PhD thesis

The effect of formin DAAM on the dynamics of actin

Szilvia Barkó

Supervisor: DR. MIKLÓS NYITRAI

Doctoral School: Interdisciplinary Medical Sciences Doctoral School D93

Head of the Doctoral School: Dr. Balázs Sümegi

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

Head of the Program: Dr. Miklós Nyitrai

University of Pécs

Medical School

Department of Biophysics

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I. INTRODUCTION

I.1. THE ACTIN AND ACTIN NUCLEATING FACTORS

The actin is one of the main components of eukaryotic cells [1, 2]. It can exist in the cell as monomer called globular or G actin [3], and in a filamentous form which is the F actin form [4]. The process during the monomeric form assembles into filaments entitled polymerisation. It is also possible that the globular actin dissociates from the end of a filament which process is termed depolymerisation. There is a very important, rate-limiting step in a polymerisation called nucleation. The actin monomers build into so called nuclei during this process which nuclei can contain two or three actin monomers. These nuclei are very unstable on their own and for this reason they can dissociate into monomers again. Compared to the other steps of polymerisation this process is slow since the nucleation defines the velocity of polymerisation.

From the point of nucleation there is an essential parameter called critical concentration which is the concentration value of actin monomers above the nucleation and polymerisation occurs spontaneously. The actin binding proteins can have effect on this concentration value.

As far as we know today there are three actin nucleating factor families in eukaryotic cells. All of them use different machineries for the nucleation.

The first discovered family was the Arp 2/3 type protein family. The members of this group cooperate with other nucleation promoting factors (NPFs) and together with them they could mimic a nucleus. These proteins generate short branched actin filaments as they create new filament-branches on the side of pre-existing actin filaments [5, 6].

The second group comprises the so called WH2 domain containing proteins. These are in close proximity with the NPFs mentioned before. The Spire, Cordon-bleu (Cobl), and Leiomodin (Lmod) families belong to this group and some other bacterial nucleator protein [7-12]. These proteins generate long unbranched filaments.

The third group is the family of formins. Similar to the previous group these create filaments without branches. These have role in the building of contractile rings, actin cables and bundles [13, 14].

The second step in polymerisation followed by nucleation is the elongation. Using the actin nuclei the actin filaments start lengthening through the assembly of actin monomers to the end of filaments. After the steady state filament length was achieved the association and dissociation of monomers at both ends of filaments occur according to the so called treadmilling process. Conferring this process the association and dissociation of monomers happen at both ends but in a different extent. The explanation is that the actin filament has polarity: it has a plus end („barbed end"), which can grow fast and a minus end („pointed end") where the dissociation is the dominant process.

The proteins which have effect on the rate of elongation termed actin elongation factors. As far as it known now there are two groups belonging here. These are the formins and the Ena/Vasp proteins.

Consequencly the formins have effect not just on the nucleation of actin but also on the elongation of it. It is interesting that the actin monomer binding protein profilin have effect on the elongation by both of these elongation factors [14].

I.2. THE PROFILINS

In most of cases the actin is found in cell as it is bound by an actin binding protein. Its most frequent binding partner is profilin which holds it in monomeric form.

Profilin is found in eukaryotes. Almost all of the profilin isoforms share a feature to be able to bind globular actin, phosphoinosites and proline rich sequences [15-17]. Their common property is that they support the nucleotide exchange on actin [18], and they sequester actin monomer [19]. The actin monomers can incorporate into filaments at the barbed end from profilin-actin complexes [20]. The different profilin isoforms have diverse affinities to proteins comprise proline rich sequences. Most of formins contains a proline rich domain called FH1 (formin homology 1). The number of prolines have determinative role in the binding affinity [21].

I.3. THE FORMINS

The first observation regarding to formins was made in mice in connection with a mutation causes limb deformity. Two alleles were described in the '60s but the appropriate gene was identified in 1985 by Woychik and co-workers in mice [22]. As they described the limb deformity is caused by an insertion mutagenesis in the *fmn* (formin) gene [22]. This gene was characterised by this lab in molecular level and the name "formin" was given by this group to the translated proteins [23].

Almost every eukaryotic formin proteins contain an FH1 (formin homology 1) [24] domain which is a proline rich sequence and has role in binding of formin to profilin and SH3-containing proteins. Aside from that there is an FH2 domain found in all the formins which plays a part in actin binding [25]. In the structure of formins this domain is the most conservative and most popularly investigated and characterised part among the different formin families.

