Structural Dynamics and Physiological Processes of Actin Filament Length Regulator Proteins

Dávid Zoltán Szatmári

Supervisors: Prof. Dr. Miklós Nyitrai, Prof. Dr. Robert C. Robinson

Interdisciplinary Medical Sciences Doctoral School D93

Head of Doctoral School: Prof. Dr. Balázs Sümegi

Program (B-130): Investigating functional protein dynamics using biophysical methods

Program leader: Prof. Dr. Miklós Nyitrai

University of Pécs, Medical School

Department of Biophysics

Pécs, 2018

Introduction

Actin is a 42 kDa globular protein. Actin forms microfilaments in eukaryotic cells that are the major component of the cytoskeletal system. In muscle cells, actin filaments are the main component of thin filaments and work as a scaffold of sarcomeres. *In vivo* actin can be presented as monomer G-actin (globular) or polymer F-actin (filamentous). Both of them are essential for important functions of a cell such as mobility, contraction, division, intracellular transport, signaling processes or maintaining the cell junctions and cell shape. A significant aspect of actin activity is that many intracellular processes are mediated by regulated interactions of actin with cellular membranes. In vertebrates there are three main actin isoforms, alpha, beta, and gamma. Alpha actins, found in muscle tissues, are the major mass of the contractile apparatus. Beta and gamma actins are represented together in many cell types as components of cytoskeleton and mediators of internal cell motility. Cells control the production of F-actin based microfilaments and their dynamical length regulation allows for rapid remodeling of cytoskeletal system, therefore allowing the cell to respond to any external or internal signals. The property of actin that it can be polymerized to long filaments endow an important roles in different cellular processes such as morphogenesis, membrane trafficking, and cell division. Strong cell adhesions, in order to form tissues, or structural proteins in a protrusion of cell membrane, can be anchored to this actin scaffold to allow processes such as endocytosis and cytokinesis. Actin can generate force by its polymerization or by the contribution of motor proteins, therefore actin plays important roles in processes of intracellular transport of vesicles and organelles as well as muscle contraction and cell migration, and is implicated in embryogenesis and invasion of cancer cells. To fulfill precisely these versatile functions, the spatial-temporal organization of actin network is controlled by a large number of actin binding proteins. These effectors can disassemble actin structures and assist in the formation of new actin networks with diverse architecture depending on the cellular process in which they are involved. To understand this actin-based machinery, it is necessary to explore the types of conformational and dynamic changes that occur in the filamentous actin due to the interaction with actin-binding proteins. The polymerization of monomeric actin into filaments is a thermal diffusion mediated process. The spontaneous association of monomers can be enhanced by high magnesium and potassium concentration. The polymerization starts with the formation of a few monomer based actin nuclei. The nucleation is proceeds by an imbalanced thermodynamical force where the entropy of dimers and trimers decreases by the local concentration of monomers. Afterward, when the actin seeds are formed the filament elongation is started immediately

with different kinetics at the ends. The fast growing end of the filament is the barbed end (+ end), and the pointed end is the slowly elongating end (- end). The kinetics of the elongation depends on the concentration of the free actin monomers (C_{free}) that are available in the solution and determines the association rate (k_a) of the actin subunits to filaments. Finally, the elongation of filaments reaches a reversible dynamical state where subsequent change in the length of the filaments does not occur. At this stage of the actin polymerization the steady-state equilibrium is maintained between the actin monomers and filaments, resulting in the continuous presence of around 0.1 μM concentration of actin monomers under in vitro conditions. However the average length of the actin filament does not change, but continuous association and dissociation of the actin subunits take place at the barbed end and the pointed end of the filaments, respectively. Actin-binding proteins (ABPs) are able to bind to actin monomers, polymers, or to both. Not only ABPs but also small molecules can bind to actin, and therefore contribute to the modulation of the dynamics of the actin cytoskeleton, and catalyze actin filament polymerization or depolymerization. This is not only integral to broader cellular processes such as cell migration and mechanosensing, but may also be exploited for experimental purposes. Proteins that bind to actin filaments affect the location rate and timing of actin filament assembly and disassembly. Actin monomers are polymerized into filaments under physiological conditions, but spontaneous depolymerization is too slow to maintain the fast actin filament dynamics observed in vivo. Gelsolin, actin depolymerizing factor ADF/cofilin, and several other actin severing/depolymerizing proteins can enhance disassembly of actin filaments and promote reorganization of the actin cytoskeleton. Many ABPs can regulate G-actin pool sizes through monomer sequestration or promotion/inhibition of nucleotide exchange. Actin-binding proteins also mediate interactions between actin and other cellular components, such as membranes, microtubules and other regulatory proteins. While some actin-binding proteins regulate the actin cytoskeleton, others use the binding of actin monomers or filaments to regulate their own activities or direct their cellular location. There is a great structural diversity in the types of proteins, which bind to actin, but the actin binding domains (ABDs) themselves can be grouped according to the conserved structures they form. Common types of actin binding domains (ABDs): calponin-homology (CH) domain, leucine rich repeat (LRR) domain, formin-homology-2 (FH2) domain, WASp-homology-2 (WH2) domain, actin-depolymerizing factor/cofilin (ADF/cofilin) domain, gelsolin-homology domain, myosin motor domain. The intracellular functions of F-actin length regulator proteins are linked to their structural dynamics and physiologically relevant complexes, and through them the whole cytoskeletal system can be remodeled or the length of

