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A Functional Recombinant Myosin II Lacking a Regulatory Light Chain–Binding Site

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Myosin II, which converts the energy of adenosine triphosphate hydrolysis into the movement of actin filaments, is a hexamer of two heavy chains, two essential light chains, and two regulatory light chains (RLCs). *Dictyostelium* myosin II is known to be regulated in vitro by phosphorylation of the RLC. Cells in which the wild-type myosin II heavy chain was replaced with a recombinant form that lacks the binding site for RLC carried out cytokinesis and almost normal development, processes known to be dependent on functional myosin II. Characterization of the purified recombinant protein suggests that a complex of RLC and the RLC binding site of the heavy chain plays an inhibitory role for adenosine triphosphatase activity and a structural role for the movement of myosin along actin.

The enzymatic and motile activities of nonmuscle myosin II from many cell types, including *Dictyostelium* and various animal cells, are regulated by phosphorylation of RLC in vitro in a manner similar to smooth muscle myosin (*1*). Fluctuations in vivo of either the phosphorylation levels of RLC or that of the RLC-kinase have been demonstrated (*2*), and these observations have led to the hypothesis that RLC phosphorylation plays a crucial role in myosin-dependent motile activities in nonmuscle cells (*1*, *2*). We tested this hypothesis here by expressing a mutant *Dictyostelium discoideum* myosin II heavy chain with an internal deletion that removes the binding site for RLC.

The NH_2-terminal half of myosin II is called subfragment 1 (S1), and S1 alone is sufficient to hydrolyze adenosine triphosphate (ATP) and move actin filaments in vitro (*3*). The S1 molecule has a globular catalytic portion, which contains ATP- and actin-binding sites, and a neck region, which connects the catalytic domain to the α-helical coiled-coil tail of the myosin molecule (*1*, *4*, *5*). Other studies have localized the light chains to the neck region of S1 (Fig. 1B) (*6*). Crystallographic analysis of S1 from chicken skeletal muscle has identified amino acid residues of the heavy chain that interact with RLC (*5*). This region contains the "IQ" motif, which is found in sites that bind calmodulin and related polypeptides such as myosin light chains (*7*). A similarity between the chicken and *Dictyostelium* sequences allowed us to identify a region of the *Dictyostelium* heavy chain that is likely to bind RLC (Fig. 1A). The nucleotide sequence that corresponds to this region was deleted from the wild-type gene for myosin II heavy chain (*mhc*A) with polymerase chain reaction (PCR) technology. This mutant gene was cloned into a self-replicating vector (*8*) and used for transformation of an *mhc*A$^-$ *Dictyostelium* cell line (*9*). We used the transformants to study the mutant phenotype and to purify the mutant myosin MyΔRLCBS (Fig. 1C).

Wild-type *Dictyostelium* cells are mostly mononucleate and grow by binary fission in suspension culture with a doubling time of 5.1 ± 0.1 hours (n = 3; mean ± SEM) (*10*). When starved, they differentiate to form fruiting bodies (Fig. 2A). Both of these processes require functional myosin II (*11*). The *mhc*A$^-$ cells, which cannot perform cytokinesis, become multinucleated in suspension culture and eventually lyse. During the development of fruiting bodies, these cells are unable to proceed beyond the "mound" stage (Fig. 2B). The transformed cells that expressed the MyΔRLCBS *mhc*A gene in place of the native *mhc*A gene regained the ability to divide and grow in suspension culture with a doubling time of 5.5 ± 0.2 hours (n = 3; mean ± SEM) and stayed mostly mononucleate. Thus, the MyΔRLCBS cells undergo cytokinesis in suspension, unlike *mhc*A$^-$ cells. Expression of MyΔRLCBS also restored the ability of the cells to form fruiting bodies, but the