In vitro the FH1 domain is dispensable to polymerisation of actin although it modulates the effect of the FH2 domain [26, 27]. As a general rule the formins slows down the elongation but they speed up in the presence of profilin [28-30].

The formins bind to the barbed end of actin filaments through their FH2 domains and they can have influence on the polymerisation in different ways:

1.) They promote the nucleation of newly evolved actin filaments.

2.) They change the elongation and depolymerisation rate of the filament.

3.) They inhibit the binding of capping proteins to actin.

4.) They can have effect on the annealing of actin.

Based on the homology of FH2 domain the mammalian formins can be divided into seven groups (the letter "m"before the name of formins denotes the origin: *mouse*, respectively *mammalian*):

Dia→ Diaphanous formins (mDia1, mDia2 és mDia3)

FRL→ „formin related gene in leukocytes” (mFRL1 mFRL2, és mFRL3)

DAAM → „dishevelled-associated activator of morphogenesis” (mDAAM1 és mDAAM2)

***INF*→** „inverted formin” (mINF1 és mINF2)

***Delphilin*→** (mDelphilin)

***FHOD* →** „formin homology domain-containing protein” (mFHOD1 és mFHOD2)

***FMN*→** formins (the firstly described formins belong in this group whose absence cause limb deformity)

The DAAM formin family

The DAAM protein was discovered in 2001 through the intervention of a protein which plays role in the PCP („*Planar Cell Polarity*) signalling pathway. This binding partner called Dishevelled consequently the DAAM name was given to this protein („*Dishevelled-associated activator of morphogenesis”*) [31]. The DAAM plays role in the β–catenin independent Wnt cell signalling and has crucial function in the reorganisation of cytoskeleton [32]. Through the detailed investigation of DAAM gene sequence it turned out that it contains formin homology domains.

The DAAM formins have various biological functions. It seems to be proven that their role in the PCP cell signalling is not crucial [33]. Matusek and co-workers have described that in the tracheal system of *Drosophila melanogaster* these formins are indispensable for the normal organisation of actin cytoskeleton [33]. The same lab confirmed that DAAM have role in the organisation of filopodia in the axons of developing neural system and that it is a very conservative function. Later on it was described that DAAM have elemental role in the growth and forming of axons [34], and that it has function in the neuronal cell differentiation of the central nervous system [35].

Preliminary *in vivo* observations show that DAAM localises to the pointed end of actin filaments in the sarcomeres of flying muscle of *Drosophila melanogaster (not published data)*. Because it is known that formins inhibit elongation and consequently they create shorter filaments it could be concluded that the DAAM formin builds short actin filaments into the pointed end of actin

II. MAIN OBJECTIVES

The formins have been investigated by different biochemical and biophysical methods for thousends of years. There are detailed results in connection with some formins families which were studied particularly (e.g. Dia formins). At the beginning of our studies there was no cleared *in vitro* study from DAAM although for the understanding of the in vivo observations it is necessary.

Our plan was describe the actin-DAAM cooperation using the methods which were suitable to characterise the members of other formin families. We have used two fragments in our experiments. One of them, a very conservative motif called DAAM FH2 domain is responsible for the actin binding. The second one contains the FH1 domain beyond the FH2 which is a proline rich sequence and it is able to bind to profilin. This second construct called DAAM FH1FH2 later on.

In our work we have planned to answer to following questions:

1. Is the DAAM FH2 domain able to bind actin and if yes, can it change its nucleation and elongation rates?

2. Does the FH1 domain have influence on the FH2-actin binding?

3. Do the DAAM FH2, respectively FH1FH2 constructs alter the depolymerisation of actin filament?

4. Do the DAAM FH2, respectively FH1FH2 constructs have effect on the critical concentration of actin?

5. What is the dissociation constant of these constructs for actin?

6. Do these construct change the average filament length, bundling properties and annealing rate of actin?

7. How the profilin modifies the dynamics of DAAM FH2 and DAAM FH1FH2 bound actin filaments? Does any cooperation exist between profilin and FH1 domain described in the literature before?

