Published in final edited form as:

Trends Cell Biol. 2010 July ; 20(7): 418–426. doi:10.1016/j.tcb.2010.04.004.

Leveraging the membrane-cytoskeleton interface with myosin-1

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Abstract

Class 1 myosins are small motor proteins with the ability to simultaneously bind to actin filaments and cellular membranes. Given their ability to generate mechanical force, and their high prevalence in many cell types, these molecules are well positioned to carry out a number of important biological functions at the interface of membrane and the actin cytoskeleton. Indeed, recent studies implicate these motors in endocytosis, exocytosis, release of extracellular vesicles, and the regulation of tension between membrane and the cytoskeleton. Many class 1 myosins also exhibit a load-dependent mechano-chemical cycle that enables them to maintain tension for long periods of time without hydrolyzing ATP. These properties put myosins-1 in a unique position to regulate dynamic membrane-cytoskeleton interactions and respond to physical forces during these events.

Myosin-1 As A Membrane-Cytoskeleton Cross-Linker

Class 1 myosins are small, monomeric members of the myosin superfamily of actin-based motors (Box 1) that are found in most eukaryotic cells [1]. Vertebrate species possess eight distinct class 1 myosin genes (Figure 1); many of these are expressed in nearly all cell types (myosin-1b, -1c, -1d, and -1e), while others are found only in specific tissues (myosin-1a in the intestine, or myosins-1f and -1g in hematopoietic cells) [2–4]. Regardless of expression profile, a defining feature of all class 1 myosins is their ability to interact directly with cell membranes. Membrane interactions are mediated by a C-terminal tail homology 1 (TH1) domain that contains a putative pleckstrin homology (PH) lipid-binding motif [5], while actin binding is localized to the N-terminal motor domain. The N- and C-terminal domains are connected by a single alpha-helix; this central ‘neck’ region is normally supported by calmodulin (CaM) light chains and acts as a mechanical lever arm, coupling conformational changes in the motor domain to a physical displacement of the membrane-binding TH1 domain (Figure 1a). Whereas most class 1 myosins are ‘short-tailed’ and possess only the TH1 domain, other variants are ‘long-tailed’ and have additional C-terminal structure that can mediate protein-protein interactions (e.g. myosins-1e and -1f, Figure 1b).

The spatial segregation of membrane and actin binding activities suggests that class 1 myosins have the potential to serve as divalent cross-linkers, physically connecting and generating force between actin filaments and membrane lipids. Indeed, numerous cell biological and biophysical studies have implicated these motors in the control of membrane movement, deformation, and bending; examples include regulation of plasma membrane tension [6], the release of extracellular vesicles from the plasma membrane [7,8], the mechanical coupling of ion channels to the underlying actin cytoskeleton [9–11], endocytosis [12], and exocytosis [13].

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Although our current understanding of myosin-1 function and mechanism rests on a strong experimental foundation provided by early studies of class 1 motors in lower eukaryotes (including *Acanthamoeba* and *Dictyostelium*; Box 2), here we will review the most recent developments in our understanding of how vertebrate class 1 myosins interact with actin filaments and membranes, as well as how these activities might be coordinated to carry out tasks at the membrane-cytoskeleton interface.

Myosin-1 Interactions With Actin Filaments

The class 1 myosin motor domain contains motifs that are conserved across the myosin superfamily, including an actin binding cleft and a nucleotide binding pocket [14](Figure 1). These two sites are functionally linked: when ATP is present, the motor has a low affinity for actin and, in contrast, affinity for actin increases if the nucleotide pocket holds ADP or is empty [15]. Because ATP hydrolysis gives rise to ADP (and Pi), this catalytic cycle is also linked to a cycle of attachment and detachment from actin filaments. Attachment to actin is also accompanied by a force generating conformational change, referred to as a ‘working stroke’ [15] (Figure 2a). Numerous biochemical studies on members of the myosin superfamily have shown that slight variations in the kinetics of the individual cycle transitions give rise to motors with divergent capabilities [16]. Indeed, compared to other myosins, class 1 motors demonstrate characteristically slow rates of ATP turnover ($0.3 - 3 \text{ s}^{-1}$) and actin movement ($10 - 150 \text{ nm/s}$). Another critical motor property is the ‘duty ratio’, defined as the fraction of the total ATPase cycle that a motor remains strongly bound to actin (i.e. $\sim$ strongly bound duration/total cycle duration). The duty ratio is important because it provides insight into the functional limitations and capabilities of a motor protein. For example, in the case of a myosin with a duty ratio of 5%, a cellular cargo (e.g. a small vesicle) would require 20 such molecules ($\sim 1/\text{duty ratio}$) to maintain continuous interactions with an actin filament. For this reason, low duty ratio motors (e.g. myosin-2) are usually envisioned working in ensembles, whereas high duty ratio motors (e.g. myosin-5) are thought to function in very small groups, or even as single molecules [17].