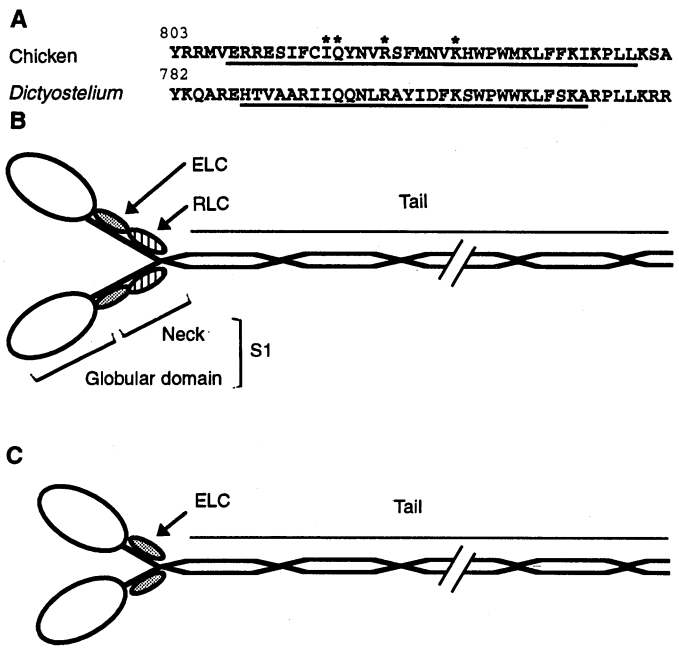

Fig. 1. (**A**) The amino acid sequences of the carboxyl portion of the neck region of S1 for chicken skeletal muscle (*30*) and *Dictyostelium* myosin II (*31*). The underlined residues of the chicken sequence interact with RLC in the crystal structure (*5*). The corresponding region in the *Dictyostelium* sequence (underlined) was deleted from the wild-type *mhc*A gene, resulting in the MyΔRLCBS *mhc*A gene. Asterisks indicate conserved residues of the "IQ" motif (*7*). (**B** and **C**) Schematic representations of the wild-type myosin II and MyΔRLCBS molecules, respectively. Numbers indicate the residue number counting from NH_2-terminus. Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

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stalks appeared thicker and a significant number of mounds did not develop further (Fig. 2C).

The MyΔRLCBS cells were grown in large quantities for the isolation (*12*) and in vitro characterization of the mutant myosin. As we expected, the MyΔRLCBS lacked the 18-kD RLC and retained the 16-kD essential light chain (ELC) (Fig. 3A). A protein immunoblot of the whole cell lysate demonstrated that the RLC polypeptide itself is present in the mutant cells (Fig. 3B).

The adenosine triphosphatase (ATPase) activity of *Dictyostelium* myosin II typically shows two characteristic activities: a Ca^{2+}-ATPase activity that is activated by a high salt concentration and is independent of actin and a Mg^{2+}-ATPase activity that is dependent on RLC phosphorylation and is activated by filamentous actin (*13*). MyΔRLCBS showed slightly higher Ca^{2+}-ATPase activity than the wild-type myosin II did (Table 1). The actin-activated Mg^{2+}-ATPase activity of MyΔRLCBS was unaffected by treatment with myosin light chain kinase and was more than twice that of the wild-type myosin II. The Mg^{2+}-ATPase activity of MyΔRLCBS in the absence of actin was also more than twice that of the wild-type myosin, but these activities are small when compared with the actin-activated activities.

Despite this higher actin-activated Mg^{2+}-ATPase activity, MyΔRLCBS moved actin filaments at only half the speed of the wild-type myosin in the presence of kinase in our in vitro motility assay (Table 2). The movement by MyΔRLCBS was somewhat discontinuous in three independent preparations. However, the quality of the movement was improved by two rounds of high-speed centrifugation in the presence of actin and Mg^{2+}-ATP so that the speed of continuous, uninterrupted movements of longer than 5 μm could then be determined.

The RLC of purified myosin has a small but significant amount of endogenous phosphorylation. This may explain why wild-type myosin had some actin-activated Mg^{2+}-ATPase activity in the absence of treatment with myosin light chain kinase (Table 1). This myosin also moved actin filaments in vitro at a slow average speed (Table 2). The slow, unidirectional movement of actin filaments was sometimes superimposed with random Brownian movement, which is typical of movement over surfaces sparsely coated with active motors (*14*) and consistent with the hypothesis that only a small fraction of molecules is phosphorylated.

