F-Y/A cells were unable to form streams, chemotaxis of individual F-Y/A cells was very similar to chemotaxis of individual WT cells and F-Y/Y cells (Table 2).

In a variation of the self-streaming assay, we mixed chemotactic competent WT cells and F-Y/A cells, plated them on chambered coverslips, allowed them to aggregate for 30 min, and then fixed the cells and stained them with anti-actin and anti-FLAG antibodies. As we had observed previously, WT cells alone formed robust streams, whereas F-Y/A cells formed heterogeneous aggregates with no detectable streams (Fig. 8). A mixture of 70% WT cells and 30% F-Y/A cells formed less robust streams than 100% WT cells, and the streams consisted almost exclusively of WT cells with the F-Y/A cells in the surrounding periphery (Fig. 8). Similarly, a mixture of 50% WT cells and 50% F-Y/A cells formed broken streams that coalesced into a mound consisting predominantly of WT cells (Fig. 8).

We reasoned that F-Y/A cells behaved like *aca*~ cells, which can readily detect cAMP and chemotax individually but cannot secrete cAMP to relay the signal to neighboring cells (25). This would explain the absence of streams in the self-streaming and needle assays with F-Y/A cells (Figs. 6 and 7), and the relative absence of F-Y/A cells in the fragmented streams formed by mixtures of WT cells and F-Y/A cells (Fig. 8). In the latter experiments, streams initiated by WT cells would be terminated when joined by F-Y/A cells if the latter could not secrete sufficient cAMP to attract neighboring cells.

To test this hypothesis, we performed needle assays with mixtures of GFP-labeled *aca*~ cells and either F-Y/Y or F-Y/A cells (Fig. 9). The *aca*~ cells, which do not form streams by themselves because they cannot synthesize and secrete cAMP, followed and attached to F-Y/Y cells, but did not follow or attach to F-Y/A cells. We propose that F-Y/A cells, unlike F-Y/Y cells, cannot secrete enough cAMP to attract *aca*~ cells or to form streams on their own, but can sense cAMP and chemotax as single cells in a CAMP gradient.

Interestingly, whereas development of F-Y/Y cells and WT cells were similar, with mature fruiting bodies forming 24 h after initiation of starvation (Fig. 10), F-Y/A cells reached only the slug stage at 24 h, and only partially matured to occasional abnormal fruiting bodies by 72 h (Fig. 10). The results with the F-Y/A cells show that the FLAG tag had little effect on the development of the amoebae to mature fruiting bodies, other than that the fruiting bodies were slightly smaller. Therefore, the failure of the F-Y/A cells to stream and form normal...
mounds and fruiting bodies must be a dominant-negative effect of the Y53A mutation. The inability of the F-Y/A cells to form normal fruiting bodies may have been a direct consequence of the multiple small mounds resulting from their streaming defects, but we cannot exclude the possibility of other defects in development subsequent to the mound stage.

Cells Expressing Y53A-Actin Have Fewer cAMP Receptors on Their Surface and Reduced Intracellular Signaling—A number of biochemical events occur when Dictyostelium amoebae in a non-nutrient buffer are pulsed with cAMP, including increased expression of cAMP receptor (33, 56) and ACA (57). We first followed the expression of endogenous actin, F-Y/Y-actin, and F-Y/A-actin during development of starved cells exogenously pulsed with cAMP for 8 h (see “Experimental Procedures”). The concentration of endogenous actin in WT cells, and endogenous and F-Y/Y-actin in F-Y/Y cells remained unchanged over the entire 8-h period, but the expression of F-Y/A-actin decreased slightly in the cAMP-pulsed F-Y/A cells (Fig. 11A).

We then followed the expression of cAR1 and ACA in the three cell lines. The expression of both cAR1 and ACA was readily detectable by 3 h in WT and F-Y/Y cells, peaking at 4–5 h (Fig. 11, B and C). However, cAR1 and ACA expression in F-Y/A cells were not detected until 5 h, although peaking at a similar level as in WT and F-Y/Y cells (Fig. 11, B and C). As expected ERK2 expression was not affected by cAMP pulsing in any of the three cell lines (Fig. 11D), which shows that the defects in cAR1 and ACA expression in F-Y/A cells reflect a specific impaired response to exogenous cAMP.

