INTRODUCTION

Myosin II is an actin-based motor, which is important for many aspects of motility in muscle and non-muscle cells (Harrington and Rodgers, 1984). Myosin is a hetero-hexamer composed of two copies each of the heavy chain, essential light chain and regulatory light chain (RLC). Phosphorylation of serine and threonine residues located near the N terminus of the RLC regulates myosin activity. In smooth muscle and vertebrate non-muscle both ATPase activity and filament formation are stimulated by RLC phosphorylation (Sellers et al., 1981). In cardiac and skeletal muscle, phosphorylation of RLC changes the calcium sensitivity of muscle fibers (Sweeney et al., 1993). Mutations in the RLC have been shown to affect the phosphorylation-based regulation of myosin activity (Bresnick et al., 1994; Ikebe et al., 1998; Kamisoyama et al., 1994). In Dictyostelium discoideum, phosphorylation of RLC by MLCK activates ATPase activity (Tan et al., 1992), although it does not appear necessary for cytokinesis or multicellular morphogenesis (Ostrow et al., 1994).

Dictyostelium cells lacking the RLC, or either of the other myosin subunits, are unable to complete cytokinesis in shaking culture (Chen et al., 1994, 1995; DeLozanne and Spudich, 1987; Knecht and Loomis, 1987; Pollenz et al., 1992). Myosin mutants are able to divide on a solid substrate through a process dubbed traction-mediated cytofission (Spudich, 1989). These mutants also have defects in cell locomotion and development, both myosin-dependent processes (Chen et al., 1994, 1995; DeLozanne and Spudich, 1987; Knecht and Loomis, 1987; Pollenz et al., 1992). Myosin mutants also show defective multicellular morphogenesis. These phenotypes provide sensitive in vivo assays for myosin function in Dictyostelium.

Although RLC null mutants are defective in cytokinesis and multicellular morphogenesis, it has been unclear whether the defects in myosin function in these mutants result from mislocalization of myosin caused by aggregation of RLC null myosin. To distinguish this from the alternative explanation that the RLC can directly influence myosin activity, we expressed three RLC point mutations (E12T, G18K and N94A) in a Dictyostelium RLC null mutant. The position of these mutations corresponds to the position of mutations that have been shown to result in familial hypertrophic cardiomyopathy in humans. Analysis of purified Dictyostelium myosin showed that while these mutations did not affect binding of the RLC to the MHC, its phosphorylation by myosin light chain kinase or regulation of its activity by phosphorylation, they resulted in decreased myosin function. All three mutants showed impaired cytokinesis in suspension, and one produced defective fruiting bodies with short stalks and decreased spore formation. The abnormal myosin localization seen in the RLC null mutant was restored to wild-type localization by expression of all three RLC mutants. Although two of the mutant myosins had wild-type actin-activated ATPase, they produced in vitro motility rates half that of wild type. N94A myosin showed a fivefold decrease in actin-ATPase and a similar decrease in the rate at which it moved actin in vitro. These results indicate that the RLC can play a direct role in determining the force transmission and kinetic properties of myosin.

Key words: Myosin, Cytokinesis, Motor function, Regulatory light chain, Myosin regulation, Dictyostelium discoideum
spectrin rod domain is capable of normal ATP hydrolysis (Anson et al., 1996). To investigate how the RLC contributes to myosin function we have produced point mutations in the RLC and determined their biochemical and phenotypic consequences. Our results indicate that RLC mutations can alter the kinetic and force generation properties of myosin, demonstrating that the RLC participates directly in defining the motor properties of myosin.

MATERIALS AND METHODS

Reagents

Unless otherwise noted, all reagents were purchased from the Sigma Chemical Co. (St Louis, MO). Potassium iodide, potassium chloride and magnesium chloride were obtained from Research Organics, Inc. (Cleveland, OH). 2-mercaptoethanol, methanol, sulfuric acid and chloroform were from Mallinkrodt (Los Angeles, CA) and sodium dodecyl sulfate was from Pierce Chemical Co. (Rockford, IL). Acrylamide and Coomasie Brilliant Blue were obtained from Bio-Rad Labs (Richmond, CA) and [γ-32P]dATP was from Amersham Corp. (Arlington Heights, IL). Restriction enzymes were purchased from New England Biolabs (Beverly, MA) and Taq DNA polymerase from Boehringer Mannheim Corp. (Indianapolis, IN).