Transient kinetic studies with myosins-1 have revealed that, in the context of solution measurements, these motors are low duty ratio and spend the majority of their time ($>90\%$) detached from actin filaments [16]. However, results from other studies dating back to the mid-1990s suggest that class 1 myosins may demonstrate more complex behavior in the actin bound state. Among the earliest were cryo-EM images demonstrating that myosin-1a bound to actin exhibits a large C-terminal tail swing ($\sim 32^\circ$) upon ADP release [18–20]. Shortly thereafter, biochemical studies with native myosin-1a revealed high ADP affinity and the existence of two kinetically distinct ADP-bound states, which the authors proposed were correlates of the two states observed in the cryo-EM studies [21]. Later single molecule studies with myosin-1a and -1b revealed that both of these motors produced a two-step working stroke: an initial $\sim 6 \text{ nm}$ step with a duration independent of ATP concentration (presumably corresponding to an ADP-bound state), followed by a second $\sim 5 \text{ nm}$ step with a duration that is sensitive to ATP levels (presumably leading to a state with ADP in rapid equilibrium)[22]. More recent optical trapping experiments with myosin-1c revealed similar two-phase behavior [23], but studies with myosin-1d were unable to detect a biphasic stroke [24].

While it may be the case that most class 1 myosins simply bind to actin and carry out their working stroke in two steps (the first coincident with Pi release and attachment to actin; the second coincident with ADP release), a more interesting interpretation of this phenomenon is that it represents a mechanism for sensing mechanical strain (Figure 2a, b). In this scenario, the first part of the working stroke would be produced upon myosin-1 binding to actin. However, an opposing load would prevent ADP release, forcing the molecule to ‘stall’ in an actin bound state until strain was relieved. This would increase the actin-attached duration, and

in turn, elevate the duty ratio. This type of ‘mechano-regulation’ would enable class 1 myosins to transition from low to high duty ratio if opposing loads were present (Figure 2b). Recent single molecule analysis of myosin-1b using a force-clamp optical trap has confirmed that this is indeed the case [25]. This study clearly shows that the detachment kinetics of myosin-1b are exquisitely sensitive to the amount of opposing mechanical load. The result is a motor that can transition from a duty ratio of ~0.2 to ~0.9 by increasing its attached duration in response to relatively small loads (<2 pN). While this study was only performed with myosin-1b, myosins-1a and -1c have similar biochemical and mechanical features suggesting comparable strain-dependence [22,23]. However, given the absence of a telltale two-step working stroke in myosin-1d [24], enhanced strain sensitivity may not be a general feature of all class 1 myosins.

In addition to being regulated by external mechanical forces, interactions between myosin-1 and actin are also governed by calcium, presumably via its effects on CaM light chains that bind to the myosin-1 neck domain. Indeed, numerous biochemical studies with myosins-1a, -1b, and -1c support the idea that CaM physically dissociates from the myosin-1 heavy chain in the presence of micro-molar levels of calcium [26–34]. When averaged across different isoforms, a general finding is that at least one molecule of CaM is released when these motors are exposed to calcium in the physiological range of 1–10 μM [28–30,32,34](Figure 2c). Moreover, the addition of $\geq 1 \mu\text{M}$ calcium to sliding filament assays universally abolishes movement [26,27,32–34]. In an almost counterintuitive manner, measurements of ATPase activity clearly show that catalytic capacity is increased in the presence of calcium over the same concentration range [26,27,29,30,32–34](Figure 2c). These data are also consistent with single molecule studies of myosin-1c, which show that the presence of calcium produces a dramatic acceleration in detachment kinetics [23]. This raises a perplexing question regarding the *purpose* of calcium regulation: what is the biological rationale for pushing myosin-1 into a state with high ATPase activity, but reduced potential for generating force?

