Actin Polymerization-Driven Molecular Movement of mDia1
in Living Cells

Chiharu Higashida, Takushi Miyoshi, Akiko Fujita, Fabian Oceguera-Yanez, James Monypenny, Yoshikazu Andou, Shuh Narumiya, Naoki Watanabe

doi: 10.1126/science.1093923

Contents
Materials and Methods
References
Movies S1–S12

Materials and Methods

Plasmids and reagents
The truncated mutants of mDia1 were described previously (S1, S2). Cytochalasin D, nocodazole, poly-L-lysine (PLL) were purchased from Sigma. Jasplakinolide and Latrunculin B were from Calbiochem. Oregon Green-phalloidin and Alexa Fluor594 anti-goat IgG were purchased from Molecular Probes.

Live cell imaging and fluorescent speckle microscopy
Speckle imaging and other live-cell imaging were carried out as described (S3). Briefly, XTC Xenopus fibroblasts were transfected with vectors using SuperFect (Qiagen). Before experiments, cells were suspended in 70% Leibovitz’s L15 medium (Invitrogen) without serum, and seeded into a hand-made flow cell with a PLL-coated glass coverslip (25 × 5 mm², depth ~1 mm). After cell spreading, the flow cell was placed on the stage of an Olympus BX52 microscope equipped with 100 W mercury illumination and a cooled CCD camera (MMX1300-YHS, Roper Scientific). Time-lapse imaging was carried out at room temperature using the MetaMorph software (Universal Imaging) up to 120 min after cells were seeded. Fluorescent speckle microscopy was carried out by observing cells expressing a low amount of EGFP-mDia1. A restricted area near the cell periphery was illuminated. Drug perfusion and wash-out were carried out by intermittently replacing the medium in the flow cell.

Speckle velocity measurement was performed by tracking individual speckles manually with Track Points in MetaMorph software. Kymograph analysis was also performed using MetaMorph.

Microinjection
Recombinant RhoA-Val14 was expressed as a GST-fusion protein in E. coli, purified and cleaved from GST as described (S1). XTC cells expressing EGFP-mDia1Full were suspended as described above, and seeded onto PLL-coated round glass coverslips (25 mm diameter) attached to a chamber, and allowed to spread for 30 min. The chamber was placed on the stage of an Olympus IX81 microscope equipped with a CCD camera (MMX-512BFT, Roper Scientific) and unattenuated 100 W mercury illumination. Only cells expressing EGFP-mDia1Full at a low level were chosen for microinjection, and time-lapse images of EGFP were acquired at 0.18-s intervals using a 100× objective (NA 1.4). Then RhoA-V14 in injection buffer (10 mM Hepes pH 7.4, 100 mM NaCl, 0.1 mM DTT, 2 mM MgCl₂) was microinjected into the cell at the concentration of 0.3 mg/ml. EGFP-mDia1Full images were intermittently acquired at 0.18-s intervals 2, 4, 7 and 12 min after microinjection.
Microscopic observation of actin polymerization

Actin was purified from rabbit muscle according to the method of Pardee and Spudich (S4). Profilin was purified from human platelets using poly-L-proline chromatography (S5). The recombinant mDia1ΔN3 was purified as follows.

*E. coli* strain BL21trxB (DE3) (Novagen) was transformed with the pGEX-4T-mDia1ΔN3 construct (S1), and grown overnight at 37°C. After subculturing into fresh media, cells were grown at 37°C for 3 h, and then grown for 24 h at 16°C with the addition of 0.5 mM isopropyl β-D-thiogalactopyranoside. Cells harvested by centrifugation were frozen, resuspended in buffer A (20 mM Hepes pH 7.4, 10% glycerol and 5 mM DTT) containing 200 mM NaCl and a complete protease inhibitor tablet (Roche), and sonicated. The sonicate was clarified at 50,000g for 30 min, and mixed with glutathione-Sepharose (Amersham Biosciences) for 1 h at 4°C. Sepharose was washed three times with an excess amount of buffer A containing 500 mM NaCl. Bound proteins were then eluted with buffer B (50 mM Tris-HCl pH 8.5, 50 mM KCl, 5% glycerol, 20 mM glutachione, 1 mM DTT), and dialyzed against buffer C (20 mM Hepes pH 7.4, 1 mM EDTA, 50 mM KCl, 5% glycerol, 1 mM DTT). Purified proteins were frozen in liquid nitrogen and stored at −80°C. Thawed proteins were used within 2 days.