Mapping and visualization of mutations

Human cardiac, chicken, scallop and Dictyostelium RLC sequences (Genbank accession numbers: M25251, Dictyostelium; M11030, chicken; M22815, human; P13543, scallop) were aligned using the DNastar modules Editseq and Megalign (Lasergene), utilizing the Clustal V algorithm. Visualization of protein structures was carried out using an Indigo II (Silicon Graphics) running the Insight 95 software package (Biosym).

Plasmid construction

The Dictyostelium expression vector pBORP (Ostrow et al., 1994) was used to express wild-type RLC and the E12T, G18K and N94A mutants in Dictyostelium. The mutant light chains were constructed using the four-primer PCR technique described previously (Ho and Chisholm, 1997). All constructs were sequenced to verify the presence of the desired mutation and the absence of additional mutations.

Cell culture and transformation

Dictyostelium discoideum RLC null cells (Chen et al., 1994) were transformed with 10-20 μg of circular plasmid DNA by electroporation. A Bio-Rad gene pulser was used, with settings of 3 μF, 1.3 kV, 5 Ω to produce a 1 millisecond time constant. 1 day after electroporation, G418 (10 μg/ml) was added to the culture medium for selection. Cells were then transferred into 96-well plates for clonal selection, and maintained in G418 after cloning.

Dictyostelium development

5×10^6 cells were washed in DB (5 mM NaH2PO4, 5 mM KH2PO4, 1 mM CaCl2, 2 mM MgCl2) and plated onto filter pads. Development was allowed to continue to the terminal phenotype: 24 hours for wild-type RLC, E12T and G18K, or 36 hours for N94A. Culminants were photographed using a stereomicroscope (Zeiss) with image capture on an LG-3 (Scion) image capture board in a Power Macintosh 7100/66 (Apple Computer, Inc.). For time-lapse videography, images were captured every 15 minutes and compiled using NIH image version 1.61 (National Institutes of Health). NIH image stacks were converted to quicktime movies for web publication using Media Cleaner Pro 2.0 (Terraform Interactive, Inc.). Stalk and spore cell microscopy was performed according to the protocol of Niswonger and O’Halloran (1997).

Biochemical assays

Acto-myosin cytoskeleton preparation and ATP extraction have been previously described (Giffard et al., 1983). Myosin levels were assessed by western blotting with the polyclonal anti-myosin antibody, NU48, followed by densitometric analysis using NIH image version 1.6.1 (National Institutes of Health). Myosin was purified according to the method of Uyeda and Spudich (1993), and phosphorylated by MLCK as previously described (Smith et al., 1996) using the T166E MLCK (a generous gift of Drs Janet Smith and James Spudich, Stanford). The phosphorylation level was assessed by urea-glycerol gel electrophoresis (Ostrow et al., 1994). All myosin used in biochemical studies carried at least 0.95 mole phosphate per mole RLC. The percentage of active heads was determined by adding 5 μg of myosin (20 pmol) to 100 μl of Actin-ATPase buffer (2 mM Tris-HCl, pH 8.0, 0.5 mM DTT, 0.2 mM CaCl2) containing 40 pmol [γ-32P]dATP. After 20 seconds, 1 mM unlabeled ATP was added. The reactions were stopped after 10 minutes, and liberated Pi measured as described by Ostrow et al. (1994). The other enzymatic assays were as previously described (Chen et al., 1994).

