Analysis of motor activity of recombinant myosin-1c

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Analysis of motor activity of recombinant Myosin-1c

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Abstract

Analysis of motor activity of recombinant Myosin-1c

Hair cells of the inner ear are responsible for hearing and balance. These hair cells are limited in number and do not regenerate following injury. To better appreciate the function of these cells it is important to understand the molecular basis of hearing and the roles of the various molecules involved in this process. The overall goal of our laboratory is to understand the molecular basis of mechanotransduction, a process that converts the mechanical sound wave to an electrical stimulus that is transmitted to the brain. We are specifically interested in the motor protein myosin-1c (Myo1c) that has been previously shown to be involved in the adaptation response, a process that makes the hair cells sensitive to prolonged stimulation. Myo1c is a mechanoenzyme that uses the energy of ATP hydrolysis to power its movement along F-actin. The goal of this research is to examine the motor properties of Myo1c, specifically the role of the neck region of the protein in its ATPase activity. Research with other myosins of the same class has elucidated the importance of the neck length of myosins in their enzyme activity and to date no such reports exist for Myo1c. In addition to hair cells, Myo1c is also expressed in other cell types, such as neurons and adipocytes in culture and is involved in motility and vesicle transport in these cells respectively. Thus understanding the regulation of Myo1c function will also enable us to understand its role in various physiological processes.
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“If I have seen further it is by standing on the shoulders of giants”

Isaac Newton
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List of Abbreviations

3THD – Myosin-3 tail homology domain
AA – Alanine/alanine
ADP – Adenosine diphosphate
ATP – Adenosine triphosphate
BAPTA – 1,2-bis(O-aminophenoxy)ethane-\(N,N,N^9,N^9\)-tetraacetic acid
BSA – Bovine serum albumin
C – Cysteine
Ca\(^{2+}\) – Calcium ion
CaCl\(_2\) – Calcium chloride
CaM – Calmodulin
Cdh23 – Cadherin 23
DFNA – Deafness autosomal dominant
DFNB – Deafness autosomal recessive
DNA – Deoxyribonucleic acid
EGTA – Ethylene glycol-bis(beta aminoethyl ether)-\(N,N,N^1,N^1\)-tetraacetic acid
EnaC – Epithelial sodium channel
F-actin – Filamentous actin
FERM – Band F ezrin-radixin-moesin homology domains
G-actin – Globular actin
HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IQ – Isoleucine/ glutamine
kbp – kilo base pair
KCl – Potassium chloride
kDa – Kilo dalton
La$^{3+}$ – Lanthanum ion
MgCl$_2$ – Magnesium chloride
MM1β – Mammalian myosin-1 beta
Myo15a – Myosin-15a
Myo1a – Myosin-1a
Myo1c – Myosin-1c
Myo3a – Myosin-3a
MyoII – Myosin II
MyoVI – Myosin-VI
MyoVIIa – Myosin-VIIa
MyTH4 – Myosin tail homology 4
NaCl – Sodium chloride
Ni$^{2+}$-NTA – Nickel nitrilotriacetic acid
NMB-ADP – N$^6$(2-methyl butyl) adenosine diphosphate
Nomp – No mechanoreceptor potential
OSM – Osmotic avoidance abnormal
P2X2 – Purinigenic receptor subunit 2
PAGE – Polyacrylamide gel electrophoresis
Pcdh15 – Protocadherin 15
PCR – Polymerase chain reaction
PDZ – Post synaptic density protein 95/ Discs-large protein/ ZO-1 protein

PHR1 – Pleckstrin homology domain retinal protein 1

P<sub>i</sub> – Inorganic phosphate

PMSF – Phenyl methyl sulphonyl fluoride

RG – Arginine/ glycine

RS – Restriction site

SDS – Sodium dodecyl sulphate

SH3 – Src homology domain 3

siRNA – Small inhibitory RNA

sw – Snell’s waltzer

Tris – Trishydroxymethylaminomethane

TRP – Transient receptor potential

Y – Tyrosine
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I. Introduction

A. Myosin – an overview

Molecular motors have fascinated researchers for decades, with the visualization of vesicle movement (Allen et al., 1982) and then filamentous tracks (Schnapp et al., 1985; Vale et al., 1985b) in the giant axons of squids by differential interference microscopy (Brady et al., 1985). Such movement was observed only in the presence of cell extracts and the nucleotide adenosine trisphosphate (ATP) (Vale et al., 1985). Subsequently these tracks within cells were identified to be microtubules (Schnapp et al., 1985; Vale et al., 1985b) and a novel “protein translocator” was identified and named kinesin (Brady, 1985; Vale et al., 1985a). The existence of actin (Halliburton, 1887) and myosin (Kühne, W, 1859) proteins have been known to researchers for even longer and experiments in the microorganism Nitella demonstrated the movement of myosin proteins along actin tracks in the cells (Sheetz and Spudich, 1983b). This movement also required ATP and could be inhibited by inactivating the myosin head domain (Sheetz and Spudich, 1983a). These exciting observations have led to years of research identifying 18 different classes of eukaryotic myosins to date (for reviews, Thompson and Langford, 2002; Foth et al., 2006).

Myosins are broadly categorized into conventional and unconventional myosins. They are actin-activated motor proteins that use ATP hydrolysis to power their movement along actin tracks (Pollard and Korn, 1973b; for review, Sellers, 2000). The catalytic structure of all myosin classes has been conserved since all the myosins hydrolyze ATP molecules by the same mechanism (for review, Sellers, 2000); however, the domain
structure differs considerably among different classes of myosins. The conventional myosins have two heavy chains consisting of the two N-terminal head domains, two light chains comprising the neck and an alpha-helical tail domain that is capable of dimerizing and forming filaments (Sellers, 1999). Conventional myosins are found in muscle cells and in the cytoplasm of animal cells and are important in cell division and muscle contraction (for review, Sellers, 2000). The first member of different classes of unconventional myosins was discovered in 1973 as a member of Myo1 family (Pollard and Korn, 1973a). The unconventional myosins, unlike conventional myosins, do not self assemble to form bipolar thick filaments. In general, they contain N-terminal globular head domain, approximately 70-80kDa, followed by the neck region consisting of variable number of IQ domains and a C-terminal cargo-binding tail domain.

B. Myosin force generation along actin

Myosins bind and hydrolyze ATP and use the energy of hydrolysis to translocate along actin, a process called power stroke (Holmes et al., 2003; Tyska et al., 2002; Fig. 1). As depicted in Fig. 1, the myosin head remains attached to the actin filament after the power stroke from the previous cycle. In the absence of ATP, the system can remain in this state for an indefinitely long period. Binding of ATP to the myosin head results in the release of the head from the actin filament (Fig. 1, state 1). The breakdown of ATP to ADP and inorganic phosphate occurs on the myosin head (Fig 1, state 2). This causes the myosin to move further along the actin filament with the products of hydrolysis attached to the head. The release of the inorganic phosphate causes the myosin to generate the power stroke and move along the actin (Fig. 1, state 3). The myosin thus docks on a new
site on actin and ADP is released (Fig. 1, state 4). This movement of myosin along actin is tightly regulated by calcium ions (Ca$^{2+}$) and light chain molecules bound to the neck domain (Uyeda et al., 1996; Purcell et al., 2002; Schott et al., 2002; Tanaka et al., 2002; Moore et al., 2004; Lin et al., 2005).

The lever arm theory (for review, Spudich, 1994) hypothesizes that for myosins, small movements of the head are amplified by the neck region that act as lever arms and thus produce a larger displacement of the myosin along actin. This theory has been tested extensively for a variety of conventional and unconventional myosins (Uyeda et al., 1996; Purcell et al., 2002; Schott et al., 2002; Tanaka et al., 2002; Moore et al., 2004; Lin et al., 2005). The lever arm hypothesis has been found to be true for the conventional muscle myosin like myosin-II (MyoII) (Uyeda et al., 1996; Purcell et al., 2002; Schott et al., 2002). There are conflicting data about the unconventional myosin, Myosin V. Several groups have demonstrated the importance of the neck length in myosin movement (Purcell et al., 2002; Schott et al., 2002; Moore et al., 2004; Sakamoto et al., 2005). In contrast, one group has shown that the neck length is not important in myosin movement (Tanaka et al., 2002).

The inner ear is notable for its repertoire of myosin proteins, both conventional and unconventional. Mutations in a number of these myosin molecules have been found to cause auditory and vestibular defects in mice and humans (Avraham et al., 1995; Mburu et al., 1997; Weil et al., 1997; Wang et al., 1998; Melchionda et al., 2001; Walsh et
al., 2002; D'Adamo et al., 2003). In the following sections, the hair cell structure and function will be discussed in brief, followed by the role of myosins in these processes.

**C. Structure and function of hair cells**

The auditory and vestibular systems owe their sensitivity to hearing and balance respectively to hair cells (Fig. 2), the epithelial receptors found in the inner ear (for review, Hudspeth, 1989b). Hair cells are sensory cells that respond to acoustic vibrations and head movements and couple mechanical stimuli to electrical signals that are ultimately sent to the brain via the nervous system (for review, Hudspeth, 1989b). The most important characteristic feature of hair cells is the hair bundle, the mechanoreceptive structure that projects outwards from the apical surface of the hair cells. The hair bundle is composed of 20-300 stereocilia, each consisting of an actin cytoskeletal core that is extensively cross-linked. Each stereocilium is made up of parallel actin bundles that confer rigidity to the hair bundle. The actin bundles are highly crosslinked by actin bundling proteins like fimbrin (for review, Tilney et al., 1992) and espin (Zheng et al., 2000) and surrounded by a plasma membrane. Like in other cell types, the actin core in hair cells is polarized, with its barbed end, also called the (+) end oriented towards the tip of the stereocilia and the pointed end or the (-) end directed towards the base of the stereocilia (for review, Tilney et al., 1992). Stereocilia are arranged in rows of increasing height giving the hair bundle a staircase-like appearance (Fig. 2). In the auditory organ, the cochlea, hair bundle height also varies allowing it to detect sounds of different frequencies (Holton and Hudspeth, 1983; for review, Tilney et al., 1992). Each stereocilium tapers towards its base where the number of actin filaments
is reduced and inserts into a dense network of actin filaments called the cuticular plate. A single true cilium called the kinocilium is present at the tall extreme of the bundle (Hudspeth, 1989a) and specifies the orientation of the hair bundle (for review, Tilney et al., 1992). The kinocilium degenerates in the adult cochlea (Hudspeth and Jacobs, 1979) and is thought to be required during development of the hair bundle (Kelley et al., 1992).

