PhD thesis

Molecular Regulatory Mechanism of Human Myosin-7a

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Abstract

Human myosin-7a is an actin-based motor protein essential for vision and hearing. It plays a vital role in the development and functionality of actin-rich stereocilia. Previous studies using the *Drosophila* homolog demonstrated that myosin-7a is a monomeric, high duty ratio motor, capable of processive movement upon dimerization. However, the characterization of full-length mammalian myosin-7a has been challenging due to difficulties in expressing and purifying a stable, intact protein.

Here, we report the production of a full-length human myosin-7a holoenzyme in insect cells and study its regulation by intra- and intermolecular mechanisms. Human myosin-7a was found to utilize the regulatory light chain, calmodulin, and calmodulin-like protein 4 (CALML4) as light chain subunits. CALML4 was recently discovered to be highly enriched in stereocilia and identified as a candidate gene for deafness. Our research indicates that CALML4 is a crucial factor in regulating the dynamic binding of calmodulin to myosin-7a in response to Ca^{2+} signaling. In the cochlea, there are two splicing isoforms of myosin-7a that are differing by a short N-terminal extension only. Using *in vitro* motility assays and biochemistry, we demonstrate that the N-terminal extension has significant effects on the mechanical and enzymatic behaviors of mammalian myosin-7a. The regulation of mechanosensitivity in hair cells is proposed to be achieved through the adjustment of expression levels of the two isoforms of myosin-7a. Our experiments using single molecule motility assays show that *in vitro*, full-length myosin-7a does not display processive movements on actin alone. However, in the presence of MyRIP (a known myosin-7a binding protein in the neuroretina), it exhibits processive movements. The motor-adaptor complex moves slowly along actin filaments and has a prolonged actin attachment time.

Our results suggests that there is a complex network of regulatory mechanisms that are working in synchrony to fine-tune the activity, structure, localization, oligomeric state, and function of myosin-7a. Our work also contributes to the understanding of retinal cell and inner hair cell function at the molecular level.

Introduction

Myosins

Myosins constitute a large superfamily of actin-based motor proteins. They are essential to various types of cellular movement, such as cytokinesis, phagocytosis, organelle trafficking, and maintaining cell shape [1, 2]. In humans, 39 myosin genes have been identified which are classified into 12 classes based on their motor and tail conformation [2, 3]. Members of the myosin superfamily comprise multiple subunits, including heavy chains and light chains. The heavy chains are usually made up of three functional domains. The *motor* or *head* domain interacts with the actin filament and binds and hydrolyzes ATP. The motor region's central sequence is conserved in all myosin classes. The N-terminal extensions also vary in length and provide the foundation for class-specific properties like membrane binding or kinase activity [2]. The *neck* domain usually contains one or more IQ motifs, which have a consensus sequence and is responsible for binding to the light chain [2, 4]. The stability of the myosin holoenzyme relies on myosin light chains, which stabilize the neck domain and enable it to serve as a stiff lever arm essential for powerstroke generation. These light chains may also have a regulatory impact on the mechanoenzymatic activity of myosin [5, 6]. The holoenzyme forms when the light chains non-covalently bind to the heavy chain. The *tail* domains exhibit the greatest diversity amongst the myosin domains. They may manifest distinct sequence and length characteristics across different myosin families. Unconventional myosins have diverse functions and are involved in multiple cellular processes. Their diversity can be observed through variations in their structure and motor properties [7, 8].

Enzymatic activity

Although the myosin isoforms have distinct structural differences, they still share a common ATPase mechanism *[9]*. In the absence of ATP, the myosin head tightly binds to the actin filament in a rigor state where the lever arm is in the post-powerstroke position. Subsequently, the presence of ATP disrupts the actin binding site, reducing the bond strength between the myosin head and the actin filament and thereby causing detachment of the myosin head from the actin. The myosin head hydrolyzes ATP into ADP and inorganic phosphate, resulting in a conformational change positioning the lever arm into the pre-powerstroke state, allowing for the rebind of the actin filament. The release of the inorganic phosphate from the ATP binding pocket facilitates the formation of the strong binding state between actin and myosin. The release of ADP causes the lever arm to perform its powerstroke, leading to the forward

movement of the tightly bound actin filament. The rigor state is restored and the cycle continues through the binding of another ATP molecule to the nucleotide binding domain [9-11].