III. EXPERIMENTAL METHODS

III.1. PURIFICATION OF PROTEINS

III.1.1. Preparation of actin

We have prepared rabbit (*Oryctolagus cuniculus*) skeletal muscle actin in our studies using the method of Spudich és Watt [36]. After an additional ultracentrifugation step (Beckman Optima MAX, MLA-80 rotor; 400000 g, 30 min, 4 °C) we have purified the actin with a gel filtration method using Sephacryl S300 column for the removal of the remaining contaminating proteins [36]. After the isolation we stored actin in the following buffer: 4 mM Tris-HCl, 0,1 mM $CaCl_2$, 0,2 mM ATP, 0,5 mM DTT, 0,005% NaN_3, pH 7,3 („buffer A"). The concentration of actin was determined using a Shimadzu UV-2100 spectrophotometer with the extinction coefficient 0,63 mg^{-1} ml cm^{-1} at 290 nm [37].

For the fluorescence spectroscopic measurements we labelled actin with N-(1-pyrene) iodoacetamide (pyrene). This fluorophore binds to Cys 374 of actin with covalent binding [38]. The determination of concentration and labelling ratio was made using the extinction coefficient of pyrene $2{,}2 \cdot 10^4$ M^{-1} cm^{-1} at 344 nm and with a correction factor.

III.1.2. Production of formin fragments and profilin

The protein sequences of the *Drosophila melanogaster* DAAM fragments were inserted into a pGex-4T-3 plasmid by József Mihály's lab (Biological Research Centre, Institute of Genetics, Szeged) for us. This plasmid contains an ampicillin resistance and the translation of a target protein is inducible with IPTG from it. There is a thrombin cleavage site between the protein sequence and the affinity tag. The protocol of preparation was made by Shimada and co-workers [39]. The determination of protein concentrations was made by the help of the „Protparam" program (http://us.expasy.org/tools/) using the following extinction coefficients: for DAAM FH2

ε_{280} = 22920 M^{-1} cm^{-1} and for DAAM FH1FH2 ε_{280} =22982,5 M^{-1} cm^{-1}. The calculated molecular weight were 47,9 kDa and 54,7 kDa, respectively. Both of the DAAM constructs were frozen in liquid nitrogen and were stored at -80 °C.

The whole amino acid sequence of yeast profilin (PDB code: 1YPR) was inserted by Pekka Lappalainen and co-workers (Institute of Biotechnology, University of Helsinki, Helsinki, Finland) in a pHAT2 plasmid. The translation of the target protein was induced by IPTG in BL21 (DE3) pLysS competent cells. The contaminating proteins were removed using Ni affinity chromatography with increasing imidazole gradient. We used a gel filtration step for further purification similar as in the case of DAAM constructs. The extinction coefficient of profilin was given as ε_{280} =19940 M^{-1} cm^{-1}. Its molecular weight is 13,68 kDa. After the preparation it was frozen in liquid nitrogen and was stored at -80 °C.

III.2. EXPERIMENTAL METHODS

III.2.1.The investigation of actin polymerisation

The polymerisation of actin can be divided into more steps. The first one is the nucleation which means that the actin monomers build nuclei and they are going to function as starting point of evolving of new filaments. We used pyrene actin as marker for following this process. The reason for that is the specific characteristic of pyrene actin: in monomeric form it has a relatively low fluorescence signal but as it incorporates into filaments its fluorescence emission increases. Using this feature in a time course experiment we can directly follow the process of polymerisation. In our experiments we have set up the final concentration of actin to 3,5 μM (pyrene labelled in 5%). The bound calcium was replaced with magnesium by adding 200 μM EGTA and 50 μM MgCl2 and incubating the samples for 5–10 min. The magnesium-actin was polymerised with 1 mM MgCl2 and 50 mM KCl in either the presence or the absence of formin fragments.

We have followed the process of polymerisation using two instruments. At lower DAAM concentrations there was a Perkin Elmer LS50B spectrofluorimeter used to

follow the intensity change of pyrene in as a function of time. The excitation and emission wavelengths were 365 nm and 407 nm, respectively. The polymerisation curves were normalised. The elongation rate was determined from the slope of the linear fit to the pyrene fluorescence curves at half-maximum polymerisation. We calculated the rate of polymerisation from these slopes to $\mu M \cdot s^{-1}$ units and these were plotted as function of formin concentration.