In vitro motility assays

Motility assays were carried out according to the protocol of Kron et al. (1991). Data collection was performed using either Image Pro (Zeiss) or MetaGFP (Universal Imaging) on an Axioskop (Zeiss) and a cooled CCD camera (Sensys, Hamamatsu). Captured images were then imported into NIH image version 1.6.1 (National Institutes of Health) for analysis of motility.

Flow cytometry

Dictyostelium cells were grown in suspension culture for five days then fixed with 70% ethanol and stained with 50 μg/ml propidium iodide. Samples were analyzed on a FACS-Calibur system (Beckton Dickinson Co.).

Other methods

DAPI staining, polyacrylamide gel electrophoresis and western blotting were performed as previously described by Chen et al. (1994).

RESULTS

Construction of RLC mutants

To investigate how the RLC contributes to myosin function we have produced mutations in the Dictyostelium RLC and expressed them in an RLC null mutant (mlcR-) (Chen et al., 1994). The three mutations presented in this paper (E12T, G18K and N94A) correspond to changes in positions in the ventricular RLC that, when mutated, cause hypertrophic cardiomyopathy (HCM) in humans (Poetter et al., 1996). Fig. 1A shows an alignment of the Dictyostelium, human ventricular, scallop and chicken skeletal muscle RLCs. The positions of the HCM mutations and the corresponding changes to the Dictyostelium RLC are indicated by shading. Despite primary sequence differences, the structures of the regulatory domains of chicken skeletal muscle (Rayment et al., 1993), chicken smooth muscle (Domínguez et al., 1998) and scallop striated muscle (Houdusse and Cohen, 1996; Xie et al., 1994) are highly similar as are the Dictyostelium myosin motor domain and the chick skeletal and smooth muscle motor domains (Domínguez et al., 1998; Gullick et al., 1997; Rayment et al., 1993). Therefore, it seems likely that the overall structure of myosin and the regulatory domain including both light chains is highly conserved despite amino acid sequence differences. Fig. 1B shows the positions of the G18K and N94A mutations as they map to the 3-D model of scallop regulatory domain
Fig. 1. Alignment of HCM mutations and the Dictyostelium RLC. (A) Sequence alignment of Dictyostelium, human cardiac ventricle, chicken skeletal muscle and scallop striated muscle RLC. Areas of sequence similarity are boxed. Residues that are mutated in HCM are shaded. (B) The Dictyostelium equivalents of the HCM/RLC mutations were mapped to the 3-D model of scallop myosin neck domain. Mutations are indicated by arrows. ELC, essential light chain; MHC, myosin heavy chain.

(Houdusse and Cohen, 1996; Xie et al., 1994). Since the N-terminal 12 residues of the RLC are not resolved in the crystal structures, the position of the E12T mutation is unknown, but likely to map quite near the other two mutations. Despite the fact that the G18K and N94A mutations are located in opposite domains of the RLC, both mapped very close to each other in the 3-D structure, near where the RLC closes around the alpha helix of the heavy chain.
Following introduction of the mutant light chains into RLC null cells, at least three cell lines expressing each RLC mutant were analyzed to ensure that observed phenotypes were due to the RLC mutation, and not a clonal phenomenon. Western blot analysis was used to determine the level of RLC expression, as a substoichiometric level of RLC could result in a functional null mutation (Chen et al., 1994; Ho et al., 1995). As can be seen in Fig. 2, cell lines expressing the three mutant RLCs or a wild-type RLC, have steady state RLC levels at least as high as wild-type cells. We have previously shown that overexpression of RLC does not interfere with normal myosin function (Chen et al., 1994). Indeed, we used the RLC null mutant expressing wild-type RLC as a control in subsequent experiments.

Biochemical properties of myosin bearing mutant RLC

Since myosin lacking the RLC is defective in filament disassembly (Chen et al., 1994), we determined the assembly state of myosin bearing the RLC mutants. Cells expressing the mutant RLCs were extracted with Triton X-100 and the insoluble material extracted with ATP and 0.5 M KCl to release monomeric myosin. The amount of myosin in the pellets and supernatants following each extraction was determined by western blotting. As shown in Table 1, the filament disassembly defects of the parental RLC null cell line (RLC−) led to a decreased percentage of myosin in the ATP/high-salt supernatant. In contrast, none of the mutant myosins differed significantly from wild type in their fractionation properties.