Regulation

Myosins require stringent control due to their vast diversity and the multitude of cell types where they are expressed. Myosins are regulated at several levels in order to tune its activity, conformation, intercellular localization, oligomeric state, and overall function, various mechanisms are employed [5, 12]. Alternative splicing produces a variety of spliced variants that possess distinct characteristics, intracellular localizations, and cell/tissue-specific functions. In many myosins, isoform splicing controls the presence or absence of an N-terminal extension. These extensions vary in size and functions. Some contain certain distinct motifs like PDZ-domain, kinase-domain, ankyrin-motifs or ATP-insensitive actin-binding sites. The extension could influence actin or nucleotide binding, protein-protein interactions, hydrolytic product dissociation, or lever arm movement/rotation [5, 6, 12]. Many monomeric myosins adopt a tightly folded conformation, leading to low actin-activated ATPase activity and affinity. This autoinhibitory state can be relieved e.g., by binding partners, phosphorylation, or cations. Divalent cations, such as Ca^{2+} and Mg^{2+}, affect myosin function in several ways, including conformational changes in the holoenzyme and alterations in motor activity. Myosin light chains have different EF hand domains that can bind cations, and regulation of the holoenzyme is achieved through specific binding of light chains [5, 6, 12]. Many unconventional myosins function as important cargo transporters. Adaptor proteins link the cargo to the cargo-binding domain of the molecular motor, mediating and specifying cargo tethering to the myosin. Myosin-cargo interactions govern the motor complex's mechanochemistry, oligomerization, and localization. Binding cargo can cause dimerization or oligomerization of the myosin's heavy chains and promote myosin processivity [5, 6, 12]. The different regulatory mechanisms do not function individually; rather, they exhibit significant interaction among themselves.

Myosin-7a

A prominent member of the myosin superfamily is myosin-7. Myosin-7a (M7a) is essential for vision and hearing [13-16]. Myosin-7a is expressed in various mammalian tissues, such as the testes, kidneys, and lungs. However, it is particularly prevalent in the inner ear and retina, specifically in the hair bundles and synapses of inner ear hair cells (IHC), photoreceptors, and

the retinal pigment epithelium. Mutations in myosin-7a lead to deafness, vestibular dysfunction, and retinal degeneration [15, 17].

The full length myosin-7a comprises of a motor domain followed by a short neck region containing 5 IQ motifs and a tail domain which has two MyTH4-FERM motifs separated by an SH3 motif [18]. Previous research using *Drosophila* homolog expressed in Sf9 cells has demonstrated that myosin-7a is a high duty ratio, monomeric motor that does not move processively on actin filaments [19, 20], however, the characterization of full-length mammalian myosin-7a has been challenging due to difficulties in expressing and purifying stable, intact protein.

A recent study discovered that the cochlea produces two myosin-7a isoforms through alternative splicing, which differ by a short N-terminal extension. The canonical long isoform (M7a-L), with an 11-amino acid extension (MVILQQGDHVW), is expressed uniformly in the inner hair cells but tonotopically in the outer hair cells (OHC) [21, 22]. Previous studies have indicated that the IQ motifs of human myosin-7a have the capability to bind RLC and ELC [23]. The identification of calmodulin-like protein 4 (CALML4) as the endogenous light chain for myosin-7b has raised the possibility of it binding to myosin-7a [24, 25]. Myosin-7a's MyTH4-FERM motifs have the ability to interact with a broad spectrum of proteins. Both motifs are targeted by binding partners [26-28].

Usher syndrome is a recessive genetic disorder that causes damage to the visual and auditory vestibular systems, it is the primary cause of combined deafness-blindness. Defects in myosin-7a can result in hair bundle disorganization, as well as changes to transduction and adaptation processes. Additionally, transportation of other stereocilia proteins may become compromised [29]. The exact physiological role of myosin-7a is not fully understood. The dimerized motor is capable of transporting cargo along the actin cytoskeleton, but recent evidence suggests that it is involved in mechanotransduction in the inner ear by tethering and generating tension [21, 26, 30]. Myosin-7a is involved in a variety of functions in the inner ear and the neuroretina, which requires complex regulatory processes. Our goal in this study is to investigate specific mechanisms responsible for these regulations.

Aims

For human myosin-7a to fulfill its physiological roles, multiple regulatory mechanisms are employed. These mechanisms, both intra- and intermolecular, adjust the myosin's localization, conformation, oligomeric state, and overall activity. The objective of this dissertation is to gain understanding of certain selected mechanisms. I wished to answer the following questions:

- **How to express and purify full-length human myosin-7a?** Only tail-truncated and motor-hybrid constructs have been utilized to investigate the motor functions of human myosin-7a due to the difficulty of expressing and purifying stable, intact full-length protein. We aimed to develop an optimize a protocol to purify full-length myosin-7a.

- **What are the molecular details of human myosin-7a`s light chain composition?** Although human myosin-7a has so far been co-expressed and purified with calmodulin, it has been demonstrated that other light chains can also bind to myosin-7a's IQ motifs. Our aim is to determine the accurate light chain composition of myosin-7a.

- **What effect does calcium exert on the structure and function of myosin-7a?** Calcium is a crucial regulatory cation in the inner ear. During the mechanoelectro transduction, myosin-7a is exposed to an influx of calcium ions. Our aim was to uncover the potential effect of calcium on the structure and enzymatic function of the myosin.

- **What is the role of the N-terminal extension?** Myosins frequently possess an N-terminal extension that modifies motor function. Following the identification of a brief N-terminal extension of myosin-7a, our research aims to examine its impact on the motor function.

- **What are the motile properties of myosin-7a?** Monomeric myosins can be activated and dimerized through binding partners. In this study, we investigated how MyRIP, a known binding partner of myosin-7a, induces the processivity of the motor protein. In addition, we assessed the intrinsic motility of myosin-7a in the dimer state by using an artificial dimer.