At higher (1μM or higher) formin concentrations it is relatively difficult to follow the process of polymerisation using a standard spectroscopic assay mentioned before because it is too fast. The stopped flow instrument (Applied Photophysics, SX.18MV-R Stopped Flow Reaction Analyser) is suitable to solve this problem because the dead time of the measurements is substantially shorter (1 ms) than in the case of manual mixing (20–40 s), and the time resolution of the instrument is higher. Actin in 7 μM concentration was mixed with buffer containing 200 μM EGTA and 50 μM $MgCl_2$ to the end that exchange the bound calcium to magnesium. After that it was diluted to 3,5 μM in the presence of polymerising buffer in the absence or presence of various concentrations of DAAM FH1FH2.

III.2.2. The investigaton of depolymerisation of actin

The depolymerisation of actin filaments (5 μM, 70% pyrenyl-labeled) in the presence of various concentrations of DAAM FH2 or DAAM FH1FH2 was followed after dilution to 0.1 μM in polymerisation buffer (buffer A supplemented with 50mM KCl and 1mM $MgCl_2$). The reason for the higher labelling ratio was this low actin concentration and the consequently low fluorescence signal.

The depolymerisation rate was determined from the linear fit to the initial (first 50 s) part of the time dependence of the pyrene fluorescence curves and normalized using the rate of actin alone as a standard. These normalised rate values were plotted as a function of formin concentration.

III.2.3. Determination of critical concentration

For the measurement of actin critical concentration there were two methods used. In the first experiments actin (5%pyrenyl-labeled) was incubated at various concentrations in polymerisation buffer overnight in the presence or absence of DAAM fragment in 100 nM concentration. The pyrene fluorescence intensities were measured between 397-417 nm, were averaged and plotted as a function of the total actin concentration. The value of the critical concentration was determined by fitting the following equation to the plots:

$$I = I_0 + ((SL + SR)([A] - cc) / 2) - ((SL - SR)\,abs([A] - cc) / 2)) \quad (1)$$

where [A] is the actin concentration, cc is the critical concentration for actin assembly, I_0 is the ordinate value at [A] = cc, and SL and SR are the slopes of the intensity versus actin concentration curves before and after the breaking point, respectively.

In the second type of experiments the actin concentration was 1 μM and it was incubated with various concentrations of DAAM fragments. After an overnight polymerisation the measurements of pyrene intensities were made as mentioned before and the averaged values were plotted as formin concentration.

III.2.4. Cosedimentation assays

The binding properties of DAAM fragments to actin played essential role in our work. To determine the affinity of DAAM-FH2 and FH1FH2 for the actin filaments, we polymerised 1.5 μM actin at room temperature in the presence of different concentrations of DAAMFH2 or FH1FH2. After a two hours long incubation the samples were centrifuged with Beckman Optima MAX bench top ultracentrifuge (TLA-100 rotor, 20 °C, 30 min at 400,000 g). The supernatants were separated from the pellets, and both were analyzed by 12% SDS PAGE. After staining with Coomassie Blue, the band intensities were determined with a Syngene bioimaging system. The

band intensities were corrected for the molecular weights of the proteins, and the ratios of the formin and actin band intensities measured in the pellets (D) were plotted as a function of the formin concentration and analyzed by using the following equation: [40]:

$$[A]_0 D^2-([A]_0 + [D]_0 + K_D) D + [D]_0 = 0 \quad (2)$$

where $[D]_0$ and $[A]_0$ are the total formin and actin concentrations, respectively, K_D is the dissociation equilibrium constant for formin binding to actin, and D is the fraction of bound formin.

III.2.5. In vitro microscopy of actin assembly

A) Investigation of bundling and length distribution of actin filaments

The bundling properties of DAAM fragments on actin filaments were measured as follows: actin was polymerised in 1 μM concentration in the presence or absence of 500 nM DAAM FH2 or FH1FH2 for two hours. After that the samples were labelled with rhodamine-phalloidin for one hour in 1:1 molar ratio and were diluted to 5 nM concentration in the following buffer : 4 mM Tris-HCl, 1 mM EGTA, 50 mM KCl, 1 mM $MgCl_2$, 0,2 M DTT, 15 mM glucose, 20 μg/ml catalase, 100 μg/ml glucose-oxidase, 0.5 % (w/v) methylcellulose), pH 7,0. The average single filament thicknesses were determined in every sample and the single filament thicknesses were divided with this value. Finally the observed relative frequencies in percent were plotted against filament thicknesses. The average filament lengths were determined similarly to this method with the only difference that the used actin concentration was 2 nM after dilution.

