Ph.D. thesis

The effect of mouse twinfilin-1 on the structure and dynamics of actin

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UNIVERSITY OF PÉCS

MEDICAL SCHOOL

Ph.D. THESIS

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INTRODUCTION

Actin

Actin is the most abundant protein in eukaryotic cells and is a major component of the microfilament system. Actin is an essential functional unit of regulation of cellular activity that plays a crucial role in processes such as skeletal formation, transport processes, or cellular movement. Actin was discovered by Ilona Banga and Albert Szent-Györgyi in 1942 [1,2,3]. The actin is an ATPase protein, its molecular weight is 42.3 kDa and it is composed of 375 amino acids. Actin exists in monomeric (G-actin) and/or filamentous (F-actin) form. The actin molecule is structurally divided into two main domains designated as large and small domain, which are divided into two further subunits. Small domain consists of subunits 1 and 2, large domain consists of subunits 3 and 4 [4,5]. Under appropriate conditions the actin monomers can bind to each other by non-covalent bonds forming a double-stranded helix that is termed actin filaments (F-actin) [6]. The actin filament has structural polarity. We can distinguish a dynamically growing positive end (barbed end) and a negative end (pointed end) where the main process is the dissociation of protomers. Three main phases of the polymerization of actin can be differentiated (nucleation, elongation, dynamic equilibrium). The first phase starts with the formation of actin nucleus containing 2-4 monomers. The next stage is the diffusion-controlled elongation where monomers incorporate into the forming filaments. In the last phase there is association and dissociation on both ends of filament with different kinetics.

The ratio of intracellular monomeric and/or filamentous actin is maintained by the control of the actin-binding proteins. The actin binding proteins can effectively modify the structural and dynamic properties of actin [7,8,9]. Actin is highly sensitive for the changes in the environmental properties and to the presence of its binding partners [9,10,11]. The actin filaments are generally susceptible to pH dependent reorganization by several actin regulatory proteins.

ADF/cofilin protein family

The members of the ADF/cofilin family can regulate the turnover of actin in many ways. This family consists of three phylogenetically distinct classes: the ADF/cofilins, the drebrin/ Abp1s and the twinfilins [12,13]. Based on sequential analysis the residues that have been indicated to be

essential for actin binding in the ADF/ cofilin family are also well conserved in other classes, which led to the conclusion that all the ADF-H domain containing proteins can interact with actin [14,15].

Twinfilin

Twinfilins (TWFs) are evolutionarily conserved actin binding proteins that can be found from yeast to mammals [13]. Twinfilin was described first by Goode and co-workers in budding yeast in 1998 [12]. Twinfilins are about 40 kDa proteins consisting of two ADF- H/cofilin-like domains connected by a short linker region and a C-terminal tail. The two domains of twinfilins display approximately 20% sequence similarity to each other and also to other cofilin-like domain containing proteins [13]. Both ADF-H domains of mammalian twinfilins are able to bind G-actin independently. The C-terminal region is capable of binding actin monomer with similar affinity as the full-length protein and about 10-fold higher than N-terminus [16]. Although twinfilins have two potential actin-binding sites the ratio of G-actin:twinfilin is usually 1:1 under *in vitro* circumstances. [16]. The crystal structure of twinfilin's C-terminal ADF- H domain in complex with an actin monomer was presented in 2008 [17]. This domain binds between actin subdomains 1 and 3 through an interface that is conserved among ADF-H domain proteins. Based on this structure a mechanism is suggested by which ADF/cofilin and twinfilin inhibit the nucleotide exchange on actin monomer and a model is presented for how ADF/cofilin induces filament depolymerisation by weakening intrafilament interactions [17]. Twinfilins are actin monomer binding and sequestering proteins and they can effectively inhibit actin filament assembly by hindering the nucleotide exchange on actin monomers. So they have a crucial role in the polymerisation/depolymerisation cycle of actin [18]. Twinfilins can form more stable complex with ADP-G-actin (K_D =0.05 μM) than ATP-G-actin (K_D = 0.47 μM) similarly to ADF/cofilins [16]. Mammalian twinfilins can interact with the barbed-ends of actin filaments by both of the ADF-H domains [14,15]. Yeast TWF displays actin severing activity at acidic pH under in vitro conditions [19]. Twinfilins are able to bind capping proteins (CP) via its conserved C-terminal tail [20]. The interaction between TWF and CP is required for proper localization of twinfilin in yeast but has no effect on twinfilin-actin binding [20,21]. Twinfilins have only one isoform in unicellular organisms and invertebrates and three isoforms (twinfilin-1, twinfilin-2a and twinfilin-2b) are differentiated in mammals. In muscle cells twinfilin-2b is expressed whereas twinfilin-1 and 2a isoforms are