Materials and methods

Cloning of myosin-7a

Over the course of this work, several different vectors containing DNA constructs were created. We engineered multiple different length myosin-7a constructs. In our attempt to express and purify full-length human myosin-7a, in expressions where multiple bacmids (heavy chain, three different light chains) were co-transfected, the purification yield was very low hence we utilized the MultiBac system – an advanced Baculovirus system designed for producing large, multimeric protein complexes in insect cells [31].

Purification of myosin-7a

FLAG-tagged myosin-7a recombinant protein was expressed in *Sf9* insect cells, which were previously infected with pACEBac1-Multi plasmid or co-infected with different pFastBac plasmids containing the heavy chain and light chains. The holoenzyme was purified from the cell lysate via FLAG-affinity chromatography.

Purification of other proteins

MyRIP construct with a C-terminal mCherry-tag followed by a FLAG-tag were cloned into pFastBac1. The purification process was similar as described for myosin-7a. CALML4 was cloned into pET15b-MHL vector and then KRX cells (Promega) were transformed with the light chain-containing plasmid. The light chain was purified from the cell lysate via His-affinity chromatography. GFP-RLC was cloned into pFastBac1 plasmid and expressed in *Sf*9 insect cells, then purified using anion-exchange chromatography. Actin was purified from Rabbit Muscle Acetone Powder (Pel-Freeze Biologicals) and prepared according to standard protocol based on [32]. Calmodulin was purified according to previously published protocols [33].

In vitro motility assay

The *in vitro* actin gliding assay provides information about the ability of surface-bound myosins to move actin. Flow chamber constructed on microscope slides was filled with 0.2 mg/ml myosin-7a and was incubated for 1 minute to allow myosin to adhere to the nitrocellulose coated surface. After thorough washing steps, rhodamine phalloidin labeled actin (20 nM) was flushed in the chamber. The actin motility was observed in the final assay buffer containing high viscosity, anti-photobleaching GOC-solution. The single molecule *in vitro* motility assay provides information about the processivity of individual myosin molecules as they travel along the surface-bound actin filaments. Flow chamber constructed on PEG covered

microscope slides was washed with NeutrAvidin then filled with actin. ~ 0.01 mg/ml myosin was added then motility was observed in the final assay buffer containing anti-photobleaching GOC-solution. Movies were collected on an inverted Nikon Eclipse Ti-E microscope with an H-TIRF module attachment, a CFI60 Apochromat TIRF 100x Oil Immersion Objective Lens) and an EMCCD camera. Processive runs were analyzed using the TrackMate plugin for ImageJ. Histograms were created using GraphPad Prism 7 software. Gaussian fits of the velocity histograms were used to determine average velocity. Characteristic run lengths and run durations were determined via exponential fits of the corresponding histograms.

Steady-state ATPase activity

Steady-state ATPase activities at different F-actin concentrations were measured in SpectraMax ID3 microplate reader at 37°C. The rate of ATP hydrolysis was calculated from the decrease of absorbance at 340 nm caused by the oxidation of NADH based on Michaelis-Menten kinetics and normalized to the myosin concentration. To get the kinetic parameters the ATPase rates were plotted against the F-actin concentration and a Michaelis-Menten curve was fitted on the data points.

Negative stain electron microscopy and image processing

Proteins were diluted then applied to UV-treated, carbon coated EM grids and stained immediately using 1% uranyl acetate. Micrographs were recorded on a JEOL 1200EX microscope using an AMT XR-60 CCD camera at a nominal magnification of 60,000x. Reference-free image alignments and K-means classification were conducted using SPIDER software.

Single molecule mass photometry

Refeyn One MP mass photometer was utilized to obtain data on single molecule landing. 20 nM of each sample or mixture was loaded into a single gasket. The data collection lasted for a duration of 1 minute. Measurements were conducted at room temperature (~24 °C), and images were processed using the manufacturer-supplied software (Refeyn, UK). The calibration between molecular mass and contrast was established using protein standards of known molecular weight [34]. For each sample, a Gaussian fit was applied to a histogram.

Isothermal Titration Calorimetry

Isothermal Titration Calorimetry (ITC) experiments were performed using MicroCal ITC-200 instrument (Malvern). 260 μM CALML4 was placed in the reaction cell, 8 mM Ca^{2+} was loaded

into the ITC syringe. Titration consisted of 20 injections. Data analysis was performed with built-in software using calcium-to-buffer as a control.

MicroScale Thermophoresis

MST measurements were conducted using a Monolith NT.115 device with the laser power set to 60%. The subsequent analysis of the MST traces was carried out employing MO.Affinity Analysis software (Nanotemper). The Monolith NT^{TM} His-Tag labelling kit was used for labelling His-CALML4 and His-calmodulin with RED-tris-NTA dye. Binding affinity assays were carried out using a 16-concentration dilution series of calcium (calmodulin: 5 mM – 150 nM, CALML4: 2 M – 60 μM). Each dilution was loaded into Monolith^{TM} capillaries (~5-7 μl). Data fitting were calculated using the built MO.Affinity Analysis based on the K_D model.

Mass Spectrometry

Samples were separated on NuPage^{TM} 4-12% Bis-Tris gel, stained with Coomassie Blue and then rinsed with distilled water. To prepare it for reduction and alkylation with DTT and IAD, the gel was destained and dehydrated using a bi-carbonate and methanol solution. Peptides were extracted with 5% formic acid and 50% acetonitrile after an overnight trypsin digestion. They were then purified with C_{18} resin containing ZipTips. Using LC-MS/MS, the peptides were concentrated and analyzed.

