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A visualized observation of calcium-dependent gelsolin activity upon the surface coverage of fluorescent-tagged actin filaments

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ABSTRACT

Gelsolin regulates the dynamics of F-actin by binding to F-actin to sever and cap. In the present study, a novel approach is introduced to observe gelsolin activity through the coverage of surface-bound F-actin. Gelsolin was immobilized on streptavidin coated surface using biotinylation and, as a result, the interaction between gelsolin and F-actin was visualized. Consequently, the coverage of F-actin reflects the activity of gelsolin as a function of free Ca^{2+} concentrations. In order to prevent non-specific binding of F-actin, the combinations of BSA and Tween-20 as blocking agents were investigated. Moreover, the measurement of the length of F-actin with actin–gelsolin mixtures at various ratios provided the verification of gelsolin activity after biotinylation. The data shows the increase in Ca^{2+} concentration leads to a proportional increase in F-actin coverage, giving to half-maximal coverage at ~2.9 μM. Furthermore, the length of bound F-actin was found to decrease along with increasing Ca^{2+} concentration, and full-length F-actin was rarely observed. This may suggest that severing and capping activities of gelsolin occur without more additional Ca^{2+} for subsequent activation after full-length gelsolin binds to a side of F-actin. This finding may provide a key to understand gelsolin activity.

1. Introduction

Gelsolin, an actin binding protein, regulates assembly and disassembly of actin in eukaryotes by severing an actin filament (F-actin) and subsequently remaining capped at the barbed end [1–4]. As a result, it plays a role in regulation of changing the shape and motility of the cell [5,6]. It is known that gelsolin is composed of six homologous domains (G1–G6) that fold together in order to form a compact globular shape in the absence of free Ca^{2+}. In this structure, the helical tail of G6 latches onto the G2 subdomain, thereby burying the actin binding domains, G1, G2 and G4, so that they are not accessible to F-actin [7]. However, in the presence of free Ca^{2+}, gelsolin undergoes conformational changes, in turn, affecting its interactions with actin [8–10].

Gelsolin activities related to free Ca^{2+} have been investigated in a number of different studies involving fluorescence [11–16] and X-ray crystallography [7,10,17–19] but there is inconsistency in literature. Lamb et al. reported that half-maximal activation of gelsolin severing and nucleating activities occurred at 10 μM free Ca^{2+} and free Ca^{2+} requirement for gelsolin activity was affected by pH [16]. Kinosian et al. reported that half maximum binding of gelsolin to F-actin occurred at ~0.1 μM free Ca^{2+} while half maximum severing and barbed end capping of F-actin by gelsolin occurred at ~0.4 μM free Ca^{2+} [13]. Pope et al. suggested that Ca^{2+} increased the hydrodynamic radius of gelsolin with half-maximum at ~30 nM free Ca^{2+}, which appeared to open the gelsolin molecule due to release of the helical tail of G6 from G2 [20]. Small-angle X-ray scattering (SAXS) analysis of full length human plasma gelsolin elucidates three states of conformational change of gelsolin in relation to free Ca^{2+} concentrations. The initial state, where the compact structure dominates, occurs from 0 to ~10 nM Ca^{2+}. The intermediate state, in which opening of gelsolin is proportional to free Ca^{2+} concentration, occurs at 0.1–10 μM Ca^{2+}. Finally, the fully activated state, in which opening of gelsolin is at a maximum, occurs at ~100 μM to 1 mM Ca^{2+} [21].

Although Bearer et al. had observed fluorescent actin filaments severing by gelsolin bound to the surface [22], it is necessary to develop an approach for direct observation of actin filament interacting with gelsolin in order to clearly understand gelsolin activity in relation to F-actin at various free Ca^{2+} concentrations as shown Fig. 1. In this heterogeneous approach, biotinylated gelsolin was immobilized on streptavidin coated surface and, as a result, the interaction between gelsolin and F-actin was visualized. Our results show that there are three activation states: Inactive state (0 to ~10 nM of free Ca^{2+}), in which no gelsolin activity was observed; Intermediate state (10 nM to 100 μM of free Ca^{2+}), in which the number of activated gelsolin was proportional to free Ca^{2+} concentrations; and active state ($\geqslant$~200 μM of free Ca^{2+}), in which the number of activated gelsolin was saturated. Further results suggest

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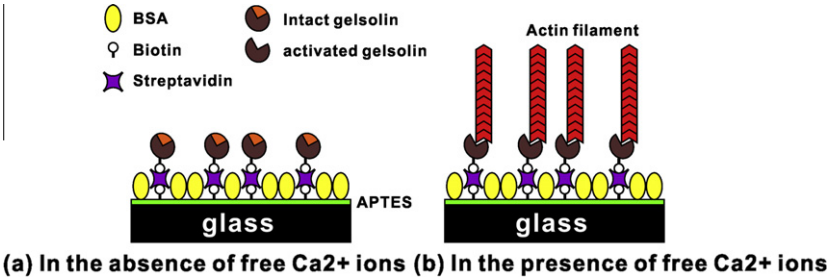

Fig. 1. Illustration of observation of F-actin coverage on a gelsolin-immobilized surface.

that, once gelsolin is activated even at low free Ca^{2+} concentrations (for instance, ~100 nM of free Ca^{2+}), it binds to F-actin and severing and capping events are followed without an additional Ca^{2+} binding.