mainly expressed in non-muscle tissues [15,18]. Several biological roles of twinfilins have been described to date. Mammalian TWF-1 was identified as a suppressor of lymphoma cell migration and a stimulator of the chemotherapeutic vincristine to confirm the role of this actin binding protein in cell migration [22]. Furthermore, twinfilin-1 has a role in heart disease as well. Increased twinfilin-1 level was exhibited in cardiac hypertrophy [23]. This observation supports the role of TWF in cell morphogenesis. Twinfilin-2a depletion inhibited the growth of neurons, whereas the overexpressed TWF-2a elevated neurite length and stimulated the varicosities development [24].

AIMS OF THE THESIS

Despite the numerous comprehensive studies several biological function and actin cytoskeleton modifying effect of twinfilin and the corresponding molecular interactions have not been clarified yet.

The central interest of my Ph.D work was to characterize the structural and conformational changes in actin monomers upon the binding of mouse twinfilin-1. In addition to better understand the function of twinfilin-1 in cells we studied the effects of mammalian twinfilin-1 on the structure and dynamics of actin filaments with different biophysical techniques.

The following questions were addressed:

1) How does mouse twinfilin-1 interact with actin?
2) How does the mouse twinfilin-1 affect the nucleotide exchange on the actin monomer?
3) What type of conformational change is promoted in the nucleotide binding cleft due to the binding of mouse twinfilin-1?
4) How does the mouse twinfilin-1 alter the structure and conformational dynamics of the small domain of the G-actin?
5) Is there relationship between the conformational state of the nucleotide binding cleft and the heat stability of the G-actin?
6) We were curious whether the mouse twinfilin-1 isoform could bind to F-actin.
7) How does the twinfilin-1 influence the actin depolymerisation?
8) We were curious whether twinfilin-1 has actin filament severing activity and this activity shows pH dependency.
9) How does twinfilin-1 change the heat stability of actin filament?

MATERIALS AND METHODS

Protein preparation

Actin was prepared from acetone-dried muscle powder from rabbit skeletal muscle according to the method of Spudich and Watt modified by Mossakowska et al. [25,26,27]. The G-actin was stored in buffer A (4 mM Tris, 0.2 mM ATP, 0.1 mM $CaCl_2$, 0.5 mM MEA, 0.005% NaN_3, pH 8.0). The concentration of the G-actin was determined by using the absorption coefficient 1.11 mg ml^{-1} cm^{-1} at 280 nm [28] .

His-tagged mouse twinfilin-1 was obtained in Escherichia coli expression system as we described earlier [29]. His-tagged twinfilin construct was grown in Luria broth medium in the presence of 100 μg/ml ampicillin until the OD of the culture at 600 nm reached 0.6. After induction with 0.2 mM isopropyl- b-D-thiogalactopyranoside the expression was performed for 3 h at 37 °C. The collected cells were lysed, and the supernatant was purified in three steps. Nickel-nitrilotriacetic acid-agarose column (Qiagen), Q-Sepharose anion-exchange column and Superdex-75 HiLoad gel filtration column (GE Healthcare) were applied to reach the purified proteins. Purified twinfilins were stored in ATP free A buffer at -80 °C. Protein concentration was determined spectrophotometrically, using the molar extinction coefficient of 39100 M^{-1} cm^{-1} determined with the ProtParam tool.

Fluorescent labelling of actin

Actin was labelled with etheno-ATP, IAEDANS, FITC, N-(1-pyrene) iodoacetamide and Alexa488SE based on previously described methods [30,31,32,33,34].