An answer to this question may come from recent studies with myosin-1c, which suggest that CaM bound to the first IQ motif (positioned closest to the motor domain) is most likely to detach from the heavy chain in the presence of calcium [28,34]. Structural studies suggested long ago that light chain binding to the first IQ motif is critical for stabilizing motor domain conformation and extension of the α -helix that comprises the neck region [14,18,35]. Thus, removal of CaM from the first IQ motif is expected to create a ‘floppy’ neck (with lower flexural rigidity) and abolish its ability to transduce force from the motor domain to the C-terminus, consistent with observed data from sliding filament assays [26,27,32–34]. However, the converse is also true: a floppier neck would be unable to ‘restrain’ (i.e. exert an opposing load on) the motor domain during catalytic cycling. From this perspective, removal of the first CaM would also enable increased ATPase cycling rates, which is again consistent with published literature [26,27,29,30,32–34]. The model that emerges suggests that CaM binding to the first IQ motif acts as a ‘clutch’ mechanism. When CaM is present, the clutch is engaged and conformational changes in the motor domain are faithfully transmitted to the C-terminus of the molecule. When CaM is released from the first IQ motif, the clutch is disengaged, which would enable the motor domain to ‘idle’, hydrolyzing ATP without producing force. Perhaps more importantly, a disengaged motor domain would be unable to feel external loads applied through the C-terminus. This gives rise to the intriguing possibility that elevating calcium levels may provide a mechanism for releasing loaded, actin-bound myosin-1 molecules from a high-force state; e.g. calcium levels increase > CaM on IQ motif #1 is released > neck stiffness falls > force on the motor is reduced > ADP is released > myosin-1 detaches from actin. This hypothesis is consistent with the proposed physiological role of myosin-1c in hair cell adaptation, a calcium dependent process [more below; 9,36].

Although the model outlined above assumes that CaM release is coupled to a dramatic change in the rigidity of the neck region, other conformational changes that effectively uncouple the mechanical and catalytic cycles of myosin-1 are equally plausible. Moreover, *in vitro* studies have shown that the calcium inhibition of myosin-1 motility can be reversed by the addition of exogenous CaM [29,32–34]. Therefore, if calcium does uncouple myosin-1 mechanical and catalytic activity, the lifetime of this uncoupled state is expected to be relatively short if free CaM in the cytoplasm is able to rapidly re-bind the exposed IQ motif. The concentration and duration of calcium exposure would also be expected to influence the lifetime of the decoupled state. These factors lead to the possibility that, by modulating either the pool of free CaM or the concentration/duration of calcium transients, cells might be able to operate the myosin-1 clutch over a wide range of timescales. Tight control of the timescale would also enable cells to minimize the energy cost imposed by a population of ‘uncoupled’ motors consuming ATP without producing mechanical work.

In combination, the data outlined above indicate that myosins-1 possess unique functional potential. Although originally viewed as slow, low duty ratio motors, more recent studies show that these motors can increase their duty ratio in response to opposing load. Moreover, association of calcium-sensitive CaM light chains with the force-transducing neck region provides the cell with an additional level of regulatory control. Thus, class 1 myosins are force generators that are uniquely positioned to respond to both the mechanical and chemical environment at the membrane-cytoskeleton interface. Below we discuss recent developments in our understanding of how these molecules bind to cellular membranes.

Myosin-1 Interactions With Membranes

Myosin-1 interacts with membranes via a C-terminal TH1 domain that is highly basic (pI ~9.0), and rich in lysine and arginine residues. Early biochemical studies with *Acanthamoeba* myosin-1c were the first to demonstrate direct binding of myosin-1 to cellular membrane lipids with a physiologically significant K_D of ~140 nM [37]. These initial studies also revealed a binding preference for acidic lipids such as phosphatidylserine and phosphatidylinositol bisphosphate (PIP₂). Later work with myosin-1a purified from chicken intestine showed comparable binding to liposomes composed of different negatively charged phospholipids [38]. Interestingly, binding of TH1 to the membrane can be long-lived; FRAP and transient kinetic measurements both suggest that the lifetime of a single TH1-membrane interaction may be several-fold longer than the duration of a single ATPase cycle in the absence of external loading [39,40].

