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Active fluctuation in the cortical cytoskeleton observed by high-speed live-cell scanning probe microscopy

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ABSTRACT

We investigated the dynamics of the cortical cytoskeleton in living cells by analyzing the motion of the endogenous components of the cytoskeleton using scanning probe microscopy (SPM). We performed molecular characterization of the microgranules visualized by SPM in living cells and analyzed the motion of these microgranules via particle tracking. Simultaneous SPM and epifluorescence microscopy observations showed that the microgranules recruited not only actin but also cortactin, which can bind to actin filaments. This indicates condensation of actin filaments at microgranules, leading us to identify them as "cytoskeletal microdomains". High-speed SPM observation and particle-tracking analysis showed that these cytoskeletal microdomains exhibit random walk-like diffusive fluctuations over a timescale of seconds. Inhibition of the molecular motor myosin II, which drives actin filaments, led to subdiffusive fluctuations of the microdomains. These results can be explained by longitudinal sliding of actin filaments stochastically driven by myosin II and the bending motion of the actin filaments in the absence of sliding. Analysis of the cytoskeletal microdomains thus revealed the intrinsic dynamics of the cortical cytoskeleton.

1. Introduction

The dynamic properties of the cortical cytoskeleton, a mechanical framework constructed from an entangled and cross-linked network mainly composed of actin filaments (AFs), are essential for a wide variety of biological cellular functions, including vesicular traffic, cell morphogenesis and extracellular matrix organization. In thermal equilibrium, such a network limits the longitudinal sliding motion of internal filaments so that the transverse bending motion of filaments is the dominant process acting on the dynamics of a network, leading to subtle subdiffusive fluctuations [1,2]. Molecular motors such as myosin II generate mechanical forces on cytoskeletal filaments by consuming chemical energy, resulting in large non-equilibrium fluctuations in the reconstituted cytoskeleton [3]. In living cells, the cortical cytoskeleton is known to undergo drastic remodeling or internal transport processes in a molecular motor-dependent manner, such as the so-called retrograde flow, which is the directional movement of AFs from the cell periphery to the center [4], and bundling of AFs to form stress fibers or contractile rings. Investigation of the microscopic mobility of the cytoskeleton in living cells can shed new light on the process that results in macroscopic reorganization of the cytoskeleton and cytoplasmic agitation of cytoskeleton-embedded materials.

Cytoskeletal motion in living cells has been investigated by the use of artificial colloidal beads bound to the cytoskeleton via the plasma membrane. These beads are often coated with a small peptide derived from the ligand of a transmembrane receptor integrin, which is known to associate with AFs on the inner surface of the plasma membrane. This technique results in a tight connection between the outer beads and the inner cytoskeleton. Particle-tracking analysis of cytoskeleton-bound beads has led to the characterization of cytoskeletal remodeling dynamics [5]. This approach is a powerful tool with which to investigate the dynamics of a fully established cytoskeleton that contains stress fibers [6]. However, the beads themselves locally recruit actin filaments around the beads [7]. Therefore, to examine the intrinsic dynamics of the cortical cytoskeleton, it is necessary to analyze the motion of endogenous cytoskeletal materials.

Scanning probe microscopy (SPM) has been successfully applied to investigate the cortical cytoskeleton in living cells. In SPM, a tip-shaped probe mechanically pushes the cell surface and then measures the forces at the piconewton scale. In principle, SPM must enable selective visualization of the materials mechanically supported by scaffold architecture and preclude detection of materials that float in the cytoplasm. Therefore, SPM is superior for observing cytoskeletal filaments that contribute to cortical mechanics. SPM provides easy access to the cortical cytoskeleton at a force scale of approximately 0.1 nN [8]. At this force scale, contact mode imaging enables visualization of a filamentous architec-

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ture, which corresponds to the AF distribution [9,10]. However, temporal resolution of conventional contact-mode SPM is generally >1 min [11] and is therefore insufficient to examine second-scale cytoskeletal behaviors. Recently, we established a method to achieve subminute temporal resolution of the cortical cytoskeleton in live-cell imaging [12]. This high-speed live-cell SPM imaging allows detailed analysis of the structural dynamics of the cortical cytoskeleton.