Within the hair bundle each stereocilium is connected to its next tallest neighbor by very fine extracellular strands called the tip links (Pickles et al., 1984; Kachar et al., 2000), that have been implicated to play an important role in hair cell transduction (Holton and Hudspeth, 1986; Howard and Hudspeth, 1988; Zhao et al., 1996; Marquis and Hudspeth, 1997). The tip links are broken in the presence of the calcium chelators 1,2–bis(O-aminophenoxy)ethane-N,N,N9,N9-tetraacetic acid (BAPTA) or ethylene glycol-bis(beta aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA; Assad et al., 1991; Zhao et al., 1996; Marquis and Hudspeth, 1997) and the cation lanthanum (La$^{3+}$; Kachar et al., 2000; Goodyear and Richardson, 2003). They are however resistant to the protease subtilisin (Goodyear and Richardson, 2003). Electron microscopic studies revealed that the tip link is a right-handed coiled-coil double filament that usually forks into two branches at the point of contact with the taller stereocilium (Kachar et al., 2000). In a recent finding, it has been suggested that the two structural proteins, cadherin 23 (Cdh23) and protochadherin 15 (Pcdh15), interact to form tip links in adult hair cells of guinea pigs (Kazmierczak et al., 2007). These two proteins are predicted to be structurally suitable to bridge the nearly 150 nm gap at the tips of the stereocilia (Kazmierczak et al., 2007).
D. Mechanotransduction

Mechanosensory transduction is the process that converts mechanical stimuli to signals that are conveyed to the brain (Hudspeth and Jacobs, 1979). This is achieved by opening channels in the membrane of the transducing cells and increasing ion influx into cells, causing the cells to depolarize and initiate neural signaling (for review, Holt and Corey, 2000). Hair bundles respond to mechanical force by pivoting at their bases but do not bend along their lengths (Hudspeth, 1989a; for review, Hudspeth, 1997). This is due to the rigid structure of the stereocilium and the change in dimension at the base where it inserts into the cuticular plate (Hudspeth, 1989a, for review, Hudspeth, 1997). The displacement of the hair bundle (Fig. 3) can either be positive or negative, termed as excitatory and inhibitory stimuli respectively (for review, Hudspeth, 1989b). Positive displacements move the bundle towards its taller edge and open transduction channels (Hudspeth, 1985, 1989a, 1992; for review, Colclasure and Holt, 2003) and depolarize the cell. In contrast, negative displacements cause the bundles to move towards the shorter edge thereby hyperpolarizing the cells by closing the transduction channels (Hudspeth, 1985, 1989a, 1992; for review, Colclasure and Holt, 2003). Displacements at right angles to the bundle’s axis of symmetry however do not produce any change in the resting potential of the cell (Hudspeth, 1985, 1989a, 1992). In a resting hair cell approximately 15% of the transduction channels remain open and the resting potential of the cell is about -60 mV (Hudspeth, 1989a). Therefore there is a resultant inward flow of current even in the absence of any stimulus.
The time of response of hair cells to mechanical stimuli is approximately 100-500 microseconds (Hudspeth, 1989b, 1997). The hair cells respond very quickly to bundle displacement suggesting a direct model of hair cell transduction rather than the involvement of conventional signaling pathways using secondary messengers (Hudspeth, 1989a). As well, it has been demonstrated that the ion channel’s gate swings 2-4 nm (Hudspeth et al., 2000) when it is open following bundle displacement. This led to the gating spring model of transduction (Corey and Hudspeth, 1983; Howard et al., 1988; Markin et al., 1995; for review, Eatock et al., 2000). According to this model, elastic gating springs transmit the mechanical force from the displacement to transduction channels located at the tips of the stereocilia and increase the open probability of the channels (Corey and Hudspeth, 1983). Thus the probability of the channel being open depends on the tension in these springs. When tension in the gating spring is high, channels remain open most of the time, while when tension is low, most of the channels close. The gating spring had been long assumed to be the tip link (for review, Hudspeth, 1989b; Assad et al., 1991; Pickles et al., 1991). However, the apparent rigidity of the tip link as determined by electron-microscopic examination of its structure, makes it unlikely to be the entire gating spring (for review, Hudspeth, 1997; Kachar et al., 2000; Howard and Bechstedt, 2004; Tsuprun et al., 2004). Instead the elastic element is thought to be lying in series with the tip link (Kachar et al., 2000; Howard and Bechstedt, 2004; Tsuprun et al., 2004).
**Mechanoelectrical transduction channels**

Transduction channels are non-selective cation pores that permit the entry of potassium ions that are present in highest concentration in the endolymph bathing the hair bundles (for review, Gillespie and Walker, 2001). The channels are also permeable to divalent calcium ions (Corey and Hudspeth, 1979; for review, Corey, 2006; for review, Ricci et al., 2006) and the concentration of the latter increases during mechanotransduction (Denk et al., 1995; Lumpkin et al., 1997; Ricci and Fettiplace, 1997). The channels also allow the passage of amino glycoside antibiotics (Kroese et al., 1989) that can obstruct the channels and thus damage the hair cells leading to impaired hearing (Imamura and Adams, 2003; Kawamoto et al., 2004). As well, the channels can be blocked with amiloride derivatives, calcium channel antagonists like D-600 and nifedipine (Engel et al., 2002). However, this inhibition profile is inconsistent with any of the known contenders for the transduction channel protein discussed below.

Initial speculations that the transduction channel may consist of the alpha subunit of the epithelial sodium channels (EnaC) were discarded when mice lacking the alpha subunit demonstrated normal hair cell transduction (Rusch and Hummler, 1999). The purinergic receptor P2X2 present in hair bundles was another attractive candidate, however its expression is delayed with respect to the onset of transduction (Glowatzki, 1997). The transient receptor protein superfamily or TRP family appears to be quite suitable for this process and it also includes two known channels involved in mechanotransduction, no mechanoreceptor potential (NompC) in flies (Walker et al., 2000) and osmotic avoidance abnormal (OSM-9) in worms (Harteneck et al., 2000). The
loss of function of nompC gene in *Drosophila melanogaster* results in the loss of sensory transduction (Walker et al., 2000; Kim et al., 2003; Gong et al., 2004). The zebrafish counterpart of nompC also encodes a TRP channel, important in mechanotransduction (Sidi et al., 2003). The kinocilium of *Xenopus laevis* expresses the Ca$^{+2}$ sensitive channel protein TRPN1, a homolog of nompC and has been suggested to be involved in mechanotransduction (Shin et al., 2005). However, TRPN1 or other homologs of nompC are not present in mammals (Sidi et al., 2003; Shin et al., 2005).

TRP channels exhibit biophysical and pharmacological properties that are similar to those of transduction channels in hair cells (Strassmaier and Gillespie, 2002). The most interesting candidate examined thus far is TRPA1. The expression profile of this gene correlates well with the onset of mechanotransduction in mammals (Corey et al., 2004) and decrease of TRPA1 expression in the mouse vestibular hair cells by siRNA also impaired mechanotransduction (Corey et al., 2004). However, TRPA1 knockout mice have normal auditory function (Kwan et al., 2006; Bautista et al., 2006). Among other attractive candidates are TRPV4 (Liedtke et al., 2000; Takumida et al., 2005; for review, Cuajungco et al., 2007) and TRPML3 (Di Palma et al., 2002), both linked to hearing loss in mammals (for review, Cuajungco et al., 2007). However, their candidacies are still questionable (for review, Cuajungco et al., 2007) and the search for the transduction channel continues (for review, Corey, 2006).
E. Adaptation

During mechanical stimulation, the hair bundle is deflected and the transduction channels open permitting an influx of cations into the bundle (for review, Eatock, 2000). The transduction channels are extremely sensitive to bundle displacements and following sustained deflection, the hair bundle adapts for the detection of the next stimulus (Holt et al., 1997). Adaptation is an extremely important process in hair cells that resets the sensitivity of the transduction channels to prolonged stimuli. Adaptation is distinguished from desensitization by the fact that following adaptation, a transduction channel can be fully opened if the mechanical force is large enough (for review, Eatock, 2000). Adaptation can be slow or fast and each modifies the mechanical properties of the hair bundle.

Fast adaptation is remarkably quick (1-2 ms) in lower vertebrates and involves active hair bundle movements (Ricci et al., 2000; Ricci, 2003). Fast adaptation is thought to involve calcium binding directly at or near the channel that alters the relation between tension in the gating springs and open probability of the channel (Wu et al., 1999). It is thought that calcium binds to intracellular sites on the channel and causes the channel to shut (Ricci and Fettiplace, 1997). As calcium concentration in the cell decreases, the channels return to their original resting state (for review, Holt and Corey, 2000).

Slow adaptation takes place over tens of milliseconds and is thought to be mediated by an adaptation motor (Howard and Hudspeth, 1987; Assad and Corey, 1992; for review, Holt and Corey, 2000). The motors are thought to attach the actin core of the
stereocilium to the transduction channel, climbing along the stereocilium to adjust the tension in the gating springs (for review, Holt and Corey, 2000). In response to a positive bundle displacement, tension in the spring increases, opening cation selective channels in the bundle and the motor slips down along the actin core thus decreasing the tension and the channels close (Gillespie and Cyr, 2004). As a result, the bundle can be deflected further by a second stimulus, thus allowing for increased sensitivity following protracted hair bundle deflection. The adaptation motor may also play a role in assembling and properly positioning the transduction apparatus in the hair cells (Zhao et al., 1996).

F. Identity of the molecular motor

The adaptation motor actively generates force and physically interacts with the actin filaments in the stereocilia. These observations indicated that a myosin might be the appropriate candidate for the motor. Also the motor activity requires the presence of Ca\(^{2+}\) and CaM (Walker and Hudspeth, 1996; Lumpkin and Hudspeth, 1999) both of which control the activity of unconventional myosins (Wolenski, 1995; Bahler and Rhoads, 2002). Furthermore, the use of ADP analogs and myosin inhibitors have been found to block slow adaptation (Gillespie and Hudspeth, 1993). Using photochemical cross-linking experiments, an unconventional myosin, myosin-1c (Myo1c), belonging to the myosin I family was identified in hair bundles in 1993 (Gillespie et al., 1993). The head domain of Myo1c binds ATP and actin and produces force for the movement along actin (for review, Mooseker and Cheney, 1995b). Next to the head lies the alpha helical neck region consisting of four isoleucine and glutamine containing IQ domains with the consensus sequence being \(<IQX_3RGX_3R>\) (Rhoads and Friedberg, 1997). These IQ
domains can bind CaM and act as regulatory switches for myosin activity. The C-terminal domain is the most variable of all domains (Mooseker and Cheney, 1995a) and for Myo1c, it has been demonstrated to bind membrane and phospholipid (Reizes et al., 1994), specifically phosphoinosotide 4,5 bisphosphate (Hokanson and Ostap, 2006). Immunolocalization studies of hair bundles revealed the presence of Myo1c near the stereociliary tips, in the pericuticular necklace and in the hair cell body (Gillespie et al., 1993, Hasson, 1997; Steyger et al., 1998; Garcia et al., 1998; Metcalf, 1998) and is absent from a distinct cap-like zone at the tips of the stereocilia (Schneider et al., 2006). In 1994, Metcalf et al., isolated a cDNA encoding myosin protein from the brain and sacculus of bullfrogs that resulted in the identification of a 119 kDa protein with high sequence homology to the adrenal mammalian myosin1 beta (MM1β). Thus the unconventional myosin, Myo1c (previously called Myo1beta) present in the tips of stereocilia where transduction takes place (Steyger et al., 1998; Garcia et al., 1998; Metcalf, 1998) has become the most well-supported candidate for the adaptation motor protein (Gillespie et al., 1993; Hasson et al., 1997; Holt et al., 2002).