Myosin was purified from the mutant cell lines and its biochemical properties characterized. The biochemical properties of the mutant myosins are shown in Table 2. The determination of active site concentration ensured that differences in activity represented inherent activity differences between the mutants and not variation between preparations in the percentage of active myosin molecules. SDS-PAGE followed by Coomassie Blue-staining of the gels was done for each myosin preparation in order to monitor purity of the myosin and the stoichiometry of ELC and RLC binding (Fig. 3). All three mutants displayed wild-type 1:1:1 stoichiometry (Table 2). The normal light chain binding and the consistently high active site concentration suggest that biochemical properties of the mutant myosins are not due to defects associated with the heavy chain. All purified myosin was treated with the Dictyostelium myosin light chain kinase (MLCK) to ensure maximal phosphorylation (Smith et al., 1996). All three mutant myosins could be phosphorylated to at least 0.95 moles phosphate/mole of RLC (Fig. 4). The

### Table 1. Myosin release from cytoskeletons

<table>
<thead>
<tr>
<th>Cell line</th>
<th>% solubility Triton-insoluble</th>
<th>Myosin ATP/ high salt soluble</th>
</tr>
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<tbody>
<tr>
<td>wtRLC</td>
<td>57±10</td>
<td>58±2</td>
</tr>
<tr>
<td>E12T</td>
<td>43±12</td>
<td>39±4*</td>
</tr>
<tr>
<td>G18K</td>
<td>51±2</td>
<td>59±11</td>
</tr>
<tr>
<td>N94A</td>
<td>56±4</td>
<td>56±3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>46±20</td>
</tr>
</tbody>
</table>

The solubility of mutant myosin was measured. Triton-insoluble cytoskeletons were prepared and the percentage of myosin in the cytosol and pellet assessed by western blot analysis. Triton insoluble pellets were then solubilized in high-salt/ATP and the percentage of myosin released was assessed by western analysis.

### Table 2. Biochemical analysis of mutant myosins

<table>
<thead>
<tr>
<th>Biochemical activity</th>
<th>Expressed RLC</th>
<th>Ca++ ATPase (nmol/min/mg)</th>
<th>% active heads</th>
<th>Relative RLC level (% of wt myosin)</th>
<th>ATPase at 10 μM actin (nmol/min/mg)</th>
<th>$K_m$ actin (μM)</th>
<th>In vitro motility (μM/second)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wtRLC</td>
<td></td>
<td>588±154</td>
<td>85±4.7</td>
<td>100±10</td>
<td>190±35</td>
<td>0.48±0.21</td>
<td>1.63±0.26</td>
</tr>
<tr>
<td>E12T</td>
<td></td>
<td>303±127</td>
<td>92±15</td>
<td>89±14</td>
<td>218±54</td>
<td>0.85±0.65</td>
<td>0.87±0.07*</td>
</tr>
<tr>
<td>G18K</td>
<td></td>
<td>359±41</td>
<td>89±4</td>
<td>129±7</td>
<td>193±34</td>
<td>0.31±0.07</td>
<td>0.92±0.05*</td>
</tr>
<tr>
<td>N94A</td>
<td></td>
<td>549±226</td>
<td>87±14</td>
<td>74±22</td>
<td>36±12</td>
<td>1.3±0.65</td>
<td>0.34±0.04*</td>
</tr>
</tbody>
</table>

Myosin purification and assays were carried out as described in Materials and methods. Each data point is the mean of three independent myosin preparations.

