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Sodium fluoride as a nucleating factor for Mg-actin polymerization

Olga A. Tsaplina, Sofia Yu. Khaitlina*

Institute of Cytology, Russian Academy of Sciences, Tikhoretsky av. 4, 194064 St. Petersburg, Russia

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ABSTRACT

Dynamic instability of actin filaments can be inhibited by Pi analogs beryllium fluoride and aluminium fluoride that mimic the intermediate ADP-Pi state and stabilize actin filaments. On the other hand, the phosphoryl transfer enzymes can be activated in the absence of aluminium by magnesium fluoride if magnesium ions and sodium fluoride (NaF) were present in the solution. Whether magnesium fluoride promotes functional activities of actin is not known. Here we show, for the first time, that sodium fluoride strongly accelerates polymerization of highly dynamic Mg-F-actin assembled from the monomers proteolytically cleaved between Gly42 and Val43 within the D-loop with actin-specific protease protealysin (Pln-actin), apparently due to stabilization of nuclei formed at the initial step of actin polymerization. Thereby, NaF did not inhibit the ATPase activity (subunit exchange) on Pln-F-actin, did not increase the amount of Pln-F-actin sedimented by ultracentrifugation, and did not stabilize the inter-strand contacts of Pln-F-actin. On the other hand, NaF diminished accessibility of the nucleotide binding cleft of Mg-G-actin to trypsin, pointing to an additional cleft closure, and additionally protected the D-loop from the protealysin cleavage in Mg-F-actin, thus indicating that the longitudinal contacts are stabilized. We also demonstrate that in cultured cells NaF can directly promote assembly of F-actin structures under conditions when the corresponding activity of the RhoA pathway is inhibited. These data suggest that the NaF-induced assembly of actin filaments is promoted by magnesium fluoride that can be formed by the NaF-originating fluoride and the actin tightly bound magnesium.

1. Introduction

Many cellular processes such as cell movement and division, endocytosis, intracellular transport are closely connected with dynamics of actin cytoskeleton, which, in turn, critically depends on assembly and disassembly of actin filaments [1–3]. Although this dynamics is modulated by numerous actin binding proteins, many functional properties of actin cytoskeleton are determined by the dynamic properties of actin itself. A polarized assembly of actin filaments from the monomers containing tightly bound ATP is coupled with ATP hydrolysis, formation of intermediate ADP-Pi-actin and subsequent release of inorganic phosphate leading to the filaments destabilization [4,5]. Hydrolysis of the tightly bound nucleotide accompanies also polymerization of tubulin to form microtubules [6] and functional activity of the DNA repair protein RecA [7]. Dynamic instability of these filaments can be inhibited by Pi analogs beryllium fluoride (BeF_x) and aluminium fluoride (AlF_4^-) that mimic the intermediate state and stabilize actin filaments [8,9], as well as microtubules [10] and RecA filaments [11]. As analog of inorganic phosphate, BeF_x or AlF_4^- also affect the activity of phosphoryl transfer enzymes such as GTPases, ATPases, protein kinases, phosphatases and nucleotidyl transferases, as well as a variety of other enzymes that bind the phosphate or nucleotide-polyphosphate [12–14]. In these enzymes, the bound AlF_4^- (or BeF_x) simulates the presence of the bound γ-phosphate of GTP and therefore confers on the protein the structure of the active GTP-state indispensable for its function [13]. The phosphoryl transfer enzymes have been also shown to be activated in the absence of aluminium by magnesium fluoride (MgF_3^-) if magnesium ions and sodium fluoride were present in the solution [15–17]. Whether magnesium fluoride promotes functional activities of actin is not known.

Previously we have shown that aluminium fluoride efficiently stabilized highly dynamic F-actin assembled from the monomers specifically cleaved between Gly42 and Val43 within the DNase-I-binding loop (D-loop) with bacterial proteases ECP32/grimelisin from *Serratia grimesii* or protealysin from *Serratia proteamaculans* [18–20] and strongly accelerated polymerization of the cleaved

* Corresponding author. Institute of Cytology RAS, Tykhoretsky pr., 4, 194064 St. Petersburg, Russia.
E-mail address: skhspb@gmail.com (S.Yu. Khaitlina).

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actin [21]. Aluminium fluoride is unstable; therefore it is formed upon incubation of actin solution with sodium fluoride (NaF) followed by addition of aluminium chloride. Surprisingly, incubation of the cleaved actin with sodium fluoride still before addition of aluminium ions seemed to promote actin polymerization. Therefore the aim of this work was to elucidate the effects of NaF on polymerization of the cleaved actin and the properties of the formed filament, in comparison with the well known effects of AlF_4^- on actin filament stability. We also aimed to correlate the observed effects with NaF-produced changes in actin cytoskeleton of cultured eukaryotic cells.

We have found that NaF strongly accelerates polymerization of the cleaved Mg-actin apparently due to stabilization of nuclei formed at the initial step of actin polymerization. We also demonstrate that in cultured Balb 3T3 cells NaF can directly promote assembly of actin filaments under conditions when the corresponding activity of the RhoA pathway is inhibited. Based on these data and comparison of the effects with the NaF-associated activity of phosphoryl transfer proteins we suggest that the NaF-induced stabilization of actin filaments may be determined by magnesium fluoride that is formed by the NaF-originating fluoride and the actin tightly bound magnesium.