Fluorescence anisotropy measurements

Steady-state fluorescence measurements were performed with a Horiba Jobin Yvon Fluorolog-3 fluorimeter (Longjumeau Cedex, France). The fluorescence anisotropy of ε-ATP-actin (1 μM) was measured in the presence of twinfilin concentration (0–8 μM). The $\Delta r/\Delta r_{max}$ was plotted as a function of twinfilin concentration and by using the appropriate equation, the affinity value was determined by fitting to the time points. In the temperature dependent FRET experiments IAEDANS labelled actin was used. The binding site of twinfilin on actin monomer is close to the position of IAEDANS. To exclude the adverse effect of labelling on the complex formation the

affinity of twinfilin for IAEDANS labelled G-actin was determined by steady state fluorescence anisotropy measurements. In these experiments the G-actin concentration was 2 μM while the twinfilin concentration was changed from 0 to 10 μM.

We applied the same method to define the affinity of twinfilin to the IAEDANS labelled Mg-F-actin (5 μM). The steady-state fluorescence anisotropy of F-actin was monitored in the presence of increasing twinfilin concentration (0-60 μM) while the excitation and emission wavelength were 350 and 479 nm, respectively.

Nucleotide exchange experiments

For the nucleotide exchange measurements an Applied Photophysics stopped-flow equipment was used. We applied 2 μM Ca-G-actin labelled with ε-ATP and varying concentration of twinfilin from 0 to 8 μM. The protein sample was mixed with buffer A supplemented with 1mM ATP. The fluorescence intensity was followed for at least 1000 s. The nucleotide exchange rate is monitored by following the decrease of fluorescence intensity of ε-ATP as it was dissociated from the actin monomers. The fluorophore was excited at 320 nm and the fluorescence emission was filtered with an FG 385 cut-off filter.

Fluorescence quenching experiments

Steady-state fluorescence measurements were carried out with a Perkin-Elmer LS50B (Waltham, MA) and a Horiba Jobin Yvon Fluorolog-3 (Longjumeau Cedex, France) spectrofluorometer equipped with a temperature-controlled holder. 5 μM ε-ATP bound actin was titrated at 22 °C with acrylamide from 0 to 0.3 M. The excitation wavelength was 320 nm and the emission spectra were recorded between 330 nm and 600 nm with 5 nm slits on the excitation and emission side as well. With the fluorescence quenching experiment it is possible to test the conformational changes around the actin bound fluorophore in the presence of twinfilin by calculating the Stern-Volmer constant (K_{SV}). The K_{SV} values can inform us about the accessibility of the fluorophore to the quencher molecules [35].

The time-resolved fluorescence quenching measurements were performed on an ISS K2 Multifrequency Phase Fluorometer (ISS Fluorescence Instrumentation, Champaign, IL) at 22 °C. The modulation frequency was varied in 10 steps from 2 to 64 MHz. The excitation wavelength

was 320 nm and the emitted light was observed through an FG 385 high-pass filter. We used freshly prepared glycogen solution as a reference (lifetime=0 ns).

Temperature dependent fluorescence resonance energy transfer (FRET) experiments

FRET measurements were carried out with Horiba Jobin Yvon Fluorolog-3 fluorimeter (Longjumeau Cedex, France) spectrofluorometer equipped with a thermostated cuvette holder. Actin was labelled with IAEDANS (donor) in subdomain 1 at Cys374 and with FITC (acceptor) in subdomain 2 at Lys61. The donor fluorescence intensity was monitored in the absence and in the presence of the acceptor. The excitation wavelength was 350 nm while the fluorescence intensity of IAEDANS was registered at 475 nm, where the contribution of acceptor to the measured fluorescence was insignificant. The measurements were performed at different temperatures from 5 to 35 °C in steps of 5 °C by employing dehumidifier machinery. The transfer efficiency, the donor acceptor distance and the *f'* parameter were determined by using the suitable equations [29,36].

Co-sedimentation assay

Co-sedimentation assay was performed to study the interaction of twinifilin and actin filaments. The samples were ultracentrifuged (300000 g, 30 min, 22 °C); the pellets and supernatant were separated and processed for SDS-PAGE analysis. The intensities of protein components were determined by Syngene bioimaging system program.