Values marked with an asterisk are significantly different from wtRLC ($P<0.05$, Student’s test).

wt, wild type.
mobility shift seen in the N94A is reproducible and likely due to charge differences introduced by the mutation. Kinase treatment ensured that any differences in activity observed were not due to varying levels of RLC phosphorylation. As can be seen in Table 2, all myosin preparations displayed normal calcium-ATPase activity, indicating that the active site of the enzyme was not disrupted by the mutant light chains. Furthermore, each myosin preparation, regardless of which RLC mutant was bound, showed at least a fourfold increase in actin-activated ATPase following phosphorylation (data not shown). This result indicates that the mutations have not interfered with the ability of the myosin to be regulated by RLC phosphorylation.

Actin binding was normal for each of the mutants (see $K_m$ in Table 2), suggesting that defects were not due to alterations in actin-myosin interaction. The actin-activated ATPase of E12T and G18K RLC bearing myosin at saturating actin concentrations (10 μM) was comparable to wild type, but only 18% of wild type for the N94A RLC. An in vitro motility assay was used to determine the ability of the mutant myosin to move actin filaments. G18K and E12T mutants translocated actin at half the normal rate, despite their wild-type actin-activated ATPase activity, while the N94A mutant displayed very poor motility, moving filaments at only 21% of the wild-type rate. These results clearly indicate that the RLC can influence both the kinetic and motor properties of myosin.

Cytokinesis in RLC mutants

To determine the phenotypic consequences resulting from the altered biochemical properties of the mutant myosins we characterized several myosin-dependent cellular functions. Suspension growth of *Dictyostelium* requires functional myosin (Chen et al., 1994, 1995; DeLozanne and Spudich, 1987; Knecht and Loomis, 1987). We therefore assayed the ability of the E12T, G18K and N94A cell lines to complete cytokinesis during suspension growth. Cells were placed in shaking culture for 5 days, and then either stained with the DNA-binding dye DAPI to determine the number of nuclei per cell or analyzed by flow cytometry to determine DNA content. Fig. 5 shows the DNA content profiles for RLC null mutant expressing wild-type and mutant RLCs. The profile for each of the mutants shows significantly increased percentages of the population with more than 2N DNA content, while the wild-type expressing line shows a profile indistinguishable from wild-type cells. Table 3 shows the percentage of cells with more than 2N DNA content and the percentage of cells with more than two nuclei as assayed by DAPI staining. Based on these two assays, all three RLC mutations increased the number of unsuccessful cytokinesis events, suggesting a defect in myosin function in these cells. However, unlike the parental RLC null cell line, none of the cell lines showed significantly decreased growth rates in suspension. By flow cytometry the three RLC mutants also showed a notable increase in a population of cells that appeared to have much less than 1N DNA content. These structures may represent cell fragments arising from defective cytokinesis.

**Development in RLC mutants**

Myosin function is also required for normal *Dictyostelium* development.

### Table 3. RLC mutant cells show increased DNA content

<table>
<thead>
<tr>
<th>Cell content</th>
<th>Cell line</th>
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<tbody>
<tr>
<td></td>
<td>wtRLC</td>
</tr>
<tr>
<td>&gt;2 nuclei</td>
<td>9.6±3.2</td>
</tr>
<tr>
<td>&gt;2.5 N DNA content</td>
<td>9.6±3.2</td>
</tr>
</tbody>
</table>

The percentage of multinucleate cells, as assessed by DAPI staining or flow cytometric analysis is shown. Cells were grown for 5 days in suspension culture, and then either fixed and stained with DAPI for microscopic analysis, or fixed and stained with propidium iodide for FACS analysis. Values marked with an asterisk are significantly different from wtRLC ($P<0.05$; Student’s test). For comparison, the RLC null mutant line shows greater than 70% of its cells with three or more nuclei by DAPI staining of the nucleus (Ostrow et al., 1994).
development as demonstrated by the observation that both RLC and MHC null cells arrest at the mound stage (Chen et al., 1994; DeLozanne and Spudich, 1987; Knecht and Loomis, 1987). Thus, development provides a sensitive assay of myosin function. When cells expressing mutant RLCs were starved and developed on filter pads, buffered agar or bacteria, both the E12T and G18K cell lines developed normally (Fig. 6B,C). In contrast, N94A cells took 36 hours to reach their terminal phenotype, consisting of small finger-like structures, or aberrant fruiting bodies with short, thick stalks and malformed spore cases (Fig. 6D). To characterize the intermediate stages of development, time lapse videos of development were taken. E12T and G18K proceeded through all stages of development with normal timing and morphology. N94A cells formed mounds with normal timing, but delays occurred after mound formation. This video segment can be accessed over the internet at http://dicty.cmb.nwu.edu/movies/chaudoir.html.