The RLC of scallop myosin II can be removed by treatment with EDTA. Scallop myosin without RLC has a reduced ATPase activity and moves actin filaments in vitro at a rate less than one-tenth that of the intact myosin (*15*). A similar experiment has been reported with skeletal muscle myosin (*16*). In skinned skeletal muscle fibers, partial extraction of RLC has been achieved, and this treatment did not inhibit the force generation (*17*). Myosin heads tend to aggregate when the RLC binding site is denuded (*18*). Our study differs from the skeletal muscle work in that total elimination of RLC binding was achieved and the recombinant molecule we generated does not contain a denuded α helix 35 amino acid residues long. We conclude from our results that the RLC is not essential for the motile and enzymatic activity of myosin II either in vitro or in vivo in *Dictyostelium*.

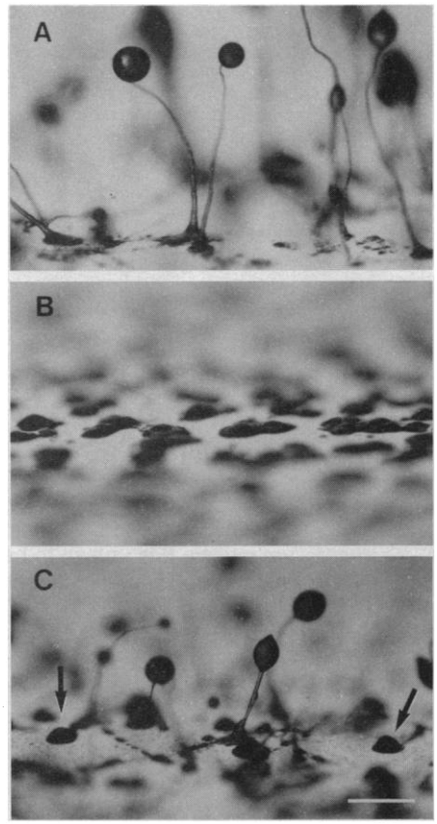

Fig. 2. Development of fruiting bodies. We allowed (**A**) JH010 (wild type), (**B**) HS1 (*mhc*A⁻), and (**C**) MyΔRLCBS cells to develop on LPS agar plates (*32*) at 22°C. Arrows indicate mounds that failed to develop further. Bar, 200 μm.

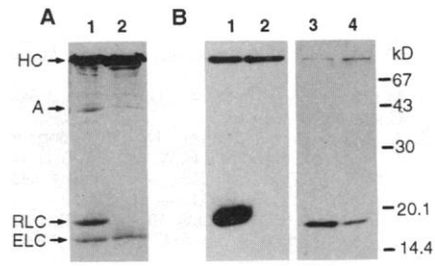

Fig. 3. SDS–polyacrylamide gel electrophoresis (SDS-PAGE) of wild-type myosin and MyΔRLCBS. (**A**) A Coomassie blue–stained 15% acrylamide SDS gel, showing 25 μg each of wild-type myosin (lane 1) and MyΔRLCBS (lane 2). (**B**) A protein immunoblot probed with a monoclonal antibody that recognizes RLC (My8) (*33*). Five micrograms each of the wild-type myosin (lane 1) and MyΔRLCBS (lane 2), as well as the whole cell lysate (derived from 0.6 mg of cells) of the wild-type cells (lane 3) and MyΔRLCBS cells (lane 4), are shown. HC, myosin heavy chain; A, actin, which is the major contaminating protein of these preparations.