Despite early progress, the molecular mechanism underlying myosin-1 binding to lipids remained undefined for nearly two decades. However, investigators recently used secondary structure homology searching to identify a novel pleckstrin homology (PH) motif in the myosin-1c TH1 domain [5]. This particular PH domain binds specifically and with high affinity to phosphatidylinositol bisphosphate (PIP₂), through direct interactions with the inositol ring headgroup in a manner reminiscent of the PH domain found in PLC δ [41,42]. Alignments with other vertebrate myosin-1 isoforms do indicate that the conserved β 1-loop- β structure required for PIP₂ binding may be a class-wide feature. Indeed, recent work with the short-tailed motor, myosin-1g, suggests that it may target to the membrane in a PIP₂-dependent manner via the putative PH domain [43,44]. Yet it remains to be seen if this and other class 1 myosins will show the specificity and tight binding to PIP₂ demonstrated by myosin-1c.

Experiments in live cells suggest that the TH1-PIP₂ interaction is solely responsible for the localization of myosin-1c [5,41], but the TH1 domain of this and other class 1 motors may contain other motifs that function in lipid binding. Indeed, a second lipid-binding motif was recently discovered in the tail domain of *Acanthamoeba* myosin-1c [45]. Referred to as a basic-

hydrophobic (BH) motif, a peptide corresponding to this region is sufficient to produce micro-molar binding affinity for acidic phospholipids (i.e. PS or PIP₂) [46]. In contrast to the putative PH domain identified in vertebrate myosin-1c, the BH motif may be poorly structured, as experiments with a scrambled peptide revealed that the order of basic and hydrophobic residues has little impact on lipid binding affinity [46]. While an alignment of class 1 myosin sequences from lower and higher eukaryotes reveals that the specific BH motif characterized in *Acanthamoeba* and *Dictyostelium* class 1 myosins is not present in vertebrate myosins-1, the BH motif detection algorithm described by Brzeska et al. [46] identifies several other possible lipid-interacting sites that await experimental validation.

In addition to direct interactions with acidic phospholipids, other findings suggest additional membrane-targeting mechanisms are at play. Data from a variety of investigations suggested that CaM release from IQ motifs in the neck region unmasks additional charged residues that may facilitate membrane association [39,47–49]. A more recent study casts doubt on the significance of this mechanism and suggest that at least a portion of the previous findings may have been influenced by calcium-induced aggregation of liposomes [41]. Other experiments indicate that the neck region could still play a role in membrane association. For example, the myristoylated EF-hand containing proteins, CIB-1 and CaBP-1, bind to the neck region of myosin-1c and co-localize with this motor in NRK and PC12 cells [50], suggesting that they may play a role in membrane targeting. Membrane binding of myosins-1 may also be mediated through interactions between the TH1 domain and other membrane-associated proteins. Studies in cultured epithelial cells revealed that myosin-1a binds to the trans-membrane protein, sucrase-isomaltase (SI), on detergent-resistant patches of brush border membrane [51]. Other recent work revealed that myosin-1c interacts with the peripheral membrane binding protein, PHR1 [52] and the transmembrane adhesion molecule, cadherin-23 [53] in the context of hair cell stereocilia. Other interactors of myosin-1c that might serve in targeting to the membrane include NEMO, which functions as a receptor for this motor in the context of TNF- α signaling [54], and RalA, a G-protein involved in the trafficking of GLUT4 to the plasma membrane [55].

Although investigators have made progress in defining the molecular basis of myosin-1 membrane binding, we still have much to learn. Depending on the molecular context, it appears that both structured lipid-binding motifs (e.g. putative PH domain) and regions that are less well ordered (e.g. BH motif) may participate in generating affinity for acidic phospholipids, the target of all class 1 myosins characterized to date. Binding to other membrane-associated proteins may also contribute to this affinity. In combination, interactions with target lipids and/or lipid-binding proteins will be critical for positioning class 1 myosins to carry out specific biological tasks.

Functions For Myosin-1 At The Membrane-Cytoskeleton Interface

All of the studies outlined above clearly indicate that myosins-1 are well-suited to provide physical connections between cellular membranes and the actin cytoskeleton, or generate mechanical forces that might enable membrane movement or remodeling relative to the underlying actin network. Below we summarize some of the most recent developments in our understanding of vertebrate myosin-1 function at the membrane-cytoskeleton interface.

