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INTRODUCTION – AIM OF THE PROJECT

The aim of the project was to characterize myosin 16 (Myo16), a vertebrate specific motor protein, a recently discovered member of the myosin superfamily. The detailed functionality regarding Myo16 is in need of elucidating or clarification, however, it appears to portray an important role in the neural development and in the proper functioning of the nervous system. The impaired expression of Myo16 has been found in the background of several neuropsychiatric disorders, including autism, schizophrenia and/or bipolar disorders. Our results based on *in vitro* experiments contribute to the better understanding of Myo16 and helps to clarify its role in the physiological and pathological processes.

HUMAN RESOURCES

At the beginning of the project the experiments were mostly conducted by András Kengyel, who successfully defended his PhD-thesis (2017) based on the research with Myo16. Nikolett Varnyuné Kis-Bicskei and Mónika Tóth PhD students have been on maternity leave. In the meantime, two young PhD-student joined the project: Elek Telek (2015) and Alexandra Holló (2017), who completed her Master's thesis earlier that year also from the Myo16 topic. We set up fruitful collaborations to conduct specific experiments: the surface plasmon resonance measurements were performed by Ferenc Erdődi (University of Debrecen), circular dichroism measurements by József Kardos (ELTE) and the experiments with the non-muscle myosin were done in James Sellers' lab (NIH, Bethesda, MD, USA).

TECHNICAL RESOURCES

As it was proposed in the budget, in the first year we completed the comprehensive electronic and software upgrade of our Applied Photophysics stopped flow instrument, which was necessary for the kinetic measurement, and for the binding assays of the different domains during the project. Based on our expertise with stopped flow technique, we were asked to contribute to a scientific book (*Thousand faces of proteins*, Semmelweis Press 2018) with a chapter entitled *Rapid kinetic methods*.

TECHNICAL PROBLEMS DURING THE PROJECT

Purifying a novel protein is always risky and challenging, especially for a ~210 kDa motor protein, like the Myo16, which was never purified and characterized *in vitro* before. Although we successfully expressed and purified the isolated ~45 kDa N-terminal ankyrin domain (Myo16Ank) and the ~86 kDa C-terminal tail domain (Myo16Tail), developing the final assay was challenging. Myo16Ank was expressed with a GST-fusion tag, which was cleaved with a TEV protease. Despite of a careful separation by fast liquid chromatography (FPLC) a small

fraction of free GST and uncleaved GST-Myo16Ank remained in the final solution, but these protein contaminations did not influence the measurements as we tested with free GST as control.

The purification of Myo16Tail which was predicted to have an intrinsic disordered structure was especially tricky because of the high potential for aggregation. The protein needed to be purified under denaturing conditioning using 8 M urea to keep the protein soluble. The urea should be removed carefully in sequential dialysis, using glycerol and sucrose transiently. Despite of the purity of Myo16Tail, we could not reach a soluble concentration above ~25 μM, because the protein started to aggregate, this limited the concentrations used later in the spectroscopic assays.

Despite the successful purification of Myo16 fragments, we got into trouble with the purification of the full-lengths Myo16 (flMyo16) and the isolated motor domain. We were able to express these proteins in the baculovirus/Sf9 system, but we could not purify them in a soluble form, even in the presence of light chains or chaperons. The motor domain may need different chaperons for proper folding. Lacking the Myo16 motor domain, we used in our kinetic experiments skeletal and non-muscle myosin 2 motor domains as a model system, acknowledging the fact, that the motor domain is the most conserved part of the myosins showing ~50% sequence homology.

The reason for the unsuccessful purification of the flMyo16 might be the intrinsically disordered structure of the tail domain, which probably requires specific cellular environment to avoid aggregation. Using denaturing conditions was out of the question, because of the sensitive enzymatic function of the motor domain.

