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Microscopic studies on severing properties of actin-binding protein: its potential use in therapeutic treatment of actin-rich inclusions

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Abstract

Actin is an important unit of the cytoskeletal system, involved in many cellular processes including cell motility, signaling, and intracellular trafficking. Various studies have been undertaken to understand the regulatory mechanisms pertaining actin functions, especially the ones controlled by actin-binding proteins. However, not much has been explored about the molecular aspects of these proteins implicated in various diseases. In this study, we aimed to demonstrate the molecular properties of gelsolin, an actin-severing protein on the disassembly of the aggregation of actin-rich intracellular inclusions, Hirano body. We observed a decreasing tendency of actin aggregation by co-sedimentation assay and transmission electron microscopy in the presence of gelsolin. Therefore, we provide suggestive evidence for the use of actin-severing protein in novel therapeutic strategies for neurodegenerative conditions.

Keywords: Transmission electron microscopy, Actin, Gelsolin, Actin-binding protein, Actin-severing protein, Hirano body

Introduction

Actin is a fundamental cytoskeletal protein abundantly found in nearly all eukaryotic cells. It is highly conserved protein mediating many processes such as cell growth, motility, structural organization, as well as intracellular trafficking (Griffin et al. 2014). Being a critical cytoskeletal factor, tight regulation of the rapidly occurring assembly and disassembly of actin filaments is required for proper cellular operation (Remedios et al. 2003). Throughout the cell, actin is found in two standard forms: globular (G-actin) and filamentous (F-actin). The distribution and organization of these filaments into functionally relevant unit is regulated by several actin-binding proteins (ABPs) that modulate the underlying polymerization and depolymerization activities (Griffin et al. 2014; Remedios et al. 2003). These actin-binding proteins actively control the overall actin dynamics to perform different functions by modifying its structure both in vitro and in vivo (Fechheimer and Zigmond 1993; Pollard 2016). One of the representative classes of an actin-binding protein includes the gelsolin family, composed of six similar domains namely G1–G6 each having a Ca^{2+} binding site (Kinosian et al. 1998). This actin depolymerizing factor is found to bind tightly to the growing side of the actin filament thus decreasing its size and increasing the amount of monomeric actin present in the actin pool (Tellam 1985). Functionally, gelsolin causes rapid disassembly of actin filaments through severing, a mechanism involving breaking of the inner non-covalent bonds to produce shortened, broken filaments (Sun et al. 1999). Besides, the regulation of gelsolin and its counterpart is mainly affected by pH, phosphoinositides, lysophosphatidic acid and

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high micromolar concentrations of Ca^{2+} under physiological conditions (Sun et al. 1999; Spinardi and Witke 2007). In certain kinds of eukaryotic cells, gelsolin has been implicated to participate in numerous cytoskeletal events such as cytokinesis, cell movement and apoptosis besides its generic role as an actin-binding protein (Lappalainen 2016). Recent studies have shown its critical role in several conditions including amyloidosis, inflammation, cardiovascular diseases and cancer (Spinardi and Witke 2007). Another class of actin-binding protein, ABP34 is a small, 34 kDa crosslinking protein organizing F-actin into condensed bundles in a calcium dependent manner (Lim et al. 1999). Several in vivo studies have reported a part of this protein involved in the formation of Hirano bodies (Maselli et al. 2003). Hirano bodies are large, eosinophilic, actin-rich intracytoplasmic structures that contain paracrystalline arrays of F-actin (Goldman 1983) and ABPs (Galloway et al. 1987). Such cytoplasmic actin-rich aggregations comprising actin and ABPs are associated with various neurodegenerative diseases and can assemble as either actin/cofilin rods or Hirano bodies (Bamburg and Bloom 2009; Hirano 1994). Hirano bodies have been found to be associated with various neurodegenerative diseases such as Alzheimer's disease (Gibson and Tomlinson 1977; Mitake et al. 1997; Schmidt et al. 1989), Pick's disease (Schochet et al. 1968; Rewcastle and Ball 1968), Guam amylotrophic lateral sclerosis and parkinsonism-dementia complex (Hirano et al. 1968), chronic alcoholism (Laas and Hagel 1994) as well as general aging (Hirano 1994). Despite a large body of literature describing the structure, incidence, and antigens associated with Hirano bodies, the mechanism of formation remains largely unexplored. Here we demonstrated the potential application of actin-severing protein, gelsolin on actin-rich intracellular inclusions Hirano body by co-sedimentation assay and TEM. In this study, we used gelsolin to compete with enormous bundled actin filaments for resolving the phenomenon of actin aggregation.

