Ph.D. thesis

The activities of the gelsolin homology domains of Flightless-I in actin dynamics

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INTRODUCTION

Flightless-I (Fli-I) is a relatively newly identified actin-associated protein, which was originally discovered in *Drosophila melanogaster*. The mutations in the *Fli-I* gene lead to abnormal myofibrillar arrangements in the flight muscles causing the loss of flight ability. The severe form of the mutations can cause incomplete cellularization during early embryogenesis (Campbell et al, 1993, Campbell et al, 1997). Fli-I is a member of the gelsolin homology (GH) domain protein family. These actin-binding proteins have multiple functions and play an important role in the remodeling of the actin cytoskeleton. The founding member of this family, the 6 GH domain gelsolin, is characterized in the most detail. It is a multifunctional protein possessing actin filament severing, filament end capping and *de novo* nucleation activities (Cowin et al., 2006, Kopecki and Cowin, 2007).

Like the other members of the gelsolin family, Fli-I is evolutionary highly conserved, implying that it has vital functions. Fli-I protein is required for actin distribution during cellularization in *Drosophila* and mice (Kopecki and Cowin, 2007). In support of this, homozygous Fli-I knockout mutation in mouse leads to early embryogenic lethality, which indicates the essential role of the protein in embryonic development (Campbell et al., 2002). Fli-I is expressed widely in human tissues; it is abundant in skeletal, myocardial and nerve cells (Campbell et al, 1993, Campbell et al, 1997, Davy et al., 2000). In cells, Fli-I presents in the nucleus, where it may act as a hormone-regulated nuclear receptor coactivator (Cowin et al., 2007). In the presence of serum, Fli-I can translocate from the nucleus to the cytoplasm, where it plays a key role in cell migration, which is thought to be linked to its negative influences on wound healing and tissue regeneration (Davy et al., 2000). The human Fli-I is involved in epidermolysis bullosa and Smith-Magenis syndrome causing developmental and behavioral abnormalities (Jackson et al., 2012, Cameron et al., 2016).

Fli-I alloys leucine reach repeats (LRR) and gelsolin homology domains (GH), which confers unique structural characteristics to the protein (Davy et al., 2000). Fli-I has been shown to interact functionally with various proteins; it participates in cell signaling, regulates cytoskeleton re-organization or influences actin dynamics and cell migration (Kopecki and Cowin, 2007). The LRR region consists of 16 tandem 23 residues long leucine-rich repeat motifs found in several proteins with diverse cellular localization and functions (Liu et al., 1998, Matsushima et a., 2010). The LRR is potentially involved in protein-protein interactions. Goshima et al. have found that *C. elegans* Fli-I binds Ras2 through its LRR region and may participate in cytoskeletal control. C-terminally to the LRR, Fli-I contains six gelsolin

homology domains which are potential actin-binding regions. Although the actin-binding ability of Fli-I was proposed, the functional outcome is still elusive (Goshima et al., 1999, Mohammad et al., 2012). Incubation of crude yeast extract containing *C. elegans* Fli-I with actin agarose suggested that Fli-I associated with G-actin even in the presence of 10 mM EGTA indicating Ca^{2+}-independent interaction (Goshima et al., 1999). In co-sedimentation experiments, Fli-I appeared in the pellet with actin filaments; also, it increased the amount of unpolymerized actin in the supernatant, interpreted as F-actin severing activity (Goshima et al., 1999). Pyrenyl polymerization experiments suggested that actin assembly is retarded by mouse Fli-I in a concentration-dependent manner, while disassembly is not markedly influenced (Mohammad et al., 2012). This led to the conclusion that Fli-I possesses actin filament capping but not severing activity. Arora et al. (2015) have shown that the GH16 domains of Fli-I inhibited actin polymerization, while the truncated Fli-I GH26 had no effect. These results suggest that the actin-related function of Fli-I is associated with its GH1 domain. Recent data suggested that Fli-I may enhance the actin nucleation activity of formin proteins, including Daam1 and mDia1 by binding to Daam1 and mDia1 through its GH46 region (Higashi et al., 2010).

