Role of formin proteins in the coordination of actin and microtubule cytoskeleton

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1. Introduction

Neuronal axon growth is one of the most remarkable cellular processes where coordinated cytoskeleton rearrangements play a pivotal role. Axons are slender projections that extend over long distances in order to reach defined targets, i.e. other neurons, muscles or glandular cells. Growth cones, located at the distal tip of axons, play a crucial role in guiding axons to their correct target sites. Growth cones respond to extracellular guidance cues which lead to coordinated changes of the two major cytoskeletal polymers, F-actin and microtubules (MT). Rearrangement of the growth cone cytoskeleton promotes shape changes that ultimately result in growth cone advance. Several proteins have already been identified with role(s) in the regulation of the neuronal cytoskeleton. Among these, formins are highly conserved proteins implicated in the regulation of multiple aspects of neuroskeleton organization and dynamics. Formins are best known for their ability to initiate actin polymerization and promote the elongation of unbranched actin filaments. They have been shown to be expressed in the nervous system and they have roles in the regulation of axonal cytoskeleton in different model organisms. The most conserved feature of the formin protein family is the presence of two formin homology domains (FH1 and FH2), that are the functional domains of formins. Besides the FH domains, most formins contain other conserved regions as well that are thought to provide means for spatial and temporal regulation of formin activity. Mammals possess seven groups of formins: (1) Diaphanous (Dia), (2) Dishevelled-associated activator of morphogenesis (DAAM), (3) Formin-related gene in leukocytes (FRL), (4) Formin homology domain-containing protein (FHOD), (5) Inverted formin (INF), (6) Formin (FMN) and (7) Delphilin. Of these, Dia, DAAM and FRL belong to the family of diaphanous-related formins (DRFs). Unique feature of DRFs is an intramolecular interaction between the N-terminal DID (diaphanous inhibitory domain) and the C-terminal DAD (diaphanous autoregulatory domain) domains which keeps the proteins in an auto-inhibited state. Binding of an activated Rho-GTPase to the N-terminally located GBD (GTPase binding domain) domain releases the protein from the inactive conformation (Figure 1.)

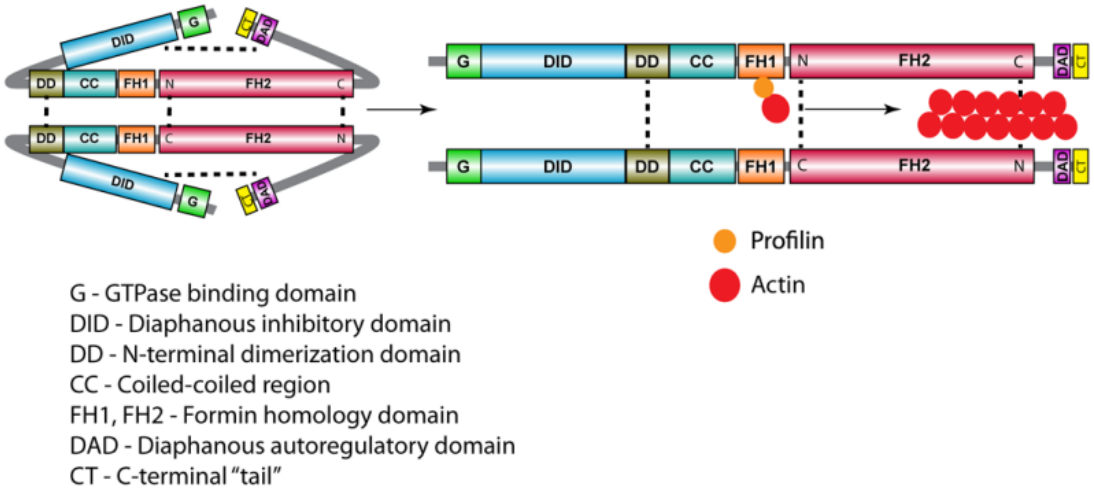

Figure 1. Domain structure of Diaphanous Related Formins.