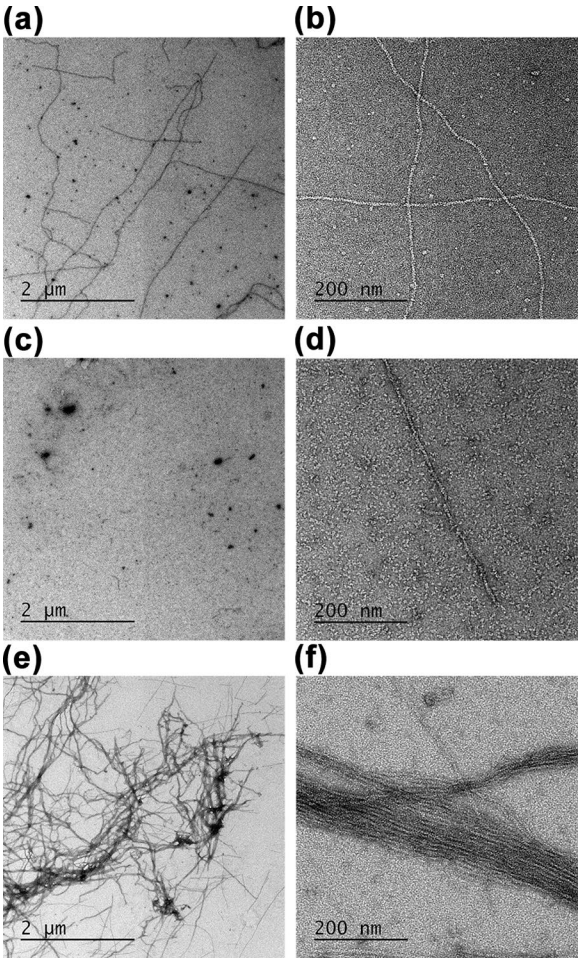

Fig. 1 Electron microscopic analysis of actin-severing and bundling activities of gelsolin and ABP34 in the presence of Ca^{2+}. Negative staining (NS) electron micrographs taken from F-actin alone (**a**, **b**); mixture of F-actin and gelsolin (**c**, **d**); mixture of F-actin and ABP34 (**e**, **f**). All the samples were mixed in low ionic strength buffer (at a molar ratio of 1:1; final concentration 3 µM). In the presence of Ca^{2+}, actin-binding proteins typically exhibited their native activity. NS-electron micrographs were taken at both low 4200 (**a**, **c** and **e**; left panels) and high 34,000 (**b**, **d** and **f**; right panels) nominal magnifications. Scale bars represent 2 µm (**a**, **c** and **e**) and 200 nm (**b**, **d** and **f**) in each field

Materials and methods

Protein sample preparation

ABP34 protein was purified from *E. coli* BL21 (DE3) cells (Novagen, USA) (Chung et al. 2017), dialyzed against buffer containing 50 mM sodium acetate, 2 mM $MgCl_2$, 1 mM EGTA, 20 mM MOPS, pH 7.0 and stored at -80 °C. Purification of gelsolin protein from bovine serum (Pel-Freez Biologicals, USA) was done as per (Kurokawa et al. 1990) which was subjected to dialysis in rigor solution (relaxing solution without mgATP), pH 6.5 and stored at -80 °C. Actin from rabbit skeletal muscle was a generous gift from M. Ikebe laboratory and stored at -80 oC.