To assess the state of differentiation in the N94A culminants the ability to form mature stalk and spores was determined by calcofluor staining. As can be seen in Fig. 7, calcofluor staining of stalk cells was not observed in the terminal N94A structures, but was readily observed in wild-type rescued cells. Spores formed by the N94A culminants had a typical ovoid spore morphology and were stained by calcofluor, suggesting they were capable of forming spore coats. However, the number of spores formed was greatly reduced. Table 4 shows the numbers of spores observed following resuspension of culminants in starvation buffer containing Triton X-100, to which only spores are resistant. When plated onto bacterial lawns, N94A cells produced only 6-13% as many viable spores as wtRLC

**Fig. 5.** RLC mutants show increased DNA content. Flow cytometric profiles of mutant (B-D) and wild-type (A) rescued cell lines. Cells rescued with wild-type RLC (A); E12T (B); G18K (C); N94A (D). N, DNA content.
expressing cells, as determined by the ability of spores to form feeding zones on bacterial lawns. The parental RLC\(^-\) line did not form detectable spores. The phenotypes of cells expressing the mutant RLCs show that alterations in the biochemical properties of myosin produce defects in some, but not all, myosin dependent functions.

Myosin localization defects typical in the RLC null mutant are corrected by expression of any of the three RLC mutants

Cells that do not express the RLC show abnormal myosin localization that is especially pronounced when the cells are grown in suspension (Chen et al. 1994, Fig. 8E). In these cells myosin is found primarily in punctate staining throughout the cytoplasm. To determine if the phenotypic defects observed in the E12T, G18K and N94A mutants might be due to mislocalization of myosin, cells expressing the mutant RLC were fixed and stained with a monoclonal antibody that recognizes the myosin heavy chain. Fig. 8 shows that expression of wild-type RLC or any of the three mutants in the mlc\(^R\)- background restores the normal cortical localization of myosin. This result supports the hypothesis that the defect the RLC mutants is due to their reduced biochemical function and not mislocalization of the myosin.

**DISCUSSION**

*Dictyostelium* cells unable to express the RLC as a consequence of targeted gene disruption fail to grow in suspension or complete multicellular development (Chen et al., 1994), suggesting that bound RLC is required for normal myosin function. However, these cells also showed abnormal myosin localization, raising the possibility that the defect in myosin function resulted from the myosin mislocalization rather than from specific effects of the RLC on myosin motor function. The role of the RLC in myosin function has been further complicated by the observation that myosin function could be restored by expression, in a myosin heavy chain null mutant, of a myosin heavy chain that lacked the binding site for the RLC and consequently had no bound RLC (Uyeda and Spudich, 1993). This result suggested that myosin could function despite the absence of RLC, providing the light chain binding portion of the MHC was deleted. Given this result, it is unclear why the presence of light chains is a general feature of myosin motors. To address this question we produced point mutations in the RLC and expressed the mutant light chains in a *Dictyostelium* RLC gene disruption. The ability of point mutations in the RLC to effect both the biochemical properties and the in vivo function of myosin strongly argues that the RLC contributes directly to myosin motor function.