ARPE19 transfection

ARPE-19, a spontaneously arising human retinal pigment epithelia (RPE) cell line, was acquired from ATCC (CRL-2302™). The cells were cultured in DMEM:F12 medium with 10% FBS in 25 cm^2 culture flasks at 37 °C and 5% CO_2. Prior to transfection, cells with a low passage number were seeded in chambered coverglass (*In vitro* Scientific). Transfection reagent Avalanche (EZ-Biosystems) and CMV plasmid DNA were diluted in Opti-MEM I Reduced Serum Medium (Gibco^{TM}), then carefully added dropwise to each well. The transfection solution was removed following a 5-hours of incubation period at 37 °C in a CO_2 incubator. Cellular observations were conducted 48 hours post-incubation.

Results

Successful expression and purification of full-length myosin-7a

Co-expressing truncated myosin-7a constructs (M7a-5IQ) with different light chains, we were able to identify multiple light chains co-purifying with myosin-7a heavy chain, hence we engineered a construct using the MultiBac system containing the full-length myosin-7a heavy chain, calmodulin, CALML4 and RLC. This approach ultimately resulted in high yield, purified myosin-7a holoenzyme, with the heavy chain and three types of light chains clearly visible on the gel.

The motor function is regulated by the C-terminal FERM domain

Using negative staining electron microscopy, we observed a bent conformation of human myosin-7a at low ionic strength, in which the tail region folds back to contact the motor domain. The contour from the probable motor domain-lever junction to the bend point measures 20.7 ± 1.9 nm. This is consistent with the expected length of the lever with 5IQ motifs (~19 nm). Since the SAH domain is expected to add an additional ~ 9 nm to the lever arm, we speculate that the SAH domain does not contribute to the length of the structure leading up to the bend but rather forms part of the return leg, along with the rest of the tail domain motifs. Increasing the salt concentration resulted in an open conformation, revealing the tail domain's high flexibility.

The interaction of the tail with the motor domain results in suppression of the enzymatic function. Actin-activated steady-state ATPase activity of myosin-7a-FL was measured by NADH-coupled assay. The maximum extrapolated ATPase rate (v_{max}) was 0.2 s^{-1} with K_{ATPase} of 54 μM. In contrast, myosin-7a-S1 containing only the motor domain and first IQ domain measured a ~7.5-fold higher maximum ATPase rate at 1.5 s^{-1} and ~5 fold lower at K_{ATPase} 11 μM. The lower K_{ATPase} represents a higher affinity to actin.

Human myosin-7a is a predominantly expressed as a monomer

Our recombinantly expressed myosin-7a-S1 forms a major mass species of approximately 103 kDa in single molecule mass photometry experiments, most closely matching the sum of one heavy chain (~89 kDa) plus one light chain (RLC, ~20 kDa). Full-length myosin-7a forms a 325 kDa major mass species, matching the sum of one heavy chain (~255 kDa) plus four of the three types of light chains (RLC: ~20 kDa, calmodulin and CALML4: ~17 kDa) indicating that the full-length myosin is a monomer with respect to heavy chains. No distinct peak was observed at a position that would correspond to dimer (or other multimer). In contrast,

artificially dimerized myosin-7a-S1SAH (myosin-7a motor and neck domain followed by a Leucine Zipper motif) formed a major mass species of approximately 406 kDa, matching the sum of two heavy chains (~141 kDa) plus six to eight light chains. The resolution of mass photometry does not allow us to provide an exact number of light chains.

CALML4 is a critical light chain for myosin-7a

The initial quality of protein expressed in *Sf9* cells was significantly improved by co-expressing CALML4 with full length human myosin-7a heavy chain. Anti-CALML4 antibody and mass spectrometry were used to confirm the presence of CALML4 in purified myosin-7a samples. We also discovered that calmodulin co-purified with our 5IQ constructs only when CALML4 was present, implying that CALML4 recruits calmodulin binding to the adjacent IQ. This hypothesis is supported by the recruitment of endogenous *Sf9* calmodulin by CALML4 as detected on SDS-PAGE after purification from experiments where no exogenous calmodulin was either co-expressed or added to the cell lysate.

CALML4 does not demonstrate a calcium-dependent shift

Calmodulin and CALML4 are 44% identical. When the amino acid sequence of CALML4 is compared to that of human calmodulin, crucial amino acids required for Ca^{2+} chelation are absent in each of the EF-hands of CALML4. The presence of some conserved amino acids in the 4^{th} EF of CALML4 hand suggests it might retain the ability to bind calcium. Therefore, we performed an electrophoretic mobility shift assay using purified calmodulin and CALML4 to directly investigate CALML4's calcium binding capability. Calmodulin exhibited altered electrophoretic mobility upon incubation with Ca^{2+}, as it undergoes calcium dependent conformational changes. However, under the same conditions, the mobility of the CALML4 samples remained unchanged following addition of calcium.