Acto-myosin preparation from scallop tissue

The adductor muscle was dissected and cleaned from live scallops which were collected from a local market and placed in rigor solution (100 mM NaCl, 5 mM $MgCl_2$, 5 mM PIPES, 5 mM Na_2HPO_4, 1 mM EGTA, 1 mM NaN_3, pH 7.0) on ice. Muscles were then teased into thin strips and permeabilized with 0.5% saponin rigor solution for 4 h at 4℃ with gentle agitation followed by

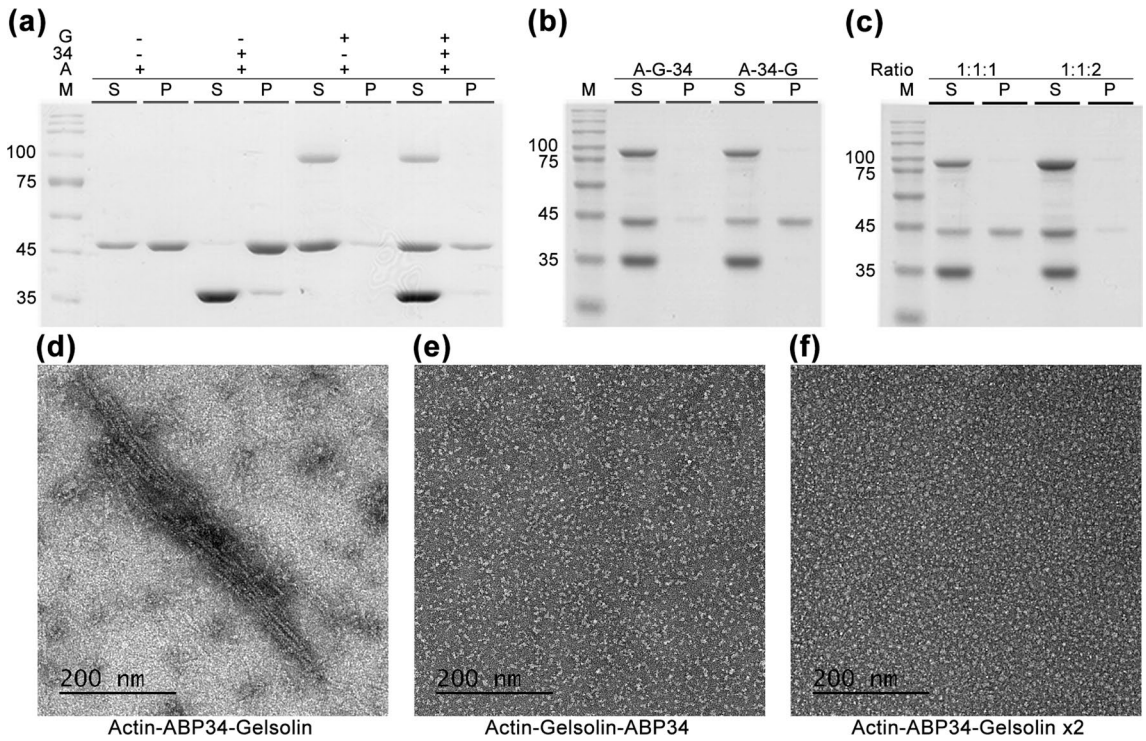

Fig. 2 SDS–PAGE and electron microscopic analysis of co-sedimentation assay. Co-sedimentation assay for gelsolin using SDS–PAGE (**a**–**c**) and negative staining electron microscopy (**d**–**f**). **a** General tendency of band migration; actin-alone, actin + ABP34 actin + gelsolin, actin + ABP34 + gelsolin (Molar ratio of 1:1:1). **b** effect of gelsolin treatment on actin-bundling activity. Gelsolin was treated (molar ratio of 1:1:1) before (A-G-34, left) and after (A-34-G, right) the formation of actin bundles. **c** Effect of concentration variation (3 μM and 6 μM) of gelsolin on actin-bundling activity [i.e., two different mixtures of A-34-G at different molar ratio of 1:1:1 (left) and 1:1:2 (right)]. S indicates supernatant, while P represents pellet. M, Marker; A, Actin; G, 34, ABP34; G, Gelsolin. **d**–**f** Negatively stained fields (at 34,000 nominal magnifications) of F-actin + ABP34 + gelsolin mixed at a molar ratio of 1:1:1 (**d**); Actin–gelsolin–ABP34 at 1:1:1 ratio (**e**); and Actin–ABP34–gelsolin 1:1:2 ratio (**f**). Here the molecular states were dramatically changed when the molar ratio was modulated to 1:1:2 and depolymerization states of the mixture were visualized in (**f**, (*c.f.* c)). Scale bars represent 200 nm in each field

