PhD thesis

Investigation of Phosphorylation and Dephosphorylation Processes in the Regulation of Conventional Myosin II and Non-conventional Myosin XVI

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Pécs, 2017

1 INTRODUCTION

Myosins are a large and diverse superfamily of actin based motor proteins, which play a crucial role in a wide variety of cellular processes including cytokinesis, cell migration, stress fibers or lamellipodia formation, anchoring of cytoskeletal proteins or vesicular transport.

Myosins share the common feature of actin binding and hydrolysing ATP to generate mechanical force. Myosins are typically constructed of three functional subdomains: (a) the motor domain which binds ATP and interacts with actin in an ATP-sensitive manner, (b) the neck domain which binds light chains, and (c) the tail domain, which is the most variable among myosins. The motor domains are relatively conserved with the exception of several surface loops and the incidental extension on the amino-terminus. The neck or light chain binding region, depending on myosin types, contains 1–7 IQ-sequence repeats, which binds calmodulin or calmodulin-like light chains (1). The tail domains are the most diverse and vary widely in length and sequence among the different myosin families.

1.1 Conventional myosins

Conventional myosins are hexamer protein complexes. The tail domains of the two identical heavy chains consisting predominantly of coiled-coil forming sequences which dimerize the myosin. The neck region binds two pairs of light chains: the ∼20 kDa regulatory light chain (RLC), which is responsible for the regulation of the motor function and the lesser known ∼17 kDa essential light chain which stabilizes the heavy chain.

The soluble form of myosin, which is well suited for kinetic studies, is the dimeric heavy meromyosin (HMM), which contains the two motor domains and the light chains, but lacks the distal two thirds of the tail domain. In our experiments we used different conventional myosins: the full lengths skeletal myosin (skMyo2), the skeletal HMM (skHMM) and recombinant non-muscle myosin HMM 2A and 2B (nmHMM2A, nmHMM2B)

1.2 Non-muscle myosin 2 isoforms

Non-muscle myosin 2 (nmMyo2) is one of the most importnant protein of the animal cells, playing a key role in the reorganization of the actin cytoskeleton and is essential during the entire life cycle of the cells. In vertebrates three isoforms are described, which are translated from different genes: nmMyo2A, nmMyo2B and nmMyo2C (2, 3). The absence of nmMyo2A or nmMyo2B leads to sever developmental diseases and embryonic lethality (4). However the isoforms show a high sequence similarity and under certain circumstances they can substitute each other's function, their main role in the vital processes and their localization are different as well as their kinetic properties.

Both nmMyo2A and nmMyo2B participate in adhesion, morpogenesis, cell division, cell migration or vesicular transport (5), but in certain cell types the isoforms appear selectively: trombocytes or spleen contain exclusively nmMyo2A, while the neural tissue contains mostly nmMyo2B (6, 7).

1.3 The myosin ATPase cycle

The common feature of myosins to catalyse the ATP hydrolysis and to convert the released energy into mechanical work. The cycle of ATP hydrolysis, actin binding and force generation consist of a series of kinetic steps with different rate constants in different myosins.

The binding of actin to myosin increase the ATPase activity with orders of magnitude. In the absance of ATP actin and myosin forms a tightly bound complex (the so called *rigor state*). Binding of ATP to myosin decreases the affinity to actin, the proteins dissociate and the myosin head changes conformation to become perpendicular to the actin filament (*pre-powerstroke*). ATP hydrolysis re-enables actin binding, which support an alternate kinetic pathway to release the hydrolysis products, thus enhances the myosin ATPase activity. Coincident with the P_i release the acto-myosin complex turns from a weak bound state to a strong bound state and force is generated (*power stroke*): the conformation change in the myosin head moves the attached actin filament. Finally, by the release of ADP, the acto-myosin complex remains in the rigor state and the ATP-binding pocket depletes (8, 9).

1.4 Phosphorylation and dephosphorylation

Numerous regulatory processes are responsible for the proper function of myosins in the living cell. In the regulation of smooth muscle and non-muscle Myo2 the phosphorylation and dephosphorylation has a high importance. The ATPase activity of these myosins is primarly regulated by the phosphorylation of the regulatory light chain. Phosphorylation on the Ser19 aminoacid induce conformational change in the RLC and the converter domain, which increases the Mg^{2+}ATPase activity of the myosin motor domain and promotes filament assembly. (10, 11). One of the most important kinase among the several enzymes responsible for the regulation is the Ca^{2+}- and calmodulin-dependent myosin light chain kinase (MLCK) (11).

Similarly to phosphorylation, its opposite process, the dephosphorylation is also essential for the proper cellular functions. Protein phosphatases, responsible for the dephosphorylation of the phosphorylated Ser or Thr aminoacids are classified, using their sensitivity against the thermostable phosphatase inhibitor as PP1 and PP2 (12). The myosin phosphatase (MLCP), a heterotrimeric holoenzyme, dephosphorylates the RLC of Myo2 and its role is essential for example to the smooth muscle relaxation (13) or to the dynamic motion of the Myo2 filaments (14). MLCP consist of a 38 kDa

catalytic domain type-1 (PP1c), a 110 kDa large targeting subunit (MYPT1) and a 20 kDa essential subunit (M20).