B) Measuring the annealing rate of actin filaments

For the investigation of annealing of actin filaments the actin was polymerised overnight in the presence or absence of DAAM fragments. These samples were labelled with Alexa 488 phalloidin for one hour and were mechanically sheared using a 26G gaugle needle. The samples were diluted after a certain time interval in microscopic buffer to 2,5 nM to stop the process of annealing. The results were analysed using ImageJ program (http://rsbweb.nih.gov/ij/index.html). For the determination of rate of annealing the following equation was used:

$$l=((l_{max}-l_{min})/(1+K/m))+l_{min} \quad (3)$$

where l, l_{min} *and* l_{max}: filament lengths in time, *m:* time interval given in minutes, *K* annealing rate constant.

The samples were analysed using an Olympus IX81 inverted fluorescence microscope using a 100x objective (NA 1.4) and a CCD camera (Orca ERG Hamamatsu) (http://rsbweb.nih.gov/ij/index.html).

IV. RESULTS AND CONCLUSIONS

IV.1. THE DAAM CONSTRUCTS HAVE EFFECT ON POLYMERISATION OF ACTIN

Drosophila melanogaster DAAM FH2 and DAAMFH1FH2 fragments were assessed for their ability to assemble actin filaments in vitro by polymerisation assays using pyrenyl labelled actin. In these experiments the intensity change of pyrene fluorophore was measured in time, which increases with the number and length of filaments, respectively For these observations there were 3,5 μM, in 5 % pyrene labelled actin used, and there was cathion exchange buffer and DAAM constructs given to that in different concentrations. We detected every fluorescence signal til their plateu phase. We observed that as higher the concentration of DAAM fragments is as steeper polymerisation curve detected. There was no detectable difference in the effectivity of FH2 and FH1FH2 domains of DAAM for the speed-up of polymerisation. So we can conclude that the FH1 domain does not change the influence of FH2 domain on actin polymerisation. As 1 μM concentration is reached the polyermisation became so fast that the usage of a stopped flow apparatus is needed. With this application we can test whether the limitations of the steady-state fluorescence spectroscopic method are responsible for the apparent saturation of the polymerisation rate. Our results showed that if we use higher DAAM concentrations as 1 μM there is no change in the polymerisation rate, which means that the DAAM fragments have saturation characteristic in their effect on actin polymerisation.

IV.2. DAAM FH2 AND FH1FH2 CONSTUCTS INHIBIT THE DEPOLYMERISATON OF ACTIN

We have also studied the effects of DAAM FH2 and FH1FH2 fragments on the depolymerisation rate of actin filaments. In these expreriments pyrenyl-labeled actin (5 μM, 70% labeled) was polymerised overnight, and then filaments were diluted to 100 nM (below the barbed end critical concentration) in the absence or presence of formins in polymerisation buffer. Similarly to the polymerisation assay there was the fluorescence intensity of pyrene detected as a function of time. Because of the

dissociation of monomers from the filaments decreased fluorescent signal was measured. There was a linear fit to the first 50 s of depolymerisation curve applied and this value was divided by the control (DAAM-free) data. Our expreriments show that both of the DAAM fragments strongly inhibit the depolymerisation of actin filaments. These results are in good agreement with the data published before in case of other formins belonging in the DRF family [30, 41]. It seems to be confirmed that the members of this formin family have very robust depolymerisation inhibitory effect. Possible explanation is for this behaviour that the DAAM fragments bind processively to the barbed end of actin filament and function as capping proteins. Its consequence is that the monomers can dissociate only from the pointed end of actin filament where the rate of depolymerisation is much lower than at the barbed end.

IV.3. THE DAAM CONSTRUCTS DO NOT HAVE EFFECT ON CRITICAL CONCENTRATION OF ACTIN

It is described that some proteins and even formins beside them have effect on critical concentration of actin [13, 27, 41-44]. To the determination of critical concentration in our experimental conditions there was pyrenyl labelled actin used. Because the signal of pyrene increases as it incorporates into filaments, the monomer-filament change is detectable. We used this fluorophore in two different methods described in details in the *Experimental methods* section.