CALML4 binds calcium with low affinity

To investigate calcium-induced changes, we conducted isothermal titration calorimetry experiments targeting CALML4 using 8 mM Ca^{2+}. The data fitting pointed out a potential binding ($K_A = 700 \pm 1730\ M^{-1}$). Nevertheless, the abnormally high standard deviation and an unrealistic binding stoichiometry ($N_{CALML4\text{-}Calcium} = 5.56 \pm 3.23$; while $N_{CaM\text{-}Calcium} = 4$) raise doubts about the binding event's veracity. MicroScale Thermophoresis (MST) was used to monitor calcium induced changes in CALML4 molecular hydration shell, charge, or size. In the range of 10^{-2} M (10 mM) to 10^{-5} M (10 μM) calcium, CALML4 samples displayed less affinity binding to calcium when compared to calmodulin. By using 1 M calcium concentration,

CALML4 calcium affinity was estimated at K_D = ~ 350 mM through sigmoidal fitting. These findings result that CALML4 is an EF-hand protein that has lost its capacity to bind to Ca^{2+}.

Light chain binding of full-length myosin-7a is calcium dependent

Using mass photometry, we were also able to visualize changes within the holoenzyme. In 1 μM EGTA conditions, full-length myosin-7a construct exhibited a major mass of 325 kDa, corresponding to one heavy chain and four light chains. Upon the addition of calcium, we observed a shift towards a smaller mass peak at 293 ± 25 kDa, which corresponds to one heavy chain plus an extra 38 kDa. Given that RLC and CALML4 are inert to calcium, we attribute this 38 kDa mass to the combined presence of one RLC and one CALM4. The mass photometer does not detect proteins less than 25 kDa and thus any dissociated, free light chains in this experiment are not detected.

Calcium influences ATPase activity and actin affinity of myosin-7a

Since calcium binding seems to promote light chain dissociation from the holoenzyme, we investigated its effect on the enzymatic activity. NADH-coupled ATPase assay was repeated with 500 nM Ca^{2+} in the reaction. Following the addition of calcium, the maximum extrapolated ATPase rate of myosin-7a-FL increased to 0.98 ± 0.27 s^{-1}.

Actin gliding velocity decreases in the presence of calcium

We carried out an *in vitro* actin gliding assay using TIRF microscopy to examine the impact of calcium on the myosin's motor activity. The surface-bound myosin molecules moved the fluorescently labeled actin at ~ 22 nm/s, however, in the presence of calcium the gliding velocity decreased to ~ 7 nm/s, suggesting that the myosin's motility is compromised as a result of calmodulin dissociation from the holoenzyme even though free calmodulin was present in the assay.

N-terminal extension regulates the location and activity of myosin-7a

The cochlea expresses two isoforms of the myosin-7a heavy chain, a canonical long N-terminal myosin, and a short N-terminal myosin, which lacks an eleven amino acid long N-terminal extension preceding the motor domain [21]. We purified the two isoforms and examined the role of the N-terminal extension in myosin-7a's function. We engineered constructs containing the motor domain and the 5IQ motifs (M7a-5IQ), but no tail to avoid the complication of tail-dependent autoinhibition. Additionally, the common use of an N-terminal GFP-tag on myosin-7a raises questions about its potential impact on the regulation by the N-terminal

extension. We therefore also engineered a long N-terminal construct that includes a GFP moiety before the N-terminal.

In an *in vitro* actin gliding assay, the canonical long isoform displayed higher gliding velocity (3 ± 1.6 nm/s) compared to the short isoform (1.3 ± 0.95 nm/s). Similar results were noted in a steady state ATPase assay, where the actin-activated ATPase activity of the longer isoform was determined to be higher (0.84 ± 0.14 s^{-1}) than the shorter isoform (0.24 ± 0.06 s^{-1}). Interestingly, the presence of an N-terminal GFP moiety on the long isoform also resulted in lower velocity values (1.6 ± 0.9 nm/s) and lower ATPase activity (0.11 ± 0.07 s^{-1}) compared to the untagged long N-terminal isoform.

Artificially dimerized tail-less myosin-7a moves processively

In order to study the mechanistic details of the myosins intrinsic processivity, we utilized single molecule *in vitro* motility assay, in which we were able to observe the processivity of individual myosin molecules using TIRF microscopy. The rhodamine phalloidin labelled actin filaments were immobilized on the surface. GFP-myosin-7a-S1SAH-Zipper was washed into the chamber then the motility of the motor proteins was initiated by adding ATP. In the presence of 150 mM NaCl and 5 mM ATP, dimerized myosin-7a moved along the surface-bound actin filaments. Using FiJi TrackMate, detailed motility characterization revealed that myosin-7a moves slowly on actin filaments (~ 4.2 nm/s) while maintaining attachment for a long duration (136 s) and traveling a long distance (379 nm). Previous studies have investigated the processivity of dimerized myosin-7a in cell cultures, but not in ARPE19 cells, which are a human retinal pigment epithelium (RPE) cell line [35]. Following transient transfection of ARPE19 cells with the dimerized construct, we observed abundant filopodia formation, the GFP-tagged motor moved along the filopodia and accumulated at the tips. By manual tracking the moving molecules using FiJi, we were able to measure the velocity of the dimered constructs. The average speed (n = 5) of the moving GFP-myosin-7a-S1SAH-Zipper was 10 nm/s.