1.5 GFP-labeled myosins

To study the localization of proteins *in vivo*, cells are often transfected with constructs in which the desired protein is fused with a fluorescent protein. The premise of these experiments is that the fluorescently labeled construct behaves identical under physiological conditions, as the wild-type protein. To follow myosins *in vivo* several fluorescent constructs are available: the heavy chain or the N- or C-terminal of the light chains can be fused to a GFP (or to an analogous protein) (15). The advantage of using labeled light chain is, that because of its universal appearance in the Myo2 family, different heavy chains (wild types or mutants) can be labeled with one fluorescent construct. In baculovirus expression system the myosin heavy chain can be coexpressed and copurified with different light chains. In cellbiological experiments, the transfected GFP-RLC transiently expresses and associates with the endogenous Myo2 and reports its location or movement within the living cells (16–19). It was shown, that C-terminal GFP-tagged RLC was activated significantly by phosphorylation and could be incorporated into myosins (17). Constructs in which GFP is fused to the N-terminus of RLC have also been used (18), however the kinetic properties of this chimeric protein has not been explored. In our present work we investigated how does the 28 kDa GFP fused to the N-terminal of a 20 kDa RLC influence the function and regulation of the nmMyo2.

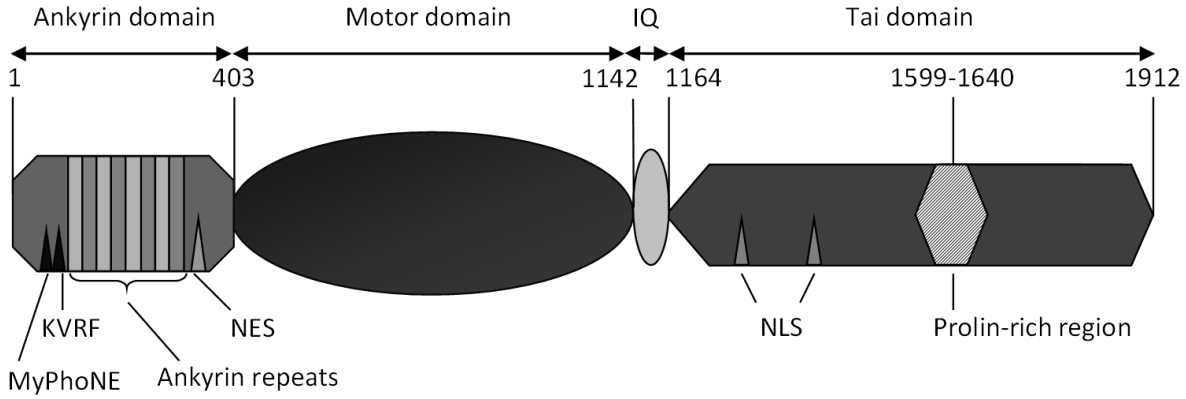
1.6 Non-conventional myosin 16

The non-conventional Myo16 draw our attention because it is one of the few myosins, which can be found in the nucleus and supposed to play a role in the dynamic reorganization of the nuclear actin cytoskeleton or in the nuclear transport. On the other hand, the N-terminal ankyrin domain (My16Ank) shows a high similarity with the N-terminal structure of MYPT1, the reagulatory subunit of myosin phosphatease, thus Myo16 is likely to be involved in dephosphorylation processes.

1.7 Structure of myosin 16b

The pre-motor domain of Myo16 differs from any other previously described myosins. It contains eight highly conserved repeating elements the so called *ankyrin repeats*. Immediately preceding the first ankyrin repeat the KVxF motif could be found, which is a conserved binding site for protein phosphatase 1 catalytic subunit (PPIc).

The motor domain contains several motifs characteristic to myosins, including the ATP and the actin binding site. The short neck domain of the Myo16 contains a single IQ motif, which is predicted to serve as a calmodulin light chain binding site (1). At present, no information is available about the possible light chain, however it would be important to know for understanding the regulation. Myo16 has two splicing variant: the shorter, tailless isoform denoted as myosin 16a (Myo16a) and the full length isoform, the myosin 16b (Myo16b). The tail domain of Myo16b contains a longer prolin-rich region, which is suggested to bind profilin, an actin regulatory protein. The tail domain of Myo16b has an intrinsically disordered structure as predicted by the IUPred bioinformatic program (20).



Schematic structure of Myo16 with the aminoacid positions

1.8 Functions of Myo16b

Little information is available to date about the functional properties of the recently discovered Myo16b. During the phylogenesis, Myo16 appeared in mammals relatively late (21), which suggest, that it has developed for specific functions.

Myo16b, which is the predominant isoform is expressed mostly in brain and in some peripheral neuronal tissues and principally in developmental time period. In rat this is the 1-2 postnatal weeks, the time, which coincides with ongoing neuronal cell migration, axonal process extension and dendritic elaboration (22).

Testing of different nuclear components, Myo16b shows colocalization with PCNA (proliferating cell nuclear antigen) and cyclin A. Overexpression of Myo16b increase the cell number being in S phase and delays progresson towards G_2 phase (23). Thus, Myo16b may have an important role in the in cell proliferation and cell cycle regulation. Myo16b tail colocalize also with profilin and it was supposed, that the Myo16b tail domain can bind to actin through profilin. A possible role for Myo16b in the nucleus could be the regulation of the polimerized state of actin, via the binding of profilin.

Previous findings suggest a key role for Myo16b during the neuronal development. Phosphorylation of the two Tyr on the tail domain recruits adaptor proteins and regulates the PI3K signal transductory pathway, thus together activates the WAVE-complex which leads to actin remodeling, mostly through the Arp2/3-induced actin polimerization (24). The PI3K signal transductory pathway is essential for the proper function of the neural system and the disorder of the PI3K pathway can be found in the background of several neurological or psychiatrical diseases (25). Genetical surveys show significant relation between a variation of Myo16 gen and schysophrenia (26), and the deletion of Myo16 gen was described in the background of autism (27).