To extend and more precisely quantify these findings, we measured cell-surface cAMP-binding sites in vegetative cells and during development. We found that prior to cAMP pulsing vegetative WT and F-Y/Y cells had the same number of cell-surface cAMP binding sites, ~13 × 10^3/cell, and vegetative F-Y/A cells had ~6 × 10^3/cell (Fig. 12, A and B), a difference that is probably not significant. When WT and F-Y/A cells were pulsed with cAMP, the number of surface cAMP receptors in both cell lines increased substantially, peaking at about 5 h, and then decreased to basal levels by
The presentation of a uniform cAMP stimulus to chemotaxis-competent Dictyostelium cells causes a transient increase in ACA activity (56), actin polymerization (40), ERK2 phosphorylation (39), and TORC2 activity (41). We assessed these cAMP-mediated responses in WT, F-Y/Y, and F-Y/A cells pulsed with cAMP for ~5 h, at which time the three cell lines expressed comparable levels of cAR1 and ACA. We found that the peak of ACA activity of F-Y/Y cells in response to a uniform stimulus of exogenous cAMP was 73% as high as the response of WT cells, but the peak ACA activity of F-Y/A cells was only 18% as high as WT cells (Fig. 12C). The peak response of actin polymerization, when cells were pulsed with cAMP, was slightly less for F-Y/Y cells than for WT cells (Fig. 12D), suggesting a possible influence of the FLAG tag, but only 50% as much actin was polymerized by F-Y/A cells (Fig. 12D), again indicative of a substantially greater inhibition than the Y53A mutation. Similarly, we found that 60 s after cells were stimulated with 100 nM or 10 μM cAMP, F-Y/Y cells had 30% less pERK2 than WT cells, but F-Y/A cells had 60–70% less pERK2 (Fig. 12E). Thus, although the FLAG tag may have made some contribution to the inhibition of cAMP-stimulated ERK2 phosphorylation in F-Y/A cells, the inhibition was mostly due to the Y53A mutation. On the other hand, activation of TORC2 activity, as measured by PKBR1 phosphorylation, was similar in the three cell lines (Fig. 12F), but somewhat more prolonged in the F-Y/A cells. These findings show that, with the exception of TORC2 activity, expression of F-Y/A-actin leads to a reduction in intracellular cAMP-mediated responses, most importantly in the current context, a substantial reduction in ACA activity.

Expression of Y53A-Actin Inhibits Enrichment of ACA Vesicles at the Rear of Chemotaxing Cells—Cell-streaming requires not only synthesis of cAMP by ACA (28) but also trafficking of ACA-containing vesicles to the rear of the cell (58). We, therefore, next determined whether intracellular trafficking of ACA vesicles was affected by the expression of F-Y53A-actin. Either F-Y/Y-actin or F-Y/A-actin and ACA-YFP were co-expressed in aca cells. The cells were fixed on coverslips, stained with anti-FLAG antibody, and the localization of FLAG-actin and ACA-YFP was determined by confocal microscopy.

As shown in Fig. 13, upper row, in polarized cells expressing both F-Y/Y-actin and ACA-YFP the F-Y/Y-actin was concentrated at the front of the cell, and the ACA-YFP was predominately concentrated at the rear of the cell with some ACA vesicles dispersed in the cytoplasm between the nucleus and the rear of the cell. In some F-Y/Y cells (Fig. 13, upper row, left) a trail of secreted ACA vesicles was observed. This behavior is in agreement with the localization and secretion of ACA vesicles in wild-type cells (58). In contrast, ACA-YFP in cells expressing F-Y/A-actin (Fig. 13, bottom row) was either concentrated with the FLAG-actin at the front of the cell or relatively uniformly dispersed around the cell periphery.

DISCUSSION

The properties in vitro of purified F-Y/A-actin are very similar to the previously reported properties of both Y/A-actin without the FLAG tag (20) and Tyr53-phosphorylated actin (14, 19). In previous papers (19, 20), we showed that phosphorylation of Tyr53 or its replacement by Ala alter the conformation of...
the adjacent D-loop and the nucleotide-binding cleft resulting in slower actin polymerization, a higher critical concentration, and polymerized actin in the form of small oligomers and imperfect filaments. We show in the present paper that the Y53A mutation similarly affects polymerization kinetics, critical concentration, and the structure of polymerized F-Y/A-actin, with no effect of the FLAG tag on any of these properties, i.e., these properties of purified F-Y/A-actin and WT-actin are indistinguishable. Although both the FLAG tag and the Y53A mutation reduce actin activation of myosin ATPase activity, the effect of the Y/A mutation is mostly reversed in copolymers of F-Y/A-actin and WT-actin, which have the same $V_{\text{max}}$ as WT-actin, but a slightly higher $K_m$ due to the FLAG tag, as shown by the similarly higher $K_m$ for F-Y/Y-actin. Neither the FLAG tag nor the Y/A mutation affects the in vitro motility velocity of phalloidin-stabilized filaments. Importantly, copolymers of F-Y/A-actin and WT-actin (in the ratio they occur in F-Y/A cells) form short, irregular filaments, like those formed by F-Y/A-actin alone, and not the typical, long, coiled-coil helical filaments formed by both WT-actin and F-Y/Y-actin.