thin filaments can be optimized. Leiomodin is muscle specific and gelsolin is more common type of actin binding protein. We are interested in their structural changes and the physiological processes, by which are activated for actin binding. Gelsolin is severing and capping, and leiomodin is nucleating and elongating actin filaments. Gelsolin localizes to PIP_2-rich areas of a membrane, thus PIP_2 inhibits interactions between free gelsolin and actin and removes gelsolin caps from actin filaments. There is a strong evidence to suggest that PIP_2 islands in the membrane lead to the uncapping of filaments, resulting a rapid, directed filament elongation pushing the membrane. The discovery of the ATP: gelsolin interaction led to the suggestion that ATP maybe important in some of the multiple functions of gelsolin. At high calcium concentrations, gelsolin is almost fully activated, implying that the loss of its ATP-binding ability is due to disruption of the interaction site within gelsolin due to a conformational change. The structure of the gelsolin: ATP complex revealed the basis for its sensitivity to calcium ion concentration. Tropomodulins and leiomodins are built from homologous domain structures. Both proteins contain tropomyosin-binding domain (TMBS1 and/or 2), an actin-binding domain (ABS1) and a leucine-rich repeat (ABS2/LRR) that also binds to monomeric actin. In addition to these homologous domains, the structure of leiomodins diverge from tropomodulins by possessing a C-terminal extension (Cterm), which contains a proline-rich region (PR, a potential recognition site of intracellular signalling), helical domains and a Wiskott–Aldrich syndrome protein (WASP)–homology 2 (WH2) domain. In cardiac sarcomeres the expression of leiomodin2 and tropomodulin1 depends on the maturation stage of myofibrils. Calcium activated gelsolin binds to the barbed-end, and leiomodin binds the pointed-end, and both can bind to the sides of filaments. These two different F-actin length regulator proteins play different roles to modify the dynamics and kinetics of actin filament polymerization. Gelsolin is mobilizing short-capped actin polymers, whereas leiomodin is stabilizing the optimal length of filaments for an adequate contractible acto-myosin complex, but their simultaneous intra-sarcomere presence and function have not studied yet. However, we can describe their muscle function as the summary of different independent data. Active gelsolin can be localized randomly along the actin filament and reduce the length in sarcomere, tropomyosin competes with gelsolin on filaments, thus tropomyosin-gelsolin complex can regulate the length of thin filaments. Gelsolin enhances the ATPase activity of actomyosin is potentiated by tropomyosin which is a Ca^{2+}-insensitive acto-myosin enhancer. Leiomodin can be found near the M-lines and also shows diffuse distribution along the entire length of the thin filaments. The expression and sarcomeric localization of leiomodins are enhanced during myofibril maturation. Leiomodins bind to

different isoforms of tropomyosin and this interaction affects the actin polymerization promoting effect of leiomodins. It was suggested that tropomyosin modifies the pointed-end interaction but not the *de novo* nucleation activity of cardiac leiomodin2, which can be explained by different structural compatibilities. There is no evidence concerning how leiomodin decorates the whole thin filament or how it changes the ATPase activity of myosin.

Aims

The aim of my work was to obtain more information about actin filament length regulator proteins by two important proteins that bind to its ends, the barbed end binding cytoplasmic gelsolin or pointed end binding cardiac leiomodin2. The description of missing steps in recycling of gelsolin in its severing cycle are needed for a better understand of cytoskeletal system remodeling nearby of the cell membrane. A deeper understanding of the function of cardiac leiomodin2 in enhancing the length of actin filaments and modifying the actin-tropomyosin-myosin complex will lead to insights to the maintenance of cardiac muscle cell contractility. The major aims were: Describe the interplay between ATP and calcium, which may modify PIP_2 binding of gelsolin. Identify the missing steps in the recycling gelsolin from the membrane to the cytoplasm. Characterize the intrinsic structural features of cardiac leiomodin2 and the differences between leiomodin and tropomodulin. Investigate the actin assembly efficiencies of leiomodin2. Explore the thin filament side binding activity of leiomodin2 and its potential functional consequences.