Actin depolymerisation assay

The depolymerisation assay was carried out with Safas Xenius FLX and Horiba Jobin Yvon Fluorolog-3 spectrofluorimeter. Pyrene labelled G-actin was applied to monitor the actin filament depolymerization kinetics. Mg-G-actin labelled with pyrene was polymerized in the presence of 2 mM $MgCl_2$ and 100 mM KCl in the sample cuvette. The actin filaments (1 μM) depolymerisation was followed in the absence or presence of twinfilin-1 (1.3 μM) after diluting the actin to 0.02 μM at different pH values whist the excitation and emission wavelength was 365 and 407 nm, respectively. The depolymerisation rate was defined from the slope of the linear fit to the initial part of the time dependence (the first 120 s) of the pyrene fluorescence curves.

Total Internal Reflection Fluorescence Microscopy (TIRFM)

TIRF microscopy technique was used to visualise and monitor the effect of twinfilin (1.53 μM) on the depolymerization of individual actin filaments (0.5 μM containing 10% Alexa488SE- G- actin). Actin filaments were bound to N-ethylmaleimide labelled myosin II treated glass surface. Images were recorded in every 10 s. Time lapse images were analysed with Fiji software. The depolymerization rates of actin filaments in μm/s were determined using the Multiple kymograph plugin and the length of filament in subunits per second was transformed by using 370 subunits per micron of filament as a reference [37].

Differential scanning calorimetry (DSC)

The DSC measurements were carried out with a Setaram Micro DSC II and Micro DSC III (Longjumeau, France) calorimeters. The heat denaturation curves were recorded between 20 °C and 100 °C with 0.3 K/min scanning rate. The actin concentration was 23 μM (0.97 mg/ml) in the experiments with actin monomer and the concentration of twinfilin was also adjusted to 23 μM. The Mg-F-actin concentration was 46 μM (2 mg/ml) while the concentration of twinfilin was 7.5 μM. In all cases, the samples were heated twice. The measurements after the second heating indicated that the denaturation of the actin during the first run was fully irreversible. The heat flow was plotted against the temperature and was further analysed by using the Origin Pro 8 software. The melting temperatures (T_m) of the proteins were determined from the peak of the heat denaturation curves of the samples. Furthermore the width of half maximum and the activation energy were calculated based on the method of Sanchez-Ruiz and the model of Lumry and Eyring [38,39].

RESULTS AND DISCUSSSION

During the whole study mainly Ca-G-actin was used in the experiments. The Ca-G-actin is more stable which makes it possible to reduce the influence of nonspecific environmental effects. Parallel to the reduced experimental errors the reproducibility of the results can be improved. Mg-G-actin was used in the temperature dependent FRET experiments and in measurements with F-actin. Another reason that can support our decision to work with Ca-actin is that it was suggested before that in some cells and in some intracellular regions of cells calcium-actin pools may be present temporary, which can be locally important in the dynamics of actin cytoskeleton [40].

I. EFFECT OF MOUSE TWINFILIN-1 ON THE STRUCTURAL AND DYNAMIC PROPERTIES OF ACTIN MONOMER

Binding of mouse twinfilin-1 to actin monomer

The first goal of our study was to investigate the interaction of mouse twinfilin-1 to actin monomer. The affinity of twinfilin for Ca-G-actin was determined by steady-state fluorescence anisotropy method. Steady-state fluorescence anisotropy is sensitive to changes in the rotation of the investigated fluorophore. One of the common sources of these changes is the change in the rotation of the whole protein to which the fluorophore is attached. The binding of a partner to a protein can thus be monitored through the change of the anisotropy of the protein bound fluorophores. The ε-ATP labelled actin monomers (1 μM) were titrated with twinfilin in different concentration (0–8 μM) the measured fluorescence anisotropy progressively increased. As the increase of fluorescence anisotropy can be related to the complex formation, the binding affinity of twinfilin for the actin monomers could be determined. The resolved equilibrium dissociation constant is 0.12 ± 0.04 μM, indicating that twinfilin-1 can tightly bind to the Ca-actin monomers.