Recombinant MyRIP is a monomeric, globular protein

Full-length MyRIP with an N-terminal mCherry-tag and C-terminal FLAG purification tag (mCherry-MyRIP) was cloned into pFastBac vector and expressed in the baculovirus/*Sf9* system, and then anti-FLAG affinity purified. Electron microscopy images revealed MyRIP as a monomeric globular protein which was further confirmed in mass photometry readings. The

major mass peak at 117 kDa most closely matches an mCherry-tagged MyRIP monomer (~120 kDa).

MyRIP binding enables myosin-7a processivity

Using a single molecule *in vitro* motility assay in TIRF microscopy to investigate the mechanistic details of myosin-7a processivity, we found that full-length myosin-7a alone is not processive; however, it becomes processive in the presence of MyRIP. mCherry tagged MyRIP co-localized with full-length myosin-7a and traveled together along the actin-filament network. Detailed motility characterization shows that the motor-adaptor complex moves on actin slowly at approx. 7.8 nm/s. The characteristic run length was 552 nm and the actin attachment time was around 156 s. At higher concentrations (~ 1 μM myosin-7a and ~ 1 μM MyRIP), we observed the clustering of moving molecules. Several fluorescent molecules were traveling together, indicating the formation of higher number of complexes. To further study this interaction, ARPE19 cells were transiently transfected with GFP-tagged full-length myosin-7a. The GFP signal showed diffuse localization of the myosin throughout the cells. The diffused localization of the full-length protein indicates that the motor is in an auto-inhibited state as opposed to the case of the artificially dimerized myosin-7a, when the motor was observed to move to the tips of the filopodia without the presence of a binding partner. Transfecting the cells with mCherry-MyRIP, MyRIP localizes along the actin cytoskeleton and we also observed red coloration on small moving vesicles. Immuno-blot analysis revealed that ARPE19 cells do not express endogenous myosin-7a or MyRIP at a detectable level. When mCherry-MyRIP and GFP-tagged full-length myosin-7a were co-transfected in ARPE19 cells, abundant filopodia formation was observed and the motor-adaptor complex moved to the tips of the filopodia.

MyBD is not sufficient to promote myosin-7a processivity

The myosin-binding domain (MyBD) with C-terminal FLAG purification tag was cloned into pFastBac vector and expressed in the baculovirus/*Sf9* system, and then anti-FLAG affinity purified. In our investigation of whether MyBD can induce myosin-7a processivity, we utilized a single molecule in vitro motility assay in TIRF microscopy. Our findings indicate that full-length myosin-7a is not processive in the presence of MyBD alone.

Summary

During our research, we have successfully purified full-length human myosin-7a and investigated its *in vitro* characteristics:

- developed a method to express and purify intact full-length myosin-7a using the MultiBac system
- myosin-7a is predominantly a monomeric myosin and it has an autoinhibitory state in physiological conditions
- the autoinhibition of myosin can be alleviated by calcium
- upon calcium addition, certain light chains dissociate from the myosin which impairs its lever rigidity, resulting in slower actin gliding velocities
- CALML4 is a calcium inert but critical light chain for myosin-7a
- the canonical (longer) isoform has a higher ATPase activity and actin gliding capability than the short isoform
- blocking the N-terminus of the myosin resulted in similar lowered activity
- the *in vitro* motility speed of the artificially dimerized myosin-7a is very slow but the motor maintained attachment for a long duration and traveled a long distance
- in ARPE19 cells, the Leucine Zipper motif containing myosin-7a construct formed filaments and the dimerized motor moved along the filopodia accumulating at the tips
- full-length myosin-7a is processive in the presence of MyRIP the *in vitro* motility assay, while it travels long distances while staying attached for a prolonged time
- in ARPE19 cells, full-length myosin-7a by itself shows a diffuse localization, while MyRIP localized along the actin cytoskeleton; co-transfecting motor and binding partner, filopodia formation was observed and myosin-7a and MyRIP traveled along the filopodia
- MyBD is not sufficient to promote myosin-7a processivity

Discussion

In order to function efficiently, a motor protein requires multiple regulatory mechanisms. Human myosin-7a has been the subject of research for many years, but several questions remain unanswered due to the complexities associated with obtaining a high-quality holoenzyme. In this study, we aimed to examine the intra- and intermolecular regulations that adjust the activity, conformation, oligomeric state, intracellular localization, and overall function of human myosin-7a.

Recombinant human myosin-7a was expressed in a baculovirus/Sf9 system and purified via FLAG affinity. By expressing constructs containing varied numbers of IQ motifs with different light chains, we successfully unveiled the light chain composition of the myosin-7a complex. Human myosin-7a prefers calmodulin and calmodulin-like protein *in vitro,* while it also binds to regulatory light chains.