1.9 Homology between myosin 16b and MYPT1

The N-terminal extension of the Myo16 (My16Ank) contains several unusual sequence elements: the myosin phosphatase N-terminal element (MyPhoNE), KVxF consensus motif, eight ankyrin repeats and a PKC phosphorylation site. The ankyrin repeat is one of the most common protein sequence motif. It is organized in clusters and the common function of this motif could be in the mediation of protein-protein interactions (28, 29).

However the My16Ank is unique among myosins, it shows a high similaraty with the N-terminal structure of the large targeting subunit (MYPT1) of myosin phosphatase. *In vivo* co-immunoprecipitation studies has shown, as predicted from the N-terminal sequences, that Myo16 binds the catalytic subunits PP1cα and PP1cγ of the myosin phosphatese. A possible role for Myo16 is the transportation of the PP1c subunits into the nucleus (22).

2 OBJECTIVES

During this present work we investigated some important aspects of the phosphorylation / dephosphorylation processes of different myosin classes.

Primarly we studied a non-muscle myosin 2 with a construct, where the regulatory light chain was fused to a GFP. We attempted to understand:

- how the GFP-RLC influences the motor function
- whether the GFP-RLC is a proper substrate for the MLCK during phosphorylation.

We also studied the biological function of the non-conventional myosin 16. We focused on the regulatory role of N-terminal ankyrin domain (My16Ank). We tried to answer the following questions:

- how does the My16Ank participate in the regulation of the motor function?
- does My16Ank bind to myosin or actin directly?
- how does My16Ank interact with PP1c isoforms?
- how does My16Ank influence the PP1c phosphatase activity?

3 MATERIALS AND METHODS

3.1 Expression and purification of nmHMM2A in baculovirus/Sf9 system

The non-muscle HMM2A with an RLC fused to GFP (GFP-nmHMM2A) was expressed in baculovirus/Sf9 system (30). For the quick and efficient purification the C-terminal of the nmHMM2A heavy chain contained a Flag-tag. The nmHMM2A together with the light chains was separated from the other cellular proteins via Flag-affinity chromatography and concetrated with MonoQ-Sepharose ion-exchange chromatography.

3.2 Expression and purification of My16Ank in *E. coli* system

The GST-(TEV)-My16Ank fusion protein was expressed in ER25.66 *E. coli* competent cells. The protein was purified from the cell lysate via GSH-affinity chromatography. The GST-tag was removed by a TEV protease and finally the cleaved My16Ank was separated from GST and TEV with Q-Sepharose anion-exchange chromatography.

3.3 Steady-state ATPase assay

Steady-state ATPase activities were measured at various actin concentrations using an NADH-coupled assay at 22 °C in a low ionic strength buffer (31). Non-muscle myosin was prephosphorylated with 10 nM MLCK in the presence of 0.1 μM calmodulin, 0.2 mM CaCl2 and 0.2 mM ATP at room temperature for 15 minutes (32). Phosphorylation was performed either before the measurement or direct in the cuvette, after iniciating the reaction. Data were collected with a Backman, Jasco or with a Cary 4000 UV-Vis spectrophotometer. The rate of reaction was plotted against substrate concentration and Michaelis-Menten equation was fitted to acquire the maximal rate of reaction (V_{max}) and the substrate concentraton requires to half maximal velocity, the so called K_{ATPase} constant (33).

3.4 Phosphorylation assay

NmHMM2A with either WT-RLC or GFP-RLC was phosphorylated at various MLCK concentations at 25 °C. After starting the reaction with the addition of MLCK, myosin samples were taken at different time points and precipitated immediately in cold acetone to stop the reaction. The unphosphorylated and phosphorylated light chains could be separated by gel electrophoresis using 40% glycerol - 10% polyacrylamide gel (34).

Phosphorylation kinetics were assayed in the same reaction conditions, but using 0.2 mM [γ-^{32}P]ATP (35). The assay was initiated by addition of HMM. Samples were taken at every time point and were applied to filter paper discs, which were immediately immersed in 5% trichloroacetic acid containing 2% sodium pyrophosphate to terminate the enzymatic reaction. The incorporation of ^{32}P into the protein was determined by counting the samples in a Beckman LS6500 Scintillation Counter.

3.5 *In vitro* motility assay

The *in vitro* motility assay provides information about the ability of surface-bound myosins to move actin (36–38). The flow chamber constructed on a microscope slide was filled with 0.1-0.2 mg/ml myosin and was incubated for 1 minute to allow myosin to adhere to the nitrocellulose coated surface. Than 20 nM TRITC-phalloidin labeled F-actin was applied to the flow chamber. Finally the chamber was washed with high viscosity, anti-photobleaching GOC-solution. Motility started only after phosphorylating the RLC using the assay mix containing 0.2 mM $CaCl_2$, 0.1 μM calmodulin and 28 nM MLCK. Images were taken with Zeiss Axioplan microscope.

3.6 Cosedimentation

In actin-My16Ank cosedimentation assay F-actin was mixed with various concentration of My16Ank in the presence or absence of skHMM and after incubation samples were ultracentrifuged. In skMyo2-My16Ank cosedimentation assay the full length skeletal myosin 2 (skMyo2) was dialyzed in a low salt buffer in the presence of My16Ank. Under low salt conditions myosins form filament through the coiled-coil region and precipitates. The coprecipitated samples were sedimented by ultracentrifugation.