Conformational changing of the nucleotide binding cleft

The interaction of actin with ADF/cofilin family proteins often resulted in the change of its nucleotide binding properties [7,35]. To test the possible conformational changes of actin monomer in the presence of twinfilin-1 we carried out nucleotide exchange experiments. Actin monomers (1 μM) were incubated with ε-ATP to allow equilibrium nucleotide binding, and then unlabelled ATP

was added at a greater concentration (1mM). Due to its large concentration it could replace the fluorescent nucleotide on actin. The displacement was accompanied by a fluorescence intensity changes allowing the measurement of the dissociation rates. When the experiments were carried out in the absence of twinfilin the first order dissociation rate constant was found to be 0.012 s^{-1}. The experiments were repeated in the presence of twinfilin in various concentrations (0–8 μM). We observed that the binding of twinfilin decreased the rate of dissociation to 0.003 s^{-1} in the presence of 8 μM twinifilin-1.

It was suggested previously that the nucleotide binding region between the small and large domains of actin is sensitive to the presence of actin binding proteins [35]. The nucleotide binding region was proved to be closed in the presence of cofilin and toxofilin [7,35]. The change of the nucleotide dissociation rate in the presence of twinfilin can be due to the altered conformation of the nucleotide binding pocket. To test this idea fluorescence quenching experiments were performed to describe the accessibility of the actin bound fluorescently labelled ATP in the presence of twinfilin-1. The Stern-Volmer constant (K_{SV}) was calculated what can get information about the accessibility of fluorophores. The K_{SV} was 0.33 ± 0.11 M^{-1} in the absence of twinfilin, its value decreased by almost ten-fold to 0.05±0.108 M^{-1} in the presence of twinfilin-1. The large decrease of the K_{SV} reflected the decrease of the accessibility of the fluorophore to the solvent and suggested that the nucleotide located in a more compact, closed nucleotide-binding pocket.

Effect of mouse twinfilin-1 on the conformation and dynamics of the small domain of actin

Fluorescence resonance energy transfer experiments were performed to study the structure and dynamics of the small domain of monomeric actin in the presence of twinfilin-1. The actin monomer was labelled with the fluorescent donor IAEDANS on cysteine 374 in subdomain 1. FITC as a fluorescent acceptor was attached to lysine 61 in subdomain 2. The binding site of twinfilin on actin monomer is close to the position of IAEDANS. The affinity of twinfilin for IAEDANS labelled G-actin was determined by steady state fluorescence anisotropy measurements to exclude the adverse effect of labelling on complex formation. The calculated K_D value was 0.16 ± 0.1 μM which is similar to the K_D value that was obtained for twinfilin binding to the ε-ATP labelled actin monomers (0.12 ± 0.04 μM). In the measurements the FRET efficiency and the R values slightly decreased while the temperature increased. The comparison of the data obtained in the presence and absence of twinfilin-1 indicated that the donor–acceptor distance was not sensitive

to the binding of the actin-binding protein at any of the applied temperatures. In addition, we determined the *f'* parameter what provides information regarding the flexibility of the protein matrix between the applied labels [36]. The relative change of the *f'* increased in case of Mg-G-actin as the temperature increased from 5 to 35 °C. In the presence of twinfilin-1 the *f'* did not significantly change as the temperature increased, indicating that the binding of twinfilin-1 made the protein matrix of the actin monomers more rigid.