SUMMARY OF THE RESULTS ACHIEVED IN THE PROJECT

Protein expression and purification

At the beginning of the project we designed and cloned the constructs to be expressed and improved the methods for purification. Myo16Ank was expressed in *E. coli* with a GST-fusion tag, which was cleaved after affinity purification by a TEV protease. Myo16Tail, which is predicted to have an intrinsically disordered structure, was cloned into baculovirus with a His-affinity tag and expressed in Sf9 cells. The protein was successfully purified under denaturing condition and after renaturation it remained soluble on physiological ion concentration. For spectroscopic experiments we successfully labelled the Myo16 fragments with fluorescent dyes: Myo16Ank was labelled with Alexa568 or pyrene, Myo16Tail with Alexa568.

Myo16Ank can regulate motor function

In our first experiments we were focusing on the Myo16Ank. Based on its close proximity to the motor domain, we supposed to have an effect on the ATPase activity and/or actin binding. Because we had difficulties by purifying the Myo16 motor domain, as a model system we used skeletal muscle myosin proteolytic fragments (heavy meromyosin, HMM and subfragment 1, S1). According to our results Myo16Ank binds to the motor domain and increases the actin-activated steady-state ATPase activity, meanwhile the actin affinity of the motor domain remains unchanged. The motor function was also tested with the *in vitro* motility assay using

total internal reflection fluorescence microscopy (TIRFM). We found, that the presence of Myo16Ank in complex with the skeletal myosin increase the motility speed of actin filaments along the myosin-coated surface suggesting, that the product release (ADP or Pi) of the ATPase cycle is the kinetic step, which is affected by the Myo16Ank, because this is the rate limiting step for the motility. This topic also leads to a successful master's thesis defense (Alexandra Holló).

To support this hypothesis, in a series of rapid kinetic experiments using the stopped flow apparatus we made an attempt to find the kinetic step, which may be directly influenced upon Myo16Ank binding. Myo16Ank had no effect on actin binding to and dissociating from myosin S1 and also the ATP binding rate was not influenced. Meanwhile the ADP-release rate from the acto-S1 complex has markedly increased in the presence of Myo16Ank, suggesting, this kinetic step can be responsible for the regulatory effect of Myo16Ank. These results highlight the importance of Myo16Ank during the regulation of the motor function.

Myo16Ank strongly binds PP1c and decreases phosphatase activity

We also confirmed and clarified the previous assumption, that the ankyrin domain interacts with the protein phosphatase catalytic subunits (PP1c). Using surface plasmon resonance technique in collaboration with Ferenc Erdődi (University of Debrecen) we stated Myo16Ank binds strongly, with nanomolar affinity to the different PP1c isoforms. Interestingly, Myo16Ank, which is homologues to the myosin phosphatase targeting subunit (MYPT1), does not enhance the phosphatase activity of the PP1c isoforms but on the contrary, it decreases the dephosphorylation of the phosphorylated myosin regulatory light chain, which is the natural substrate of MYPT1. Summarizing these facts, Myo16Ank may participate more likely in the transportation of PP1c subunits and not in the regulation of the catalytic function. The results based on the measurement of Myo16Ank were published (Kengyel et al., 2015).

Myo16Tail posses intrinsically disordered structure

The other protein fragment we were focusing on, the Myo16Tail supposed to have an intrinsically disordered structure, based on sequence prediction. We performed tryptophan fluorescence quenching, steady-state anisotropy and time correlated single photon counting to determine the structural flexibility and the hydrophobic core accessibility of Myo16Tail.

In the fluorescence spectroscopic experiments, the tryptophan emission spectrum of Myo16Tail was measured at increasing guanidine concentration. The results showed only a minor change in fluorescence emission and in wavelength shift comparing to globular actin used as control. This can be explained, with a higher solvent accessibility of tryptophans, which did not change during denaturation. The steady-state anisotropy of Myo16Tail showed only a slight change upon denaturation, indicating a low unfolding cooperativity. Time-correlated anisotropy measurements used to determine the rotational correlation time of Myo16Tail revealed a flexible structure. However, the shallow decrease in tryptophan fluorescence lifetime, which correlates with denaturation indicate that Myo16Tail can undergo conformational transition, supposing Myo16b possess molten-globule or pre-molten-globule structure.