Material and methods

Protein preparation and labelling

Calcium bound G-actin was prepared from rabbit muscle acetone powder according to the method of Spudich and Watt with a slight modification introduced by Mossakowska and co-workers. Skeletal muscle tropomyosin (Tpm1.1/2.2) was purified as described earlier then applied to hydroxyapatite chromatography and stored frozen. The human cytoplasmic gelsolin and its mutants were expressed by plasmid constructs containing the nucleotide sequence of His-tagged human wild type gelsolin in a pSY5 plasmid, were in *E. coli* Rosetta2 (DE3) pLyS cells.

Rattus norvegicus full length cardiac leiomodin2 and the C-terminal fragment (373-549 aa; Cterm) were expressed and purified using the Twin-CN chitin-intein self-cleavage and

purification system. DNA constructs were obtained from Roberto Dominguez's lab and cloned into pTyB1 vectors.

The Cys-374 residue of actin was labeled with Alexa488-maleimide or Alexa532-maleimide and was labelled with either IAEDANS (5-((((2-iodoacetyl) amino) ethyl) amino) naphthalene-1-sulfonic acid) as a FRET donor or IAF (5-iodoacetoamidofluorescein) as FRET acceptor as described earlier. Pyrene (N-1-pyrene-iodoacetamide) labelling was carried out by a standard protocol as described earlier. Actin concentration and labelling ratios were determined from the absorption spectra. Alexa488-maleimide and Alexa532-maleimide were used to modify the cysteine residues of gelsolin (Cys93, Cys188, Cys201, Cys304 and Cys645).

Membrane vesicle preparation

Phospholipid vesicles were prepared by a modified protocol from James H. Morrissey (Protocol from James H. Morrissey , Dept. of Biochemistry, University of Illinois at Urbana-Champaign, Urbana, IL 61801, USA). A mixture of 1% PIP_2 (PtdIns-(4,5)-P_2(1,2-dipalmitoyl)) 79% PC (L-α-phosphatydilcholine) and 20% PS (3-sn-phosphatidyl-L-serine) was dissolved in 20 μM rhodamine 590 N-succinimidyl ester contained chloroform.

Fluorescent spectroscopy methods

Intrinsic tryptophans of leiomodin2 were used as intrinsic probes for the fluorescence emission coupled structural dynamics measurements. Steady-state fluorescence quenching method was used to characterizing the structural dynamics of mutant gelsolin was labeled by Alexa488-maleimide. The quenching of Alexa488 emission refers to the accessibility of the fluorophore exposed by the binding of calcium, ATP or PIP_2. Steady-state anisotropy of gelsolin binding fluorescent derivatives of ATP and PIP_2 was measured for characterizing the dynamics of their complexes. The steady-state anisotropy resulting from intrinsic fluorescence of the tryptophans in leiomodin2 was measured to describe their intradomain flexibility. Fluorescence lifetime and anisotropy decay resulting from intrinsic fluorescence of the tryptophan of leiomodin2 were measured using the cross-correlation phase-modulation method (ISS K2 multi-frequency phase fluorimeter). Interdomain FRET of an engineered mutant gelsolin was measured to provide more information about interdomain flexibilty of gelsolin under different calcium ATP and PIP_2 conditions. Inter-monomer FRET measurements between actin monomers were carried out to characterize inter-monomer flexibility of the leiomodin2 decorated actin filaments. To study the effect of leiomodin2 on

actin polymerization the time dependent intensity of pyrene-labelled actin was measured. To determine the critical concentration of actin assembly, we measured the fluorescence emission of different concentration of pyrene labeled actin in the absence or presence of leiomodin2. To measure the binding kinetics of leiomodin to F-actin, the real time change of pyrene F-actin fluorescence mixed with leiomodin2 was measured by a stopped-flow fast kinetic system.

X-ray diffraction, crystallography

Crystals of calcium-free, human gelsolin mutant and Alexa488-maleimide labeled gelsolin were obtained by using the sitting-drop vapor diffusion method. Their structure was determined by X-ray diffraction.

Critical micelle concentration of PIP2

The critical micelle concentrations of PIP_2 and 1-(1-octadecanoyl-fluorescein-2R-octadecanoylphosphatidyl)inositol-4,5-bisphosphate were determined by dynamic light scattering.

Microscopy imaging

Confocal fluorescence microscopy imaging was carried out to explain the effect of ATP on Alexa488-labeled gelsolin binding to PIP_2 contained rhodamine590-filled vesicles.

Cosedimentation assays

High-speed cosedimentation assays used to describe the salt dependent binding of leiomodin2 to F-actin.

Coupled assay

Mg2+-ATPase activity of HMM in the presence of leiomodin2 was measured with coupled assay.