Structural analysis of myosin showed that it is predominantly monomeric *in vitro,* and under low ion conditions, EM images showed a bent structure. In accordance with the EM images, mass photometry measurements also proves that the full-length myosin is a monomer with respect to heavy chains. The peak of the distribution corresponds best to a lever with four light chains bound. The autoinhibited conformation, in which the fifth IQ motif may be compromised and unable to bind a light chain, results in the mass photometry histogram showing a holoenzyme with four light chains bound, as indicated by the Gaussian fit. Myosin-7a-S1 displayed a distinct peak at 103 kDa, which corresponds most closely to a truncated heavy chain consisting of the motor domain and first IQ motif, along with a regulatory light chain. In contrast to the monomeric full-length myosin-7a, the Leucine Zipper motif containing construct formed a major mass peak in mass photometry readings that was consistent with a dimer.

Calmodulin is a well-known calcium binding protein. As part of the myosin-7a holoenzyme it suggests that the myosin may be influenced by calcium through calmodulin light chains. A steady-state ATPase assay in the presence of calcium revealed increased ATPase activity of myosin. However, calcium addition reduced the motor`s actin gliding abilities. Calcium binding causes a conformational change in human calmodulin, resulting in a shift from an apparent molecular mass of 17 kDa to 14 kDa. In the case of purified CALML4 samples, this mobility shift was not observed. Calmodulin dissociation in calcium conditions may alter the flexibility of the lever arm and thus the motor properties of myosin, allowing for calcium-sensitive regulation.

Alternative transcription and translation start sites generate multiple isoforms of myosin-7a in the cochlea. The hair cell types show different expression patterns of the isoforms. In OHCs the expression pattern of the two isoforms is inversely correlated, the canonical long N-terminal myosin-7a is expressed mostly at the apex, while the short N-terminal myosin-7a is expressed at the base of the cochlea. The IHCs are thought to only express the canonical long N-terminal isoform [21]. The two isoforms differ only in their N-terminal domain, the canonical isoform has an 11-amino acid long extension preceding the motor domain. Through an *in vitro* actin gliding experiment, surface-bound myosin propelled fluorescently labeled actin filaments, revealing a significant difference between the canonical long isoform and the short isoform. The actin filaments moved ~2 times faster on the long N-terminal isoform. It is noteworthy that the GFP-tagged long isoform, where the GFP-tag was attached to the N-terminal of the myosin-7a, showed motile properties similar to the short isoform. By utilizing a steady-state ATPase assay to assess the activated ATPase activity of the myosin-7a isoform, we have observed that the canonical long isoform exhibits a greater ATPase activity as compared to the short isoform. Similarly, to the gliding assay, the GFP-tagged long isoform exhibited a lower ATPase activity, closely matching the activity of the short isoform. According to structural predictions, the N-terminal extension of myosin-7a is closely situated near the nucleotide binding pocket of the motor domain [22]. Our observations suggest that the extension may play a role in the allosteric regulation of ATP hydrolysis, most likely in ADP or phosphate release.

Myosin-7a is believed to directly control the tip link resting tension and thereby regulate the open probability of the MET channel [21]. Intriguingly, recent studies on hair bundle mechanics have revealed a tonotopic variation in the tip-link tension of OHCs, wherein the tension gradually increases towards the base [36]. The short N-terminal isoform, which concentration similarly increases towards the base exhibit lower ATPase activity with higher affinity to actin comparing to the long N-terminal isoform. These observations collectively suggest that the two myosin-7a isoforms may have a distinct mechanochemistry that contributes to the tonotopic gradients of the tip link tension. Based on the unique expression pattern and differing enzymatic activity we propose that the hair cell regulates its mechanosensitivity by adjusting the expression levels of the two myosin-7a isoforms. The significance of this N-terminal extension regarding deafness is still unknown.

To study single molecule motile properties of myosin-7a and observe possible processivity, which is required for an efficient transport, we used single molecule motility assay methods *in vitro* and in ARPE19 cell cultures. Processivity usually requires a motor protein to

dimerize or work in ensemble. However, the short alpha-helical motif is not able to dimerize myosin-7a and functions as a SAH domain and not as coiled-coil. Dimerization of monomeric motor proteins is also possible through the binding of partner proteins. We constructed a GFP-tagged myosin-7a Leucine Zipper dimer with a truncated tail to reveal its intrinsic motility in the dimeric state. In order to study the kinetic details of the dimerized myosin, we performed single molecule *in vitro* motility assays using TIRF microscopy. The forced dimerized motor slowly moved (~ 4.4 nm/s) along single actin filaments, travelling long distance while staying attached for a prolonged time. Following transient transfection of ARPE19 cells with the dimered construct, we observed abundant filopodia formation, the GFP-tagged motor moved along the filopodia and accumulated at the tips. Given the high flexibility and mobility of the protrusions, only a small number of motor protein velocities have been observed and measured. However, our calculations (~ 10 nm/s) are in good agreement with previous observations.

Since the C-terminal FERM domain of myosin-7a folds back to the motor, molecules targeting this area are predicted to be able to alleviate the inhibition. MyRIP, a known binding partner of myosin-7a and Rab27a has been shown to activate the myosin *in vivo* [37]. Myosin-7a alone did not exhibit processivity in a single molecule *in vitro* motility assay. However, with the inclusion of MyRIP, myosin-7a began to exhibit processive behavior. The analysis of the motility assay of full-length myosin-7a and MyRIP shows that the motor and binding partner move together at low speed (~ 7.8 nm/s) for the entire duration of the recorded movement. At higher concentrations, we observed larger fluorescent complexes. Interestingly, the complex became part of other motor-adaptor clusters as it traveled along the actin filaments until it reached the end of the given filament. This observation suggests the potential for oligomerization of myosin-7a, although no additional evidence has been discovered to confirm the formation of large complexes by the myosin. Our findings also indicate that full-length myosin-7a is not processive in the presence of MyBD alone.