In an experimental approach to examine the role of Myo1c in adaptation, a mutant Myo1c was designed (Holt et al., 2002) with increased affinity of binding to an ADP analog (Gillespie et al., 1999). The mutant Myo1c has a key tyrosine residue replaced by glycine in its ATP binding groove (Gillespie et al., 1999), making it sensitive to non-hydrolyzable ATP-analogs like N^6(2-methyl butyl) adenosine diphosphate (NMB-ADP) that maintains the Myo1c in rigid conformation and inhibits its motor activity and thus inhibits adaptation. However, in the presence of ATP, the mutant Myo1c behaves similar
to the wild-type protein (Gillespie et al., 1999). Thus the transgenic mice bearing this mutation allowed for targeted disruption of Myo1c function when NMB-ADP was introduced into vestibular hair cells concomitant with mechanical stimulation of the hair bundle (Holt et al., 2002). The transgenic mice bearing the mutant Myo1c had normal transduction and adaptation in the presence of ATP (Holt et al., 2002). However, when the ADP analog was introduced into the hair cells, adaptation was completely blocked (Holt et al., 2002). It is thought that in the presence of NMB-ADP, the mutant Myo1c molecules are unable to translocate along actin and freeze in their tracks thus abolishing adaptation (Holt et al., 2002; Stauffer et al., 2005). In addition, another finding implicates Myo1c in fast adaptation as well (Stauffer et al., 2005). The researchers show that the rate of fast adaptation is dependent on Myo1c and that fast adaptation is also blocked in mutant Myo1c knock-in mice. Myo1c is suitably positioned near the transduction channels and it is hypothesized that following transduction, rapid conformational change of Myo1c causes calcium to bind directly to the Myo1c-CaM complex and causes the channels to close (Stauffer et al., 2005; Manceva et al., 2007).

G. Myo1c and Mechanotransduction

In the proposed model of transduction, mechanical force increases tension in the gating spring, causing the transduction channels to open, permitting influx of cations including Ca\(^{2+}\) into the hair bundle (Corey and Hudspeth, 1979; for reviews, Corey, 2006; Ricci et al., 2006). Accumulation of calcium and its interaction with CaM in the stereociliary cytoplasm is expected to deplete CaM from Myo1c binding sites. The gating spring can thus pull down the myosin molecules alleviating the tension in the spring and
it shortens. When the spring reaches its resting tension, the channel returns to its resting state with a decrease in intracellular calcium (reviewed in Gillespie and Cyr, 2004). This restores motility and the balance between the upward force produced by Myo1c and downward tension in the springs (for review, Gillespie and Corey, 1997).

Myo1c binds to its ‘receptors’ that are specific intracellular binding sites for Myo1c located near the stereociliary tips and other subcellular locations (Cyr et al., 2002). This interaction is thought to be vital for the process of transduction and is regulated by calcium and CaM (Cyr et al., 2002). It is believed that the receptors are not endogenous CaM or self-aggregated Myo1c molecules (Cyr et al., 2002). As well, it was concluded that the receptors were not membrane phospholipids (Phillips et al., 2006), although Myo1c has been shown to bind phosphoinosotide 4,5 bisphosphate (Hirono et al., 2004; Hokanson and Ostap, 2006). The Myo1c receptors are thought to couple Myo1c to the rest of the transduction apparatus. It has been shown that Myo1c binds to its intracellular receptors through the IQ domains in its neck region (Cyr et al., 2002). This suggests that the interaction of Myo1c with the transduction complex is regulated by CaM. As well, CaM-free IQ2 domain of Myo1c can bind intracellular receptors (Cyr et al., 2002; Phillips et al., 2006) and it is thought that CaM bound IQ3 may enhance the above interaction (Phillips et al., 2006).

Removal of CaM from the hair bundle halted the movement of the adaptation motor and thereby abolished adaptation (Walker and Hudspeth, 1996). However, CaM also binds to plasma membrane calcium ATPase in hair cells and depletion of CaM could have also depleted the calcium pump, abolishing adaptation. Also CaM binding calcium
may control adaptation by speeding up the release of ADP from Myo1c. This has important implications during mechanotransduction. The enhanced calcium concentration during transduction leads to dissociation of CaM but permits Myo1c to go through its ATPase cycle with reduced force production by the motor. This in turn helps Myo1c to perform its role in adaptation better by exerting less force on the transduction channel (for review, Gillespie and Cyr, 2004).

**Other proteins of the transduction complex**

Years of research focused on identifying the proteins other than Myo1c in the assembled transduction complex has led to the identification of Cdh23 and Pcdh15 as components of the tip link (Siemens et al., 2004; Sollner et al., 2004; Kazmierczak et al., 2007). Of these, one potential candidate for the Myo1c receptor is the protein Cdh23 (Phillips et al., 2006). It has been shown that Myo1c interacts with Cdh23 in heterologous expression systems (Siemens et al., 2004), although such interactions have not yet been observed in hair cells. Cdh23 is mutated in humans with Usher syndrome type 1D (USH1D) that leads to both deafness and blindness and also to age-related hearing loss (Bolz et al., 2001; Bork et al., 2001; Di Palma et al., 2001; Noben-Trauth et al., 2003). Studies in the waltzer mouse with mutations in Cdh23 showed developmental defects in the stereociliary organization and implicated Cdh23 in hair cell differentiation (Di Palma et al., 2001). However its abundance in hair cells of adult animals was very low, persisting only near the kinociliotial links in the vestibular organs (Lagziel et al., 2005; Michel et al., 2005) as compared to younger ones, suggesting its importance in development (Boeda et al., 2002). Siemens et al. (2003) have suggested that Cdh23 is a
component of the tip link in adult animals. They have shown the persistence of Cdh23 in the stereocilia of older animals at the stereociliary tips where transduction actually occurs. The zebrafish mutant *sputnik* that has a mutation in the gene encoding Cdh23 (Sollner et al., 2004), associated with loss of tip links in these animals. Myo1c receptor interaction is absent in hair cells of Cdh23 mutant mice (Phillips et al., 2006), suggesting that these proteins interact either directly or in the presence of a third molecule that is missing in Cdh23 mutant mice (Phillips et al., 2006).

Pcdh 15 was identified in the context of the human disorder Usher 1F (Ahmed et al., 2001) and the protein was found to be present in the stereocilia of the cochlea and the vestibule (Ahmed et al., 2003). The Ames waltzer mice deficient in protocadherin 15 were found to have defects in the polarity of the hair bundle (Senften et al., 2006) and degeneration of the stereocilia, suggesting a role for Pcdh 15 in development and maintenance of the hair bundles (Alagramam et al., 2001). In addition, these mice were also found to be defective in mechanotransduction thus explaining their deafness and circling phenotype (Senften et al., 2006). As well, the tip link antigen (TLA) present in the tip link and the kinocilial links of hair bundles (Goodyear and Richardson, 2003) has been identified by mass spectrometry to be Pcdh15 (Ahmed et al., 2006). Although Pcdh15 and Cdh23 have been shown to interact in vitro (Kazmierczak et al., 2007), further studies are necessary to put the missing pieces of the transduction complex puzzle together.
Another hair cell protein pleckstrin homology domain retinal protein 1 (PHR1) has been shown to interact with Myo1c (Etournay et al., 2005), however the fact that it interacts with IQ4-tail region of the protein excludes it from being the receptor (Phillips et al., 2006). In addition, PHR1 knockout mice do not demonstrate any sensory defects (Xu et al., 2004).

The model for Myo1c regulation suggests that an increase in calcium concentration near the stereociliary tips causes the CaM bound to Myo1c to dissociate and enables the interaction of Myo1c with its receptors, thus facilitating adaptation (for review, Gillespie and Cyr, 2004). The paucity of the hair cells in the inner ear, however, makes it extremely difficult for molecular identification of the receptors.

H. Other myosins of the inner ear

Myosin-VI

Myosin-VI (MyoVI) (Fig. 4) is an unconventional myosin first identified in Drosophila and demonstrated to play an important role in embryogenesis (Kellerman and Miller, 1992). It is also expressed in adult flies and shown to be required during fly spermatogenesis (Rogat and Miller, 2002). MyoVI was first identified in mammals in the porcine kidney cell line LLC-PK1 (Hasson and Mooseker, 1994). Like its Drosophila homolog, this mammalian enzyme has a N-terminal head domain consisting of ATP and actin binding motifs, a 50 amino acid linker domain that is unique to MyoVI immediately following the head, a neck domain consisting of one IQ consensus region for binding CaM and a tail domain consisting of coiled-coil regions that is predicted to form
homodimers (Hasson and Mooseker, 1994). MyoVI is different from other classes of unconventional myosins in that it moves along actin towards the pointed ends instead of barbed ends (Wells et al., 1999). It has been recently shown that the insert at the end of the motor domain binds to Ca\(^{2+}\)-CaM (Bahloul et al., 2004) and determines the reversality of movement of MyoVI on actin (Park et al., 2007). This feature allows MyoVI to move cellular cargo from the surface of the cell to the inside, towards the pointed end of the actin filaments. MyoVI has also been demonstrated to be a processive motor that is bound to actin for longer intervals during the actin-crossbridge formation (Rock et al., 2001). This enables the protein to carry its load further along actin with fewer dissociations and reassociations.

Subsequent characterization of the Snell’s waltzer (sv) mice, that exhibit profound deafness and circling behaviour, revealed that the mutation mapped to a region of the chromosome coding for MyoVI. The mutation deleted a 130 bp region of the gene inducing a shift in the reading frame and a premature stop codon after the neck domain (Avraham et al., 1995). Hair cell degeneration in the sv mice is visible at three weeks of age and Myo VI immunoreactivity was absent from cytoplasm and the cuticular plate of the inner and outer hair cells of the cochlea in the mutant mice compared to wild type (Avraham et al., 1995). In a later study by Self et al., (1999), it was found that the sv mice had gross abnormalities of the stereocilia by postnatal day 7, revealing fused stereocilia also called giant stereocilia. As reported years earlier (Deol and Green, 1966), the organ of Corti develops normally in the sv mice up to postnatal day 12 followed by a progressive loss of hair cells (Avraham et al., 1995). The apparent discrepancy in the
ages could be because of the use of light microscopy (Deol and Green, 1966; Avraham et al., 1995) compared to electron microscopy by Self et al. However, both studies reported that the sv mice had gross stereociliary defects by postnatal day 20), indicating that MyoVI may play a role in maintaining the hair cells in these animals and not in their development. Because MyoVI is found at the base of the hair cells in the cuticular plate, it is speculated to be important in the transport of cellular components and anchoring the stereocilia in the dense actin network (Self et al., 1999). It has been shown that MyoVI is associated with the terminal web in brush border epithelia (Hasson and Mooseker, 1994), a polarized cell and thus thought to play a role in endocytosis. MyoVI has also been shown to associate with uncoated vesicles in clathrin-dependent endocytosis in polarized cells (Aschenbrenner et al., 2004). Thus the distribution and role of MyoVI are quite similar across different cell types.