Control of plasma membrane tension

Recent work has established that class 1 myosins are capable of controlling cellular plasma membrane tension [6](Figure 3a). Membrane tension enables membranes to resist deformation and regulates many critical cellular processes that involve changes in membrane shape such as endocytosis, exocytosis [56], and cell motility [57]. Biophysical studies have established that cellular membrane tension is governed in large part by adhesion to the actin cytoskeleton

[58]. Indeed, “blebbing” occurs when membrane detaches from the cytoskeleton and is forced outward by intracellular hydrostatic pressure [59]. The enrichment of class 1 myosins in actin-supported membrane specializations such as microvilli and podocyte pedicels may reflect a need for high levels of cytoskeletal adhesion, which would be required to maintain the morphology of these highly-ordered structures. Indeed, myosin-1a knockout mice develop “herniations” or large membrane blebs that extend outward from enterocyte microvilli [60], while myosin-1e knockout mice display extensive effacement of podocyte pedicels that results in severe proteinuria [61]. Given their exquisite strain dependence and ability to switch into a long-lived actin-bound state, class 1 myosins are ideally suited for contributing to adhesion between the membrane and the actin cytoskeleton [6].

Dynamic gating of ion channels

Transduction of mechanical stimuli (such as sound or head acceleration) into electrical signals interpretable by our nervous system depends on the maintenance of a constant level of tension across “tip links” that connect adjacent rows of hair cell stereocilia in the inner ear [reviewed in 62]. Following a stimulus and stereocilia deflection, tension is rapidly restored to a baseline level by an “adaptation” response that is thought to be mediated by myosin-1c [63] (Figure 3d). The strongest evidence in support of this model is derived from a chemical-genetic approach that enabled investigators to specifically inhibit myosin-1c in functioning hair cells [64]. While impairing the function of myosin-1c abolished both slow and fast adaptation [9, 65], the approach was based on a dominant-negative effect that trapped the motor in a strongly bound state. Thus, one cannot rule out the possibility that strongly bound myosin-1c was also blocking the function of other proteins or other myosins known to play critical functions in stereocilia, including myosin-3a [66,67], myosin-6 [68], myosin-7a [69], and myosin-15a [70]. Nonetheless, the size, polarity, timescale, and calcium-dependence of the adaptation response are generally consistent with myosin-1c being the adaptation motor [9].

Microvillar vesicle shedding

In light of their kinetic limitations, we do not expect individual myosin-1 molecules to be able to “walk” processively along actin filaments. However, ensembles of myosin-1 may be able to produce directed movement if bound to a common substrate. Evidence for this type of movement was recently observed in brush borders isolated from intestinal epithelial cells, where myosin-1a powers the sliding of apical membrane along microvillar core actin bundles, towards distal tips [7] (Figure 3c). Myosin-1a probably exerts substantial plus-end directed force *in vivo* as well and in the case of intact enterocytes, this force may contribute to the production of vesicles that are released from the tips of microvilli [8]. The finding that myosin-1a knockout mice produce fewer vesicles with abnormal morphology and protein composition suggests that this motor plays a role in either delivering the proper lipids and/or proteins to the nascent vesicle, or preventing certain cargoes (e.g. sucrase-isomaltase [51]) from enriching at microvillar tips. Intriguingly, preliminary ultrastructural studies suggest that hair cell stereocilia also shed vesicles, indicating that this activity may represent a more general aspect of epithelial cell biology (B. Kachar and P. Gillespie, personal communications).

Endocytosis and exocytosis

Given their association with membranes and ability to generate force, it is not surprising that myosins-1 have been implicated in endo- and exocytosis, activities that involve extensive membrane deformation [71–74] (Figure 3b). Myosins-1 do not appear to be required for vesicle transport, but instead are critical for the formation and fusion of vesicles at the plasma membrane [75]. Although the precise mechanism behind these functions is currently unclear, recent findings suggest a few possibilities. For example, myosin-1e interacts with synaptojanin-1 and dynamin indicating that this motor is part of a critical complex that