In our fist experiments we used a constant DAAM concentration and the quantity of actin was changed. As the amount of monomers increased there were higher signal detected but there was only a linearly proportional increasing observed. As it is summarized in the *Experimental methods* section the pyrene fluorescence emission was measured. The average fluorescence intensities were plotted against actin concentration and the *Equation Nr.1*. was fitted to the points. The critical concentration was given as the intersection of the linear line in the equation. Our results show that neither DAAM FH2 nor DAAM FH1FH2 changed the critical concentration intensely; there is only a mild increasing detectable.

To confirm these data there was a second, alternative measurement introduced. In these experiments the quantity of actin was constant and different DAAM concentrations were used. The experimental setup was similar as it is stated before. As

an analysis there was the filamentous actin concentration-values plotted against DAAM concentrations. Our data show that as concentration of formin increases the concentration of actin filaments decreases with approximately 100 nM. The possible reason for that is the increasing of critical concentration of actin with this extent. We can accomplish from these results that the formin fragments had effect on actin filaments even in this 20-40 nM concentration value.

As a summary it can be concluded that our results using DAAM fragments are in good agreement with previous observations made in case of other formins. Completed with our previous observations with the depolymerisation the DAAM seems to be a so called „leaky capper". It means that it can bind the barbed end of filaments as a capping protein but it does not close it very strongly.

IV.4. THE AFFINITY OF DAAM FRAGMENTS IS IN A MICROMOLAR RANGE

To determine the binding constant of formin fragments to actin filaments there were co-sedimentation experiments made. This method is described in the *Experimental methods* section detailed. The results showed that formin fragments sedimented with the actin filaments. In control samples, neither DAAM FH2 nor DAAM FH1FH2 appeared in the pellets in the absence of actin. The ratio of the formin and actin band intensities measured in the pellets were determined and plotted as a function of the formin concentration, and the plots were analyzed by hyperbola fits using *Equation Nr. 2.* The analyses gave equilibrium dissociation constants of 7.0 ±2.5 and 2.1±0.7 μM for DAAM FH2 and DAAM FH1FH2, respectively.

Because the concentration of the DAAM fragments in the pellets was much higher (100–900 nM) than would be expected from their sole binding to the filament ends (8–15 nM), these observations showed that formin fragments bound to the sides of the actin filaments. The binding of DAAM fragments to the filaments can have role in the bundling of actin filaments. This phenomenon was described before in connection with other formins [45, 46], so we have planned to prepare some investigations in connection with the bundling efficiency.

IV.5. THE EFFECT OF DAAM FH2 AND FH1FH2 FRAGMENTS ON THE BUNDLING PROPERTIES AND FILAMENT LENGTH DISTRIBUTION OF ACTIN FILAMENT

IV.5.1. The DAAM FH2 and FH1FH2 fragments induce actin bundling

As it was described before some formins are able to bundle actin filaments [47, 48]. It was known that DAAM has role in the nucleation and elongation of actin in the central nervous system [49], but there was no evidence for its bundling effect. With this object our aim was to investigate the influence of DAAM FH2 and FH1FH2 constructs on the morphology of actin filaments using fluorescence microscopic methods. The experiments were performed in the presence or absence of 0,5 μM DAAM FH2 or FH1FH2 on 1 μM actin concentration to achieve that DAAM could bind even to the sides of filaments in appropriate amount for bundling. Our observations showed that in absence of DAAM the number of bundled actin filaments was relatively low; most of the filaments were single. In the presence of 500 nM DAAM FH2 the ratio of the bundled filaments increased in the sample. Investigating these preparates we have noticed that next to these thick, high intensity bundles the single actin filaments were obviously shorter that in the control sample. Our data were analysed using ImageJ program. The results showed that in presence of DAAM high ratio of filaments cross-linked wiht another one, approximately 30-40% of filaments is bundled. Using 500 nM DAAM FH1FH2 instead of FH2 we noticed similar bundling ratio but the filaments seemed to be even shorter. To clarify these observations there were some experiments made measuring the average filament length.