Mutation in human MYOVI was first identified in an Italian family with an early onset of progressive loss of hearing but normal vestibular functions (Melchionda et al., 2001). By the age of 50, all adults in the family with the mutation in MYOVI were found to be suffering from profound deafness (Melchionda et al., 2001). The mutation (C442Y) results in the replacement of a crucial cysteine (C) residue by tyrosine (Y) in the motor domain of MyoVI that is thought to destabilize the protein and result in its degradation. In addition the zebrafish mutant satellite has been shown to carry mutations in myo6b, that results in defective stereocilia and decreased endocytosis (Seiler et al., 2004). Another zebrafish mutant ru920 lacking important structural motifs of MyoVI has also been shown to be defective in mechanotransduction (Kappler et al., 2004).
**Myosin-VIIa**

Mutations in the motor domain of Myosin-VIIa (MyoVIIa) (Fig. 4) have been linked to the shaker phenotype in mice (Mburo et al., 1997) characterized by cochlear and vestibular defects as also in humans with Usher (USH) 1B syndrome (Weil et al., 1995) or non-syndromic deafness disorders DFNB2 (Weil et al., 1997) and DFNB11 (Friedman et al., 1999). Myo VIIa is an unconventional myosin consisting of an N-terminal head domain that can bind actin and ATP (Sellers, 2000). It is an actin plus-end directed motor protein (Udovichenko et al., 2002; Inoue and Ikebe, 2003). The head is followed by five IQ domains predicted to bind CaM (Udovichenko et al., 2002; Inoue and Ikebe, 2003) followed by a very long tail region consisting of several protein-protein interaction domains consisting of FERM, MyTH4 and SH3 domains (Sellers, 2000).

Several binding partners of the MyoVII tail have been identified over the years, a significant number of which were identified in the context of the Usher (USH) syndrome in humans, a genetic abnormality characterized by deafness and later onset of blindness associated with retinitis pigmentosa. These include the scaffold protein harmonin (Boeda et al., 2002), SANS (Adato et al., 2005), vezatin (Kussel-Andermann et al., 2000) and the recently identified tight junction protein shroom–2 (Etournay et al., 2007). Myo VIIa also binds to the structural protein protocadherin 15 to maintain cohesion among the bases of the stereocilia (Senften et al., 2006). It is suggested that MyoVIIa by virtue of its protein cross-linking ability plays a very important role in the stereociliary development and organization (Adato et al., 2005; Etournay et al., 2007).
In addition to MyoVIIa’s role in stabilizing the hair bundle, it has also been suggested that it plays an important role in mechanotransduction in the cochlea (Kros et al., 2002). The MyoVIIa mutant mouse *shaker 1* have seven different mutations, out of which five have been mapped to the motor domain, thus potentially interrupting its motor properties. These mice had a very high bundle deflection threshold of about 150 nm and had abnormal transduction currents (Kros et al., 2002). As well, the hair bundles in these animals were grossly disorganized (Kros et al., 2002). The authors hypothesized that MyoVIIa, by virtue of its distribution in the stereocilia and its interactions with the scaffold proteins, would be an ideal candidate to maintain tension in the transduction channels at the tips of the stereocilia (Kros et al., 2002).

**Myosin-15a**

Myosin-15a (Myo15a) is an unconventional myosin localized to the tips of stereocilia (Fig. 4; Belyantseva et al., 2005). Like all other myosins of this class, Myo15a has a N-terminal motor domain containing ATP and actin-binding motifs, followed by two IQ domains predicted to bind CaM and a C-terminal tail containing potential protein-interacting domains like myosin tail homology 4 domain, (MyTH4), band F ezrin-radixin-moesin homology domains (FERM), src-homology domain 3 (SH3) and post synaptic density protein 95/ Discs-large protein/ ZO-1 protein (PDZ) domains. Preceding the motor domain is a long stretch of proline-rich amino acids of unknown function (Anderson et al., 2000). As well, Myo15a is the only unconventional myosin known to contain a PDZ domain in its C-terminal (Belyantseva et al., 2005). Myo15a is an actin
plus-end directed motor protein demonstrated to move cellular cargo to the tips of filopodia (Belyantseva et al., 2005).

Myo15a is thought to contribute to the maintenance of the staircase pattern of the stereocilia by transporting another PDZ domain–containing protein whirlin to the tips (Belyantseva et al., 2005). Whirlin has been demonstrated to be responsible for maintenance of hair bundle structure (Mburu et al., 2003). The graded and dynamic expression of whirlin at the tips of stereocilia is thought to be part of a molecular mechanism required to terminate actin polymerization at the tips (Kikkawa et al., 2005).

Mutations in Myo15a cause the non-syndromic deafness disorder DFNB3 in human patients (Wang et al., 1998) and gross cochlear and vestibular defects in the mouse mutant shaker 2 (Liang et al., 1999). The inner ear of shaker 2 mice revealed very short stereocilia and the appearance of abnormal actin-rich processes called cytocauds (Anderson et al., 2000). Shaker 2 mice have similar phenotype as whirler mice lacking the protein whirlin. Immunofluorescence studies of homozygous whirler mice revealed disorganized hair bundles and absence of whirlin, but Myo15a immunoreactivity was still present in these animals (Belyantseva et al., 2005). As well, heterologous expression and localization of GFP-myo15a and DsRed-whirlin in Cos7 cells demonstrated co-localization of the two proteins at the filopodia, whereas expression of a mutant Myo15a containing a mutation in the motor domain, failed to transport either proteins to the tips of the filopodia, indicating a role for Myo15a in the translocation of whirlin (Belyantseva et al., 2005). Despite abnormal architecture of the hair bundles in these mutants,
mechanotransduction and adaptation are unaffected in young whirlin mutant mice as well as shaker 2 mutants before the onset of degeneration of the stereocilia (Stepanyan et al., 2006). Thus the principal role of this motor protein seems to be the transport of crucial cellular cargo and also in maintaining the structural integrity of the stereocilia at the tips where mechanotransduction occurs (Belyantseva et al., 2005).

**Myosin-3a**

Myosin-3a (Myo3a) is an unconventional myosin (Fig. 4), first identified in *Drosophila* and shown to play a role in photoreceptor structure and function (Porter and Montell, 1993). Analysis of the structure of the vertebrate Myo3a revealed the presence of a motor domain and a neck domain containing two IQ motifs and a third IQ motif in the tail domain, also predicted to contain two Myo3 tail homology domains, 3THDI and 3THDII (Dose and Burnside, 2000). Preceding the motor domain, lies an N-terminal kinase domain that undergoes autophosphorylation (Ng et al., 1996). Human Myo3a has been demonstrated to be an actin-activated molecular motor that moves toward the barbed-end of actin filaments (Komaba et al., 2003).

Mutation in the motor domain of human Myo3a has been linked to nonsyndromic hearing disorder DFNB30 characterized by late-onset of deafness, but balance and vision are unaffected in these individuals (Walsh et al., 2002). Subsequent immunolocalization studies of Myo3a in rats demonstrated that it is expressed around the stereociliary tips in a “thimble-like” pattern (Schneider et al., 2006) and is predicted to be involved in protein
translocation to the tips, perhaps facilitating the assembly of the mechanotransduction apparatus, or in maintaining stereociliary tension (Schneider et al., 2006).

**Myosin-1a**

Myosin-1a (Myo1a) was identified as a 110 kDa protein in the brush border of the renal microvilli (Coluccio, 1991) and also in the brush borders of the intestinal epithelial cells (Mooseker, 1985). Like other class I myosins, it has a N-terminal motor domain, three IQ-motif containing consensus regions and an acidic tail domain (Mooseker and Cheney, 1995a). It has been demonstrated to maintain the highly ordered actin-rich brush borders on the apical surface of intestinal enterocytes (Tyska and Mooseker, 2002). Subsequent development of Myo1a knockout mice did not reveal any overt phenotypes in the intestinal brush-border cells, although some fusions of the microvilli in the enterocytes and disorganization of the brush border structures were observed (Tyska et al., 2005). The most striking observation of this study was the up-regulation of Myo1c in the brush border, suggesting that the lack of phenotype in the knockout could be due to compensation of Myo1a function by Myo1c (Tyska et al., 2005).

In a recent study, non-syndromic deafness DFNA48 was mapped to the chromosome locus coding for Myo1a (D'Adamo et al., 2003). Eight different mutations of Myo1a were identified, of which six were predicted to be responsible for hearing loss in these patients (Donaudy et al., 2003). Expression of Myo1a in the cochlea was confirmed by reverse transcriptase PCR analysis from mouse cochlear tissue (Donaudy et al., 2003). However, Myo1a protein expression and localization in the inner ear is
unknown and the Myo1a knockout mice have no apparent auditory or vestibular defects, although detailed examinations of inner ear defects have not been performed thus far. In another study researchers created a recombinant Myo1a with mutation in a key aspartate residue (Yengo et al., 2007) in the active site of the protein that has been shown to be associated with non-syndromic deafness (D'Adamo et al., 2003). It was found that the mutant protein had lower ATP hydrolyzing capacity compared to the wild type protein and failed to slide along actin in *in vitro* motility assays (Yengo et al., 2007). As well, unlike the wild type protein, the mutant proteins failed to localize to the apical protrusions in the polarized LLC-PK1 cell types, thus indicating that the mutation affected its ability to target to appropriate cellular compartments (Yengo et al., 2007). It is possible that in the human patients, inability of the mutant protein to localize properly affects the stability of the cells in the sensory epithelium. However, more in depth studies are necessary to definitively address that point.

I. Significance

Myo1c has been shown to play a role in glucose transporter recycling (Bose et al., 2002) and exocytosis (Bose et al., 2004), lamellipodial growth of neuronal cells (Diefenbach et al., 2002) and powering transcription in the nucleus together with RNA Pol II (Pestic-Dragovich et al., 2000), in addition to its role in adaptation in hair cells. Myo1c has also been localized in brush border epithelium in kidney cells (Boyd-White et al., 2001) and is implicated in sodium transport in kidney cell lines (Wagner et al., 2005). The motor properties of Myo1c have been shown to be regulated by the Ca\(^{+2}\) and CaM bound to the IQ domains (Barylko et al., 1992; Zhu et al., 1996; Zhu et al., 1998).
is the slow adaptation motor in hair cells (Holt et al., 2002) and one of the functions attributed to the motor is proper positioning of the mechanotransduction apparatus in the hair bundle (Holt et al., 2002). It is thought that Myo1c being an actin-based motor climbs up the actin cytoskeleton in hair bundles exerting force, thus maintaining the transduction channel at the position of maximum sensitivity (reviewed in Gillespie and Cyr, 2004). The importance of the lever arm in force production has not been investigated for Myo1c, although the CaM molecules have been clearly shown to confer rigidity on this myosin molecule (Zhu et al., 1996; Zhu et al., 1998). It is the long-term goal of this research to further investigate the role of the neck length of Myo1c to better understand its role in hair cell function and various other physiological processes.
Figure 1. *Myosin force generation along actin*. Myosin head is bound to actin in a rigor state. In (1), binding of ATP to the myosin head causes it to dissociate from actin. In step (2) hydrolysis of ATP causes myosin to bind actin at a new site with ADP and Pi still bound to it. Release of Pi from the head (3) causes myosin to exert the power stroke along actin and dock to a new site on the latter. In step (4) the bound ADP dissociates from the head. Adapted by permission from W.H. Freeman & company, Molecular Cell Biology, 5th Edition.
Figure 2. Differential interference contrast image of an isolated bullfrog saccular hair cell showing the stereocilia, kinocilium, the cuticular plate and the cell body. Used with permission from Dr. A.J. Hudspeth, The Rockefeller University, NY.
Figure 3. Model for hair cell transduction. A short and a tall stereocilium are depicted in light grey, connected by the gating spring. Red lines represent the actin cytoskeleton. The yellow boxes are the transduction channels and Myo1c is shown in blue. The cuticular plate is depicted in dark grey. Resting state refers to the absence of stimulus. Positive displacement pushes the shorter edge towards the taller edge of the stereocilia, tenses the tip links and opens transduction channels in the membrane. Negative displacement pushes the taller edge of the stereocilia towards the shorter edge, slackens the tip links and closes the channels. Adapted by permission from Macmillan Publishers Ltd: Nature 431, © 1999.
Figure 4. Distribution of myosins in the hair cell stereocilia: Cartoon of two neighboring stereocilia connected by the tip link in blue. The transduction channel complex is depicted in bright pink. Stereociliary membrane is in pink. The different myosin molecules are shown in the top right panel. MyoVI is localized to the cuticular plate region shown in purple. MyoVIIa and Myo1c are depicted in yellow and distributed throughout the stereocilia. Myo3a is localized in a thimble-like pattern at the tips shown in green and Myo15a is also present at the tips depicted in orange. The actin cytoskeleton is shown in grey. Diagram is not drawn to scale. Used with permission from Kelli R. Phillips, 2007, Characterization of Myosin I in the inner ear.
References