Beyond their role in the control of actin cytoskeleton, formins have also been implicated in the regulation of MT dynamics. Formins can directly interact with MTs, they promote the formation of stabile MTs and they can crosslink F-actin filaments and MTs. In addition, several formins have been shown to be able to interact with MT (+)-end.binding proteins, such as end-binding protein (EB) and CLIP-170. By these interactions formins are implicated in the coordination of actin and MT cytoskeletal changes.

2. Preliminary results

Our group already showed that the *Drosophila* formin, DAAM, is highly expressed in the nervous system from the embryonic stages till adulthood, and the lack of this protein results in impaired axonal development [1–4]. By using *in vitro* assays, we proved that the functional domains of DAAM act as *bone fide* FH domains [5]. Whereas recently we demonstrated the CT region of DAAM is also implicated in actin interactions and it seems that this region tunes the nucleating activity and filament-end binding capacity of DAAM [6]. We also investigated the MT-binding capacity of DAAM and our results revealed that the FH1-FH2 and the CT regions of DAAM can directly bind to taxol-stabilzed MTs. Mutant analysis showed that a mutation of a conserved amino acid located in the centre of the primary actin-binding surface of the FH2 domain does not influence MT-binding [3]. Similar to other formins, *in vitro* DAAM is able to stabilize MTs against cold-induced depolymerization. In accordance with this data, we showed that the lack of DAAM significantly reduces the lifetime of axonal MTs in *Drosophila* primary neurons. A detailed analysis was performed in *DAAM* mutant primary neurons that helped to reveal a role in the regulation of MT dynamics both in axons and growth cones [3]. Furthermore, our results showed that DAAM is able to crosslink F-actin and MTs. Although it is not clear how the co-alingment of these polymers takes place, we noted that it requires the presence of both the FH2 and CT domains [3]. In addition to the previously summarized formin/MT direct interactions, we collected evidence for the involvement of DAAM in +TIP complex formation, which is consistent with an indirect mode of MT association. For example, a significant portion of the DAAM protein localizes to MT plus-ends, and DAAM co-purifies with EB1 as judged by

co-immunoprecipitation [3]. Nonetheless, the interaction between DAAM and EB1 has not been studied in details, and although the presence of an SxIP motif in the FH2 domain of DAAM supports the possibility of direct binding, further studies are required to resolve this issue.
Along with DAAM, our group investigates the functions of other *Drosophila* formins. We showed that another DRF-type of formin, FRL, is expressed in the embryonic and adult nervous system of *Drosophila*. In addition, FRL has an effect on axonal growth in the mushroom bodies of the adult brain, and *frl* mutants dominantly enhanced the axon growth phenotype of a *DAAM* hypomorphic allele [7]. Despite these advances, whether FRL affects both actin and MT organization, or exhibit a selective effect with regard to neuronal cytoskeleton regulation, remained an open question.
In summary, our group showed that at least two of the six *Drosophila* formins contribute to axonal growth. Curiously, formins can interact with both major cytoskeletal elements, which puts them into an ideal position to serve as key regulators of actin and MT cytoskeleton coordination during growth cone advance.

3. Aims

Despite the accumulated data on formin/MT interactions, at the molecular level it is still unclear how they bind to each other. According to literature and our data, the FH2 domain is key in MT-binding, although the mode of interaction is not deciphered yet. It is known that the FH2 domain contains at least five subdomains (lasso, linker, knob, coiled-coil, post). Our plan was to find the possible MT-binding sites within the FH2 domain of DAAM. However, due to the fact that FH2 domain is also implicated in actin binding, it is possible that the binding surfaces are overlapping, and the MT- and actin-binding capabilities of the FH2 domain cannot be discriminated. Since the DAAM/F-actin interaction is fully characterized, mutants are available which selectively impairs the actin-processing capacity of this formin. It would be interesting to see whether these mutations influence the effect of DAAM on MT dynamics, especially during axonal growth.
Since we had already shown that DAAM and FRL act together in the mushroom body of adult *Drosophila* brain, we aimed to investigate the interaction between these formins in the development of embryonic nervous system.
Beyond focusing on the direct binding between formins and MTs, investigating the interactions between formins and MT-associated proteins, mainly +TIPS, is also of a great importance. Some formins have been shown to directly interact with +TIPs and our data also revealed that DAAM purifies with EB1 in the same complex. In this study we aimed to characterize the interaction between DAAM and EB1. We expected that out results would help us to understand a potentially important mode of cytoskeleton coordination in neurons as well as in other cellular contexts.