In this study, we examined the dynamics of the cortical cytoskeleton by using high-speed live-cell SPM imaging. SPM allowed visualization of submicron-size microgranules on the cortical cytoskeleton that moved and spontaneously disappeared. Simultaneous fluorescence microscopy and SPM showed localization of cytoskeletal proteins at the microgranules, indicating that the microgranules are AF-rich "microdomains" of the cortical cytoskeleton. We analyzed the motion of these microdomains through single-particle tracking. The stochastic fluctuating motion of the microdomains was not subdiffusive but diffusive. Pharmacological inhibition of myosin II abolished the diffusive behavior of microdomains and led to a subdiffusive behavior. The transport dynamics of microdomains are partly consistent with the previously reported cytoskeletal dynamics investigated using artificial beads and also reveal new characteristics of cytoskeletal fluctuation. We also found that vesicle-bound proteins were recruited into the microdomains.

2. Materials and methods

2.1. Cell culture

The mouse embryonic fibroblast cell line NIH3T3 (RIKEN Cell Bank, Tsukuba, Japan) was cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma Aldrich, St. Louis, MO) supplemented with 10% bovine serum (Invitrogen, Carlsbad, CA) and 1% antibiotic solution (Sigma–Aldrich) at 37 °C in a 5% CO_2 atmosphere on a chambered glass coverslip or a polystyrene Petri dish. The mouse myoblast cell line C2C12 (RIKEN Cell Bank) was cultured in DMEM supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA) and 1% antibiotic solution at 37 °C in a 5% CO_2 atmosphere on a polystyrene Petri dish.

2.2. Plasmid construction and transfection

mRNA was extracted from NIH3T3 cells or C2C12 cells cultured on a polystyrene dish by using TriPure isolation reagent (Roche Molecular Biochemicals, Basel, Switzerland) according to the manufacturer's instructions. Through reverse-transcription polymerase chain reaction (RT-PCR) using the ReverTra Ace qPCR RT Kit (TOYOBO, Osaka, Japan) and KOD-FX (TOYOBO), we obtained complimentary DNA (cDNA) for target proteins by using the following primers (sequences 5′–3′): cgaattcgatggatgacgatatcgctg (forward) and aaactcgagctagaagcacttgcggtgcac (reverse) for β-actin, aaggtac catgtggaaagcctctgcag (forward) and ggaattcctactgccgcagctccacatag (reverse) for cortactin, aaggtaccatggaccattatgattcccag (forward) and ccgctcgagaggtcgctctcgccatac (reverse) for α-actinin, aaggtaccatgagcgagacggtcatctg (forward) and ggaattctcaaggagaaccccgcttc (reverse) for vasodilator-stimulated phosphoprotein (VASP), aaggtaccatggacgacctcgatgccctg (forward) and ggaattcctagcagaagagcttcacgaag (reverse) for paxillin, aaggtaccatgggcagcaacaagagcaag (forward) and ggaattcaggttctccccgggctggtac (reverse) for cytoplasmic Src (c-Src), aaggtaccatgaacattctggcgcccgtg (forward) and ccgctcgagtcagggacaacatgtggctttttc (reverse) for Rah/Rab34, aaggtaccggcgggaagttcggtgtg (forward) and ggaattcctactgccgcagctccacatag (reverse) for cortactin mutant lacking the N-terminal acidic region (cortactin-dNTA) [13], and aaggtaccatgtggaaagcctctgcag (forward) and ggaattctaggcgatggctgtgatgcccag (reverse) for cortactin mutant lacking the Src homology 3 domain (cortactin-dSH3) [14]. We processed RT-PCR products with the Wizard SV Gel PCR Clean-Up System (Promega, Madison, WI). cDNA was digested using the following pairs of restriction enzymes: *Kpn*I/*Eco*RI for cortactin, VASP, paxillin, c-Src, cortactin-dNTA and cortactin-dSH3; *Kpn*I/*Xho*I for α-actinin and Rah/Rab34; and *Eco*RI/*Xho*I for β-actin. The digested cDNAs were purified using gel electrophoresis and the Wizard SV Gel PCR Clean-Up System. By using the DNA Ligation Kit Mighty Mix (TaKaRa Bio, Shiga, Japan), the digested cDNAs were ligated into the multiple cloning site of a phmAG1-MC-Linker or phmAG1-MN-Linker vector purchased from MBL (Woburn, MA) to label the proteins with the green-fluorescent protein Azami-Green (AG) [15]. For α-actinin and c-Src, we used the phmAG1-MN-Linker vector. For all other proteins, we used a phmAG1-MC-Linker vector. We cloned and amplified the constructs by using homemade competent *Escherichia coli* cells on neomycin-containing Luria–Bertani plates. We then transfected cells with the constructs by using Lipofectamine 2000 reagent (Invitrogen).