II. Designing truncated constructs of Myosin 1c and purification of proteins

Introduction

All unconventional myosins studied to date have IQ consensus sequences (IQX$_3$RGX$_3$R) (Rhoads and Friedberg, 1997) in their neck domains following the motor heads. These IQ domains bind the cellular protein calmodulin (CaM) either in the presence or absence of Ca$^{+2}$ (Garcia et al., 1989; Espreafico et al., 1992; Bahler et al., 1994; Porter et al., 1995; Rhoads and Friedberg, 1997). The IQ domains regulate the functions of most of the myosin classes studied (Uyeda et al., 1996; Zhu et al., 1996; Zhu et al., 1998; Purcell et al., 2002; Schott et al., 2002; Moore et al., 2004; Sakamoto et al., 2005). The lever arm hypothesis (for review, Spudich, 1994) postulates that for all myosins, small movements of the head domain are amplified by the myosin neck regions to longer distances traveled along the actin molecules in the cell. This hypothesis is supported in the case of the conventional myosin or Myosin-II (MyoII). As the length of the neck domain of MyoII was shortened by successively deleting the regulatory domains in the protein, the velocity of its movement became slower (Uyeda et al., 1996). In contrast, addition of regulatory domains resulted in a faster moving MyoII compared to the wild type protein (Uyeda et al., 1996). This relationship between the neck length and velocity of myosins is also defined by the mathematical equation $v = d/t_{on}$ (Huxley, 1990; Uyeda et al., 1996; Moore et al., 2004; Lin et al., 2005) where $v$ is the velocity, $d$ is the step size of the myosin along F-actin, and $t_{on}$ is the time myosin is strongly bound to F-
actin (Uyeda et al., 1996; Lin et al., 2005). Assuming $t_{on}$ to be constant, velocity is directly proportional to the step size that in turn is dependent on the lever arm length.

The unconventional myosin Myo1b consists of several alternatively spliced isoforms that have varying numbers of IQ motifs (Ruppert et al., 1993; Lin et al., 2005). Biochemical characterization of these proteins revealed differential CaM binding affinities of the IQ domains among splice variants (Lin et al., 2005). While such differences did not cause a change in ATPase rate of Myo1b isoforms, it was reported that the motility increased with more CaMs bound to Myo1b (Lin et al., 2005). Thus the IQ domain is predicted to act as a lever arm for this myosin as well.

However this may not be the case in all unconventional myosins. Testing the lever arm hypothesis in Myosin V has been controversial. MyoV is a two-headed unconventional myosin (Cheney et al., 1993) consisting of one motor domain in each of the two heads and a large neck region consisting of six IQ motifs (Cheney et al., 1993). Laser trap studies using purified wild type and mutated MyoV with truncated IQ domains demonstrated similar step sizes along F-actin molecules (Tanaka et al., 2002). It was hypothesized that the neck of this myosin did not function as a lever region, instead the head domain was responsible for the large step sizes associated with the truncated molecules (Tanaka et al., 2002). Several other groups investigating the cell biological or biophysical properties of this myosin maintain that the neck domain of MyoV acts as an amplifier of the movements of the motor domains (Purcell et al., 2002; Schott et al., 2002; Moore et al., 2004; Sakamoto et al., 2005). In yeast, the movement of secretory
vesicles is dependent on the presence of MyoV (Schott et al., 1999). Expression of mutant proteins lacking either two or four IQ domains showed reduced velocity of movement compared to the wild type or the mutant expressing eight IQ motifs (Schott et al., 2002). Subsequent studies with purified proteins using chicken MyoV or the murine isoform demonstrated the importance of the neck length in the step size of this myosin as well (Purcell et al., 2002; Moore et al., 2004; Sakamoto et al., 2005).

The importance of the lever arm in force production has not been investigated for Myo1c, although the CaM molecules have been clearly shown to confer rigidity on this myosin molecule (Zhu et al., 1996; Zhu et al., 1998). To further understand this regulation, the length of the neck region of Myo1c was altered by generating truncated forms of the protein as outlined in the methods section. These truncated constructs, along with the wild-type Myo1c were used to generate recombinant baculoviruses that were subsequently used to infect Sf9 insect cells to produce wild type and truncated proteins.

**Materials and Methods**

**Generation of truncated constructs of Myo1c containing the head, tail and each successively lacking IQ domains 4, 3 and 2:** The truncated forms of Myo1c (Fig. 1 & 4) were generated using high-fidelity polymerase chain reaction (PCR) with Pfu polymerase (Invitrogen, Carlsbad, CA) and PCR sewing techniques (Horton et al., 1989; Fig. 2) with the frog full-length Myo1c cDNA as the template. Due to lack of suitable restriction sites between the neck and tail regions of Myo1c, PCR sewing allowed us to join the two regions without the use of restriction endonucleases. The annealing temperatures
employed for each set of primers varied between 55°C-75°C as indicted in Table 1 and the inserts were amplified 1 minute for every kilobase pair for a total of 30 cycles. The steps in this reaction are briefly:

(1) The individual pieces (head-neck and tail) were amplified using high fidelity PCR and purified using PCR purification columns (Qiagen, Valencia, CA). The primers (Table 1) to the neck and tail regions have sequences that are complementary to the tail or the neck respectively (Fig. 3).

(2) The PCR products were analyzed on a gel (Fig. 4), purified by gel extraction and combined in a molar ratio of 1:1 for a second PCR reaction without added primers, to get one contiguous piece of “sewn” DNA.

(3) The sewn piece of DNA from step 2 acted as a template for an additional PCR reaction to obtain enough DNA for transforming bacterial cells. The DNA from this step and the pFastBac HTA plasmid (Invitrogen) are subjected to restriction enzyme digestion using restriction enzymes Bam H1 and Spe1 (New England Biolabs, Ipswich, MA). The plasmid is treated with calf intestinal phosphatase (New England Biolabs) to prevent reclosure of the DNA during ligation. The plasmid and the inserts were purified by gel extraction and ligated using T4 DNA ligase (New England Biolabs). The ligated plasmid was used to transform DH5α cells (Invitrogen) according to the manufacturer’s protocol. The constructs contain a His₆ tag and an Xpress-tag at the N-terminus and were sequenced in their entirety to screen for any mutations in the open reading frame. Purified plasmids were used to obtain recombinant baculoviruses. Recombinant
baculoviruses expressing CaM were co–infected along with Myo1c for protein expression in Sf9 insect cells.

In addition, a second set of constructs were made that have a c-myc tag (Sundaresan et al., 1987) (amino acid sequence-EQKLISEEDL) after the tail domain (Fig.1). These constructs will enable us to use commercially available antibodies against c-myc for future in vitro motility assays. The c-myc constructs were generated as follows:

(1) A c-myc cassette was generated with the use of 5’ phosphorylated primers complementary to the c-myc sequence (Table 1 & Fig. 3), also consisting of overhangs of the restriction sites for the enzymes Kpn 1 and Spe 1 as color coded in Table 1. Briefly, the two phosphorylated primers were incubated together at 94°C for 2 minutes, 37°C for 5 minutes and room temperature thereafter. This cassette was then ligated using T4 DNA ligase to the pFastBac plasmid digested with restriction enzymes Kpn1 and Spe1 (Table 1; New England Biolabs) and the plasmid was used to transform DH5α cells using the manufacturer’s protocol.

(2) The head-neck-tail region generated by PCR is ligated to the c-myc-plasmid, by the use of suitable restriction enzymes (Table 1). The constructs were sequenced in their entirety to ensure preservation of the reading frame and expressed as previously outlined.

**Protein purification:** The recombinant frog Myo1c proteins, both wild type (baculovirus stock received from Dr. Peter Gillespie, OHSU) and truncated, were purified as described previously (Gillespie et al., 1999). Briefly, Sf9 cells infected with wild type Myo1c and
CaM viruses were sedimented and the cell pellets were resuspended in the lysis buffer containing 25 mM Tris pH 8, 0.5 mM MgCl₂, 0.5 mM EGTA, 2.5 mM 2-mercaptoethanol, 0.2 µM PMSF, 1 µM leupeptin, and 1 µM pepstatin, and lysed by passing twice each through 22-g and 25-g needles using a 10-ml luer lock syringe. The lysate was adjusted to final NaCl and ATP concentrations of 400 mM and 1 mM respectively and centrifuged at ~400,000g for 30 minutes at 4°C to sediment cellular debris. The supernatant containing the soluble protein was passed through a Nickel nitrioloacetic acid (Ni²⁺-NTA) affinity column (Qiagen, CA) at 4°C. His₆-tagged proteins were retained on the column bound to the Ni²⁺ and the other proteins were discarded as they flow through the column. The column was washed with wash buffer containing 300 mM NaCl, 25 mM Tris pH 8, 0.5 mM MgCl₂, 0.5 mM EGTA, 2.5 mM 2-mercaptoethanol, 0.2 µM PMSF, 1 µM leupeptin, and 1 µM pepstatin to discard non-specifically bound proteins. The bound proteins were eluted with an elution buffer containing 125 mM imidazole pH 8, 200 mM KCl, 15 mM HEPES pH 8, 1 mM MgCl₂, 0.1 mM EGTA, 2.5 mM 2-mercaptoethanol, 0.2 µM PMSF, 1 µM leupeptin, and 1 µM pepstatin, which replaced the protein with the imidazole on the column. Several fractions were eluted and protein concentration was assayed using Bradford reagent (Bradford, 1976) using BSA as a standard. Fractions with the highest concentration of protein were combined together. Gel-filtration chromatography was used to check for aggregation of a part of the protein, using 25 ml Superdex 200HR 10/300 column with an AKTA/FPLC system (Amersham, NJ) in a buffer consisting of 15 mM HEPES, 400 mM KCl, 1 mM MgCl₂, 1 mM EGTA, based on the elution profile at A₂₈₀.
**Actin purification from rabbit muscle acetone powder:** The wild type and the truncated Myo1c proteins were used in ATPase assays as outlined in chapter III. These assays require purified F-actin protein. Actin was purified from rabbit muscle acetone powder (Pel Freez, Rogers, AR) according to the protocol of Pardee and Spudich (1982) with modifications. Briefly, the acetone powder was dissolved in buffer A containing 2 mM Tris pH 8, 0.2 mM ATP pH 7, 0.5 mM DTT, 0.1 mM CaCl$_2$ and 1 mM sodium azide and subjected to high speed centrifugation at 300,000g for 20 minutes to separate non-actin binding proteins in the preparation. The actin present in the supernatant was polymerized by the addition of KCl to a final concentration of 800 mM and 2 mM MgCl$_2$ centrifuged at 400,000g for 30 minutes at 4°C to sediment the F-actin. This actin was depolymerized by dialyzing into buffer A devoid of KCl and MgCl$_2$ at 4°C over a period of 2 days. The G-actin thus formed was centrifuged at 400,000g for 30 minutes to sediment co-purifying proteins, polymerized with the addition of 50 mM KCl and 1 mM MgCl$_2$ and dialyzed into the ATPase buffer containing 15 mM HEPES pH 7.5, 50 mM KCl, 1 mM MgCl$_2$ and 1 mM sodium azide. The dialyzed protein was sedimented at 400,000g for 30 minutes at 4°C and resuspended in ATPase buffer and stored up to 2-3 weeks at 4°C.