Contradictory results have apparently been obtained in *Drosophila melanogaster* (*19*). In *Drosophila*, reduced amounts of the RLC gene transcripts for the nonmuscle and muscle myosin II's result, respectively, in a failure of cytokinesis in larvae and in flightless behavior in adults. *Drosophila* myosin II's may be different from *Dictyostelium* myosin in that they require a complex of RLC and the RLC binding site of the heavy chain to function. Alternatively, it may be that *Drosophila* myosin II's with the denuded RLC binding site denature or form aggregates in vivo and that the phenotype is a consequence of reduced amounts of intact or free myosin molecules. Consistent with the latter of the two possibilities, it has been reported that the removal of the RLC gene in *Drosophila* results in a phenotype

Table 1. ATPase activities of the wild-type myosin and MyΔRLCBS (*29*) with (+) and without (−) myosin light chain kinase treatment (*12*). Results of two independent preparations are shown. ATPase activities are given in molecules of P_i liberated per molecule of myosin S1 per second.

Myosin type	Kinase	Ca^{2+}-ATPase (high salt)	Mg^{2+}-ATPase (no actin)	Mg^{2+}-ATPase (with actin)
Wild type	−	2.5	0.09	0.23
		1.6	0.0	0.27
	+	3.4	0.05	0.86
		2.0	0.02	0.72
MyΔRLCBS	−	3.9	0.16	1.9
		4.3	0.17	1.9
	+	4.1	0.20	1.8
		3.5	0.27	2.1

Table 2. Sliding speeds of wild-type myosin and MyΔRLCBS in vitro with (+) and without (−) myosin light chain kinase treatment. The sliding velocities of 20 filaments were measured (*25*) for each condition (mean ± SEM).

Myosin type	Kinase	Sliding speed (μm/s)
Wild type	−	1.6 ± 0.2
	+	2.4 ± 0.3
MyΔRLCBS	−	1.3 ± 0.1
	+	1.2 ± 0.1

similar to that caused by the *mhc*A null mutation in *Dictyostelium* (*20*).

The observation that MyΔRLCBS has a higher actin-activated Mg^{2+}-ATPase activity than does native myosin suggests that the role of RLC on the enzymatic activity is purely inhibitory and that phosphorylation does not fully remove the inhibition. The reason why MyΔRLCBS moves actin more slowly than wild-type myosin is not clear. One explanation for this is that MyΔRLCBS may be more unstable in vitro and that the resultant denatured molecules interfere with the normal movement caused by other active molecules. Another possibility is that the shorter neck sterically or structurally interferes with motility in our in vitro assay system.

Alternatively, it is possible to explain the slower movement in terms of a structural role for the neck region. A classic model (*21*) for myosin motility proposes that the driving force for movement derives from cyclic changes of the binding angle between S1 and actin while they are strongly bound. However, failure to observe gross changes in the binding angle of the actomyosin crossbridge led to a modification of this "swinging crossbridge" model. In a modified model (*22*), the globular domain of S1 stays strongly bound to actin without grossly changing the binding angle during the entire power stroke. Instead, the neck region of S1 swings relative to the globular domain of S1, resulting in a net displacement of the myosin tail. This model is supported by physical measurements that suggest that S1 changes its shape during the enzymatic cycle from a relatively bent and compact shape to a more straight and elongated conformation (*23*). This conformational change is large enough to explain a proposed displacement of at least 5 nm per cycle (*24*). In the context of this "swinging neck lever" model, removal of the binding region for RLC effectively reduces the length of the lever by about a factor of two (*5*). This would halve the displacement per stroke. If the duration of the stroke is unaffected by the mutation, a smaller displacement per stroke translates to slower movement (*22–25*), consistent with the movement observed here.

The mutant MyΔRLCBS is functional in vivo; however, the absence of the RLC makes it impossible for this mutant myosin to be regulated by RLC phosphorylation. Such regulation has been postulated to be essential for myosin II function in nonmuscle cells (*1, 2*). Our study argues against the obligatory role for this type of regulation in *Dictyostelium* cells. This does not mean, however, that the wild-type myosin that has RLC does not require RLC phosphorylation to be functional in vivo.