2. Materials and methods

2.1. Cell culture and growth conditions

The cell line Balb 3T3 was obtained from the Russian Cell Culture Collection (Institute of Cytology, St Petersburg, Russia). Cells were grown on glass cover slips in culture dishes in DMEM containing 10% fetal bovine serum in incubator supplemented with 5% CO_2 at 37° C for the time required to form a monolayer (~48 h).

2.2. Protein preparations

Rabbit skeletal muscle actin was isolated from acetone dried muscle powder according to a standard procedure [22]. G-actin was stored in buffer G (0.2 mM ATP, 0.1 mM $CaCl_2$, 5 mM Tris-HCl, pH 7.5, 0.02% NaN_3) on ice during a week. ATP-Ca-G-actin was transformed into ATP-Mg-G-actin by a 3–5 min of incubation with 0.2 mM EGTA/0.1 mM $MgCl_2$ at room temperature. Actin was stabilized with aluminum fluoride (AlF_4^-) as described previously [21]. The protealysin cleaved actin was obtained by incubation of G-actin with purified protealysin [21,23].

2.3. Limited proteolysis assay

Mg-G- or Mg-F-actin (1 mg/ml) was incubated with protealysin or trypsin at various enzyme/protein mass ratios at 22 °C. The digestion was stopped by addition of an equal volume of the electrophoresis sample buffer containing 4% SDS, 125 mM Tris-HCl, pH 6.8, followed by a 3-min boiling. The digestion products were analyzed by SDS-PAGE.

2.4. ATP hydrolysis measurements

Protealysin-cleaved Mg-G-actin, polymerized with 0.1 M KCl for 40 min to steady state, was further incubated at 22 °C. The aliquots of the F-actin solutions withdrawn at time intervals were quickly mixed with an equal volume of 0.6 M ice-cold perchloric acid to quench the ATPase reaction; precipitated protein was removed by centrifugation, and released P_i was determined in supernatants by the Malachite Green method [24].

2.5. Sedimentation of F-actin in the ultracentrifuge

Protealysin-cleaved Mg-G-actin, polymerized with 0.1 M KCl for 40 min to steady state, was stabilized with fluorides and collected by a 30 min centrifugation in Beckman TLA120.2 ultracentrifuge at 200 000×*g*.

2.6. Cross-linking experiments

Actin was cross-linked with *N*, *N*′-*p*-phenylene-dimaleimide [25], at a cross-linker/actin molar ratio of 0.5: 1, for 15 min. The cross-linked products were analyzed by SDS/PAGE.

2.7. RhoA pathway inhibition

Balb 3T3 cells in DMEM medium were serum-starved for 24 h and treated with 10 μM Y27632 dissolved in DMSO or DMSO alone for 30 min. Then 5 mM NaF or NaCl was added to the Y27632 treated cells for 30 min [26].

2.8. Fluorescence microscopy

To visualize actin cytoskeleton, the cells were washed with PBS, fixed with 3.7% formaldehyde for 10 min and permeabilized with 0.1% Triton X-100 for 5 min. Cell nuclei were visualized by staining with DAPI for 5 min. To visualize F-actin cells were then stained with Rhodamine-phalloidin for 15 min. Samples were analyzed using a fluorescence Leica SP5 TCS inverted microscope.

2.9. Fluorescence and light-scattering measurements

All fluorescence and light-scattering measurements were performed in a Fluorat-02-Panorama spectrofluorimeter (Lumex, St. Petersburg, Russia). Fluorescence intensity of pyrenyl-labeled actin was monitored at 407 nm after excitation at 365 nm [27]. Intensity of light scattered at 90° was measured at 350 nm.

3. Results

3.1. Stabilization of protealysin cleaved F-actin with NaF

Globular actin cleaved between Gly42 and Val43 within the DNase-I-binding loop is virtually non-polymerizable if it contains Ca^{2+} as a tightly bound cation [18]. Replacement of the tightly bound Ca^{2+} by Mg^{2+} restores polymerization of the cleaved actin, although both the rate and extend of the reaction are lower than those for intact actin [21,28] primarily because the nucleation step of the filament formation is inhibited. Fig. 1 shows that the presence of 5 mM NaF results in disappearance of the long lag phase in the time course of protealysin-cleaved Mg-actin (Pln-Mg-actin) polymerization measured as the KCl-induced increase in the intensity of pyrenyl-fluorescence (Fig. 1A) or light scattering (Fig. 1B). This effect is apparently due to stabilization of nuclei formed at the initial step of the reaction, similarly to the effect produced by aluminium fluoride [21]. However, in contrast to the effect of AlF_4^-, the NaF-accelerated polymerization of Pln-Mg-actin was observed only in the presence of 0.1 M KCl, which may be caused by the difference in binding of sodium and aluminium ions with actin.

A twofold increase of NaF concentration did not produce any further acceleration of Pln-Mg-actin polymerization (not shown).

3.2. Dynamics of Pln-F-actin in the presence of NaF

Assembly of the cleaved Mg-actin monomers results in formation of highly dynamic filaments due to the enhanced turnover rate

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