**Actin cycling of the purified protein to assay the amount of functional Myo1c:** A portion of the Myo1c protein was further subjected to a second step of purification following elution from the Ni$^{2+}$-NTA column to assess the percentage of functionally active enzyme molecules in the preparation. The purified protein was incubated with 10 µM F-actin stabilized with 15 µM unlabeled phalloidin (Invitrogen) on ice for 30
minutes, followed by centrifugation at 400,000g for 30 minutes at 4°C. The F-actin pellet containing any bound Myo1c was resuspended in a buffer containing 15 mM HEPES pH 7.5, 50 mM KCl, 1mM MgCl₂, 0.1 mM EGTA, 2.5 mM 2–mercaptoethanol, 0.2 µM PMSF, 1 µM leupeptin, and 1 µM pepstatin, 0.3% Tween-20 and 5 mM ATP. This step removed enzymatically dead Myo1c that was not released by the ATP and remains bound to F-actin and was discarded (Gillespie et al., 1999). The solution was centrifuged at 400,000g for 30 minutes at 4°C and the supernatant consisted of the enzymatically active Myo1c protein. Actin-cycling and subsequent release by ATP ensured that the recovered protein preparation was enzymatically active (Gillespie et al., 1999).

**Analysis of protein purity:** The proteins were run on 12% SDS polyacrylamide gels and stained with colloidal blue (Invitrogen). Gels were destained and scanned using Personal Densitometer SI (Molecular Dynamics, Sunnyvale, CA) equipped with ImageQuant software (v5.2; Molecular Dynamics). As well, densitometry was performed on scanned gels to compare the percentage of Myo1c that bound and released from F-actin after resuspension in an ATP-containing buffer with the Myo1c eluted from the Ni²⁺-NTA column. Thus the protein concentration of the Ni²⁺–NTA elute obtained by Bradford reagent was normalized for percentage purity and percentage of active Myo1c. This normalized Myo1c protein concentration was used in all subsequent assays performed with the purified proteins. Details on the analysis of purity and determination of functional Myo1c are discussed in the appendix.
**Western blot analysis:** Protein samples were run on a 12% SDS-PAGE gel in tris-glycine buffer and transferred overnight onto Immobilon PVDF membrane (Millipore, Bedford, MA). The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline-Tween 20 (TBS-T; 1.5M NaCl, 0.5 M Trizma base, 0.1% Tween-20, pH 8.0) for 1 hour at RT. Primary antibody (anti-Xpress, Invitrogen) was added at a 1:2500 dilution in 5% nonfat dry milk in TBS-T for 1 hour. Membranes were washed 3X10 minutes in TBS-T and incubated with goat anti-mouse secondary antibody conjugated to horseradish peroxidase at a 1:20,000 dilution (Jackson Laboratoreies Inc, West Grove, PA) for 1 hour at RT. The proteins were detected by ECL plus Western Blotting Detection system (GE Healthcare, Piscataway, NJ).
<table>
<thead>
<tr>
<th>#</th>
<th>Primer name</th>
<th>Sequence 5'–3' (Overhangs)</th>
<th>Melting temperature used (°C)</th>
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<tr>
<td>1</td>
<td><strong>HeadFwd</strong> BamH1</td>
<td>TAACCGGATCCAGGTATGGCTAGCATGACTGGTGGA</td>
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<tr>
<td>2</td>
<td><strong>TailRevSTOP</strong> Spe1</td>
<td>CATGACTAGTTTCATCGTAATTCAGACGAGGAGC</td>
<td>72</td>
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<td>3</td>
<td><strong>IQ2RevTail</strong></td>
<td>CAGGACTTGTCAGACAACGTGCTTTTCTCTGCC</td>
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</tr>
<tr>
<td>4</td>
<td><strong>TailFwd</strong> IQ2</td>
<td>GGCAGACGAAAAAGCTGCAAGTGTGCTGGACAAGTCTCTG</td>
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</tr>
<tr>
<td>5</td>
<td><strong>IQ3RevTail</strong></td>
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<td>50</td>
</tr>
<tr>
<td>6</td>
<td><strong>TailFwd</strong> IQ3</td>
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</tr>
<tr>
<td>7</td>
<td><strong>IQ1RevTail</strong></td>
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</tr>
<tr>
<td>8</td>
<td><strong>TailFwd</strong> IQ1</td>
<td>CAACGACAGAAATTCCTGAGTGTGCTGGACAAGTCC</td>
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<tr>
<td>9</td>
<td><strong>Spe1-c-mycKpn1</strong></td>
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<td></td>
<td></td>
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</table>
Results and Discussion

**Analysis of protein purity:** To obtain purified Myo1c protein from Sf9 insect cells we employed affinity chromatography using Ni\(^{2+}\)-NTA agarose beads (Qiagen, CA). Myo1c has a His\(_6\) tag at the N-terminus that binds to the Ni\(^{2+}\) in the affinity column, thus enabling selective purification of the protein over other proteins in the insect cell homogenate. The purified Myo1c as shown in Fig. 5 co-purified with a number of other contaminating proteins. As a result, determination of protein concentration by Bradford assay is not reflective of solely Myo1c in the preparation. To account for this, we have determined the percentage of purity of our protein of interest compared to other non-specific proteins. CaM co-purifies with Myo1c, hence the CaM molecules were included in determination of protein purity of the sample. In addition, the percentage of functional enzyme in the protein preparation was estimated based on the amount of Myo1c that bound and released F-actin in the presence of ATP. It is evident from our results that a substantial fraction of the purified protein was not enzymatically active. It has also been reported previously that for the rat Myo1c isoform, about 50% of the protein that bound F-actin released in the presence of ATP (Gillespie et al., 1999). Our analyses suggest that nearly 12% of the frog enzyme was active, which is lower than that reported for the rat isoform. This could be because the frog isoform is less stable and the protein loses activity faster than other isoforms. Another possibility is that the protein expression in Sf9 insect cells is low resulting in decreased yield of functional protein.

**Purification of mutated Myo1c constructs:** To determine the effect of truncation of Myo1c neck domains on its function, we generated several constructs successively
lacking IQ domains 4, 3 and 2. As well, other constructs were generated by mutating key residues in the IQ domains responsible for binding CaM. One of these is FL2 consisting of the mutation IQ→AA in the 2nd IQ domain of Myo1c (K.R. Phillips, unpublished data). All of these mutated proteins have a His\textsubscript{6} tag at the N-terminus for protein purification by Ni\textsuperscript{2+}-NTA affinity chromatography, similar to the wild type protein. The truncated proteins HIQ12T (Fig. 1; Row B; left) and FL2 were purified as outlined in the methods section and run on 12\% SDS-PAGE. Both HIQ12T and FL2 had low expression in the insect cell culture systems and we were unable to definitively identify the myosin protein on the stained gel. To examine if the proteins were being expressed at all, we performed immunoblots on the high speed supernatant, flow through and the Ni\textsuperscript{2+}-NTA elute of the HIQ12T and FL2 proteins. As indicated in Fig. 6, HIQ12T and FL2 could only be detected in the elutes at very low levels. It is possible that the proteins are being expressed at very low levels in the insect cells and require further optimization of expression for each construct.
Figure 1. *Deletion constructs of Myo1c*. The wild-type construct is shown at the top. The numbers indicate the positions of the amino acids in the protein. Approximate molecular weights are indicated in parentheses. Each of the constructs has a His<sub>6</sub>-tag for purification and a Xpress tag at the N-terminus for detection. The constructs in rows A, B and C lack IQ domains 4, 3 or 2. Each of the constructs in the left panel has a c-myc tag after the tail for immobilization. Construct in row D has mutation in the 2<sup>nd</sup> IQ domain (isoleucine/glutamine to alanine residues). Diagram is not drawn to scale.
Figure 2. **Outline of PCR sewing technique.** The two DNA strands are amplified in separate reactions with primers that have sequences complementary to the other strand as shown in step 1. RS, restriction site. In step 2 the two amplified fragments with overhangs at 5’ and 3’ ends are combined in another PCR reaction without added primers. This results in the joining of the two fragments as one contiguous piece of DNA. In the last step this DNA strand is amplified with the 5’ primer of the head and the 3’ primer of the tail from the very first step. Diagram is not drawn to scale.
Figure 3. *Sequence of frog wild type Myosin 1c.* Highlighted regions represent the primer sequences used to amplify the various truncations as outlined in Fig. 1. The numbers correspond to the primers in Table 1 in the text. The wild type and the truncated constructs have a His$_6$ tag indicated in bold at the start of the sequence. The start codon for Myo1c is indicated in green. c-myc constructs represent the insertion of the 10 amino acid c-myc tag (in violet) before the stop codon in the wild type construct.
Figure 4. *Ethidium bromide stained DNA gels of the truncated construct HIQ12T.* HIQ12T construct consists of the head domain of Myo1c followed by IQ domains 1 and 2. A. Head-IQ2, PCR amplification of the head through IQ2 domains. Reverse primers consist of overhangs complementary to the tail; B. IQ2-tail, PCR amplification of the tail consisting of nucleotide overhangs in the forward primer complementary to the IQ2 domain; C. Purified products shown in panels A and B are combined in the PCR sewing reaction without added primers, followed by addition of primers to amplify the full insert (HIQ12T); D. To generate HIQ12Tc-myc, the HIQ12T construct is ligated to a plasmid containing the c-myc tag. The c-myc tag is inserted after the tail in the HIQ12T construct. DNA mass markers (kbp) are indicated to the left of each gel.
Figure 5. Purification of frog wild type Myo1c: Colloidal blue stained 12% SDS-PAGE gel of wild-type Myo1c. Proteins were purified on a Ni\textsuperscript{2+}-NTA column. 