functions at sites of endocytosis [12]. Expression of a myosin-1e tail construct containing both the SH3 and TH1 domains inhibited transferrin uptake in HeLa cells, but this inhibition was not observed when these domains were expressed on their own. This suggests that both membrane-binding (mediated by TH1) and protein-protein interactions (mediated by SH3) are necessary to displace the endogenous myosin-1e from endocytic machinery [12]. The fact that the myosin-1e tail domain acts in a dominant negative manner shows that motor domain interactions with the actin cytoskeleton are required for proper function in the context of endocytosis [12]. Generation of force at sites of either vesicle formation or vesicle fusion could be directly coupled to deformation of the plasma membrane, a model that has been suggested based on studies in adipocytes where myosin-1c was shown to promote the insulin-stimulated exocytosis of the GLUT-4 glucose transporter [76]. Alternatively, studies in *Xenopus* oocytes suggest that myosin-1c and myosin-1e control cortical granule exocytosis and the subsequent compensatory endocytosis by directly regulating actin polymerization [13,77,78], although the mechanism behind this regulation is still unclear. Intriguingly, myosin-1f has also been shown to regulate the actin cytoskeleton: neutrophils from myosin-1f knock out mice possessed denser cortical actin that inhibited granule exocytosis [4]. This cellular phenotype caused myosin-1f knock out mice to mount a significantly decreased immune response to *Listeria* infection, highlighting the physiological importance of these myosins in the regulation of cytoskeletal architecture at the membrane interface.

Concluding Remarks and Directions for Future Studies

Cytoskeletal biologists have made great strides in defining the mechanism of myosin-1 interactions with actin, the basis of membrane binding, and the specific functional responsibilities these motors carry out in cells. Indeed, class 1 myosins appear to have their hand in a wide-range of functions at the membrane-cytoskeleton interface. In some cases, these motors are playing roles in dynamic events, where membranes are moving/remodeling relative to the underlying actin cytoskeleton (e.g. vesicle shedding, endo- and exo-cytosis, channel gating). In other cases, myosins-1 generate force for maintaining cellular mechanical properties (such as plasma membrane tension), which are not expected to undergo large fluctuations. Thus, understanding if/how class 1 myosins are able to switch between dynamic and stationary functions should be one critical focus of future experimental efforts. One possible switching mechanism may relate to the nature of myosin-1 attachment to the membrane. Mechanical experiments with class 1 myosins have only been performed with motors attached to rigid substrates such as beads or coverslips. Thus, a critical first step in understanding the function of these motors *in vivo* will be to determine if attachment to their native substrate (i.e. a fluid lipid bilayer) can provide sufficient resistance to load the motor and in turn, alter its kinetic properties. If class 1 myosins do experience load-induced stall while attached to cellular membranes, it will be important to define the time scales over which this stalled state persists and how it contributes to specific cellular functions. The intriguing possibility that calcium-induced CaM release could act as a 'clutch' to release motors from a loaded state also has important implications for how these motors may operate in cells, particularly in temporally regulated processes such as hair cell adaptation and vesicle budding/fusion. To fully appreciate how factors such as mechanical load and calcium impact the cellular function of myosins-1, investigators will need to develop techniques that enable us to probe the mechanics and conformational dynamics of these motors, while operating at the membrane-cytoskeleton interface in living cells.

Box 1**The myosin superfamily**

Myosins are a diverse family of actin-based motors that convert chemical energy in the form of ATP into mechanical work. All myosins share a common body plan, consisting of an N-terminal motor domain coupled to a C-terminal cargo binding or dimerization domain, via an α -helical neck region that binds CaM or CaM-like light chains. Currently, myosins are grouped into ~ 35 distinct classes based on sequence similarity [83]. The first myosin to be identified was isolated from muscle nearly 70 years ago [84], where it was shown to power contraction through its interaction with actin filaments [85,86]. Studies on this muscle myosin revealed that a globular “motor” domain binds actin filaments and couples the hydrolysis of one molecule of ATP to a force-generating conformational change [for an excellent overview of this extensive literature see 87]. In the late 1960 s and early 1970 s, myosin isoforms were found in non-muscle cells such as macrophages [88], platelets [89], and in invertebrates such as *Physarum* [90]. Although not organized into sarcomeres, these motors were similar to muscle myosin in that they homo-oligomerized to form thick filaments. In 1973, a new type of myosin that did not form thick filaments was isolated from a biochemical screen of fractions from *Acanthamoeba castellanii*. Unlike muscle-type myosins, this protein consisted of a single heavy chain that was relatively small (~140 kD) [91], and was subsequently found to bind directly to cellular membranes [92]. This motor was eventually named myosin-1, based on the fact that it had one motor domain, whereas the other two-headed muscle and non-muscle myosins would become known as myosins-2. While the first myosins were discovered through biochemical purification [84,88–91], the availability of myosin gene sequences in the 1990 s accelerated the identification of new myosins using PCR-based approaches. One early study utilizing degenerate PCR primers against conserved sequences in the motor domain identified six myosins from the same vertebrate cell type [93]. The advent of genomics and computational biology has hastened myosin identification in a wide range of species, and allowed a more comprehensive system of classification [1,94]. Currently, there are ~40 known myosin genes in humans [3]. As more genomes are sequenced and additional branches are added to the myosin family tree, biophysicists, biochemists, and cell biologists struggle to keep pace in characterizing the properties and biological significance of these novel motors.