3.7 Steady-state anisotropy

Fluorescence anisotropy measurements were carried out with a Horiba Jobin-Yvon fluorimeter. For actin binding experiments IAEDANS-labeled monomeric G-actin or IAF-labeled filamentous F-actin was added to various concentrations of My16Ank. Anisotropy was observed at 20 °C.

3.8 Surface plasmon resonance

Interactions of PP1c isoforms or skHMM with My16Ank was monitored by surface plasmon resonance using the Biacore-3000 instrument (Biacore AB, Sweden). My16Ank was immobilized on the surface of the sensorchip with covalent amine-coupling, while the GST-My16Ank domain was immobilized via binding to anti-GST antibody covalently attached to the sensorchip surface by amine-coupling. Different concentrations of PP1cα, PP1cδ or skHMM in was injected above the surface and the resonance unit signals were detected as a function of time.

3.9 Phosphatase activity measurement

The PP1c phosphatase activity was assayed with 1 µM radioactive [γ-^{32}P]ATP phosphorylated smooth muscle myosin regulatory light chain (^{32}P-RLC) (39). PP1c was preincubated for 10 minutes with different concentrations of My16Ank and the reaction was initiated by the addition of substrate. The reactions were terminated by the addition of 200 µl of 20 % TCA plus 200 µl of 6 % BSA and after centrifugation $^{32}P_i$ was determined in the supernatant by measuring the radioactivity in a scintillation counter (Perkin). For the measurements recombinant PP1cα, PP1cδ and native PP1c purified from tissue were used.

3.10 Statistical analysis

In case of small sample size (e.g. the V_{max} or K_{ATPase} values of ATPase activity), the significance was determined by two-sample Student's t-test (40). Comparing normally distributed, independent smples with known variance (e.g. velocities from the *in vitro* motility assay) two-sample z-test was used. The kinetical processes was compared with Chi-square test. Computed parameters was compared to the significance values of the appropriate degree of freedom (41).

4 RESULTS

4.1 GFP-RLC decreases ATPase activity of nmHMM2A

To examine the enzymatic function of GFP-nmHMM2A, steady-state Mg^{2+}ATPase assays were performed as a function of actin concentration. Conventional myosins share a common feature, that their ATPase activity dramatically increse in the presence of actin. In addition, smooth muscle and non-muscle Myo2 also require phosphorylation on the RLC for the activation (42).

In the unphosphorylated state the GFP-nmHMM2A has a basal ATPase activity similar to WT-nmHMM2A, with $\sim 0.03 \pm 0.01\ s^{-1}$. After phosphorylation of the GFP-RLC by MLCK it increases in an actin dependent manner. The V_{max}, was determined to be $0.20 \pm 0.02\ s^{-1}$ (mean ± SE, n=9 individual series of experiments) which was significantly lower than we measured for WT-nmHMM2A and correlates with the literature (43): $0.39 \pm 0.02\ s^{-1}$ (mean ± SE, n=8; $p \ll 0.001$ with the pooled t-test). There were no significant difference in the K_{ATPase}, which is actin concentration at half maximal velocity and is an approximation of the actin affinity (9.53 ± 1.37 μM for GFP-nmHMM2A compared to 7.97 ± 0.84 μM for WT-nmHMM2A: $p>0.1$ using the pooled t-test).

4.2 GFP-RLC is phosphorylated slower than wild type

To test whether the GFP-fusion altered the phosphorylation of RLC by myosin light chain kinase (MLCK) we measured both the extent and the rate of phosphorylation of HMM bearing the GFP-RLC construct and compared these parameters to HMM that contains WT RLC. The results acquired with different methods indicate, that the GFP-RLC is able to fully phosphorylate, but the rate of phosphorylation is somewhat slower, than for WT-RLC.

The phosphorylation kinetics were tested for the GFP-nmHMM2A using [γ-^{32}P]ATP incorporation at different HMM concentrations where the initial rate of phosphorylation was measured. There was no significant difference in the extrapolated maximal velocity, V_{max}, for myosin phosphorylation using MLCK (18.4 ± 1.9 s-1 for GFP-nmHMM2A versus 20.6 ± 0.3 s-1 for WT-nmHMM2A), however there is a slight difference in the K_M (2.1 ± 0.2 μM for WT-RLC versus 3.6 ± 0.6 μM for GFP-RLC). This different was not significant using the Chi-square test ($\chi^2(60) = 18.96$; $p>0.05$). Thus at low HMM concentrations the GFP-nmHMM2A will be phosphorylated more slowly than WT-nmHMM2A.

4.3 *In vitro* motility assay of the GFP-labeled nmHMM2A

The myosin motor function was tested for the ability to translocate fluorescently-labeled actin filaments on single molecule level in an *in vitro* motility assay. Without MLCK treatment no movement of actin filaments were detected with either the surface bound GFP-nmHMM2A or WT-nmHMM2A in the presence of ATP. Following phosphorylation on Ser19, the actin filaments were moved at 0.29 ± 0.05 μm/s for the GFP-nmHMM2A (n=155 individual filaments were counted) versus 0.35 ± 0.06 μm/s for the WT-nmHMM2A (n=79). The difference, albeit small, was significant by the two sample z-test ($p<0.01$).