*Dictyostelium* cells lacking any myosin subunit, ELC, RLC or MHC, are defective in cytokinesis and multicellular morphogenesis (Chen et al., 1994, 1995; DeLozanne and Spudich, 1987; Knecht and Loomis, 1987; Pollenz et al., 1992). Expression of E12T, G18K or N94A mutant RLC in

![Figure 6](image)

N94A cells develop abnormally. 5\(\times\)10\(^6\) cells were washed in starvation medium, and plated onto black filter pads. Cells were then allowed to develop until the terminal phenotype was observed (24 hours later for wild-type RLC, E12T and G18K, and 36 hours later for N94A). For details, see Materials and methods. (A) Wild-type RLC; (B) E12T; (C) G18K; (D) N94A. Bars, 1 mm.

**Table 4. N94A cells form reduced numbers of viable spores**

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<tr>
<td>Spores/sorocarp</td>
<td>(1.62\pm0.12) (\times10^6)</td>
<td>0 (\times10^6)</td>
<td>2.1(\pm0.4) (\times10^3)</td>
<td>1.1(\pm0.4) (\times10^3)</td>
<td>1.3(\pm1.1) (\times10^3)</td>
</tr>
<tr>
<td>% of wild type</td>
<td>100%</td>
<td>0</td>
<td>13%</td>
<td>7%</td>
<td>8%</td>
</tr>
</tbody>
</table>

The number of viable spores was assessed for wtRLC, E9 RLC\(^-\) and three different N94A cell lines.
suspension-grown cells led to increased DNA content and increased numbers of multinucleate cells, suggesting a defect in cytokinesis. Although these cells clearly divide their cytoplasm more effectively than the RLC null parent, these results suggest that mutant RLC-bearing myosins do not produce sufficient myosin function for efficient completion of cytokinesis. In contrast, expression of E12T and G18K RLCs resulted in sufficient myosin function to support the normal completion of multicellular morphogenesis. These results led to the conclusion that cytokinesis is a more sensitive phenotypic indicator of myosin function than is development.

Unlike the E12T and G18K mutants, cells expressing N94A RLC were unable to complete normal morphogenesis. Development of N94A cells proceeded further than the RLC null parent, but produced only 10% of the normal number of viable, mature spores. This phenotype is similar to that reported for a cold-sensitive mutant MHC shifted to the non-permissive temperature after mounds had been allowed to form (Springer, 1994), or cells in which the expression of RLC is restricted to prespore cells (Chen et al., 1998). These phenotypic observations suggested that the function of the N94A mutant was more severely impaired than was E12T- or G18K-bearing myosin, a conclusion confirmed by the biochemical characterization of purified myosin. Both E12T and G18K had nearly normal actin-ATPase activities, but reduced ability to translocate actin filaments in vitro. In contrast, N94A myosin showed actin-ATPase activities of 18% of wild type and in vitro motility rates were reduced to a similar extent. Comparison of the actin-ATPase and in vitro motility activities with the phenotype suggests that the in vitro motility assay is the better predictor of phenotypic consequences of myosin mutations.

Based on the structures of the chicken S1 and scallop regulatory domains, all three mutations are located quite near to each other in space. As noted by Poetter et al. (1996), the proximity of these mutations to the phosphorylation site raised the possibility that they could reduce myosin function by affecting RLC phosphorylation and thereby some aspect of myosin regulation. However, since all three of the Dictyostelium mutants were phosphorylated in vitro by purified myosin light chain kinase, alteration of phosphorylation is unlikely to be the basis of reduced myosin function observed in these studies.

We do not yet have a thorough understanding of the role played by the light chains in myosin function, but at least three, non-exclusive mechanisms have been proposed. The first is that the ELC and RLC serve a structural role to stiffen the light chain binding region of the heavy chain so that it can effectively serve as a lever arm. A second possibility is that the light chain-binding domain together with the bound light chains provides an elastic element that contributes to or enhances force production. The third possibility is that the light

Fig. 7. N94A cells fail to form a mature stalk and produce reduced numbers of spores. Wild-type RLC-rescued cell line (A,C) and N94A (B,D) culminants were stained with calcofluor to detect mature stalk and spore cells and visualized by fluorescence (C,D) or DIC (A,B). Note the absence of stalk cells in N94A culminants. Bars, 10 μm.
chain-binding domain, together with bound light chain, communicates directly with the active site to influence myosin kinetics.