4. Results

4.1.Characterization of the interaction between DAAM and MTs.

Recombinant proteins containing domains or subdomains of the C-terminal region of DAAM were made to perform microtubule (MT) - and tubulin-binding assays.

In order to characterize the interaction between DAAM FH1-FH2 and MTs, five different constructs were made. As a first step, FH1-FH2 domains were split and constructs were generated containing only either the FH1 or FH2 domain. The results showed that the FH1 domain alone cannot interact with either taxol-stabilized MTs or α/β-tubulin heterodimers. In contrast, the FH2 domain showed strong interactions with both MTs and α/β-tubulin. The FH2 domain was then separated into two halves, one contained the Lasso-Linker-Knob (La-Li-K) subdomains and the other contained the Coiled-coil-Post (CC-Post) regions. Binding assays revealed that only CC-Post was able to interact with both MTs and α/β-tubulin. The La-Li-K region had a rather weak binding-capacity for alpha/beta-tubulin, however, MT-co-sedimentation assay showed that the La-Li-K could bind to MTs. Since only the CC-Post region was able to interact with MTs and alpha/beta-tubulin in both binding assays, we decided to split this fragment into two halves in order to map the MT- and tubulin-binding surface accurately. The C-terminal half of this region, called CC-Post-2 was expressed successfully, but the purification of the N-terminal half, called CC-Post-1 was not feasible possibly due to protein instability issues. MT-binding assays revealed that CC-Post-2 was not able to interact with MTs, which indirectly suggests that the MT-binding surface of CC-Post should be in the CC-Post-1 region. Protein structure analysis showed that there is a basic amino acid cluster in CC-Post-1 which gives a positively charged surface to that region. This part of the FH2 domain could be favorable for ionic interactions with the negatively charged MT surface and the acidic C-terminal region of the α/β-tubulin heterodimers. Mutations affecting the surface charge of the DAAM FH2 domain were generated (FH2R/K-A) by site-directed mutagenesis. Mutant FH2 was purified and then tested in both MT co-sedimentation and GST pull-down. The results showed that the both α/β-tubulin- and MT-binding affinity of mutant FH2 reduced significantly as compared to wild type FH2. Immunostaining experiments were also performed to analyze the intracellular localization of a constitutively active form of DAAM (CDAAM) containing only the C-terminal functional domain (FH1, FH2, DAD and C-terminal region - CT). GFP-tagged CDAAM displayed a filamentous organization at the cell cortex after immunostaining and the filaments showed a strong co-localization with MTs. A very same staining pattern was detected when CDAAM was carrying a mutation (I732A) affecting the actin-assembly of DAAM. In contrast, filamentous organization of CDAAM staining disappeared when FH2R/K-A mutation was present.
As a summary, we can conclude that the FH2 domain of DAAM alone can interact with both MTs and alpha/beta-tubulin heterodimers. We successfully identified a short, basic amino acid reach sequence in the CC-Post-1 region, which is essential in MT-binding.

4.2. Investigation of the role of DAAM during axonal growth in Drosophila primary neurons and embryonic nervous system.

Our previous showed that in DAAM mutant neurons the axonal growth was altered and MT dynamics increased significantly as compared to control cells. We have also found that DAAM directly binds to and stabilizes MTs and can crosslink F-actin and MT filaments *in vitro*. These results together suggested that DAAM regulates MT dynamics directly and it has an important actin and MT cytoskeleton coordinator function. However, we have also noticed that the effect of an actin destabilizing reagent (latrunculinA) on MT growth was similar to what we measured in *DAAM* mutant neurons. Therefore, further investigations were needed to clarify the role of DAAM in axonal growth. We aimed to separate the actin- and MT-related functions of DAAM. To this end, we performed rescue experiments by using wild type and mutant *DAAM* transgenes,

expressed at comparable levels. Two of the mutant *DAAM* transgenes have decreased actin-processing activity (I732A and K881A) and one of them has reduced MT-binding capability ($FH2^{R/K-A}$). We expressed these transgenes in *DAAM* mutant primary neurons ($DAAM^{Ex4}$) by using a pan-neuronal ElavGal4 driver.