4.4 My16Ank enhance ATPase activity of skeletal myosin

The functional properties of the non-conventional Myo16 is poorly understood. The information available to date about the Myo16b indicated that it may play essential roles in the phosphorylation regulatory processes, prilmarly through its N-termnal ankyrin domain (My16Ank). By investigating the recombinant My16Ank, we found that surprisingly, the My16Ank not only participiate in the phospho-rylation/dephosphorylation processes, but it also takes part in the regulation of the motor function.

Based on its close proximity in sequence to the Myo16 motor domain it was hypothesized that My16Ank may play a role in the regulation of the motor domain function. The expression and purification of the motor domain of Myo16 has not been achieved so far, so we used skeletal muscle HMM (skHMM) and non-muscle myosin 2B HMM (nmHMM2B) as model systems. Myo16 motor domain shows ~52% similarity to skeletal muscle myosin motor domain, and the actin and ATP binding regions are highly conserved.

The basal ATPase activity of the skHMM was obtained in the absence of actin and My16Ank and was found to be 0.05 s^{-1} in accordance with previous results (44). This basal activity did not change in the presence of 13 μM My16Ank.

Because the most important partner protein of myosins is the filamentous actin (F-actin) and the binding of F-actin to myosin increases the ATPase activity dramatically, we also measured the effect of My16Ank on the actin-activated Mg^{2+}-ATPase of skHMM. The V_{max} of the ATPase reaction in the absence of My16Ank was 0.41 s^{-1}, which is consistent with the literature, measured under standard relaxation conditions in the absance of Ca^{2+} (45). This increased to 0.66 s^{-1} in the presence of 15 μM My16Ank, while the corresponding K_{ATPase} values changed from 5.1 ± 0.7 μM to 7.1 ± 0.4 μM.

4.5 The effect of My16Ank on the ATPase activity of the nmHMM2B

We repeated these measurements using a non-muscle myosin fragment, nmHMM2B. We chose this myosin primarly because it requires not only actin, but also phosphorylation on the RLC to get fully activated, similar to nmHMM2A (11). On the other hand, nmHMM2B is expressed mainly in brain, just like Myo16.

First we observed that My16Ank had no effect on the ATPase activity in the unphosphorylated state of nmHMM2B and the presence of My16Ank did not influence the phosphorylation of the RLC. When nmHMM2B, after addition of MLCK, calmodulin and Ca^{2+}, the RLC became phosphorylated and was premixed with My16Ank the ATPase rate increased considerably on a My16Ank concentration dependent manner.

To determine V_{max} and K_{ATPase}, mesurements were performed at different My16Ank concentrations. The actin-activated Mg2+-ATPase activity markedly increased in the presence of My16Ank. The V_{max} of the reaction changed from 0.17 s^{-1} in the absence of My16Ank (which is in consistence with the literature (31)) to 0.23 s^{-1}, while the corresponding K_{ATPase} values did not change significantly. These changes in the ATPase activity correlated well with those we observed with skHMM.

4.6 My16Ank binds skeletal myosin

The effect of My16Ank on the actin activated ATPase activity of myosins can only be manifested if My16Ank interacts with either actin or myosin, or with both of these proteins. These interactions can come into effect through binding. First we tested the interaction between My16Ank and the full length skeletal muscle myosin (skMyo2). We found that My16Ank cosedimented with skMyo2, while My16Ank did not sediment alone. This observation indicated that My16Ank can bind to full length skeletal muscle myosins at an approximate 1 : 1 stochiometry. The dissociation equilibrium constant (K_D) was found to be 3.01 ± 0.20 μM.

We also investigated which part of the full length myosin is responsible for My16Ank binding. We tested the interaction between My16Ank and skHMM using surface plasmon resonance. The dissociation equilibrium constant (K_D) for the binding of My16Ank domain to skHMM and was found to be 2.4 ± 1.4 μM. This value correlates well with the result obtained in the experiments with the skMyo2 in the cosedimentation assay.

Based on these observations we concluded that My16Ank binds to skeletal muscle myosin. Considering that the binding of My16Ank was observed with HMM, the truncated fragment of myosin, we also concluded that the binding site for My16Ank is located on the motor domain or on the light chain-binding neck region of myosin.

4.7 My16Ank does not bind to actin

Based on our observation that My16Ank influences the actin-activated ATPase activity of skHMM and nmHMM2B, raise the possibility that My16Ank can bind the actin filaments too. We tested this possibility by using cosedimentation assay, which showed, that My16Ank did not sediment with F-actin, indicating that My16Ank did not bind to the actin filaments.

We also used steady-state fluorescence anisotropy measurements to see whether the My16Ank can bind to actin monomers or filaments. Neither the anisotropy of the IAEDANS-labeled G-actin nor that of the IAF-labeled F-actin changed when My16Ank was present in increasing concentration. These results strenghten our previous finding, that My16Ank did not bind directly to actin.

4.8 My16Ank strongly binds PP1c isoforms

Previous studies have shown that in rat postnatal cerebellum lysate Myo16 coimmunoprecipitate with the catalytic subunits of the protein phosphatase: PP1cα and PP1cγ, but not PP1cδ (22). The fact of the interaction was not surprising, knowing that the amino acid sequence of My16Ank shows homology with the protein phosphatase 1 targeting subunit (MYPT1).

To understand whether My16Ank has a function in phosphatase activities we assayed the interaction between My16Ank and the different isoforms of the PP1c under in vitro conditions using surface plasmon resonance techniques. Different concentrations of the purified PP1cα and PP1cδ (0.5 - 5 μM) were injected over the immobilized My16Ank surfaces and association and dissociation (when free PP1c was removed) phases were recorded as resonance signals.