Biochemical analysis of the E12T and G18K mutations showed that these mutants did not significantly affect rates of ATP hydrolysis, but reduced the in vitro motility rates by 50%. This observation is consistent with a decreased ability of the light chain binding domain to participate in conversion of conformational changes in the head into movement. This could result from either decreased rigidity in the lever arm or a decreased elasticity in this domain of the protein. Movement of the light chain binding domain has been demonstrated using electron paramagnetic resonance (Baker et al., 1998) as well as X-ray scattering (Wakabayashi et al., 1992). During such a movement, the light chain binding domain must be rigid enough not to dissipate the force generated by a non-elastic deformation under the load.

Alternatively, if this domain is effectively an elastic element, as has been suggested by Howard and Spudich (1996), the nature of the bound light chains is likely to be an important contributor to the properties of such an elastic element. The magnitude of deformation ($y_{max}$) of a rigid structure in response to load can be described by $y_{max} = -PL^3/3EI$ (Beer and Johnston, 1981), where $P$ is the force (load) on the lever arm, $L$ the length of the lever arm, and $E$ and $I$ are the elastic modulus and moment of inertia of the structure, respectively. It is likely that the load, the length of the lever arm, and the moment of inertia are not effected by the RLC mutations we have produced. Changes in the modulus of elasticity of the light chain binding domain could result in increased deformation in the light chain binding domain. Increased $y_{max}$ would therefore reduce the effective motion of the proposed lever arm during the powerstroke. The decreased filament motility seen in E12T and G18K mutants could be explained by alterations in the stiffness or elastic properties of the neck resulting from changes in the modulus of elasticity. A similar explanation has been suggested by Lowey et al. (1993) to explain reduced in vitro motility of myosins lacking bound light chains. An important prediction of this hypothesis is that the step size or amount of force generated by E12T or G18K would be reduced relative to wild type. Application of the technology to measure the single molecule mechanics of E12T and G18K myosin should allow a direct test of this idea.

A corollary of this hypothesis is that increasing the stiffness or elasticity of the light chain binding domain could produce a more efficient motor, producing a larger motion for each ATP hydrolyzed. It is interesting that myosin with an artificial lever arm 24% shorter than wild-type myosin, but which may be more rigid than wild-type myosin, moves filaments at a slightly increased rate in a motility assay (Anson et al., 1996).

In contrast to the E12T and G18K mutants, the N94A mutant results in decreased actin-activated ATPase along with a corresponding decrease in in vitro motility rates. Based on their analysis of the S1 structure, Rayment and his colleagues have suggested that the entire myosin molecule may be linked through a series of non-covalent interactions (Rayment et al., 1993). The RLC has an extensive interface with both the ELC and the light chain binding domain of the heavy chain. The ELC in turn interacts with the portion of the MHC involved in ADP product release (Gulick et al., 1997; Houdusse and Cohen, 1996). We have recently provided evidence in support of this idea through ELC mutations that alter the actin-activated ATPase of myosin (Ho and Chisholm, 1997). In this study, we have shown that the N94A RLC mutation also dramatically affects actin-ATPase. One possible mechanism for this effect...
stems from the extensive non-covalent interactions between the RLC, ELC and MHC. The N94A mutation could be envisaged as disrupting or altering the interface between the RLC and the ELC such that the ELC would then display an altered interaction with the active site. This result could also cause the N94A mutation to alter the modulus of elasticity, as discussed above.

In summary, all three RLC mutations reported here result in a decreased ability of the myosin to move actin filaments, regardless of their effect on actin-activated ATPase. These studies demonstrate the importance of the RLC for myosin motor function. We hypothesize that the reduction of in vitro motility seen in these mutants is likely to result from a decreased ability to participate in processes necessary for the efficient translation of ATP-hydrolysis into movement of actin filaments. Higher resolution of the underlying myosin defects in these mutants will require either single molecule kinetic analysis, structural studies or a combination of the two.

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