Effect of mouse twinfilin-1 on the heat denaturation of G-actin

Previously described that there is connection between the conformation of the nucleotide-binding cleft and the heat stability of the actin. Therefore, to get further information the conformational changes in actin molecule differential scanning calorimetry assay was performed. The heat transitions of 23 μM Ca-G-actin was recorded in the absence or presence of 23 μM twinfilin-1. We found that the melting temperature of twinfilin-1 bound actin (T_m = 59.04 °C) was higher than that of the twinfilin-1 free actin monomers (T_m = 56.33 °C) and higher than twinfilin alone (Tm = 54.01 °C). These data suggest that parallel to the conformational transitions responsible for the more compact nucleotide binding cleft of actin the thermal stability of the actin increased after the binding of twinfilin-1. We further investigated the basic changes behind the complex formation between the actin and twinfilin- 1. The $T_{1/2}$ (the denaturing temperature width at half-height of the of the heat flow at the transition peak) and the activation energy were calculated for actin in the absence and in the presence of twinfilin- 1. The $T_{1/2}$ values were used to get information about the cooperativity of the melting of actin [41]. The 22% increase in the $T_{1/2}$ was observed in the presence of twinfilin-1 what can suggest that the cooperativity during the phase transition is smaller for the actin-binding protein bound actin monomers compared to the un-complexed ones. The activation energy of the phase transitions was also calculated for the Ca-G-actin and the twinfilin-1 as well. The E_A value for Ca-G-actin was smaller in the presence of twinfilin-1. This observation may suggest that in case of the actin–twinfilin complex, a little bit less energy is needed to initiate the denaturation process than in the case of the un-complexed Ca-bound actin monomers.

Investigation of the actin-twinfilin-1 interaction with co-sedimentation assay

It was described before that twinfilins have sequestering function [19]. Co-sedimentation experiments were performed to confirm the sequestering activity of twinfilin- 1 and to investigate the twinfilin-F-actin interaction. It was found that the density of actin in the supernatant increased

with increasing twinfilin-1 concentration, suggesting an increase in the concentration of actin monomers. Analyzing the pellet, it can be stated that the density of actin in the pellet decreased with the increase of the concentration of twinfilin, thus the concentration of F-actin shows a decreasing tendency. Furthermore, the twinfilin-1 was detected in pellets at higher twinfilin-1 concentrations but did not sediment itself. Consistent with literature data, the results of co-sedimentation studies suggest that twinfilin-1 has sequestering activity and may interact with actin filaments.

II. INTERACTION OF MOUSE TWINFILIN-1 WITH F-ACTIN

Binding of mouse twinfilin-1 to F-actin

The affinity of twinfilin-1 for Mg-F-actin was determined by steady-state fluorescence anisotropy method. The IAEDANS labelled actin filaments (5 μM) were titrated with twinfilin- 1 in different concentration (0–60 μM). A decreasing tendency was observed of anisotropy value with increasing twinfilin-1 concentration. The decrease of the steady-state anisotropy can be related to the binding of twinfilin to F-actin. The resolved equilibrium dissociation constant was 25,7 ± 8,5 μM, indicating that twinfilin-1 can tightly bind to F-actin. In agreement with previous reports [18] we concluded that the decrease of the anisotropy was related to the sequestering function of twinfilin-1. Parallel to the isolation of the actin fragments the direct severing activity of twinfilins can also be involved in the appearance of the decreased steady state anisotropy values.

The effect of twinfilin-1 on the dynamics of actin filament depolymerisation

Dilution-induced depolymerization assay was also performed to verify the effect of twinfilin-1 on the dynamics of actin depolymerisation. During the experiments 1 μM pyrene labelled F- actin was diluted to 20 nM to trigger the net disassembly of the actin filaments. As the 20 nM is below the critical concentration of actin ($actin_{cc}$ ~ 100 nM) the actin filaments are depolymerised. Under these conditions the reversal effect of the free G-actin on the kinetics of F-actin depolymerisation can also be excluded. As the actin monomers do not affect the depolymerisation the sequestering function of an actin binding protein can also be eliminated by these experiments. The severing function of an actin binding protein can be more specifically explored by this assay. It was described previously that yeast twinfilin can function as an actin filament severing protein at pH values below 6.0 [19]. In our measurements the effect of mouse twinfilin-1 on actin

depolymerisation was studied at different pH values. The measurements were performed at different pH values between pH 5.0 and 7.8. Latrunculin-A was used as a control with sequestering function and this actin-binding protein did not cause any effect in the depolymerisation of actin filament as we expected. Twinfilin-1 induced a more prominent decrease in the fluorescence signal compared to sample where actin was present alone. We determined the depolymerisation rate constants what does not change significantly in the case of actin with increasing pH. However, in the presence of twinfilin-1 a decreasing tendency was observed with increasing the pH and a significant difference ($p < 0.05$) was observed at pH 5.5 and 6.0. These results suggest that the applied twinfilin isoform has a pH sensitive actin filament severing activity.