Although several studies have been performed regarding the effects of Fli-I on actin dynamics, the underlying molecular mechanism is still controversial and incomplete. To get more insight into the biological functioning of Fli-I, we aimed to analyze the actin activities of its different regions, including the gelsolin homology (GH) domains, as well as the leucine-rich repeat segment (LRR) by protein biochemical and biophysical approaches.

OBJECTIVE AND AIMS

Although several studies have been performed regarding the effects of Flightless-I on actin dynamics, we still face a lack of understanding of the underlying molecular mechanisms. We aimed to investigate the activities of Fli-I underlying its cytoplasmic functions. We studied the interactions of different, recombinantly produced Fli-I constructs with actin by using protein biochemical and biophysical approaches *in vitro*. Gelsolin homology domains of Fli-I are responsible for the actin-binding activities; therefore, we primarily investigated the effects of the GH domains on actin dynamics. The small actin-binding protein, profilin plays an important role in the building of the cellular actin structures; thus, we also studied whether profilin influences the actin activities of Fli-I. We also performed a comparative analysis of Fli-I and gelsolin to reveal the similarities and differences between these GH domain proteins.

My work addressed the following questions:

- What are the activities of Flightless-I in actin dynamics?
- How each region (LRR/GH domains) of the protein contributes to these activities?
- What are the similarities and differences between the regulation and actin interactions of gelsolin and Flightless-I?
- Does profilin influence the actin activities of Flightless-I? If it does so, what are the effects of profilin on the actin activities of Flightless-I?

MATERIALS AND METHODS

Protein expression and fluorescence labeling

For bacterial protein expression, cDNAs of *D. melanogaster* Flightless-I (GenBank accession no. Q24020) were inserted into pGEX-6P1 vector encoding the GH16 domains and into pGEX-2T vector encoding the LRR, GH13 and GH46 domains by our collaborator (József Mihály, Hungarian Academy of Sciences, Biological Research Centre, Szeged). Constructs were expressed as glutathione S-transferase (GST) fusion proteins in the *Escherichia coli* BL21(DE3)pLysS strain (Novagen). Fli-I was purified with affinity chromatography using Glutathione Sepharose 4B (Sigma-Aldrich). Actin was extracted and purified from acetone-dried rabbit skeletal muscle powder (Feuer et al., 1948) and modified with fluorescence probes (pyrene, Alexa488NHS and Alexa568NHS) according to standard protocols (Bugyi et al, 2006; Barko et al., 2010; Bugyi et al, 2010; Bugyi, Didry et a., 2010; Toth et a., 2016). Gelsolin (human) and profilin 1 (mouse) was purified as described previously (Perelroizen et al., 1994; Nag et a., 2009). DAAM (*D. melanogaster*) fragments were purified according to Vig and co-workers (2017).

Fluorescence spectroscopy experiments

In **polymerization/depolymerization** measurements gel filtered actin (2.5 µM, containing 2% or 5% pyrenyl actin in the presence and absence of profilin, respectively) were used in the presence or absence of different concentrations of Fli-I proteins. The polymerization was initiated by the addition of 1 mM $MgCl_2$ and 50 mM KCl. The profilin:G-actin samples contained 6 µM profilin. Actin assembly and disassembly were measured by monitoring the change in pyrenyl fluorescence emission using a Safas Xenius FLX spectrofluorimeter ($\lambda_{excitation}$ = 365 nm, $\lambda_{emission}$ = 407 nm). The polymerization rate was derived from the slope of the initial linear part of the pyrenyl fluorescence traces.