We confirm in this paper the earlier observation that expression of Y53A-mutated actin in Dictyostelium amoebae blocks the developmental cycle with formation of small mounds that cannot develop further to mature fruiting body. We now show that this developmental block is preceded, and presumably caused, by a profound inhibition of head-to-tail streaming of amoebae expressing F-Y/A-actin to the extent of 30% of the total actin. The dominant-negative effects of F-Y/A-actin are due to the Y53A mutation, and not the FLAG tag, as they do not occur in cells expressing equivalent amounts of F-Y/Y-actin. Furthermore, individual F-Y/A cells chemotax normally in a cAMP gradient and the behavior of mixtures of F-Y/A cells with either WT cells or aca$^+$ cells suggest that F-Y/A cells do not form head-to-tail streams because they cannot secrete sufficient cAMP to relay the cAMP signal to neighboring cells. Consistent with this conclusion cAMP activation of adenylyl cyclase, translocation of ACA vesicles to the rear of the cell, and ACA vesicle secretion are all severely inhibited in cells expressing F-Y/Y-actin.

The only significant difference between the properties of purified F-Y/A-actin and F-Y/Y-actin is that F-Y/A-actin forms small aggregates and short fragmented filaments when polymerized alone or copolymerized with WT-actin, whereas F-Y/Y-actin forms normal filaments that are indistinguishable from filaments of WT-actin. We have shown that expressed F-Y/Y-actin and F-Y/A-actin colocalize, and presumably copolymerize, with endogenous WT-actin. Importantly, the cytoskeleton of F-Y/A cells is characterized by short, bundled and aggregated filaments, and extensive empty spaces that are not present in the cytoskeletons of WT and F-Y/Y cells, consistent with the products of copolymerization of WT-actin and Y/A-actin in...
Inhibition of cAMP Signaling by Y53A-Actin Mutant

**FIGURE 13.** Expression of F-Y/A-actin inhibits trafficking of ACA vesicles to the rear of the cell. aca− cells were doubly transfected with ACA-YFP and either F-Y/Y-actin or F-Y/A-actin. Differentiated cells were plated on glass coverslips in starvation buffer, allowed to polarize, then fixed and stained with anti-FLAG antibody, and localization of FLAG-actin (red) and ACA-YFP (green) observed by confocal microscopy. Only about 10% of the cells were doubly transfected (the two panels on the left of the upper row show one doubly transfected cell and one cell expressing only the FLAG-actin). The upper row shows two representative cells transfected with F-Y/A-actin with actin and ACA vesicles concentrated at opposite ends of the cells. One cell is releasing ACA vesicles one cell expressing only the FLAG-actin. The cells were doubly transfected (the localization of FLAG-actin (red) and ACA-YFP (green) observed by confocal microscopy. Only about 10% of the cells were doubly transfected (the two panels on the left of the upper row show one doubly transfected cell and one cell expressing only the FLAG-actin). The upper row shows two representative cells transfected with F-Y/A-actin with actin and ACA vesicles concentrated at opposite ends of the cells. One cell is releasing ACA vesicles (upper row, second panel). The bottom row shows two representative cells transfected with F-Y/A-actin with ACA vesicles either concentrated at the same end as actin or ACA uniformly distributed around the cell periphery in addition to cytoplasmic vesicles.

in vitro. In fact, the cytoskeleton of F-Y/A cells may actually be even more fragmented than indicated in Fig. 4 because phalloidin, which is present in the cell extraction buffer to stabilize the actin filaments, converts the fragmented filaments formed by polymerization of purified Y/A-actin in vitro into long, normal filaments (20).