Real-time visualization of the direct effect of twinfilin-1 on individual actin filaments

The spectroscopic experiments showed that the twinfilin-1 dependence of the depolymerisation was most prominent at pH 6.0 while no twinfilin-1 dependence was observed at higher pH value (7.8). To see the maximum difference in the effect of twinfilin-1 the TIRFM measurements were performed at these two pH values. The length of actin filaments and the rate constants of actin depolymerisation were determined in the absence and presence of twinfilin-1. The average filament lengths were compared in the absence and in the presence of 1.53 μM twinfilins-1. At pH 7.8 the average filament length was not shown significant difference after depolymerisation in the absence and presence of twinfilin-1, respectively. At pH 6.0 the value of this parameter significantly decreased in the presence of twinfilin-1. The average rate of depolymerisation was calculated based on the length change during the available time period in the absence and presence of twilfilin-1 at both pH values. The average rate of depolymerisation was observed a small increasing in the presence of twinfilin-1. at pH 7.8. Contrarily the depolymerisation rate significant grown in the presence of twinfilin-1 at pH 6.0. Based on these results it was concluded that twinfilin-1 can effectively sever the actin filaments at low pH values. With these TIRFM experiments it is possible to verify the existence of the pH dependent severing activity of mouse twinfilin-1.

The effect of twinfilin-1 on the thermodynamic stability of Mg-Factin

To complete to describe the effect of twinfilin on the thermodynamic stability of Mg-Factin differential scanning calorimetry experiments were performed. When only 46 μM (2 mg/ml) F- actin was present in the solution only a single denaturation peak was identified at 66.5 °C. This

T_m value is in good agreement with our previously recorded data [42]. The heat denaturation curve of actin filaments was also recorded in the presence of twinfilin-1. When 7.5 μM twinfilin-1 was added to 46 μM (2 mg/ml) F-actin a complex peak was obtained that could be resolved into the three individual curves of the components. The peak with the lowest melting temperature can be related to the presence of actin monomers [29] while the peak with
59.2 °C can be connected to the G-actin-twinfilin complex [29]. The peak with the highest Tm value (63.23 °C) is a new component that was not observed before. It is reasonable to assume that this peak is a descendant of F-actin based on the closed proximity of its T_m value to that observed for the actin filaments in the absence of twinfilin-1 (66.5 °C). The result would be a population of actin polymers which are partially disintegrated and bind the effector molecule in a certain quantity. Based on these considerations the 63.23 °C Tm value probably belongs to the twinfilin–F-actin complex and the partially disintegrated actin filament in the sample. As the T_m in this case is lower than in case of the F-actin it seems that twinfilin-1 loosen the molecular connections between the neighbouring actin protomers within the actin filaments just before the separation of the protomers from the polymeric actin.

SUMMARY

In living cells actin is responsible for many biological functions. The key molecular mechanisms behind the reaction of the actin cytoskeleton can help to understand the nature of the interactions of actin-binding proteins, and thus deserves future investigations**.**

Based on the results of this work we state that:

- We determined the affinity of twinfilin for Ca-G-actin by steady-state anisotropy experiments. The resolved equilibrium dissociation constant is 0.12 ± 0.04 μM, indicating that twinfilin-1 can tightly bind to the Ca-actin monomers.
- Mouse twinfilin-1 inhibits the nucleotide exchange process on the actin monomer.
- We observed that the accessibility of the etheno-ATP located in the cleft decreased in the presence of twinfilin-1.
- We determined FRET efficiency and the R values in the presence and absence of twinfilin-1. The donor–acceptor distance was not sensitive to the binding of the twinfilin-1 at any of the applied temperatures. In the presence of twinfilin-1 the f' did not significantly change as the temperature increased, indicating that the binding of twinfilin made the protein matrix of the actin monomers more rigid.
- DSC data suggest that the thermal stability of the actin increased after the binding of twinfilin.
- Steady-state anisotropy revealed that twinfilin can bind to F-actin with a K_D of 25.7 ± 8.5 μM.
- The results of the dilution-induced depolymerisation assay was indicated that mammalian twinfilin-1 has a pH sensitive actin filament severing activity as well.
- TIRFM measurements further supported the existence of the pH dependent severing activity of mouse twinfilin-1.
- Our DSC measurements demonstrated that after binding the filaments mammalian twinfilin-1 reduced the rigidity within the actin filaments. It seems that twinfilin-1 loosen the molecular connections between the neighbouring actin protomers within the actin filaments just before the separation of the protomers from the polymeric actin.