Based on our results, the My16Ank domain bound strong, with submicromolar affinities to PP1cα (K_D = 540 ± 209 nM) and also to PP1cδ (K_D = 606 ± 173 nM). This finding is in contrast to the previous report that the myosin 16b coimmunoprecipitate with PP1cα or PP1cγ only, but not with PP1cδ (22).

4.9 My16Ank decreases phosphatase activity of PP1c

In protein phosphatase-1 holoenzymes (PP1) the catalytic subunit (PP1c) is responsible for dephosphorylating activity, while the targeting subunit for the regulation. The dephosphorylating activity of the PP1c is markedly increased toward the substrate if the catalytic subunit is bound to the targeting (also called: regulator) subunit, which also ensures the specific substrate to be in close proximity. On the other hand, association of PP1c with the targeting subunit could also inhibit activity towards other substrates preferentially dephosphorylated by the free PP1c.

Considering the homology between My16Ank and MYPT1, we also tested whether My16Ank can enhance the dephosphorylation of smooth muscle P-RLC upon binding to PP1c. Radioactive ^{32}P-RLC was produced with phosphorylation by MLCK using [γ-^{32}P]ATP and Mg^{2+} in the presence of Ca^{2+} and calmodulin. PP1c activity was measured via the release of $^{32}P_i$ after dephosphorylation. We tested different types of PP1c: recombinant PP1cα and PP1cδ, or native PP1c, an isoform mixture purified from rabbit skeletal muscle.

Despite of our expectations, the presence of My16Ank significantly decreased the phosphatase activity for all types of PP1c in a concentration dependent manner. The phosphatase activity was reduced to ~40% by 1 μM of My16Ank compared to that observed in the absence of My16Ank. These data imply that the dephosphorylation of P-RLC by PP1c is inhibited when PP1c is in complex with My16Ank. A possible explanation for these observations is that My16Ank may cover the binding surface for the P-RLC on PP1c.

5 SUMMARY

During our research we concluded, that non-muscle HMM2A with a regulatory light chain fused to a GFP on its N-terminal (GFP-nmHMM2A):

- can be coexpressed and purified in a baculovirus/Sf9 system – similar to the previously described WT-nmHMM2A
- the GFP does not impair the regulation of the GFP-nmHMM2A, in the dephosphorylated state of the RLC, the myosin is inactive
- upon phosphorylation, the steady-state Mg^{2+}ATPase activity of the GFP-nmHMM2A increased, but the V_{max} remained lower (0.20 ± 0.02 s^{-1}) comparing to the WT-nmHMM2A (0.39 ± 0.016 s^{-1}), while the K_{ATPase}, representing the affinity, did not changed significantly
- the GFP-RLC is a substrate of the MLCK and is able to be fully phosphorylated, but with a slower kinetics
- the GFP-RLC is a less suitable substrate of the MLCK than the WT-RLC and the K_M of the phosphorylation reaction is higher, but not significantly (3.6 ± 0.6 μM for GFP-RLC, 2.1 ± 0.2 μM for WT-RLC)
- on single molecule level, the *in vitro* motility speed of actin filaments on a GFP-nmHMM2A surface is significantly lower (0.29 ± 0.05 μm/s), than for WT-nmHMM2A (0.35 ± 0.06 μm/s).

During the investigation of the N-terminal ankyrin domain of myosin 16 (My16Ank), using skeletal myosin or non-muscle myosin 2B as a model system, we concluded, that the My16Ank:

- binds to the skMyo2 (K_D = 3.01 ± 0.2 μM) and to the skHMM fragment (K_D = 2.4 ± 1.4 μM)
- does not alter the basal activity of the skHMM, but enhances the actin-activated steady-state ATPase activity, the V_{max} changed from 0.42 ± 0.012 s^{-1} measured in the absance of My16Ank to 0.66 ± 0.009 s^{-1} in the presence of 15 μM My16Ank.
- does not influence the phosphorylation of nmHMM2B
- enhances the actin-activated steady-state ATPase activity of the nmHMM2B, the V_{max} changed from 0.17 ± 0.06 s^{-1} measured in the absance of My16Ank to 0.23 ± 0.008 s^{-1} in the presence of 11.7 μM My16Ank
- neither binds globular actin, nor filamentous actin and does not influence the polimerization dynamics of actin
- shows ~50% homology to the myosin phosphatase regulatory subunit (MYPT1)
- strongly binds a PP1cα($K_D = 540 \pm 228$ nM) and PP1cδ ($K_D = 606 \pm 249$ nM)
- decreases the phosphatase activity of different PP1c isoforms towards the phosphorylated RLC to ~40% of the original activity.

6 DISCUSSION

Phosphorylation, and its opposit, the dephosphorylation are the key elements of the regulatory and signal transductory mechanisms in the living cell. For the appropriate function of myosins, the phosphorylation of the regulatory light chain or the heavy chain is essential, also the proper function and balance between the regulatory enzymes. During our work, we investigated certain aspects of the phosphorylation-dephosphorylation processes behind the regulation of different myosins. In our first series of experiments, we investigated the phosphorylation dynamics of the conventional non-muscle myosin 2A (nmMyo2A), when its regulatory light chain is fused to a GFP fluorescent protein. The aim of our second project was to characterize the ankyrin domain of the non-conventionl myosin 16 (My16Ank), which can bind to the catalytic subunit (PP1c) of protein phosphatase and it is supposed to participate in the targeted dephosphorylation of intracellular proteins.