Microscopy of phallloidin-labeled filaments was carried out as follows. GST-mDia1ΔN3 (740 ng) was labeled by mixing with 5 µg GST-specific polyclonal antibody (Amersham) at 4°C for 30 min, and then with Alexa Fluor594 secondary antibody directed against goat IgG for another 30 min in buffer C. Actin (1.9 µM), profilin (1.3 µM) and 1/50 (Fig. 4, A to D) or 1/10 (Fig. 4, E to H) of the labeling mixture were mixed in 40 µl of buffer [20 mM imidazole (pH 7.0), 2 mM Tris-HCl, 0.2 mM ATP, 2.1 mM MgCl₂, 2 mM EGTA, 100 mM KCl, 10.5 mM DTT, 0.1 mg/ml glucose oxidase, 3 µg/ml glucose oxidase, 20 µg/ml catalase, 0.5% methylcellulose, Oregon Green-phalloidin]. From the mixture, 2 µl was immediately placed on a glass slide, and squashed with a PLL-coated (2 µg/ml) glass coverslip. Time-lapse images were acquired at 22°C using an BX52 Olympus microscope and a 60× objective (NA 1.4). Image acquisition was started after finding actin cloud, which typically took 5 to 10 min.

References for Supplemental Material


Legends for Supplemental Movies

**Movie S1.** Single-molecule observation of fast movement of EGFP-mDia1ΔN3 in a live XTC cell. Time is in seconds. Scale bar, 2 µm.

**Movie S2.** Intracellular molecular movement of EGFP-mDia1F2. Time is in seconds. Scale bar, 2 µm.

**Movie S3.** Fast molecular movement of EGFP-mDia1Full induced by RhoA-V14 microinjection. Time-lapse images were taken before (top) and 2 min after (bottom) microinjection. Time is in seconds. Scale bar, 2 µm.

**Movie S4.** The movement of EGFP-mDia1ΔN3 was not inhibited by 10 µM nocodazole. Time-lapse images were taken before (left) and 60 min after (right) the treatment. Scale bar, 2 µm.

**Movie S5.** The movement of EGFP-mDia1ΔN3 was blocked by 1 µM jasplakinolide. Time-lapse images were taken before (left) and 14 min after (right) the treatment. Note that all speckles were stopped after the treatment. Scale bar, 2 µm.

**Movie S6.** Gradual, synchronous decrease in the speed of EGFP-mDia1ΔN3 speckles upon 1 µM latrunculin B treatment. The drug was added at about 5 s. Scale bar, 2 µm.

**Movie S7.** Speed of EGFP-mDia1ΔN3 speckles decrease gradually with 1 µM jasplakinolide treatment. The drug was added at about 6 s. Scale bar, 2 µm.

**Movie S8.** Sudden stoppage of the EGFP-mDia1ΔN3 speckle movement upon 1 µM cytochalasin D treatment. Note that cessation of individual mDia1ΔN3 speckles occurred at various times and places in cells. The drug was added at about 5 s. Scale bar, 2 µm.

**Movie S9.** Massive actin filament outgrowth emanating from GST-mDia1ΔN3-containing protein aggregates. Time is in minutes. Scale bar, 5 µm.

**Movie S10.** Speckle imaging of actin filament growth emanating from GST-mDia1ΔN3. An area around mDia1ΔN3-containing protein aggregates (Fig. 4E) is shown. A low concentration of Oregon Green-phalloidin was used to label actin filaments. Time is in minutes. Scale bar, 5 µm.

**Movie S11.** Movement of actin filaments around the area used for kymograph (Fig. 4F) is shown. Time is in minutes. Scale bar, 2 µm.

**Movie S12.** Growth of isolated actin filaments anchored to mDia1ΔN3. Arrows indicate the position of small GST-mDia1ΔN3-containing aggregates. The movie demonstrates persistent association of a growing single actin filament end to mDia1ΔN3. Time is in minutes. Scale bar, 5 µm.