thorough rinsing in rigor solution (Jung and Craig 2008). The weighed muscle tissue (approx. 0.4 g) was washed thrice using wash buffer (40 mM NaCl, 5 mM phosphate buffer). It was efficiently chopped and homogenized using HG-15D homogenizer (Daihan Scientific, R.O.K) at 15,000 rpm for 1 min on ice. The resulting suspension was re-homogenized with a glass homogenizer (Wheaton, U.S) under "low ionic buffer" condition (20 mM PIPES, 50 mM KCl, 5 mM EGTA, 1 mM $MgCl_2$, pH 7.0) on ice with 3–5 full passes (Jung et al. 2008a). The preparation was immediately centrifuged using Micro 17TR (Hanil Science, R.O.K) at 15,000 rpm for 10 min. The resulting pellet containing acto-myosin was resuspended up to 4 ml with relaxing solution, pH 6.5 and equally distributed into 4 tubes.

Gelsolin treatment in scallop muscle

In the first place, 1 mM mgATP was added to each tube containing the resuspended acto-myosin suspension. Next, each tube was treated for 30 min with different conditions involving 5.1 mM $CaCl_2$ and 3 uM gelsolin either alone or in combination, except for the control. This was then centrifuged to confirm the migration of actin band by SDS-PAGE. The gels were stained with Coomasie Brilliant Blue R-250 (Bio-Rad) and analyzed using ImageJ software (Schneider et al. 2012) whereas the statistical procedures were performed using SigmaPlot (Systat Software, San Jose, CA, USA).

Actin co-sedimentation assay

Co-sedimentation assay was conducted to understand the binding activity of actin with ABP34 and gelsolin. Actin was first polymerized under low ionic buffer (20 mM PIPES, 50 mM KCl, 5 mM EGTA, 1 mM $MgCl_2$, 1 mM mgATP, pH 7.0) for 1 h at room temperature forming F-actin. The F-actin was then mixed in equal proportion with ABP34 protein (3 μM) for 30 min at RT to form the actin bundles (Kim et al. 2015). Gelsolin (3 μM) was mixed with the former F-actin/ABP34 mixture for 30 min at RT followed by centrifugation in Micro 17TR