Box 2**Class 1 myosins in lower eukaryotes**

Our current understanding of myosin-1 function began developing many years ago with Pollard and Korn s discovery of a small myosin-like protein in *Acanthamoeba castellanii*. While this protein demonstrated actin-activated Mg^{2+} ATPase activity, it lacked the ability to form bipolar thick filaments, a hallmark activity of myosin-2 [91]. Originally suggested by some to be a proteolytic fragment of myosin-2 [95], peptide sequencing data and molecular biological studies would eventually establish that myosins-1 were *bona fide* gene products, distinct from myosin-2 [96,97]. *Acanthamoeba* expresses three isoforms of myosin-1, referred to as myosins-1A, 1B, and 1C [98]; all three of these motors are ‘long-tailed’ as their C-terminus extends beyond the basic TH1 domain with additional glycine-proline-alanine rich (GPA) and SRC homology 3 (SH3) motifs that enable interactions with other proteins. The GPA domain is capable of ATP independent actin binding, allowing long tailed myosins-1 to crosslink actin filaments; this feature also underlies the unique ‘triphasic’ activity observed in the steady-state ATPase assays with *Acanthamoeba* myosins-1 [99]. Early biological studies showed that *Acanthamoeba* myosins-1 localize to a variety of cellular domains including pseudopodia, phagocytic cups, and the contractile

vacuole [100,101]. Intriguingly, these motors were also shown to move vesicles along actin filaments *in vitro* [102] and bind directly to membrane lipids [37]. Some of the first data to indicate that myosin-1 might function at the membrane-cytoskeleton interface in cells was provided by classic experiments which demonstrated that *Acanthamoeba* loaded with anti-myosin-1C antibodies were unable to expel water via their contractile vacuole [103].

Concurrent with the initial characterization of myosins-1 from *Acanthamoeba*, experiments with the slime mold *Dictyostelium discoideum* revealed the presence of small, monomeric myosin variants in this organism as well [104]. Yet further studies would show that *Dictyostelium* express an even larger collection of class 1 myosins [105,106]; seven in total, now referred to as myosins-1A-G [80]. *Dictyostelium* has both long-tailed (1B, 1C, 1D) and short-tailed (1A, 1E, 1F) class 1 myosins [80]. Like the class 1 motors from *Acanthamoeba*, *Dictyostelium* myosins-1 localize to the actin-membrane interface. Cell biological studies quickly linked these motors to the internalization of membrane, including pinocytosis [74,107] and phagocytosis [105,107]. Other studies highlighted roles in secretion [108] and recycling to the cell surface [109]. The importance of class 1 myosins in membrane trafficking would gain additional support through further investigations in *Acanthamoeba*, *Saccharomyces*, and *Aspergillus* [71,110–112]. A more general function in the control of cell morphology and movement was suggested by studies showing that the extension of pseudopodia [113], cell motility and chemotaxis [114–118], and cortical tension [119] are all compromised in *Dictyostelium* cells with an abnormal complement of myosin-1.

Experimentation with the complex collection of myosin-1 motors in *Dictyostelium* also revealed an important and eventually, recurring theme: the concept of overlapping function. For example, deletion of certain myosin-1 isoforms would produce little if any phenotype, suggesting that other class 1 myosins were compensating for functions that would otherwise be lost [120,121]. In other cases, phenotypes produced by deleting two different myosins-1 were very similar, suggesting that both myosins are responsible for the same function [105,114,116]. The potential for overlapping myosin-1 function in vertebrate cells was realized much later with the analysis of the myosin-1a knockout mouse [60,122]. Nevertheless, the very existence of multiple motors that are capable of carrying out similar tasks suggests that the subcellular responsibilities performed by myosin-1 include functions that are fundamental to cell growth, differentiation, and survival.