Furthermore, circular dichroism measurements, which were performed in collaboration with József Kardos (ELTE), revealed α/β type secondary structure elements beside the considerable amount of disorder content in Myo16Tail, suggesting the secondary structure contains ordered regions. Proving the presence of ordered structure elements beside intrinsically disordered regions may help to explain how Myo16 behave during interactions with binding partners. The results describing the preparation and structure of Myo16Tail are summarized in a manuscript ready for submission.

Binding partners of Myo16Tail

Several possible binding partners of Myo16Tail has been described earlier using colocalization or coimmunoprecipitation techniques. We tried to test the interactions *in vitro* between purified Myo16Tail and some of the supposed partner proteins, such as profilin and filamentous actin (F-actin). In contrast to the previous findings we found no direct binding between Myo16Tail and F-actin.

Myo16Tail contains a proline-rich region, which is a canonical binding site of profilin. However, we found no direct interaction between Myo16Tail and fluorescently labeled profilin-1 using steady-state and time-resolved anisotropy measurements. The profilin-1 regulated actin polymerization was also not influenced by the tail domain. We suppose, maybe only selected profilin isoforms are able to bind to Myo16Tail, a candidate could be the brain specific profilin 2 isoform.

Autoregulation mechanisms of Myo16

By examining the Myo16Ank and Myo16Tail simultaneously, we discovered an interesting phenomenon, namely that these two fragments of the same motor protein are able to bind to each other. Performing steady-state anisotropy measurements we confirmed the moderate interaction, using either labeled Myo16Ank or labeled Myo16Tail. However, the K_D was markedly higher in case of labelled Myo16Tail, suggesting the fluorescent dye may interfere with the binding interface. The surface plasmon resonance (SPR) measurements, using label-free proteins confirmed the interaction, however, we were not able to determine kinetic parameters from the resonance curves, because of the unorganized binding of the intrinsically disordered Myo16Tail to the surface.

We hypothesize that a key step in the regulation of Myo16 might be the backfolding and binding of the C-terminal tail domain to the N-terminal ankyrin domain. This type of regulation is not unusual among myosins (e.g. myosin 5, 7 or 10). However, the interaction is weak, the affinity falls in a micromolar range, but this is optimal for a regulatory function. When the tail is backfolded Myo16 may express a more compact conformation. These findings can explain the previously described different intracellular localization of Myo16Tail and flMyo16.

***In vivo* microscopic experiments**

We have only preliminary results from the *in vivo* microscopic experiments. A fluorescent mCherry-fused Myo16Ank and a tailless Myo16 (the so-called Myo16a splice variant) was constructed and transfected into Cos7 cells. After cellular expression the cells were visualized with Zeiss LSM710 confocal microscope. The Myo16a, as expected, showed exclusive

cytoplasmic localization, while Myo16Ank showed a diffuse cellular localization. This latter is controversial with the literature, (which states almost exclusive nuclear localization for the ankyrin domain). We acquired fluorescent actin marker to perform double labelling and visualize the intracellular interaction between Myo16 and the actin cytoskeleton.

PUBLICATIONS

In appreciation of our efforts in connection with this unusual motor protein Myo16, we were asked to contribute to a scientific book (*Myosins - A Superfamily of Molecular Motors*, Springer Nature CH) with a chapter dedicated to Myo16.

Apart from directly working on Myo16 fragments we implemented several studies related to the interactions and regulation of actin and actin binding proteins, which are indispensable to understand the cellular environment necessary for the optimal function of Myo16. During the project we published 11 papers based on the results. All these papers are listed in the appropriate page of the OTKA web site.

Publications in progress:

Beáta Bugyi and András Kengyel: Myosin XVI (submitted manuscript in: *Myosins - A Superfamily of Molecular Motors*, Springer Nature CH)

Elek Telek, Kristóf Karádi, József Kardos, Beáta Bugyi, András Lukács, Miklós Nyitrai and András Kengyel: The C-terminus of Myosin 16 possess intrinsically disordered structure (manuscript before submission)