Dictyostelium myosin II is also phosphorylated on the heavy chain; this phosphorylation regulates the extent of filament assembly. However, removal of the phosphorylation sites on the heavy chain either by truncation or by substitution (*26*) does not render the mutant myosin totally nonfunctional. Furthermore, cells expressing a mutant myosin that lacks both the binding site for RLC and the phosphorylation sites on the heavy chain can still divide in suspension culture, albeit inefficiently (*27*). It appears that the regulatory role of the phosphorylation of myosin in *Dictyostelium* in vivo is subtle and modulatory, rather than essential for function.

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Isolation of *ORC6*, a Component of the Yeast Origin Recognition Complex by a One-Hybrid System

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Here a method is described to identify genes encoding proteins that recognize a specific DNA sequence. A bank of random protein segments tagged with a transcriptional activation domain is screened for proteins that can activate a reporter gene containing the sequence in its promoter. This strategy was used to identify an essential protein that interacts in vivo with the yeast origin of DNA replication. Matches between its predicted amino acid sequence and peptide sequence obtained from the 50-kilodalton subunit of the yeast origin recognition complex (ORC) established that the gene isolated here, *ORC6*, encodes this subunit. These observations provide evidence that ORC recognizes yeast replication origins in vivo.

The replication of DNA in eukaryotic cells is tightly controlled and coordinated with other events in the cell division cycle. This control is thought to be exerted primarily at the initiation of DNA replication. Replication initiation depends on the completion of earlier events in the cell cycle that commit the cell to a new round of cell division, and reinitiation is prevented until later events are completed, particularly mitosis.

Eukaryotic chromosomal replication initiates at multiple sites in the genome and proceeds bidirectionally. The position of these sites is believed to be specified by DNA elements called origins of replication. Much of our knowledge about the initiation of bidirectional replication comes from prokaryotic and viral systems, most notably the replication systems of *Escherichia coli*, phage λ, and SV40. In these systems, replication origins have been identified, and in vitro systems are available to dissect the initiation reaction (*1*). Studies on the initiation of eukaryotic DNA replication, however, have been hampered by difficulty in the identification of origin sequences. Putative origins have been isolated in a number of eukaryotic systems (*2*), but proof of origin function has remained elusive, and the definition of these elements at the nucleotide level has proven frustrating.

Only in the yeast *Saccharomyces cerevisiae* have eukaryotic origin sequences been clearly identified [reviewed in (*3*)]. Yeast origins were first detected as DNA elements that allow plasmids to be maintained autonomously in yeast cells and were called autonomous replicating sequences (*ARS*s). *ARS*s act as replication origins on plasmids and, in many cases, behave as origins in their native chromosomal location [reviewed in (*4*)]. *ARS*s have a bipartite structure. Domain A is primarily composed of a degenerate 11-bp *ARS* consensus sequence (ACS), 5′-(T/A)-TTTA(T/C)(A/G)TTT(T/A)-3′ found in virtually all *ARS*s (*5*). Domain B, which is approximately 100 bp in size and positioned 3′ to the T-rich strand of domain A, exhibits little sequence similarity among *ARS*s and appears to be organized from multiple partially redundant sequence elements (*6*). Because the ACS is the only sequence motif common to all known *ARS*s, and because single point mutations in this sequence can abolish *ARS* activity (*5*), proteins that specifically recognize the ACS are prime candidates for proteins that initiate DNA replication.

In order to identify potential yeast initiators, we developed a genetic strategy (Fig. 1), the one-hybrid system, to find proteins that recognize a target sequence of interest. This strategy was derived from the two-hybrid system for detecting protein-protein interactions (*7*). The one-hybrid system has two basic components: (i) a hybrid expression library, constructed by fusing a transcriptional activation domain to random protein segments, and (ii) a reporter gene containing a binding site of interest within its promoter region. Hybrid proteins that recognize this site are expected to induce expression of the reporter gene because of their dual ability to bind the promoter region and activate transcription (*8*). This association may be indirect because hybrids that interact with endogenous proteins already occupying the binding site can also activate transcription (*7*). Nevertheless, as long as the association is sequence-specific one may expect the protein incorporated in the hybrid to be functionally relevant.

We have used this method to look for proteins from the yeast *Saccharomyces cerevisiae* that recognize the ACS of yeast origins

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