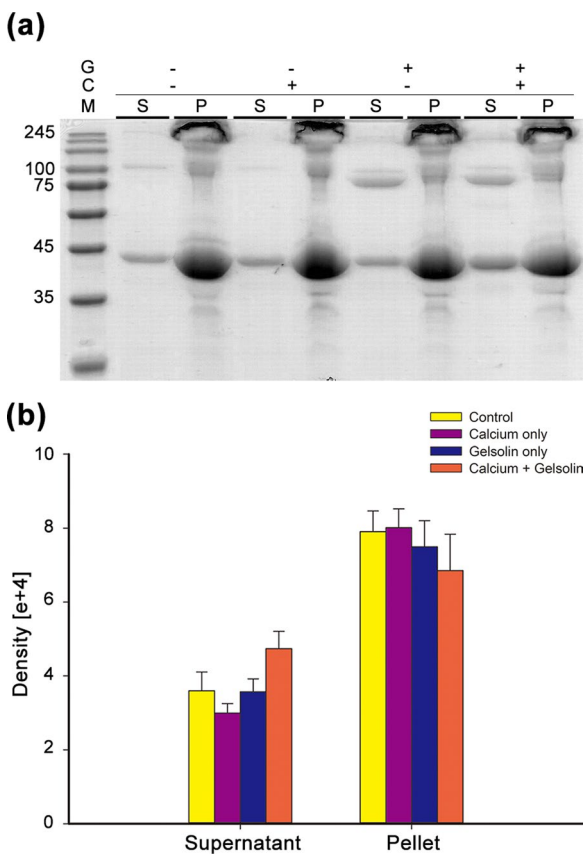

Fig. 3 Gelsolin activity in scallop muscle. Denote changes in the migration pattern of actin band from homogenized scallop muscle to confirm actin recover activity. SDS-PAGE result of homogenized scallop muscle with different conditions; control, with calcium, gelsolin and both (See tendency of actin band in each column) (**a**) and statistically analyzed intensity of each band (**b**). The graph represents the average intensity values of migrated bands (mean ± SD., $n = 8$). In the presence of activated gelsolin (i.e., with calcium ion), actin showed prominent movement from pellet to supernatant. S indicates supernatant, while p represents pellet. M, Marker; C, Calcium; G, Gelsolin;

(Hanil, R.O.K) at 15,000 RPM for 10 min (Kinosian et al. 1998). The obtained supernatant and pellet fractions were collected and analyzed by gel electrophoresis. Low ionic buffer conditions were maintained throughout the experiment.

Transmission electron microscopy

An aliquot of 5 µl of the sample under investigation was applied to a carbon-coated grid formerly glow-discharged (Harrick plasma, USA) for 3 min in air. The grid was negatively stained with 1% uranyl acetate (Jung et al. 2008b). The prepared grids were examined by Technai 10 TEM system (FEI, USA) operating at 100 kV. Images were acquired with UltraScan 1000 CCD camera (Gatan, USA). Instrumentation was used in Kangwon Center for Systems Imaging, Chuncheon, Republic of Korea.

Results and discussion

Activity of actin-binding proteins in the presence of calcium

Negatively stained electron micrographs were analyzed to understand the activity of actin in the presence of Ca^{2+} with or without ABPs such as ABP34 and Gelsolin. In the presence of Ca^{2}, G-actins were polymerized to from a filamentous actin called F-actin (Fig. 1a, b). However, when the F-actins were treated with gelsolin, they were depolymerized causing the formation of relatively shorter and fewer F-actin molecules (Fig. 1c, d). Compared to that, ABP34 effectively caused the formation of F-actin bundles in the presence of Ca^{2} (Fig. 1e, f). These implies that in the presence of Ca^{2+}, both gelsolin and ABP34 effectively execute their functions over F-actin such severing and bundling activities, respectively.

Poor tendency of actin aggregation in the presence of gelsolin

To understand the effect of gelsolin on the actin-bundling activity of ABP34, co-sedimentation assay was conducted (Fig. 2). When actin was incubated with gelsolin, it was found in the supernatant, while the band were predominantly seen in the pallet when it was treated with ABP34 (Fig. 2a). And also, the actin band significantly shifted to the supernatant when the two APBs were mixed together along with actin (Molar ratio 1:1:1), indicating that the severing activity of gelsolin somehow affect the actin-bundling function of ABP34. To evaluate the mechanism of the inhibition effect of gelsolin on the actin-bundling activity of ABP34, actin was incubated with both ABPs in different orders. In the experiment, the inhibition effect of gelsolin was more effective when it was incubated with actin before adding ABP34, suggesting that both ABP34 and gelsolin share same binding sties on F-actin (Fig. 2b, d, e). Notably, the actin bundles formed by ABP34 was eliminated by the doubled concentration of gelsolin, indicating that gelsolin has stronger binding affinity then ABP34 (Fig. 2c, f).