The **steady-state anisotropy** of monomeric actin (0.2 µM Alexa488NHS labeled G-actin, incubated with 4 µM LatrunculinA (LatA)) was measured to study its interaction with Fli-I. The anisotropy measurements were performed in a Fluorolog-3 spectrofluorometer (Horiba Jobin Yvon, $\lambda_{excitation}$ = 488 nm, $\lambda_{emission}$ = 516 nm). In profilin-containing samples, profilin (4 µM) was added to actin after the incubation with LatA. LatA binds to actin monomers and prevents them from polymerizing; thus, the increase in steady-state anisotropy is expected to result from the binding of Fli-I to G-actin.

Total internal reflection fluorescence microscopy (TIRFM) experiments

The effects of Fli-I and profilin on the assembly/disassembly were studied by TIRF microscopy, which allowed us to observe the activities at the level of individual actin filaments. A mixture of G-actin (0.5 µM, 10% Alexa488NHS labeled) and different concentrations of Fli-I constructs was injected into the flow cell. Two-color TIRF experiments were performed to follow the filament growth in the absence and presence of Fli-I and profilin (2 µM). G-actin (0.5 µM, 10% Alexa488NHS labeled) was polymerized in the flow cell to form "green" actin filaments, and then unpolymerized actin was washed out. A mixture of G-actin (0.5 µM, 10% Alexa568NHS labeled), profilin (2 µM) and Fli-I in TIRF buffer was transferred into the flow cell. In disassembly assays, G-actin (0.5 µM, 10% Alexa488NHS labeled) was polymerized in the flow cell, then different amounts of Fli-I or gelsolin were added. In end-to-end annealing assays, G-actin (1 µM, containing either 10% Alexa488NHS or 10% Alexa568NHS labeled actin) was polymerized for 1 h at room temperature, F-actin was stabilized by unlabeled phalloidin (1:1 molar ratio) overnight. Actin filaments labeled with different fluorophores were mixed in the absence or presence of Fli-I and then fragmented by a 26G syringe (10×). Samples were diluted to 2 nM F-actin into TIRF buffer, adsorbed onto poly-L-lysine-functionalized coverslip and processed for microscopy analysis. To measure the number of actin filaments at steady-state, G-actin (2 µM, unlabeled) was polymerized overnight either in the absence or presence of Fli-I GH16 (800 nM). Filaments were stabilized by Alexa Fluor^{TM} 488 phalloidin (1:1 molar ratio), diluted to 5 nM into TIRF buffer and adsorbed onto poly-L-lysine-functionalized coverslip for imaging.

Imaging and image analysis

Images were captured with an Olympus IX81 microscope equipped with a laser-based TIRF module (laser lines: 491 nm, 561 nm, APON TIRF 60x NA1.45 oil immersion objective, Hamamatsu Orca-ER high-resolution digital B/W CCD camera). Time-lapse images of actin assembly/disassembly were captured every 10.5 s. The number and length of actin filaments were derived from a 66×66 µm^2 region of the images by using Fiji.

Statistical analysis

The data represented in the dissertation were derived from at least two independent experiments. Values are displayed as mean ± standard deviation. Statistical analysis (Student's

t-test) was performed by Microsoft Excel (ns $p > 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$).

RESULTS AND DISCUSSION

The GH domains of Flightless-I interact with actin and affect actin dynamics in a Ca^{2+}-independent manner

The actin interactions and activities of Fli-I were investigated in the absence and presence of Ca^{2+} ions in fluorescence spectroscopy experiments. For this purpose, constructs encompassing all the six gelsolin homology (GH16) domains, as well as an N-, and a C-terminal fragment of Fli-I comprising the first and second half of the GH domains, Fli-I GH13 and GH46, respectively, were studied.

The interaction of Fli-I GH domains with G-actin was tested in steady-state anisotropy measurements by monitoring the anisotropy of fluorescently labeled G-actin (0.2 µM Alexa488NHS-actin). In control measurements, we found that gelsolin binds extremely weakly to G-actin in the absence of Ca^{2+} (1 mM EGTA condition), while the addition of $CaCl_2$ (1 mM) profoundly strengthens the interaction. The addition of Fli-I GH16 to monomeric actin, even in the absence of $CaCl_2$, resulted in an increase in anisotropy. This suggests direct binding of the GH domains of Fli-I and G-actin. A similar response in a broader concentration range could be detected in the case of Fli-I GH13. A similar binding trend and affinity were detected in the presence of the divalent cation, indicating that the interaction of Fli-I with G-actin is not affected by the presence of Ca^{2+} (K_D ~ 500 nM). Importantly, no significant change in anisotropy was found when Fli-I GH46 was added to G-actin, suggesting that this region does not bind actin.