IV.5.2. TheA DAAM fragmentums construct short actin filaments

As it was mentioned before we have observed that the average filament length seemed to be shorter investigating the bundling properties of actin filaments in the presence of DAAM fragments. To confirm these observations there were some additional experiments made where we have polymerised the actin filaments in presence of DAAM FH1FH2 at various concentrations. To exclude the disturbing influence of bundling we have diluted the filaments to even lower concentrations for the microscopic investigations as in the previous experimental setup. The average

filament lengths were measured using ImageJ program. As it was expected in the presence of DAAM fragments the average filament length was shorter than in the control one. Besides that it was noticed that the average length was affected by the concentration of DAAM: the higher the DAAM concentration was the shorter filaments were appeared. The possible explanation is the capping property of DAAM. Because after the nucleation step the DAAM fragments remain bound at barbed ends they could have effect on the association of actin monomers to the filament. The evolved shorter average “steady state” filament length can be a possible consequence for this capping function.

IV.5.3. The DAAM FH1FH2 fragments effect the annealing rate of actin filaments

On the basis of previous publications and observations made by our collaborating partners we have planned to investigate the effect of DAAM FH1 FH2 on the annealing rate of actin filaments. Correspondingly to the previously described experiments we measured the average filament lengths using ImageJ program. In these experiments actin was polymerised in presence of 1 μM DAAM FH1FH2 and after stabilisation with phalloidin the filaments were fragmented using mechanical shearing. The average filament length was measured in time. Our results showed that coincidently with the previous observations the average filament length is shorter is presence of DAAM that in case of spontaneous polymerised actin filaments. Using the *Equation Nr. 3* detailed in the *Experimental methods* section we have concluded that the short actin filaments evolved by DAAM anneal faster as the control.

IV.6: THE ROLE OF FH1 DOMAIN IN INTERACTION BETWEEN DAAM FH1FH2 AND PROFILIN

IV.6.1: The profilin interacts with the FH1 domain of DAAM for increasing the rate of polymerisation

It was described before that the FH1 domain of formin attached to the end of filament is able to interact with an actin monomer bound profilin and using this cooperation it can support the association of monomers at the barbed end of filament. The rate of polymerisation could be higher in case of profilin bound monomers as using free actins [21, 50, 51]. It raised the question if a similar cooperation exists between DAAM FH1 and profilin. These experiments were made on the same lines with the polymerisation assays described before the only difference was that after giving polymerisation buffers and formins additionally profilin was given in 5 μM concentration.

Our results showed that in the presence of DAAM FH2 and profilin the polymerisation decreases to a great extent. In contrast, using DAAM FH1FH2 construct this rate dramatically increases even compared to the maximal rate of polymerisation in presence of DAAM alone. The only possible explanation is the cooperation between the FH1 domain of DAAM and profilin. Namely profilin decreases the rate of polymerisation on its own, because it inhibits the incorporation of monomers into filament [52]. Because DAAM FH2 domain is not able to bind profilin, the affect of profilin dominates and the net polymerisation rate is much lower as in presence of FH2 alone. Contrarily, the FH1 domain of DAAM interacts with profilin and the net effect is the observed increased polymerisation rate. Based on these results we have concluded that the previously described FH1-profilin interaction [28-30] is valid even in case of DAAM.

IV.6.2: The DAAM fragments decrease the dissociation of monomers from actin filament even in presence of profilin

As it is known the profilin supports the dissociation of actin monomers from the end of filaments [53, 54]. Our aim was investigate if the DAAM fragments are able to modulate this effect. For this purpose there were depolymerisation assay fulfilled in presence of 5 μM profilin and various concentrations of formins.

Our data showed that the effect of profilin showed up but at higher formin concentrations the dissociation of actin monomers caused by profilin decreased. Interestingly, there was no difference in the depolymerisation rate observed in presence of the two DAAM constructs, even in the presence of profilin. Consequently, it seems to be possible that the previously described FH1 domain-profilin interaction has no role in the affect of DAAM on the depolymerisation of actin.

IV.7.3: The FH1 domain of DAAM interacts with profilin to maintain the critical actin concentration

We have repeated the previously described critical actin concentration measurements in presence of profilin to clarify if there is effect of FH1-profilin interactrion on the critical actin concentration. Our data showed that profilin decreased the actin filament concentration even in presence of DAAM FH2 domain but using FH1FH2 domains the filament concentration reappeared, indicated that the FH1 domain –profilin interaction has role even in maintaining the critical actin concentration. A possible explanation is for this observation that the FH1 domain of DAAM supports the dissociation of profilin from actin monomer and thus it is able to incorporate into actin filament.