6.1 The effect of GFP on the function of the RLC

The physiological functions and properties of the nmMyo2A were intensively investigated during the past decades using many different techniques *in vitro* and *in vivo*. One of the most useful *in vivo* methods to follow nmMyo2A involves cellular expression of the RLC fused to GFP. The GFP-RLC associates with the endogenous nmMyo2A, incorporates into filaments and reports its location in living cells. However, the question of whether this fusion protein behaves similarly to endogenous RLC with respect to its effect on nonmuscle myosin's enzymatic and motile properties has not been explored.

It was found that phosphorylation of the regulatory light chain of nmMyo2A regulates not only the enzymatic activity of the molecule, but also the state of assembly (46). In the absence of phosphorylation, nmMyo2A forms filaments at physiological ionic strength, when no ATP is present. Addition of ATP results in a depolymerization of the filaments and the soluble myosin adopts a conformation in which the tail folds back on the myosin heads to give a compact structure, the off-conformation (46–48). Phosphorylation of the RLC stabilizes the filaments in the presence of ATP.

The GFP molecule, which has a molecular weight approximately one and a half times that of the RLC itself, might possibly interfere with normal myosin function when fused to the amino-terminus of RLC. It might block the ability of MLCK to phosphorylate Ser-19, prevent the myosin from adopting the off state conformation, or interfere with normal enzymatic or mechanical function of the myosin. We found that the myosin containing the GFP-RLC has a very low Mg^{2+}ATPase activity in the presence of actin when it is in the unphosphorylated state, suggesting that the molecule is able to adopt the folded off state. The GFP-RLC bound to HMM2A was phosphorylated by MLCK with virtually the same V_{max},

albeit with a higher K_M as was obtained using WT-nmHMM2A. This means that at low myosin concentrations, the GFP-RLC would be phosphorylated more slowly than the WT-RLC, however at high myosin concentrations this difference would not be apparent.

The Mg^{2+}ATPase activity of GFP-nmHMM2A was markedly activated by phosphorylation, again suggesting that the chimeric molecule is well regulated by phosphorylation. However, the V_{max} of the actin-activated Mg^{2+}ATPase activity for phosphorylated GFP-nmHMM2A was only about half of the WT-nmHMM2A. The K_{ATPase}, which is an approximation of the affinity of the HMM for actin, was not greatly affected by choice of RLC. The rate of actin filament sliding generated by phosphorylated GFP-nmHMM2A was also somewhat slower than that of WT-nmHMM2A. The observation that the actin-activated Mg^{2+}ATPase activity is more compromised by the GFP fusion, than the rate of actin filament sliding is explained by the fact that these two measurements are likely controlled by two different kinetic steps. The actin activated Mg^{2+}ATPase rate of nmHMM2A is limited by phosphate release (49), whereas the rate of actin filament sliding is thought to be limited by ADP release.

The differences we found in the kinetics and phosphorylation dynamics of the GFP fused RLC are not large and should not preclude the use of this fluorescent fusion protein in cell biological studies. The fact that the enzymatic activity of the GFP-nmHMM2A used in this study is still regulated by phosphorylation implies that full length nonmuscle myosin should be able to adopt the off state that may be necessary for dynamic myosin localization in cells. Thus, the N-terminal GFP-tagged RLC does not drastically affect the *in vitro* properties of nmMyo2A and that its use as a fluorescent-fusion protein for imaging the localization of myosin in cells is justified.

6.2 Biochemical characterization of the myosin 16 ankyrin domain

The information available to date about the functional properties of Myo16b indicated that it may play essential roles in key physiological processes. Primarly we chose motor domains from skeletal muscle (skHMM) and non-muscle (nmHMM2B) myosin as a model system to characeterize My16Ank. We found that My16Ank did not modify the basal ATPase activity. Interestingly, the actin activated ATPase activity of skHMM or nmHMM2B was enhanced by My16Ank. Because the K_{ATPase} did not change significantly, the weakening of the actin-myosin interaction and therefore a faster dissotiation is not probable. We speculate that the altered kinetic step was probably the release of the products of the ATP hydrolysis from myosin that made the observed V_{max} values greater in the presence of My16Ank.

We showed with different techniques, that My16Ank can bind to the myosin. However the binding was relatively weak, it indicates, that the myosin contains an My16Ank binding site and based on the measurements with skHMM, it can be found most likely on the motor domain or on the light chains.

We showed, that My16Ank did not bind to actin either in its monomeric or filamentous form, so we excluded that My16Ank modified the myosin binding sites on actin. Together with the elevation of the actin-activated activity it is suggested that the coupling between actin and myosin was modified by My16Ank making this protein-protein interaction more effective. One possibility is that My16Ank built into the motor domain and became part of the protein surface that was responsible for the binding of actin. This would imply that in the native structure My16Ank plays an important role in the manifestation of interaction between actin and Myo16b.

Protein phosphatase (PP1) enzymes play important roles in regulating the phosphorylation levels of proteins in cells through their ability to dephosphorylate key proteins. In our in vitro experiments we found that the isolated My16Ank strongly bound to PP1cα and also to PP1cδ with nearly identical affinities and we can conclude, that My16Ank in complex with the PP1c can mimic a protein phosphatase holoenzyme. In our experiments, we used the P-RLC as a possible substrate for My16Ank-PP1c complex. But our surprising observation that the phosphatase activity of the different PP1c preparations (i. e. recombinant or native) has markedly decreased toward P-RLC in the presence of My16Ank suggested that the P-RLC is not a preferred substrate for the complex. To explain the structural aspects of this observation we assume that My16Ank competes for the binding site of P-RLC on PP1c. In terms of the functional indications the decreased PP1c activity in the presence of My16Ank suggests that the specific target protein for the My16Ank–PP1c complex is not the P-RLC.