Results and discussion

Activation cycle of gelsolin

ATP and PIP_2 have been shown to compete in binding to K_{ATP} channels. Here, we have demonstrated that ATP can displace PIP_2 from gelsolin in solution under physiological buffer conditions *in vitro*. Furthermore, ATP is able to release PIP_2-bound gelsolin from the surface

of phospholipid vesicles. These observations suggest that ATP is likely to dissociate gelsolin from PIP_2 at plasma membranes, and this ATP-driven dissociation is the missing step in recycling of gelsolin during its actin filament remodeling cycle. In a background of high cellular ATP, PIP_2 will not generally bind to gelsolin. However, in the situation where gelsolin-capped filaments point at the plasma membrane, the filament barbed-end bound gelsolin becomes greatly reduced in its mobility, and it is in close proximity to membrane-bound PIP_2 that can move within the membrane and increase its local concentration by forming clusters. All these factors favor the binding between gelsolin and PIP_2, and hence filament uncapping. We propose that effective competition by ATP will dominate following filament uncapping and the dissociation of PIP_2-bound gelsolin is increased. The stages of this remodeling cycle under standard cellular conditions. Activation: elevation of calcium levels above 10 nM leads to a conformational change in gelsolin that releases ATP and allows gelsolin to recognize an actin filament. Severing: competition for actin-actin interactions by gelsolin-actin interactions leads to the severing of the filament. Capping: gelsolin remains bound to the barbed-end of the severed filament, preventing its elongation. Uncapping: when a gelsolin-capped filament encounters PIP_2 in the plasma membrane, the cap is removed through an unknown mechanism. The uncapped filament is then free to elongate and exert force on the plasma membrane. Release: gelsolin is released from PIP_2 at the plasma membrane through ATP competition, leading to diffusion of the gelsolin: ATP complex away from the plasma membrane. Gelsolin will return to its inactive state in low calcium environments. Thus, gelsolin is likely removed from PIP_2 at the plasma membrane in an ATP-dependent manner that distinguishes it from other PIP_2-sensing actin-regulating proteins, allowing gelsolin to cycle in a background of elevated PIP_2.

Structural dynamics and function of cardiac leiomodin2

We have shown that *Rattus norvegicus* cardiac leiomodin2 (Lmod2) possesses similar structural and functional features as human leiomodin2. *Rattus norvegicus* Lmod2 contains highly flexible, intrinsically disordered regions, similar to its Tmod homologues and other Lmods. In terms of actin dynamics, we demonstrated that *Rattus norvegicus* Lmod2 influences the rate of actin polymerization in an ionic strength dependent manner. This indicates that leiomodin2 may play a role in the regulation of thin filament generation in locations where tropomyosin and matured actin filaments are present, and new actin filaments are not needed. Apart from its already known colocalization with filament ends, Lmod2 was detected along the length of thin filaments in rat cardiomyocytes, as well as in M-lines.

Consistently with these observations, we demonstrated that Lmod2 can bind to the sides of actin filaments *in vitro*. The binding of leiomodin to actin filaments made the structure of the filaments more flexible. As a functional consequence, Lmod2 decreased the actin enhanced Mg^{2+}-ATPase activity of myosin upon F-actin binding. These novel interactions of leiomodin have functional implications. The localization of leiomodin in living muscle cells is not limited to the ends of the thin filaments, the presence of Lmod along the actin filaments, as well as in regions where myosin II is localized indicates that Lmod may influence acto-myosin activity in the cellular context. Leiomodin by reducing the activity of myosin, as we detect in our experiments even in the presence of lower amount ($\leq$ 1 μM) of Lmod2, may decrease the generated force during the interaction of thin and thick filaments. Our findings on the Lmod2 dependent activity of myosin II can supplement the recently published new model of cyclic activities of Lmod in sarcomeric functions and provides an *in vivo* support for the interpretation of our results. Considering that previous data indicated important roles of leiomodin in the developing heart, the regulation of the acto-myosin cross-bridge activity by leiomodin maybe essential in developing heart muscle cells, where larger than optimal forces may perturb the proper formation of the sarcomeric structure.

Plasticity of actin filaments provided by different length regulator proteins

The functions of actin polymers varies within a wide range of activities, and this variation is based on the plastic dynamics of filaments controlled by the effects of ABP proteins. Capping the barbed-end or pointed-end of a filament modifies the kinetics and dynamics of filamental turnover, allowing actin oligomers to be mobilized to remodel the whole cytoskeleton by gelsolin or for long polymers to be built in the stiff structural system of sarcomeres by leiomodin. Gelsolin and leiomodin are both recycling and implicated in crucial steps of cell migration or maturation of embryonic muscle. They bind the opposite ends of filament and show opposite effects on filament length, but both bind to the sides of filaments for two different purposes. Their physiological functions are composed by several different types of complexes for a well-tuned process of actin filament length regulation.

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