Gelsolin activity on actin aggregation in muscle

To understand the effect of gelsolin on the actin aggregation in the physiological environment, co-sedimentation assay was demonstrated using scallop muscle tissue. Based on the analysis of migration pattern of actin band, addition of Gelsolin in the presence of Ca^{2+} showed actin-band shift from pallet to supernatant significantly,

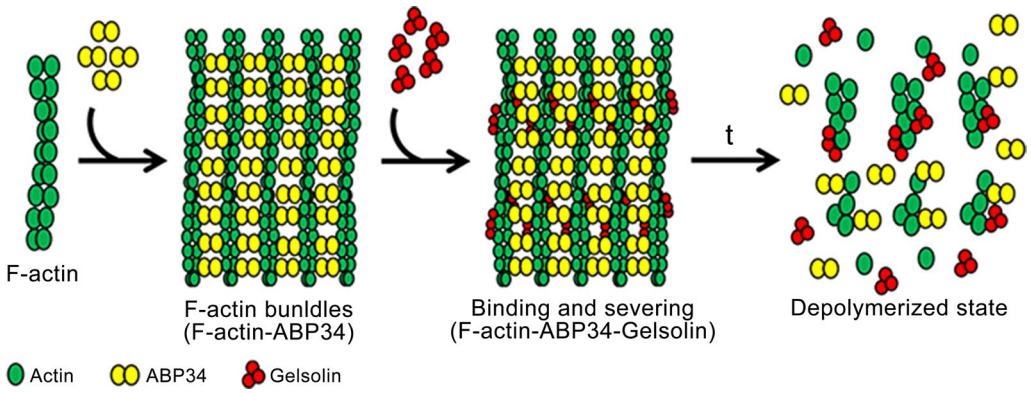

Fig. 4 A schematic outline of actin-bundling and severing activity from this study. This schematic model elucidates a consolidated approach and correlation of the three key proteins involved in this study shown as actin-bundling (F-actin-ABP34) to actin-severing (F-actin-ABP34-Gelsolin) activities. t, time; Green circle, actin; yellow circle, ABP34; red circle, gelsolin

and this indicates that gelsolin enable its inhibition effect on the actin bundling formation in the physiological conditions (Fig. 3a, b).

In this study, a decreasing tendency of aggregated actin by gelsolin molecules was determined through co-sedimentation assay and EM analysis. Taken together, we proposed that addition of gelsolin can eliminate aggregated form of actin, and this can be used to purify actin more efficiently from muscle tissue (Fig. 4). Furthermore, this finding can also provide fundamental clue on further studies devising a novel therapeutic approach to treat various actin aggregation related diseases (Carnell and Insall 2011; Gourlay and Ayscough 2005). A previous study demonstrating the role of actin-severing proteins in a variety of neurodegenerative conditions showed increased vulnerability to excitotoxicity when gelsolin deficiency was present in hippocampal neurons, both in cell culture and in vivo (Furukawa et al. 1997). Moreover, a study investigating the effect of antibiotics on the actin aggregates concluded that the actin aggregates are significant therapeutic target site not only for the treatment of various neurovegetative diseases, but also neurodevelopmental disorders (Pathak et al. 2016).

Although, this is in very early stage of studying new therapeutic strategies for neurogenerative conditions associated with actin aggregation, it provided a fundamental knowledge of how actin-severing proteins could be used to solve problems involved in the neurovegetative diseases (Furukawa et al. 1997; Pathak et al. 2016; Moseley et al. 2006).

Abbreviations

EM: Electron microscopy; TEM: Transmission electron microscopy; ABP: Actin-binding protein; F-actin: Filamentous actin; G-actin: Globular actin.

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Authors' contributions

Conceptualization was done by HSJ. Data curation was done by KHU and JHS. Formal analysis was performed by H-UK and JHS. Methodology was done by H-UK, JMC, AVB, JCM, and HSJ. Software was provided by H-UK. Validation was carried out by H-UK, JMC, and HSJ. H-UK carried out the investigation. Writing—original draft was done by H-UK, JMC, and HSJ. Writing—review and editing was done by H-UK, AVB, DJ, JMC, and HSJ. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Competing interests

The authors declare that they have no competing interests.

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