The nucleotide binding cleft shifted into a more closed and stable conformational state of actin in the presence of twinfilin-1.

Mouse twinfilin-1 is able to accelerate the monomers dissociation and/or sever the filaments effectively at low pH values. Twinfilin-1 can have an important regulatory effect on F-actin decomposition in some special biological processes or under stress situations where the pH is markedly lower than the physiological value.

LIST OF PUBLICATIONS

Publications related to my Ph.D. work

Veronika Takács-Kollár, Miklós Nyitrai, Dénes Lőrinczy, Gábor Hild: Resolving the similarities and differences between the effect of structurally different actin binding proteins on the thermodynamic properties of G-actin. JOURNAL OF THERMAL ANALYSIS AND CALORIMETRY 127: pp. 1261-1266. (2017)

IF: 2.209

All public citation: 1, Independent citation: -

Veronika Takács-Kollár, Dénes Lőrinczy, Miklós Nyitrai, Gábor Hild: Spectroscopic characterization of the effect of mouse twinfilin-1 on actin filaments at different pH values. JOURNAL OF PHOTOCHEMISTRY AND PHOTOBIOLOGY B-BIOLOGY 164 pp. 276 - 282., 7 p. (2016)

IF: 2.673

All public citation: -, Independent citation: -

Veronika Takács-Kollár, Miklós Nyitrai, Gábor Hild: The effect of mouse twinfilin-1 on the structure and dynamics of monomeric actin. BIOCHIMICA ET BIOPHYSICA ACTA-PROTEINS AND PROTEOMICS 1864: 7 pp. 840-846. , 7 p. (2016)

IF: 2.773

All public citation: 5, Independent citation: 2

Conference talks related to my Ph.D. work

Kollár Veronika, Kardos Roland, Nyitrai Miklós és Hild Gábor: A twinfilin-1 hatása az aktin szerkezeti és dinamikai tulajdonságaira. II. Interdiszciplináris Doktorandusz Konferencia, Pécs, 2013. május 15-17.

Veronika Kollár, Roland Kardos, Miklós Nyitrai and Gábor Hild: The effect of mouse Twinfilin-1 on the structure and dynamics of actin

27th European Cytoskeletal Forum Meeting, Pécs, Hungary, 3-7 November 2012.

Veronika Kollár, Roland Kardos, Pekka Lappalainen and Gábor Hild: The effects of mouse twinfilin 1 on the structure and dynamics of monomeric actin. 8th European Biophysics Congress, Satellite Conference: Intracellular Fluorescence Spectroscopy, Pécs, Hungary, 20-22 August 2011. Winner of poster competition.

Conference posters related to my Ph.D. work

Veronika Kollár, Roland Kardos, Pekka Lappalainen Miklós Nyitrai and Gábor Hild**:** The effects of mouse Twinfilin-1 on the structure and dynamics of actin. 28th European Cytoskeletal Forum Meeting, Fribourg, Switzerland, 1-5 September 2013

Kollár Veronika, Kardos Roland, Nyitrai Miklós és Hild Gábor: Twinfilin-1 hatása az aktin szerkezeti és dinamikai tulajdonságaira. A Magyar Biofizikai Társaság XXIV. Kongresszusa, Veszprém 2013. augusztus 27-30.

Veronika Kollár, Roland Kardos, Pekka Lappalainen Miklós Nyitrai and Gábor Hild**:** The effect of mouse Twinfilin-1 on the structure and dynamics of actin. 27th European Cytoskeletal Forum Meeting, Pécs, Hungary, 3-7. November 2012

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