Despite of our investigations several major questions remain to be answered in the future to understand the cellular functions of Myo16b. Myo16b may act as a protein phosphatase subunit in complex with the PP1c catalytic subunit and the Myo16b itself may be responsible for targeting PP1c to certain, yet unidentified substrate. Finding the natural target for the My16Ank-PP1c complex will probably give an immediate insight into the intracellular functions of Myo16b. This target may be a protein still to be identified, or may simply be the Myo16b itself.

Previously the N-terminal myosin domains were assumed to act as subunits enhancing the interactions between myosin heads and actin. The observations presented in our work clearly showed that the ankyrin domain of Myo16b can play important role in the overall function of the Myo16b protein by being responsible for its interaction with PP1c, and to fulfill a central function in the cellular regulatory processes.

7 LIST OF PUBLICATIONS

7.1 Publications related to the thesis

1. Kengyel A, Bécsi B, Kónya Z, Sellers JR, Erdődi F, Nyitrai M. Ankyrin domain of myosin 16 influences motor function and decreases protein phosphatase catalytic activity. *European Biophysical Journal*. 2015 May; 44(4): 207-18. (IF: 2,22)
2. Kengyel, A., Wolf W.A., Chisholm, R. and Sellers JR. Nonmuscle Myosin IIA with a GFP Fused to the N-terminus of the Regulatory Light Chain Is Regulated Normally. *Journal of Muscle research and Cell Motility*. 2010 Sep; 31(3): 163-70. (IF: 1,93)

7.2 Posters and presentations related to the thesis

1. Kengyel, A., Telek, E., Nyitrai, M. Biochemical Characterization of Myosin 16 Domains. *15th Alpbach Motors Workshop*, Alpbach, Austria, Márc. 13–18, 2016
2. Kengyel, A., Kónya, Z., Bécsi, B., Erdődi, F., Nyitrai, M. Ankyrin domain of myosin 16 influences motor function and decreases protein phosphatase catalytic activity. *The 30th European Cytoskeletal Forum Meeting*, Postojna, Slovenia, Aug. 30 – Sept. 3, 2015
3. Kengyel, A., Kónya, Z., Bécsi, B., Erdődi, F., Nyitrai, M. A miozin 16 ankyrin domén szabályozza a motor funkciót és csökkenti a protein foszfatáz katalitikus aktivitását. *MBFT XXV. Kongresszusa*, Budapest, Hungary, Aug. 25–28, 2015
4. Kengyel, A, Kónya, Z., Bécsi, B., Erdődi, F., Nyitrai, M. A miozin 16 ankyrin domén szabályozza a motor funkciót és csökkenti a protein foszfatáz katalitikus aktivitását. *45. Membrán-transzport Konferencia*, Sümeg, Hungary, Május 19-22, 2015
5. Kengyel A, Bécsi B, Kónya Z, Erdődi F, Nyitrai M (2013). The role of the ankyrin domain in the function of the myosin 16b. *29th Annual Meeting of European Cytoskeletal Forum*, Stockholm, Sweden, Sept., 2014
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7. Kengyel, A, Bécsi, B., Erdődi, F., Nyitrai, M. Az ankyrin domén szerepe a miozin 16b funkciójában. *43. Membrán-transzport Konferencia,* Sümeg, Hungary, Május 21-24, 2013
8. Kengyel, A, Nyitrai, M. Binding properties of the Myosin 16b Ankyrin Domain. *The 27th European Cytoskeletal Forum Meeting*, Pécs, Hungary, Nov. 3-7, 2012
9. Kengyel, A, Sellers, J. Regulation and kinetic characterization of a GFP-fused non-muscle myosin IIA. *8th European Biophysics Congress, Intracellular Fluorescence Spectroscopy*, Pécs, Hungary, Aug. 20-22, 2011

10. Kengyel, A, Sellers, J. GFP-vel jelölt nem-izom miozin IIA regulációja és kinetikai karakterizációja. *41. Membrán-transzport Konferencia,* Sümeg, Hungary, Május 17-20, 2011

11. Kengyel, A. and Sellers, J. Kinetic Characterization of the Myosin IIA with an N-terminal GFP Fused Regulatory Light Chain. *48th Annual Meeting of The American Society for Cell Biology*, San Francisco, CA, USA, Dec 13-17, 2008

12. Kengyel, A. and Sellers, J. Kinetic Characterization of the Myosin IIA with an N-terminal GFP Fused Regulatory Light Chain. *Gordon Research Conference of the Molecular Motors,* New London, NH, USA, Jun. 30 – July. 4, 2008

13. Kengyel, A. and Sellers, J. Kinetic Characterization of the Myosin IIA with an N-terminal GFP Fused Regulatory Light Chain. *52nd Annual Meeting of the Biophysical Society,* Long Beach, CA, USA, Febr. 2-6, 2008

7.3 Other publications

1. Kellermayer M.S.Z., Bianco, P., Mártonfalvi, Zs., Nagy, A., Kengyel, A., Szatmári D., Huber, T., Linari, M., Caremani, M., and Lombardi, V. Muscle Thixotropy: More than Just Cross-Bridges? Response to Comment by Campbell and Lakie. *Biophysical Journal.* 2008 January 1; 94(1): 329–330. (IF: 4,68)

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Cumulative impact factor: 28,21

Independent citations: 122

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