The calcium response of the effects of Fli-I on actin assembly was monitored in pyrenyl polymerization experiments. In control measurements, we found that gelsolin does not significantly affect actin polymerization in a Ca^{2+}-free environment (1 mM EGTA condition), while it accelerates actin assembly kinetics in the presence of $CaCl_2$ (1 mM). Fli-I GH16 and GH13 (5 nM) inhibited actin assembly both in the absence and presence of Ca^{2+}. In contrast, Fli-I GH46 (5 nM) did not have any effect on actin assembly, independently from the presence of calcium. *Altogether, these observations suggest that in contrast to gelsolin, the actin activities of Fli-I are Ca^{2+}-independent.*

The GH domains of Flightless-I affect actin assembly from free G-actin in a biphasic manner which relies on the GH13 regions

To address the biochemical activities of Fli-I in actin dynamics, the effects of Fli-I on actin assembly kinetics from free G-actin were investigated in bulk pyrenyl polymerization

experiments. The data revealed that the effects of Fli-I GH16 on polymer formation follow a biphasic concentration-response. At lower concentrations (< ~ 10 nM) it inhibits the overall rate of actin polymerization; in contrast, higher concentrations of Fli-I GH16 (> ~ 25 nM) accelerate the assembly kinetics of free G-actin above the rate of spontaneous assembly. The biphasic concentration dependence of the influence of the GH domains of Fli-I on polymerization kinetics suggests multiple activities in actin dynamics. We found that Fli-I GH13 can influence actin dynamics in a qualitatively and quantitatively similar manner as Fli-I GH16. In contrast, the GH46 region does not have any effect on actin polymerization. Actin assembly at the level of individual polymers was visualized by using TIRFM microscopy in the absence and presence of different regions of Fli-I. In agreement with the results of pyrenyl polymerization experiments, the effects of Fli-I GH16 on polymer assembly can be recapitulated by Fli-I GH13; as 10 nM Fli-I GH13 resulted in similar assembly inhibition as observed for 10 nM Fli-I GH16. Consistently with the observations made in the fluorescence spectroscopic experiments, we found that Fli-I GH46 does not have a significant effect on either polymer number or growth rate in TIRFM assays. Altogether, TIRFM data support the results obtained from pyrenyl fluorescence experiments; when present at a low amount, Fli-I inhibits actin assembly, and this activity relies on the GH13 segment of the protein. *It is interesting to note that both G-actin interaction and actin assembly inhibition of the Fli-I GH16 domains are reconstituted by GH13, suggesting the lack of direct interaction between Fli-I GH46 and actin.*

We also investigated the effects of the LRR region of Fli-I on actin dynamics. In steady-state anisotropy experiments, no significant change was detected upon titration of actin monomers (0.2 µM Alexa488NHS-G-actin) with Fli-I LRR; Fli-I LRR did not affect actin assembly in pyrenyl polymerization measurements either. *These findings indicate that the isolated LRR does not interact with actin.*

The GH domains of Flightless-I inhibit actin filament growth by barbed end capping