Acknowledgments

The authors thank the members of the Tyska Laboratory and the Reviewers of this manuscript for helpful suggestions and advice. We also thank Dr. Peter Gillespie and Dr. Bechara Kachar for sharing unpublished data during the preparation of this review. This work was supported in part by grants from the National Institutes of Health (R01-DK075555, MJT) and the American Heart Association (09GRNT2310188, MJT; Pre-doctoral Fellowship, REM).

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Figure 1. An introduction to class 1 myosins

All myosins-1 share a common structural arrangement, with a globular N-terminal motor domain (blue) that is connected to a membrane-binding tail (red) by an α -helical neck region (yellow) that binds 1–6 CaM light chains. (A) Predicted structure of a prototypical class 1 myosin, myosin-1a, based on a Phyre server homology search [79,80] against the solved structures of other myosins [80]. (B) Domain structures of the eight vertebrate class 1 myosins. The most variable domain is the neck region, with different numbers of IQ motifs. Additional IQ motifs will not only bind more CaM light chains, they also increase the length of the neck region, which may increase the size of the working stroke [27]. The two long-tailed vertebrate class 1 myosins (-1e and -1f) are frequently referred to in the literature as ‘amoeboid’ myosins-1 because they share the extended C-terminal tail structure first observed in the *Acanthamoeba* myosin-1 sequences. These additional domains may mediate ATP independent interactions with actin (i.e. TH2/GPA) [81] and direct binding to other proteins (i.e. SH3) [12].

Figure 2. Regulation of class 1 myosin activity

(A) The myosin-1 mechanochemical cycle. Myosin-1 bound to actin in a nucleotide-free rigor state is released when it binds ATP (T). Hydrolysis of ATP to ADP (D) and phosphate (Pi) allows myosin to bind to the actin filament, which triggers Pi release and the initial portion of the working stroke. If the motor is unloaded, ADP is rapidly released and the cycle can repeat; however, if an opposing load is present, ADP release is inhibited, and the motor stalls in a strong actin-binding state. (B) Myosin-1 is mechanically regulated. In the context of a small ensemble of myosin-1 molecules, opposing mechanical load will raise the duty ratio and thus, the fraction of motors bound to actin at steady-state [25]. Reducing load will lower the duty ratio and reduce the number of myosin-1 molecules bound at any instant. (C) Myosin-1 is

regulated by Ca^{2+} /CaM interactions. Studies from a number of laboratories have shown that Ca^{2+} can reduce the affinity of CaM for IQ motifs in the neck region of myosins-1a, -1b, and -1c [26–34]. CaM bound to the first IQ motif (proximal to the motor domain) is likely the first to dissociate [34]. The consequences of Ca^{2+} exposure are somewhat counter-intuitive: ATPase activity increases [26,27,29,30,32–34], yet motility is dramatically inhibited [26,27,32–34]. These results may indicate that CaM binding to the first IQ motif plays a role as a clutch, to control the transmission of conformational changes in the motor domain to the C-terminus of the molecule. It is important to note that myosin-1d appears to be divergent from the other characterized class 1 myosins, as it is not expected to be load-sensitive (it lacks a two-step working stroke)[24] and shows a different response to Ca^{2+} (ATPase is inhibited by Ca^{2+}) [82].

Figure 3. Cellular functions of class 1 myosins

(A) A general function of class 1 myosins is to regulate membrane tension by dynamically linking the plasma membrane to cortical actin [6]. (B) Myosins-1 function in both endo- and exo-cytosis, where their motor activity may be used to actively deform the membrane or to regulate dynamics of the actin meshwork surrounding vesicles near the cell surface [4,12,13, 74,76–78]. (C) Myosin-1 molecules in microvilli generate tip-ward forces that contribute to the production of vesicles carrying specific cargos [7,8]. (D) In hair cell stereocilia, myosin-1 actively maintains a constant tension across tip-links, which optimizes the sensitivity of tip-link associated cation channels [9,63,65].