2. Materials and methods

2.1. Actin purification

G-actin was purified from a rabbit muscle acetone powder (Pelfreez Arkansas, LLC) by repeated filtration and ultracentrifugation in 1 mM $NaHCO_3$ buffer [23]. G-actin was then polymerized into F-actin in 1 mM $NaHCO_3$ with 30 mM KCl at 4 °C for 12 h. The concentration of purified F-actin was determined using spectrophotometer at 290 nm wavelength (BioMate 3, Thermo electron Corp.) using the extinction coefficient of 0.633 mg^{-1} mL cm^{-1}. Rhodamine-phalloidin (R415, Invitrogen) was dissolved in methanol and the solvent was evaporated immediately before use. 0.25 mg/mL of F-actin was labeled with rhodamine-phalloidin with a molar ratio of 1:1 in M-buffer (25 mM KCl, 0.2 mM $CaCl_2$, 2 mM $MgCl_2$, and 25 mM Imidazole at pH 7.0) for 12 h.

2.2. Biotinylation of gelsolin

Gelsolin (G8032, Sigma) was biotinylated using NHS-PEG_4-Biotin (Thermo Fisher Scientific Inc.) with the following conjugation protocol. Gelsolin was dialyzed against a crosslinking phosphate buffer (B-PBS buffer, 150 mM NaCl and 100 mM sodium phosphate at pH 7.2). NHS-PEG_4-Biotin was dissolved in deionized water immediately before use at a concentration of 2 mg/mL. The two solutions were mixed together with a molar ratio of 60:1 for 2 h on ice. Unattached biotins were removed by dialysis in a gelsolin storage buffer (75 mM NaCl, 20 mM Tris, 0.2 mM EGTA and 10 mM Imidazole at pH 7.4). Gelsolin concentration was measured using Bradford protein assay (Thermo Fisher Scientific Inc.) and biotinylated gelsolin was stored at −80 °C until use.

2.3. Measurement of F-actin length

50 μg/mL of F-actin was incubated with gelsolin in various molar ratios for 30 min at room temperature. Then, gelsolin-actin complexes were adsorbed electrostatically on 0.0001% Poly-L-Lysine (PLL) coated cover slips. Length measurement was performed using a plug-in program called manual tracking in ImageJ. More details can be found in the supporting information.

2.4. Fabrication of biotinylated surface

Cover slips (No. 1, 18 × 18 mm) were cleaned using Summa solution and immersed in aminopropyltriethoxysilane (APTES) solution (5% APTES in 95% ethanol). As a result, the primary amine groups were covalently formed on the cover slip that was confirmed using X-ray photoelectron spectroscopy (XPS). More details can be found in the supporting information. 2 mg/mL of NHS-PEG_4-Biotin in dimethylformamide (DMF) was incubated on APTES coated coverslip for 2 h to create biotinylated surface.

2.5. Gelsolin activity in various free Ca^{2+} ions

A flow cell (10 mm × 18 mm × 0.15 mm) was constructed from the biotinylated coverslip (top) and a clean microscope slide (bottom), separated by two double sided tapes. The solution in the flow cell was changed by introducing new solution at one end while drawing it with a tissue paper. After 5% BSA with 0.05% T-20 in B-PBS buffer was introduced into the flow cell for 2 h in order to block non-specific binding sites, 0.1 mg/mL of fluorescent dyed streptavidin (Thermo Fisher Scientific Inc.) in B-PBS buffer was incubated on biotinylated surface for 1 h. In order to achieve a better blocking effect, another 1 h of the blocking agent incubation was followed and then 5 μg/mL of biotinylated gelsolin was incubated for 30 min in 25 mM KCl, 2 mM EGTA, 2 mM $MgCl_2$ and 25 mM Imidazole at pH 7.0. After washing away unattached gelsolin, 0.25 μg/mL of F-actin was incubated for 30 min in 25 mM KCl, 2 mM EGTA, 2 mM $MgCl_2$, 25 mM Imidazole with desired free Ca^{2+} concentrations at pH 7.0. The buffer composed of 25 mM KCl, 2 mM EGTA, 2 mM $MgCl_2$, and 25 mM Imidazole was considered a zero free Ca^{2+} buffer. After F-actin incubation, unnecessary F-actin was washed away for observation. All experiments were performed at 24 ± 1 °C.

2.6. Measurement of coverage

To investigate interaction between gelsolin and actin, the area occupied by fluorescent F-actin was measured. Each image was converted to a black and white image and pixels with black color were calculated using Matlab. More details can be found in the supporting information.

2.7. Calculation of free Ca^{2+} concentration

Free Ca^{2+} concentration in buffers were estimated using the Maxchelator program (http://www.stanford.edu/~cpatton/maxc.html).

2.8. Microscopy and recording

F-actin was viewed in a Nikon Eclipse TE-200 inverted fluorescent microscope with a Plan Apo 60×/1.40 oil objective and a Nikon G-2E/C filter. Images were captured with an ORCA-AG camera (C4742-80-12AG, Hamamatsu, Japan) and recorded using Wasabi imaging software (Hamamatsu, Japan).