It seems highly likely that the disrupted actin cytoskeleton revealed by electron microscopy in F-Y/A cells is responsible for the inability of F-Y/A cells to accumulate ACA vesicles at the rear of the cell and to secrete cAMP. Although ACA vesicles move to the rear of the cell along microtubules, vesicle trafficking also requires an intact actin cytoskeleton (58). The shorter and bundled actin filaments may interfere with the movement of ACA vesicles along microtubules (58), or disorganize the microtubules impeding their ability to serve as tracks for the translocation of ACA vesicles. It is also possible that actin filaments and a myosin work in conjunction with microtubules and a microtubule motor in the accumulation of ACA vesicles at the rear of the cell, similar to the cooperative interaction of actin- and microtubule-based systems in the trafficking of melanosomes in melanocytes (59) and contractile vacuoles in Dictostelium (60). Also speculatively, an actomyosin might be required for the secretion of ACA vesicles at the rear of the cell, as is the case for the secretion of enzyme-containing vesicles by epithelial cells of the intestinal brush border (61).

The fragmented and aggregated cytoskeleton of F-Y/A cells may also be responsible for the impaired responses of F-Y/A cells to extracellular cAMP. Although differentiated F-Y/A cells express similar levels of total cAR1 as WT cells and F-Y/Y cells, they have only half as many cell surface cAMP receptors, suggesting that trafficking of cAMP receptors to the cell surface, like trafficking of ACA vesicles to the rear of the cell, may be inhibited by the disorganized cytoskeleton in F-Y/A cells.

Binding of extracellular cAMP to cell surface cAR1, a heterotrimeric G protein-coupled receptor, causes the release of Gβγ subunits which, through a sequence of events involving Ras, PI3K, and TORC2, results in the activation of ACA (62). Also, and independently of Gβγ, cAMP binding to cAR1 initiates a series of events resulting in phosphorylation of ERK2 (63). Possibly, the 50% fewer cell surface cAR1 receptors in F-Y/A cells results in the substantial reduction in cAMP-induced ACA activity that could contribute to the decrease in cAMP secretion by chemotaxing F-Y/A cells. Yet, the more modest decreases in F-actin assembly and ERK2 phosphorylation observed in F-Y/A cells have no deleterious effects on chemotaxis of individual cells.

Alternatively, the disrupted actin cytoskeleton of F-Y/A cells might impair the mecanochemical properties of the cytoskeleton, which comprises F-actin, myosin II, and actin cross-linking proteins (64). For example, a recent paper (65) reports that activation of Ras, PI3K, and TORC2 are mis-regulated in myosin II null cells, and that complexes of IQGAP and the actin-bundling protein cortexitn (66) are involved in the regulation of PI3K and TORC2. Furthermore, and remarkably similar to our observations, disruption or deletion of cortexitin I prevents Polysphondylium, a close relative of Dictystelium, from forming aggregation streams under starvation conditions and blocks normal development to fruiting bodies (67). It is also relevant that ACA activation in wild-type Dictystelium is inhibited by latrunculin A (33), which causes dissolution of the actin cytoskeleton. A dysfunctional actin cytoskeleton might also interfere with the recently reported (55) requirement for a class VII myosin, MyoG, in early events of cAMP-induced chemotaxis.

It is remarkable that the cAMP signal relay is much more sensitive to alterations in the cytoskeleton due to actin polymerization defects than are other actin-dependent processes such as cytokinesis (cell growth), phagocytosis, pinocytosis, and cell motility. Finally, one wonders if the biological functions of Tyr53-phosphorylated actin, whose properties so closely resemble those of Y53A-actin, in vivo so closely resemble those of Y53A-actin, involve cAMP signaling.

Acknowledgments—We gratefully acknowledge the assistance of Dr. James Sellers in the in vitro motility assays and the mass spectrometry performed by Dr. Rodney Levine.

**REFERENCES**

1. Chisholm, R. L., and Firtel, R. A. (2004) *Nat. Rev. Mol. Cell Biol.* 5, 531–541
2. Weijer, C. J. (2009) *J. Cell Sci.* 122, 3215–3223
3. DeLozanne, A., and Spudich, J. A. (1987) *Science* 236, 1086–1091
4. Knecht, D. A., and Loomis, W. F. (1987) *Science* 236, 1081–1086
5. Neujahr, R., Heizer, C., and Gerisch, G. (1997) *J. Cell Sci.* 110, 123–137
6. Zang, J. H., Cavet, G., Sabry, J. H., Wagner, P., Moores, S. L., and Spudich, J. A. (1997) *Mol. Biol. Cell* 8, 2617–2629
7. Noegel, A. A., and Schleicher, M. (2000) *J. Cell Sci.* 113, 759–766