- **HSS**, total cell lysate after high-speed centrifugation;
- **FT**, flow through containing proteins that do not bind to the Ni\textsuperscript{2+}-NTA column;
- **Wash**, proteins non-specifically bound to the column;
- **Elute**, protein from Ni\textsuperscript{2+}-NTA column;
- **A+M**, Elute + 10\(\mu\)M F-actin-Phalloidin;
- **S1**, Supernatant after centrifugation of A+M;
- **P1**, actomyosin pellet after centrifugation of A+M;
- **S2**, Myo1c released after P1 is dissolved in 5mM ATP;
- **P2**, Myo1c molecules not released by resuspension in ATP. (*) indicates Myo1c, (•) indicates actin and (#) indicates CaM. Molecular weight markers (kDa) are indicated to the left.
Figure 6. *Purification of mutated Myo1c constructs.* Colloidal blue stained 12% SDS-PAGE gel of mutated Myo1c proteins. Proteins were purified on a Ni$^{2+}$-NTA column. HSS, total cell lysate after high-speed centrifugation; FT, flow through containing proteins that do not bind to the Ni$^{2+}$-NTA column; Wash, proteins non-specifically bound to the column; Elute, protein from Ni$^{2+}$-NTA column. On the right are representative western blots of each protein to demonstrate protein expression in the elute. A. Purification of HIQ12T. B. Purification of FL2. Molecular weight markers (kDa) are indicated to the left.
Figure 7. Purification of F-actin from rabbit muscle acetone powder: Colloidal blue stained 12% SDS-PAGE gel of F-actin protein. F-actin was purified by repeated polymerization and depolymerization. A+ABP indicates a mixture of actin and actin-binding proteins. Polymerization and high-speed centrifugation led to precipitation of the F-actin and ABP in the supernatant. Polymerized actin was depolymerized by dialysis for 48 hours at 4 °C, denoted by globular or G-actin. The G–actin is polymerized and dialyzed overnight to separate polymerized actin and actin incapable of polymerizing denoted by Unpol. The polymerized actin is (Actin) sedimented, homogenized and stored at 4 °C. Molecular weight markers are indicated to the left.
References


III. Determination of ATPase activity of Myo1c

Introduction

All known myosin proteins bind F-actin and ATP in their N-terminal motor domains and their interaction with actin is modulated by ATP binding (Barylko et al., 1992). Binding of ATP in the ATP binding cleft in the head domain causes the myosin to release the bound F-actin molecules and hydrolyze ATP to ADP and inorganic phosphate (P_i). Subsequent release of the P_i reorients its motor head domain exerting the power-stroke and the myosin reattaches to a new site on the actin (Lynn and Taylor, 1971). It has been shown for a number of myosins that the power stroke leads to a conformational change in the myosin lever domain that aids in the forward motion of myosins (Uyeda et al., 1996; Schott et al., 2002; Moore et al., 2004; Lin et al., 2005; Sakamoto et al., 2005).

Determination of ATPase activity of members of the myosin-1 family is fairly common and provides important insights into the enzymatic characteristics as well as regulation of this class of unconventional myosins. Among one of the well-characterized myosin-1 proteins is myosin-1b (Myo1b). It is expressed in a large variety of tissues and plays a role in membrane trafficking (Ruppert et al., 1993; Lin et al., 2005). It has six alternatively spliced isoforms containing varying numbers of IQ domains that bind CaM (Lin et al., 2005). It was found that the ATPase activities of each of these isoforms were independent of the number of bound CaMs, however the motility along actin was linearly related to the number of CaMs associated with the IQ domains of Myo1b (Lin et al., 2005), thus implying a role of the IQ domains in Myo1b function. Studies in rat myosin-
Id, another unconventional myosin containing two IQ motifs with two bound CaMs, revealed that the actin-activated and basal ATPase rates of this protein were inhibited by an increase in Ca$^{2+}$ concentration (Kohler et al., 2005). Another rat Myo1 isoform termed Myo1e (previously called Myr 3), containing a single bound CaM, also showed decreased ATPase activity in the presence of Ca$^{2+}$ (Stoffler and Bahler, 1998). Thus the regulation of motor activity for various members of the myosin-1 family varies widely and a comprehensive explanation for the basis of such variation is still lacking.

Myo1c, another widely-studied member of the myosin-1 family was first purified from the bovine brain medulla and found to have characteristics similar to other members of the myosin-1 family (Barylko et al., 1992). Its lever arm consists of four IQ consensus sequences that bind CaM (Barylko et al., 1992; Zhu and Ikebe, 1994). This was further confirmed by western blot analysis of the purified protein with anti-CaM antibodies (Barylko et al., 1992). Gel-filtration analysis of the purified protein revealed that it was a single subunit protein with 4 bound CaM light chains (Barylko et al., 1992). Because medullary tissue is also enriched in conventional MyoII, Myo1c was further purified by ion exchange chromatography followed by elution in a high potassium ion (K$^+$) containing buffer. Under these conditions the purified Myo1c also demonstrated ATPase activity, consistent with it being an ATPase, like other myosins of its class (Barylko et al., 1992). The purified protein showed a low basal rate of Mg$^{2+}$-activated ATPase reaction (1-3 nmol min$^{-1}$mg$^{-1}$). However the rate increased significantly upon addition of F-actin (50 nmol min$^{-1}$ mg$^{-1}$) (Barylko et al., 1992). It was also observed that free Ca$^{2+}$
ions (100 µM) also increased the basal rate of ATP hydrolysis (10nmol min⁻¹ mg⁻¹) in an actin-independent manner (Barylko et al., 1992).

In an attempt to express enzymatically active recombinant Myo1c, Sf9 insect cells were infected with baculoviruses containing a bovine Myo1c insert (Zhu et al., 1996). It was observed that soluble Myo1c could only be expressed if CaM was coexpressed in the insect cells, lending credence to the fact that it was indeed an associated light chain molecule (Zhu et al., 1996). It was also reported that the CaM molecules bound to Myo1c regulate its motor properties i.e. its ATPase activity and in vitro motility (Zhu et al., 1996; Zhu et al., 1998). Consistent with previous observations, the basal ATPase activity of the recombinant Myo1c increased in the presence of Ca²⁺, however, the actin-activated ATPase activity remained unchanged in the presence (0.189 µmol min⁻¹ mg⁻¹) or absence (0.185 µmol min⁻¹ mg⁻¹) of Ca²⁺. The movement of Myo1c along actin was completely abolished under conditions of high Ca²⁺ (Zhu et al., 1996). However, this could be reversed by adding excess exogenous CaM to the in vitro motility preparation (Zhu et al., 1996). Thus it was hypothesized that this regulation was achieved by Ca²⁺ binding to the CaM molecules associated with Myo1c IQ domains. Further support for this comes from experiments where key Ca²⁺–binding residues in CaM were mutated (Zhu et al., 1998). These mutations abolished the Ca²⁺ binding of CaM completely and provided important insights into the regulation of Myo1c, when the mutant CaM and Myo1c recombinant baculoviruses were co-expressed in Sf9 insect cells. Under such conditions, Myo1c basal ATPase activity was unaffected by increase in Ca²⁺ concentration and inhibition of motility along actin was also absent in the presence of excess Ca²⁺ (Zhu et al., 1998).
Expression of recombinant rat Myo1c in Sf9 cells also demonstrated the actin-activated ATPase rate (1 s\(^{-1}\)) of Myo1c compared to its basal ATPase rate (0.1 s\(^{-1}\)) (Gillespie et al., 1999; Gillespie and Cyr, 2004).

The importance of the lever arm of Myo1c has not been investigated thus far. The aim of the following experiments was to investigate the role of the lever arm length in Myo1c function. The long-term goal of these studies is to measure the basal and actin-activated ATPase assays of the mutant Myo1c proteins generated in chapter II, to determine the role played by each IQ domain in Myo1c function. In the present work, analysis of the ATPase activity of frog wild type protein was performed, which acts as a control for comparing the rates of the mutants. These experiments will help us to assess the importance of the neck domain of Myo1c as a lever arm. In addition, constructs were made consisting of mutations in putative CaM-binding amino acids in the individual IQ domains. These constructs will enable us to address the role of CaM bound to individual IQ domains in Myo1c function.

**Materials and Methods**

**ATPase assay:** Myo1c ATPase activity was determined as described previously (Gillespie et al., 1999) in a buffer containing 15 mM HEPES pH 7.5, 1 mM MgCl\(_2\), 0.1 mM EGTA and a final KCl concentration of 50 mM, in the presence or absence of 25 µM F-actin. Assays also included 250 µM ATP (Roche, IL) containing γ\(^-32\)P ATP (6000 Ci/mmol, GE Healthcare, NJ) to a total count of ~300000cpm/sample. Each reaction was carried out in a total volume of 10 µl, in a siliconized 1.7 ml microfuge tube, initiated by
the addition of ATP. Each tube was centrifuged briefly for 5 seconds, followed by incubation at 30°C for 10 minutes and the reaction was terminated by the addition of a mixture of silicotungstic and sulphuric acids. The released $\gamma^{-32}P$ was extracted into 250 µl of a solution of benzene and isobutanol (1:1) followed by the addition of ammonium molybdate (Pollard and Korn, 1973). 100 µl of the organic layer was counted for $\gamma^{-32}P$ in a scintillation counter (LS 5000TD Liquid Scintillation Systems, Beckman Coulter, Fullerton, CA). Figure 1 represents the enzymatic reaction for ATP hydrolysis by Myo1c. Control samples included F-actin only and ATP only in the absence of any added proteins. Myo1c ATPase activity is represented as data ± SE of at least 9 samples done in triplicates. Details of the calculation for the rates of hydrolysis are discussed in the appendix.

**Results and Discussion**

**ATPase activity of frog wild type Myo1c:** Myo1c is a mechanoenzyme that hydrolyzes ATP to produce ADP and Pi. The energy of hydrolysis is used to power movement along F-actin molecules. The goal of the present experiment was to determine the rate of ATP hydrolysis of frog wild type Myo1c. We have used the recombinant wild type frog isoform of Myo1c to determine its rate of hydrolysis in the presence and absence of 25 µM F-actin filaments. The Ni$^{2+}$-NTA elute from the purification of wild type Myo1c protein as the starting enzyme to hydrolyze ATP added was added in excess to each reaction. The number reported here is the turnover number defined as number of moles of ATP hydrolyzed per unit time per mole of enzyme. The basal Myo1c ATPase activity is $0.15 \pm 0.03 \text{s}^{-1}$ (Table 1). In the presence of 25 µM F-actin this increases about 8 fold to
1.26 ± 0.30 s⁻¹, thus demonstrating that the recombinant frog protein is an actin-activated ATPase. It has been demonstrated previously that Sf9 cells do not express any other actin-activated ATPase proteins (Zhu et al., 1996). Also in our hands two of the expressed mutant Myo1c proteins had no actin-activation of their ATPase rates, although they were expressed at low levels. Thus the actin-activation of Myo1c ATPase rates that we observe in our experiments is due to the functional expression of the recombinant protein.