V. CONCLUSIONS

The main findings of our work are as follows:

- The DAAM FH2 and FH1FH2 fragments increase the rate of polymerisation. This rate depends on the concentration of formin, but it shows saturation: upon achieved a certain concentration it is not intensified any more.

- The FH1 domain has no effect on actin polymerisation in a profilin-free system.

- In presence of yeast profilin and DAAM FH1FH2 the rate of polymerisation increases dramatically, although in case of DAAM FH2 domain only the effect of profilin dominates. Based on previous results it can be explained with the interaction between FH1 and profilin.

- The DAAM constructs inhibit the dissociation of monomers from the actin filament, and at higher concentrations ($1\ \mu M \leq$) they can even abolish the depolymerisation effect of profilin.

- The investigated DAAM fragments do not have effect on critical concentration of actin.

- The DAAM FH1FH2 construct eliminates the increasing of critical concentration of actin casused by profilin, wherefore the profilin-FH1 cooperation could be responsible.

- The affinity of DAAM FH2 to actin filaments is approximately $7 \pm 2{,}5\ \mu M$, in case of DAAM FH1FH2 fragment it is $2{,}1 \pm 0{,}5\ \mu M$.

- Both of these DAAM constructs are able to bundle actin filaments.

- The average filament length is shorter in presence of DAAM and it is caused by its effect on actin polymerisation and end-to-end annealing of filaments.

VI. REFERENCES

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VII. PUBLICATIONS

I. Publication related to the Thesis

Barkó, Sz., Bugyi, B., Carlier, M-F., Gombos, R., Matusek, T., Mihály, J. and Nyitrai, M. (2010).

Characterization of the Biochemical Properties and Biological Function of the Formin Homology Domains of Drosophila DAAM.

Journal of Biological Chemistry, 285 (17), 13154–13169.

Impact factor: 5.328; Citations:3

II. Other publications

1. Orbán, J., Halasi Sz., Papp, G., Barkó, Sz. and Bugyi, B. (2005).

Thermodynamic characterization of different actin isoforms.

Journal of Thermal Analysis and Calorimetry, 82, 287-290.

Impact factor 1.425; Citations:1

2. Halasi, Sz., Papp, G., Bugyi, B., Barkó, Sz., Orbán, J., Ujfalusi, Z. and Visegrády, B. (2006).

The effect of pyrene labelling on the thermal stability of actin filaments.

Thermochimica Acta, 445, 185-189.

Impact factor 1.417; Citations:2

3. Papp, G., Bugyi, B., Ujfalusi, Z., Barkó, Sz., Hild, G., Somogyi, B. and Nyitrai, M. (2006).

Conformational changes in actin filaments induced by formin binding to the barbed end.

Biophysical Journal, 91 (7), 2564–2572.

Impact factor 4.757; Citations:9

4. Orbán, J., Pozsonyi, K., Szarka, K., Barkó, Sz., Bódis, E. and Lőrinczy D. (2006).

Thermal characterisation of actin filaments prepared from ADP-actin monomers.

Journal of Thermal Analysis and Calorimetry, 84, 619–623.

Impact factor 1.438; Citations:2

5. Ujfalusi, Z., Barkó, Sz., Hild, G. and Nyitrai, M. (2010).

The effects of formins on the conformation of subdomain 1 in actin filaments. *Journal of Photochemistry and Photobiology B: Biology*, 98 (1), 7-11.

Impact factor: 2.116 Citations:2

Cumulative impact factor:16,481

Sum of independent citations: 19

III. Conference presentations related to the Thesis

1. Characterization of the Biochemical Properties and Biological Function of the Formin Homology Domains of Drosophila DAAM

A FEBS/EMBO lecture course in conjunction with European Cytoskeletal Forum and SACR: The Cytoskeleton in Development and Pathology

Stockholm, Sweden, 19 – 24 June, 2010.

2. Characterization of the Biochemical Properties and Biological Function of the Formin Homology Domains of Drosophila DAAM

EBSA Satellite Meeting

Pécs, 20-22 August, 2011.