2.3. SPM and epifluorescence microscopy

NIH3T3 cells cultured on a chambered glass coverslip were used for experiments. The cell culture medium was replaced with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-buffered DMEM (pH 7.4; Sigma–Aldrich) with 10% bovine serum and 1% antibiotic solution. We observed live cells by SPM using a NanoScope IIIa BioScope (Veeco, Santa Barbara, CA) mounted on an inverted optical microscope (TE2000, Nikon, Tokyo, Japan) and cantilevers with a spring constant of 0.01 N m^{-1} (MLCT series, Veeco) at 37 °C. Detailed procedures for SPM have been described previously [12]. To improve the temporal resolution to the second scale, we reduced the SPM imaging slow-scan length to 1/4 of the fast-scan length. The pixel size of the raw SPM images was set to 78 nm. To simultaneously observe epifluorescence and SPM, we combined SPM with optical microscopy by using a ×100 objective lens (Nikon) and a cooled charge-coupled device camera (ORCA, Hamamatsu, Japan). We measured the position of the SPM tip by optical microscopy and, with the help of the measured tip position, we overlaid the fluorescence images on the SPM images using different pseudocolors.

2.4. Single-particle-tracking analysis

Prior to tracking, we extended raw SPM images four times to a pixel size of 20 nm and processed them with a Gaussian filter. We then tracked single microdomains through a built-in method in the Image-Pro 5.2 software (Media Cybernetics, Silver Spring, MD) based on a center-of-mass algorithm and obtained two-dimensional trajectories for each microdomain, described as $[x_i(r), y_i(r)]$, where r is a discrete time step corresponding to 1, 2, 3,..., ρ_i and i is the index number for each microdomain. The time t is equal to r multiplied by the temporal resolution. We calculated the displacement vector of the microdomains as $\mathbf{d}_i(r, \Delta r) = [x_i(r + \Delta r) - x_i(r), y_i(r + \Delta r) - y_i(r)]$. The mean square displacement (MSD) was calculated as follows:

$$\delta_i^2(\Delta r) = \langle |\mathbf{d}_i(r, \Delta r)|^2 \rangle_r \quad (1)$$

In a system with a directional bias, variance of displacement (VOD) approximates the MSD of bias-free behaviors when $\Delta r/\rho_i \ll 1$ (see Supplementary data). We used a modified form of VOD, as follows:

$$\sigma_i^2(\Delta r) = \langle |\mathbf{d}_i(r, \Delta r) - \langle \mathbf{d}_i(r, \Delta r) \rangle_r - \mathbf{h}_i(\Delta r)|^2 \rangle_r \quad (2)$$

where $<\ldots>_r$ denotes time average and $\mathbf{h}_i(\Delta r)$ is a term to correct hysteresis of piezoelectric actuators of SPM. Hysteresis is caused