Morphological analysis of primary neurons showed that *DAAM* mutant cells have significantly longer central MT bundles in their axons. In a good harmony with this data cytoskeletal organization of growth cone changed remarkably indicating that axonal growth of *DAAM* mutant cells altered as compared to wild type control. Rescue experiments showed that wild type DAAM successfully restored the wild type phenotype in mutant neurons. In contrast, the I732A actin-processing mutant form did not rescue at all, while the K881 mutant provided a partial rescue. These results suggest that the actin-processing activity of DAAM is indispensable in axonal growth. The difference between the two mutants (I732A vs K881A) might indicate a differential effect on actin-assembly. Positions of the mutations were in good accordance with their efficiency. According to this, mutation located in the primary actin-binding surface (I732A) of DAAM had the strongest effect on the activity of DAAM as compared to the mutations located in the secondary actin-binding surface (K881A). In contrast to actin-processing mutants, the $FH2^{R/K-A}$ form of DAAM successfully restored the wild type phenotype in mutant cells, which suggests that the MT-binding capacity of DAAM is less important in axonal growth. Based on these results, we conclude that the function of DAAM in axonal growth mainly depends on its actin-related activity. It is very likely, that the direct action of DAAM on MTs is less important.

Besides rescue experiments, a constitutively active form of DAAM (CDAAM) was also expressed in *Drosophila* embryos. In the first place, the development of the embryonic nervous system was analyzed. Immunostaining showed that the overexpression of CDAAM dramatically disrupted the structure of both the central and peripheral nervous system as compared to the wild type. In contrast, mutation affecting the actin-assembly of FH2 (I732A) significantly reduced the effect of CDAAM. In order to see the effect of these CDAAM constructs on the axonal cytoskeleton, primary neuronal cultures were made. Quantitative analysis of the axons of primary neurons revealed that in CDAAM expressing cells the axonal MT length reduced significantly. In contrast, the strong effect of CDAAM on axonal growth was weakened significantly by I732A mutation. These results suggest that the effect of CDAAM on neuronal cytoskeleton and axonal growth depend on its actin-regulatory functions.

Live imaging experiments were also carried out to measure MT growth velocity by tracking EB1::GFP signal. The results revealed that CDAAM has only a very weak effect on MT growth speed and this effect was completely abolished by I732A mutation. This suggests that despite its MT-binding ability DAAM has a very weak effect of MT dynamics and this effect relies on its actin-processing activity.

Results obtained from both primary neuronal cultures and whole body embryos strongly suggest that the actin related activity of DAAM is indispensable in axonal growth while its direct MT-binding capacity is less important.

4.3.Investigation of the interaction between DAAM and FRL in Drosophila primary neurons and embryonic nervous system.

We have already shown that the *Drosophila* formin DAAM has a crucial role in development of both the embryonic and adult nervous system. It has been proved that DAAM and FRL

formins have redundant functions in the development of the mushroom body of *Drosophila* adult brain. Our preliminary data showed that FRL is moderately expressed in the embryonic central nervous system. Thus, it suggests that the redundant function of DAAM and FRL may be present in the development of the embryonic nervous system, as well.

Immunostaining experiments showed that FRL is expressed in both the cell body and in the axon of primary neurons. We analyzed the cell morphology of primary neurons obtained from *FRL* (*FRL^{59}*) and *DAAM* (*$DAAM^{Ex4}$*) single mutant and *$DAAM^{Ex4};FRL^{59}$* double mutant embryos. Our analysis revealed that the axonal MT bundles were significantly longer in *$DAAM^{Ex4}$* and *$DAAM^{Ex4};FRL^{59}$* cells as compared to wild-type control and *FRL^{59}* single mutant cells. We also studied the cytoskeleton organization of the growth cone and we found significant alterations in both actin and MT cytoskeleton of *$DAAM^{Ex4}$* and *$DAAM^{Ex4};FRL^{59}$* cells as compared to wild-type control and *FRL^{59}*.