The polymerization inhibition that we observe at low nM concentrations reflects high-affinity interactions and can result from the prevention of subunit addition to filament ends. This can be manifested by sequestration upon binding to monomeric actin and/or by capping upon filament end interactions. Based on the experimental conditions in our TIRFM assays (0.5 µM free G-actin), actin polymer growth is dominated by barbed end assembly. Considering the dissociation equilibrium constant of the Fli-I:G-actin interaction derived from anisotropy measurements, 10 nM Fli-I – which causes polymer growth inhibition in TIRFM experiments – is expected to bind to ~ 1% of monomeric actin, i.e., ~ 5 nM. This would result in a negligible

reduction (~ 1.4%) in polymer growth rate. Thus, G-actin sequestration that relies on monomer binding by Fli-I does not explain the marked polymerization inhibition that we observe at nanomolar protein concentrations. Consequently, our data points towards filament end-related activities. To experimentally address barbed end capping activity, the end-to-end annealing kinetics of pre-formed, mechanically fragmented filaments were monitored both in the absence and presence of Fli-I by dual-color TIRFM. In the presence of either Fli-I GH16 or GH13 (120 nM) the filaments remained short and were characterized by homogeneous fluorescence emission indicating that annealing is inhibited. No such inhibitory effect was detected when Fli-I GH46 was added to actin, in agreement with our previous observations. *Altogether, the above data indicate that Fli-I inhibits end-to-end annealing by binding to filament barbed end. Therefore, we propose that inhibition of actin assembly by Fli-I results from the prevention of actin incorporation at barbed ends upon direct capping of filament ends.*

The GH domains of Flightless-I do not depolymerize/disassemble filaments but facilitate the formation of nucleation intermediates

In pyrenyl polymerization experiments, we noted that the addition of a relatively large amount of Fli-I resulted in facilitated polymerization, which can be manifested through enhanced nucleation and/or cutting of actin filaments and generating more ends for elongation. To test the fragmentation ability of Fli-I, dilution-induced bulk disassembly/depolymerization kinetics measurements were performed. In control experiments, we found that the spontaneous disassembly/depolymerization of actin filaments is relatively slow; in contrast, gelsolin (5 nM, in the presence of 1 mM $CaCl_2$) accelerated disassembly kinetics. In the presence of Fli-I at a concentration that can enhance actin polymerization in pyrenyl fluorescence experiments (105 nM), no significant increase in the rate of filament disassembly was observed. Alternatively, the disassembly/depolymerization efficiency of gelsolin and Fli-I was visualized in TIRFM experiments as well. The presence of gelsolin (0.5 nM, in the presence of 1 mM $CaCl_2$) resulted in a marked decrease in the actin filament area as compared to the control. In contrast, the addition of either the GH16 or GH13 fragments of Fli-I (105 nM) to actin filaments does not significantly influence this parameter. Based on these results, *we conclude that, in contrast to gelsolin, neither of the GH domains of Fli-I possess filament disassembly/depolymerization activity.*

Considering the monomer binding ability of Fli-I revealed by anisotropy measurements, we hypothesize that the assembly-promoting activity of Fli-I results from its ability to *de novo* nucleate actin filaments. To test the nucleation ability of Fli-I, the number of actin filaments

formed in the absence and presence of Fli-I GH16 (800 nM) was measured at steady-state by TIRFM. Fli-I GH16 increased this parameter significantly. On the other hand, the steady-state length of filaments was markedly reduced in the presence of Fli-I GH16 as compared to the control samples, further supporting barbed end capping by Fli-I. Nonetheless, *the above observation clearly indicates that Fli-I can promote actin assembly by facilitating the de novo formation of nucleation intermediates.*