The actin-activated rate of Myo1c reported here is slightly higher than that reported for the rat isoform (1 s⁻¹) (Gillespie et al., 1999; Gillespie and Cyr, 2004) or that reported for other myosins of the same class e.g. Myo1b (0.6 s⁻¹) (Lin et al., 2005). This could be due to the normalization of Myo1c concentration to the amount of active enzyme in each protein sample. We consider active Myo1c to be the protein that can bind and release actin in the presence of excess ATP. Since the Ni²⁺-NTA elute consists of a mixture of enzymatically active and inactive Myo1c molecules, the normalization of protein concentration to the amount of active enzyme is a better way to represent the data when reporting the rate of enzyme hydrolysis. It is possible that some amount of active enzyme was lost in the process of recovering active Myo1c through several cycles of sedimentation. As a result these numbers are not representative of absolute rates, rather they are wild type controls which will be compared to the rates of mutant Myo1c proteins as discussed in chapter II.
**ATPase activity of mutant Myo1c proteins:** We analyzed a mutant Myo1c protein HIQ12T as discussed in chapter II. The basal ATPase rates for this protein was very low and there was no activation of ATPase rates in the presence of 25 µM F–actin. In the case of HIQ12T, it is possible that deletion of the IQ domain 3 resulted in loss of stability of the protein and thus loss of activity. It has also been shown that IQ3 domain of Myo1c binds tightly to CaM (Manceva et al., 2007). However, since the yield of HIQ12T protein was very low, it is also possible that the absence of activity was due to very low amounts of active protein initiating the reaction. At this point we decided to analyze another mutant protein FL2 as described in chapter II. This recombinant protein has mutations in key residues in the IQ2 that are predicted to cause dissociation of CaM from IQ2. It has been shown that CaM-free IQ2 domain of Myo1c can bind intracellular receptors, in a truncated Myo1c protein lacking the head domain (Phillips et al., 2006). As such we did not expect FL2 to have considerably different rates than the wild type protein, since the bound CaM is capable of dissociation and reassociation. However, analysis of at least two such FL2 protein pellets did not demonstrate any actin-activation of the ATPase rates. The yield of this protein was low as well and that could be a potential reason why we had no activity. Thus further optimization of expression is necessary for both of these proteins to increase the yields. Once we have satisfactory expression of these mutant proteins, we can process them similarly to the wild type control to obtain their ATPase rates.
Table 1. ATPase activity of recombinant Myo1c

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Turnover number for wild type Myo1c (n=9)</th>
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<tr>
<td>Basal ATPase rate ± SE + EGTA</td>
<td>0.15 ± 0.03 s(^{-1})</td>
</tr>
<tr>
<td>Actin-activated ATPase rate ± SE + EGTA</td>
<td>1.26 ± 0.30 s(^{-1})</td>
</tr>
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Assayed with 250 µM ATP; 25 µM F-actin for actin activation
Figure 1. Hydrolysis of ATP by Myosin-1c: ATP hydrolysis is initiated by the addition of \( \gamma^{-32}P \) ATP to a mixture of F-actin and Myo1c, forming the products ADP and \( \gamma^{-32}P_i \), while the enzyme Myo1c and F-actin remain unchanged (step 1). The enzymatic reaction is stopped by the addition of silicotungstic and sulphuric acids (step 2). The released \( \gamma^{-32}P_i \) formed in step 1 is converted to \( \gamma^{-32}P \)-phosphomolybdate by the addition of ammonium molybdate solution. The \( \gamma^{-32}P \)-phosphomolybdate thus formed is extracted into a solution of isobutanol and benzene (step 4) and counted in a scintillation counter (step 5).
References


APPENDIX

Analysis of percentage purity of purified Myo1c: The wild-type Myo1c Ni\(^{2+}\)-NTA elute (region of interest ROI, Fig. 1 app; denoted Elute in Fig. 5, chapter II) and the functional Myo1c (Functional Myo1c, Fig. 1 app; denoted S2 in Fig. 5, chapter II), obtained after binding and releasing F-actin in the presence of ATP, were resolved on a 12% SDS-PAGE gel. The gel was stained with Colloidal blue (Invitrogen) and scanned using a laser-based scanner (Personal Densitometer SI, Molecular Dynamics). To determine the total protein, a region of interest (ROI) was analyzed (ImageQuant, v5.2, Molecular Dynamics) as shown in dotted lines in Fig. 1 app. A graph of ROI was generated depicting each band as a peak. The area under each peak was quantified and subsequently summed to determine the total area. The peaks for Myo1c and CaM were identified by exact positioning of the cursor on the Myo1c and CaM bands on the gel.

The percentage of total purity represented by the Myo1c: CaM complex:

\[
\frac{\text{Area under the peak for Myo1c} + \text{Area under the peak for CaM}}{\text{Total area under the peaks for ROI}} \times 100
\]

For example, as represented in Table 1 app, the calculated percentage purity is:

\[
\frac{3.857 + 2.022}{30.572} \times 100 = 19\%
\]

Analysis of Functional Myo1c: Myo1c band (Box 2) in the Ni\(^{2+}\)-NTA elute was compared to functional Myo1c (Box 3) directly to determine the percentage of functional or enzymatically active Myo1c in the Ni\(^{2+}\)-NTA elute. Boxes of identical dimensions were drawn around the Myo1c band (Box 2) and the functional Myo1c band (Box 3). A
third box was placed in an empty lane for background correction (Box 4). The pixel intensity for each of the boxes were quantitated with ImageQuant (v5.2, Molecular Dynamics) and the percentage of functional Myo1c was calculated as:

\[
\frac{\text{Pixel intensity for Box 3}}{\text{Pixel intensity for Box 2}} \times 100
\]

For example, as represented in Table 2 *app*, percentage of functional Myo1c is calculated as:

\[
\frac{266.37}{1788.34} \times 100 = 14.8\%
\]

Total protein concentration by Bradford assay = 0.834 mg/ml

Molecular weight of Myo1c = 168000 Da

Corrected concentration for functional Myo1c = 0.834 mg/ml X 0.19 (percentage pure Myo1c) X 0.148 (percentage functional Myo1c) = 0.022 mg/ml

Molarity of functional Myo1c in Ni-NTA elute = \(0.022/168000\) M

\[= 130 \text{nM}\]

**Table 1 *app*: Quantitation of area under the peak**

<table>
<thead>
<tr>
<th>Area under the peak</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Myo1c</td>
<td>3.857</td>
</tr>
<tr>
<td>CaM</td>
<td>2.022</td>
</tr>
<tr>
<td>Total</td>
<td>30.572</td>
</tr>
</tbody>
</table>
Table 2 *app*: Calculation of pixel intensity

<table>
<thead>
<tr>
<th>Pixel intensity</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Box 2</td>
<td>1788.34</td>
</tr>
<tr>
<td>Box 3</td>
<td>266.37</td>
</tr>
</tbody>
</table>

**Calculation of ATPase rate for wild type Myo1c**: To determine the rate of hydrolysis of ATP by Myo1c, the ATPase assay is performed as described in chapter III, fig. 1. Table 3 *app* represents the counts in cpm in triplicates obtained by the enzymatic hydrolysis of ATP by Myo1c and other controls as indicated in each row.

**Table 3 *app*: Counts for ATP hydrolysis**

<table>
<thead>
<tr>
<th>Counts in cpm</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>8477.09</td>
</tr>
<tr>
<td>Actin</td>
<td>9478.29</td>
</tr>
<tr>
<td>Myo1c</td>
<td>10749</td>
</tr>
<tr>
<td>Actin+ Myo1c</td>
<td>15518</td>
</tr>
<tr>
<td>Total counts</td>
<td>332889.1</td>
</tr>
</tbody>
</table>

Average (Avg) Total counts = 339413

Total organic layer of each ATPase reaction = 250 µl

Total organic layer read = 100 µl

Total ATP in the reaction = 250 µM

Volume of each reaction = 10 µl

\[
\text{Total count} = \frac{339413 \text{ cpm} \times 1000}{250 \mu\text{M} \times 10 \mu\text{l}} \times \frac{100 \mu\text{l}}{250 \mu\text{l}} = 54306 \text{ cpm/nmol of ATP}
\]
Avg ATP count = 8900 cpm

Avg actin count = (Actin counts – Avg ATP counts) = 809 cpm

ATP hydrolysed (in nmol) by Myo1c alone (basal/ no Actin):

\[
\text{Avg Myo1c counts in cpm} - 8900 \text{ cpm} = \frac{54306 \text{ cpm/nmol}}{54306 \text{ cpm/nmol}} = 0.0259 \text{ nmol}
\]

ATP hydrolysed (in nmol) by Myo1c + Actin (actin-activated):

\[
\text{Avg Actin + Myo1c counts in cpm} - 8900 \text{ cpm} - 809 \text{ cpm} = \frac{54306 \text{ cpm/nmol}}{54306 \text{ cpm/nmol}} = 0.1002 \text{ nmol}
\]

Total reaction time = 10 min

The Ni-NTA elute was diluted 1:1 with elution buffer without KCl = 0.022mg/ml/2

= 0.011 mg/ml

Volume of Myo1c used per reaction = 2.5 µl

Molecular weight of Myo1c = 168000 Da

ATP hydrolysed (in nmol) per minute per mg of Myo1c (actin-activated)

\[
\frac{0.1002 \text{ nmol}}{0.0025 \mu l \times 0.011 \text{ mg/ml} \times 10 \text{ min}} = 364.4 \text{ nmol/min/mg}
\]

Turnover number or moles of ATP hydrolysed per second per mole of Myo1c

\[
\frac{364.4\text{ nmol/min/mg} \times 10^{-9} \times 10^{-3} \times 168000 \text{ g}}{60 \text{ sec}} = 1.02 \text{ mol/sec/mol}
\]

= 1.02 s^{-1}
Figure 1: Determination of percentage purity and percentage functional Myo1c. Colloidal blue stained 12% SDS-PAGE gel of wild type Myo1c. Myo1c Ni^{2+}-NTA elute is denoted as ROI in the text. The Myo1c band is denoted as Box 2. Box 3 depicts the functional Myo1c band and Box 4 represents the background correction for boxes 2 and 3. CaM is indicated to the right of the gel. Molecular weight markers are indicated (in kDa) to the left.
Curriculum Vitae
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• 2000-2002: MS, Biochemistry, University of Calcutta, India.

• 1997-2000: BS, Chemistry (major) with Physics and Mathematics (minors),
  University of Calcutta, India.

Appointments:
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Technical skills:

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- General cell culture techniques
- Insect cell culture
- Cloning using bacterial systems
- Transient transfection in tissue culture cells
- Expression of proteins in bacteria
- Purification of recombinant proteins using nickel-nitriloacetic acid column
- Gel filtration chromatography
- SDS PAGE
- Western blotting
- Limited experience in immunofluorescence using confocal microscopy
- Immunoprecipitation/Pull down assays
- Enzyme assay

Teaching Experience:

Biochemistry 339 laboratory (Introduction to Biochemistry; # of contact hours: 2)
CCMD 730 Human function (First year Medical Students; # of contact hours: 1)