Besides the primary neuronal cell cultures, immunostainings were carried out in stage 16-17 embryos to analyze the morphology of the ventral nerve cord and motor axons, particularly the intersegmental nerve b (ISNb). Anti-Fascilin II staining was applied to detect fasciclin-positive axons. Our analysis revealed that the ventral nerve cord of either the single (*$DAAM^{Ex4}$* and *FRL^{59}*) or double mutant (*$DAAM^{Ex4};FRL^{59}$*) embryos looked normal. However, in *$DAAM^{Ex4}$* and *$DAAM^{Ex4};FRL^{59}$* embryos a significant portion of ISNb axons were stalled in growth as they failed to reach their proper innervation sites in the body wall muscles. No striking difference was found between wild type control and *FRL^{59}* single mutant embryos.

Based on these results we can conclude that FRL has a negligible effect on axonal growth of primary neurons and the development of the embryonic nervous system. These results together with the data from DAAM rescue experiments and biochemistry data on DAAM/MT interaction have already been summarized in a manuscript which has been submitted for publication in *Cells.*

4.4.Characterization of the interaction between DAAM and EB1.

End-binding protein 1 (EB1) is the core MT (+)-end tracking protein (+TIP), which can directly bind to the growing end of MTs. EB1 can interact with a relatively wide panel of proteins and recruits them to (+)-end of MTs. This so called MT (+)-end-binding complex has a pivotal role in the regulation of the changes of MT cytoskeleton.

A couple of formins have been shown to be able to interact with EB1 or other +TIPs. However, the molecular basis of the binding between formins and +TIPs is unclear. In this project, we aimed to characterize the interaction between DAAM and EB1.

First, by using a new anti-DAAM antibody produced in rat, we were able to perform DAAM/EB1 double staining in primary neuronal cells to get a direct evidence for the colocalization of these two proteins. Our previous data showed that DAAM is highly expressed in primary neurons and a significant portion of DAAM signal is located at the (+)-end of MTs. However, without a good double staining we were not able to detect the colocalization of DAAM and EB1 proteins in the axonal growth cones. Our recent DAAM/EB1 double staining and subsequent image analysis revealed a medium level of colocalization between DAAM and EB1 (Sperman r-value = 0. 527±0.15). We compared this r-value to control experiments. On one hand we detected EB1 with two different dyes which gave us the experimental maxium level of correlation (r = 0.88±0.05). On the other hand, we applied Enabled (Ena) staining with which we could compare the correlation of DAAM/EB1 double staining to the colocalization between

another actin-cytoskeleton regulator, Ena and EB1 (r = 0.352±0.17). Our data showed that the correlation of DAAM/EB1 staining is significantly stronger as compared to Ena/EB1, which suggest that the DAAM/EB1 colocalization in the primary neuronal axons is not an accidental phenomenon.

Detailed biochemistry experiments were performed to characterize the molecular basis of the interaction between DAAM and EB1. We performed GST pull-down experiments in which fragments of DAAM were GST-tagged and used as bait, while the 3xFlag-tagged EB1 was expressed in S2 cells and treated as prey. The results revealed that EB1 precipitated with CDAAM, but no interaction was detected when FH1 or FH2 fragments were used as bait. In addition, EB1 did not bind to CDAAM if the basic amino acids in the CT were mutated to alanine ($CDAAM^{CTR/K-A}$). These results together strongly suggest that the main EB1-binding surface of DAAM is located in the CT and the basic amino acids in this region are important in EB1-DAAM interaction. In addition, equivalent, GST-tagged fragments of FRL (FH2 and FH2-DAD-CT) were also used and no interaction was detected between these recombinant proteins and EB1. It suggests that the interaction between DAAM and EB1 is specific and EB1-binding capacity is not a general feature of all formins.