Profilin directs the activities of Flightless-I towards barbed end capping

Cellular actin structures are built from profilin:G-actin; therefore we aimed to investigate whether the presence of profilin influences the actin assembly activities of Fli-I. In pyrenyl polymerization experiments, we found that both Fli-I GH16 and GH13 inhibit the assembly of profilin:G-actin at subnanomolar concentrations. This indicates that Fli-I prevents the assembly of profilin:G-actin with high-affinity barbed end capping. Similarly to the lack of the effect of Fli-I GH46 on actin assembly from free G-actin, this construct failed to influence the bulk polymerization of profilin:G-actin. The results of dual-color TIRFM experiments performed to study profilin:G-actin assembly corroborated our observations made in pyrenyl spectroscopic assays. In the presence of Fli-I GH16 or GH13 (10 nM) the number of elongating barbed ends, as well as the rate of profilin:G-actin association to preassembled F-actin seeds, was severely inhibited. The lack of the polymerization-promoting effects that we detected in the presence of profilin indicates that the interaction of Fli-I with monomeric actin is compromised by profilin. This was corroborated by steady-state anisotropy measurements, which revealed that the G-actin affinity of Fli-I GH13 is significantly decreased in the presence of profilin (K_D ~ 4500 nM), as compared to that we measured in the absence of it (K_D ~ 500 nM). Altogether *our results indicate that profilin does not influence the barbed end capping activity of Fli-I but it interferes with its monomer binding suggesting that profilin and Fli-I competitively bind to monomeric actin.*

The GH46 domains of Flightless-I interact with the C-terminus of DAAM and inhibit its actin assembly activities

In the above experiments, we did not detect any direct actin interaction or activity of the GH46 domains of Fli-I. In previous studies, this region of the human Fli-I protein was identified to interact with the C-terminal region of formins, including human mDia1 and Disheveled-associated activator of morphogenesis (Daam) 1 (Higashi et al., 2010). The interaction seems to be specific to Fli-I since the binding was not detected for the GH46 domains of gelsolin.

Based on this, we sought to investigate the effects of Fli-I GH46 on *D. melanogaster* DAAM-catalyzed actin assembly. An N-terminally truncated DAAM construct comprising the formin homology (FH) domains FH1FH2 and the C-terminal Diaphanous autoinhibitory domain (DAD)-CT regions, as well as the isolated DAAM FH1FH2 domains were studied in pyrenyl polymerization experiments. We found that while Fli-I GH46 did not influence FH1FH2-mediated actin polymerization, it inhibited the cDAAM-catalyzed actin assembly in a concentration-dependent fashion. *This indicates that Fli-I GH46 interacts with DAAM, and its main binding site is the DAD-CT region corroborating previous findings. Altogether, besides its activities directly targeting actin, this interaction can provide an indirect way for Fli-I to regulate actin dynamics through the DAAM-mediated pathway.*

CONCLUSION

In my Ph.D. work, I investigated the effect of Flightless-I on actin dynamics. Like the other members of the gelsolin family, the protein has multiple functions and plays an important role in actin cytoskeleton organization. To better understand the activities of Fli-I underlying its cytoplasmic functions, I studied the interactions of different, recombinantly produced Fli-I constructs with actin by using protein biochemical and biophysical approaches *in vitro*.

My main results are as follows:

- Flightless-I LRR and GH domains were successfully expressed and produced in the *E. coli* system.
- Flightless-I interacts with both actin monomers (low-affinity interaction) and polymer barbed ends (high-affinity interaction).
- Flightless-I affects actin assembly in a biphasic manner. Flightless-I GH16 and GH13 inhibit actin polymerization at lower concentrations (high affinity capping activity) and increase the rate of actin assembly at higher concentrations (low-affinity nucleation activity).
- Unlike gelsolin, the GH domains of Flightless-I have no severing activity.
- The actin interactions and activities of Flightless-I rely on the GH13 domains; the GH46 and LRR regions have no direct effects on actin dynamics.
- The actin-binding protein, profilin allows Flightless-I to cap barbed ends and block actin polymer growth, but it inhibits its interaction with actin monomers and, thereby, its nucleation activity.
- In contrast to gelsolin, the actin activities of Flightless-I are not calcium-dependent, which can be explained by the lack of the conservation of type II calcium-binding sites between the two proteins.
- Flightless-I GH46 binds to the DAD-CT region of DAAM and thereby tunes the actin assembly activities of DAAM.

In conclusion, our results indicate that in the cytoplasmic environment, Flightless-I interferes with actin dynamics by capping polymer ends, which may explain its negative effects on cell migration and, thus, wound healing and tissue regeneration.

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