Following transient transfection of ARPE19 cells with full-length myosin-7a, we have observed the diffuse localization of the GFP-tagged motor protein. The lack of filopodia formation and no observed motility suggest that the myosin is in the autoinhibited conformation. After transiently transfecting MyRIP, the mCherry-tagged protein localized along the cytoskeleton and we also observed red coloration on small, moving vesicles. When co-transfecting mCherry MyRIP and GFP-tagged full-length myosin-7a into ARPE19 cells, we observed abundant formation of filopodia and subsequent translocation of the motor-adaptor complex to the tips of the filopodia. The autoinhibition was alleviated through co-transfecting the binding partner.

Myosin-7a is involved in a variety of functions in the inner ear and the neuroretina. Based on a generally accepted theory, a myosin motor is involved in adjusting tension through the tip-link between two stereocilia. A slow myosin, such as myosin-7a is an excellent candidate as it is able to exert force on the links connecting stereocilia whilst translocating along the actin core and it localizes at the appropriate position to fulfill this role. Moreover, the tonotopic distribution of the myosin-7a isoforms along the basilar membrane of the cochlear duct helps to maintain the varying tension. In the retina, melanosomes play an important protecting role. The distribution of melanosomes in the RPE changes throughout the light cycle therefore, proper trafficking of melanosomes is necessary for physiological function. The transfer of melanosomes requires a tripartite complex comprising Rab27a and myosin-7a on actin bound by the linker protein MyRIP.

The majority of the measurements were performed *in vitro* and, to a lesser extent, on model cells. The kinetics measurements aimed to simulate the processes occurring in the inner ear hair cells, while the processivity studies were primarily intended to model the transportation of retinal melanosomes. Naturally, the investigated systems cannot be equated to the complexity of human cells, where the function of myosin-7a may be affected by other factors beyond the scope of this thesis. We examined the roles of light chains, Ca^{2+}-regulation, isoform-related differences, and MyRIP binding, however several other proteins in the Usher complex can also associate to and likely regulate myosin-7a.

Our results indicate that there is a complex network of regulatory mechanisms that are working in synchrony to fine-tune the activity, structure, localization, oligomeric state, and function of this unconventional myosin. We gained some insight into these mechanisms' however, there are more to uncover. The successful production of intact and functional full-length human myosin-7a enables future studies to understand the molecular details of human vision and hearing loss caused by myosin-7a defects. Our results also contribute to the understanding of retinal cell and inner hair cell function at the molecular level, which may facilitate further cellular or *in vivo* studies.

Publications Related to the Dissertation

Journal article

Holló A, Billington N, Takagi Y, Kengyel A, Sellers JR, Liu R. Molecular regulatory mechanism of human myosin-7a. *J Biol Chem*. 2023 Oct;299(10):105243. doi: 10.1016/j.jbc.2023.105243. Epub 2023 Sep 9. PMID: 37690683; PMCID: PMC10579538.

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Conference abstracts and posters

1. **Holló, A.,** Billington N., Kengyel, A., Sellers, JR, Liu, R. (2021): Molecular Regulatory Mechanisms of Human Myosin-7a. *ASCB Cell Bio Virtual.* Virtual, USA, December 2021, Mol. Biol. Cell 28, page 546 (Abstract #P908)

2. **Holló, A.,** Billington N., Kengyel, A., Sellers, JR, Liu, R. (2022): Molecular Regulatory Mechanisms of Human Myosin-7a. *66th Annual Meeting of the Biophysical Society*, San Francisco, CA, USA, February 2022, 1406-Plat

3. **Holló, A.,** Billington N., Kengyel, A., Sellers, JR, Liu, R. (2022): Molecular Regulatory Mechanisms of Human Myosin-7a. Cytoskeletal Motors Gordon Research Seminar and Conference, Dover, VT, USA, July 2022.

4. **Holló, A.,** Billington N., Kengyel, A., Sellers, JR, Liu, R. (2022): Mechanistic Insight into the Regulation of Human Myosin 7a. ASCB Cell Bio. Washington DC, USA, December 2022, Mol. Biol. Cell 33, page 485 (Abstract #P1801)

5. **Holló, A.,** Billington N., Kengyel, A., Sellers, JR, Liu, R. (2023): Mechanistic Insight into the Regulation of Human Myosin 7a. *67th Annual Meeting of the Biophysical Society*, San Diego, CA, USA, February 2023, 1258-Pos

6. **Holló, A.,** Billington N., Kengyel, A., Sellers, JR, Liu, R. (2023): Human myosin-7a has multiple distinct regulatory mechanisms that makes it an excellent candidate as tension adaptation motor. ASCB Cell Bio. Boston, MA, USA, December 2023, Mol. Biol. Cell 35, page 518 (Abstract # P1833)

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