Steady-state anisotropy experiments revealed that CDAAM and DAD-CT fragments of DAAM bind to Alexa488-labelled EB1. In contrast, no interaction was found for DAAM FH2 domain and $CDAAM^{CTR/K-A}$. Therefore, these results entirely confirmed the outcome of the previously described GST pull-downs. In addition, by applying different conditions in the reaction buffer, we found that the interaction between EB1 and DAD-CT is salt sensitive, which proves the electrostatic nature of the binding.

Besides protein-protein interaction studies, pyrene-actin assays were also performed to see if EB1 affects the *in vitro* activity of DAAM. To this end, the actin assembly activity of DAAM FH2 and CDAAM fragments were analyzed in the absence and presence of different forms of EB1. Interestingly, we found that EB1 significantly inhibited the activity of CDAAM in a concentration-dependent manner. In contrast FH2 did not show any strong reduction in its potency to accelerate actin assembly in the presence of EB1. These results also prove that DAAM interacts with EB1 via its CT region and more importantly pyrene-actin assay revealed a regulatory interaction between these two proteins. Further experiments showed that the C-terminal truncation of EB1, by removing the EB-homolog domain did not disturb its DAAM inhibitory effect. In contrast, the N-terminal truncated form of EB1 failed to inhibit CDAAM, which strongly suggests that the N-terminal calponin-homolog domain of EB1 is important in DAAM binding.

Further experiments are on the way to fully characterize the interaction between EB1 and DAAM. According to literature, the actin-binding capacity of EB1 can be an important factor in the regulation of DAAM-binding. In order to investigate this, a mutant form of EB1 (K89D) was created which has a reduced actin-binding capability. In addition, to investigate whether the inhibitory effect of EB1 on formin activity is a general feature, we plan to test the effect of EB1 on FRL, another formin belonging to the diaphanous related subfamily. Finally, we want to assess the *in vivo* importance of the DAAM-EB1 interaction. We know that overexpression of CDAAM in Drosophila embryos has a very strong dominant negative effect, and development of the central nervous system is significantly compromised. Exploiting this strong effect, experiments will be performed in which CDAAM and EB1 will be co-expressed in embryos to see if EB1 can inhibit the effect of CDAAM.

5. Deviations from the original project plan

5.1. Characterize the interaction between Drosophila DAAM and Myo1D proteins.

Left-right (LR) asymmetry is an evolutionary conserved developmental process, which is important in organ positioning, shape and function. Recently, Stéphane Noselli and his co-workers showed that a particular myosin, Myo1D is a major player in asymmetry in *Drosophila* and vertebrates. Genetic interaction studies revealed that the Drosophila formin, DAAM is an essential factor in LR asymmetry development. In the second year of this project, I investigated the protein-protein interaction between DAAM and Myo1D within the framework of cooperation between our research groups. My studies revealed that Myo1D specifically co-purifies with Flag-tagged DAAM in a co-imunoprecipitation assay. More detailed analysis showed that the main Myo1D interaction surface of DAAM is located in the N-terminus. These findings are in a good correlation with immunostainings which revealed strong co-localization between DAAM and Myo1D in the *Drosophila* hindgut. As a summary, we can conclude that DAAM and Myo1D act in a concert to regulate LR asymmetry via the F-actin network. Our results were published in *Plos Genetics* in 2020 [8] .

5.2. Characterization of the interaction between the FH1-FH2 domain of Formin3 and MTs.

Properly developed dendritic architecture is important in the integration and propagation of sensory and synaptic information. Our collaborators, Daniel N. Cox and Ravi Das (Georgia State University, Atlanta, USA) found that Formin 3 is a crucial cytoskeletal regulator in nociceptive sensory neurons of *Drosophila* larvae. They showed that dendritic growth and branching was significantly reduced in mutant cells. Interestingly, the main alterations they found were linked to MT cytoskeleton organization. Within the framework of collaboration between our research groups, I investigated the MT-binding capacity of Formin3 by using standard MT co-sedimentation and GST pull-down assays. The results showed that the FH2 domain of Formin3 has an MT-binding surface, and similarly to other formins, it can directly bind to MTs. This study, published in *Development* provides another evidence for the importance of formins in regulation of the MT cytoskeleton [9].

6. References

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