Targeted disruption of the *Dictyostelium* myosin essential light chain gene produces cells defective in cytokinesis and morphogenesis

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SUMMARY

We have previously demonstrated that the myosin essential light chain (ELC) is required for myosin function in a *Dictyostelium* cell line, 7-11, in which the expression of ELC was inhibited by antisense RNA overexpression. We have now disrupted the gene encoding the ELC (mlcE) in *Dictyostelium* by gene targeting. The mlcE− mutants provide a clean genetic background for phenotypic analysis and biochemical characterization by removing complications arising from the residual ELC present in 7-11 cells, as well as the possibility of mutations due to insertion of the antisense construct at multiple sites in the genome. The mlcE− mutants, when grown in suspension, exhibited the typical multinucleate phenotype observed in both myosin heavy chain mutants and 7-11 cells. This phenotype was rescued by introducing a construct that expressed the wild-type *Dictyostelium* ELC cDNA. Myosin purified from the mlcE− cells exhibited significant calcium ATPase activity, but the actin-activated ATPase activity was greatly reduced. The results obtained from the mlcE− mutants strengthen our previous conclusion based on the antisense cell line 7-11 that ELC is critical for myosin function. The proper localization of myosin in mlcE− cells suggests that its phenotypic defects primarily arise from defective contractile function of myosin rather than its mislocalization.

The enzymatic defect of myosin in mlcE− cells also suggests a possible mechanism for the observed chemotactic defect of mlcE− cells. We have shown that while mlcE− cells were able to respond to chemotacticant with proper directionality, their rate of movement was reduced. During chemotaxis, proper directionality toward chemotacticant may depend primarily on proper localization of myosin, while efficient motility requires contractile function. In addition, we have analyzed the morphogenetic events during the development of mlcE− cells using lacZ reporter constructs expressed from cell type specific promoters. By analyzing the morphogenetic patterns of the two major cell types arising during *Dictyostelium* development, prespore and prestalk cells, we have shown that the localization of prespore cells is more susceptible to the loss of ELC than prestalk cells, although localization of both cell types is abnormal when developed in chimeras formed by mixing equal numbers of wild-type and mutant cells. These results suggest that the morphogenetic events during *Dictyostelium* development have different requirements for myosin function.

Key words: myosin essential light chain, *Dictyostelium*, cell motility

INTRODUCTION

Among the expanding family of myosin isoforms, conventional myosin (myosin II) has been shown to be the mechanochemical force transducer that powers muscle contraction and non-muscle contractile activities such as cytokinesis (Chen et al., 1994; De Lozanne and Spudich, 1987; Karess et al., 1991; Knecht and Loomis 1987; Mabuchi and Okuno, 1977; Manstein et al., 1989; Pollenz et al., 1992), cell motility (Wessels et al., 1988) and morphogenesis in multicellular organisms (Young et al., 1993). In this hexameric molecule, each of the two myosin heavy chains (MHC) binds an essential light chain (ELC) and a regulatory light chain (RLC). The binding sites of the light chains have been located to a stretch of alpha helix emerging from the C-terminal region of the MHC globular head domain, with the ELC binding more proximal to the nucleotide binding site than the RLC (Rayment et al., 1993).

The function of ELCs has received extensive study mostly through in vitro approaches. They were named ‘essential’ because their removal resulted in loss of actin binding and ATPase activity (Dreizen and Gershman, 1970; Gershman and Dreizen, 1970; Weeds and Lowey, 1971). Later experiments challenged this point, showing that skeletal muscle myosin retained actin activated ATPase activity after removal of both light chains (Sivaramakrishnan and Burke, 1982; Wagner and Giniger, 1981). Analyzing myosin enriched with a specific ELC isoform prepared by light chain exchange suggested that isoforms of ELC could modulate the kinetic properties of skeletal muscle S1 (Chalovich et al., 1984; Wagner and Weeds, 1977) and aortic smooth muscle myosin (Hasegawa and Morita, 1992), although this effect was not as profound when full length skeletal muscle myosin was used (Pope et al., 1981). Indeed, Okamoto et al. (1986) used photoaffinity labeling to demonstrate a possible contribution of ELC to the nucleotide
binding site in smooth muscle myosin. Using an in vitro motility assay, Lowey et al. (1993) have shown that removal of both light chains from skeletal muscle myosin severely damaged its motor function while the enzymatic activity was only slightly reduced. Since full restoration of motility required reconstitution of both light chains with the heavy chain, these results suggested that motor function of myosin requires all three component polypeptides. Complementary to this in vitro evidence, we have reported an ELC deficient mutant in *Dictyostelium* (7-11) that allowed us to combine in vivo and in vitro approaches to explore the role of ELC (Pollenz et al., 1992). Created by antisense mRNA over-expression, the 7-11 cells were unable to divide when cultured in suspension, failed to cap cell surface receptors following exposure to concanavalin A and did not complete development. In addition, myosin purified from 7-11 cells lacked actin-activated ATPase activity despite significant calcium ATPase activity. Our results provided in vivo evidence for an essential role of ELC in myosin function.

Several limitations of the antisense inactivation approach complicated the interpretation of the 7-11 cell line: the antisense expression construct may integrate at multiple chromosomal locations. Residual ELC was present in 7-11 cells at 2-5% of the wild-type level. In addition, the mode of antisense inactivation limited the use of antisense mutants as hosts for re-introducing engineered genes to pursue structure-function studies. Here we report the generation of ELC null mutants (mlcE-) by homologous gene targeting. The mlcE- cells exhibited defects in cytokinesis similar to those observed in MHC mutants (DeLozanne and Spudich, 1987; Knecht and Loomis, 1987; Manstein et al., 1989), RLC null mutants (mlcR-) (Chen et al., 1994), as well as 7-11 cells (Pollenz et al., 1992). Myosin purified from mlcE- cells also exhibited enzymatic properties similar to the 7-11 myosin, supporting our previous conclusion based on results using 7-11 cells, that ELC is essential for myosin function.

We have now used the mlcE- cells to analyze chemotactic movements and morphogenetic events during development. The mlcE- cells showed delayed aggregation and many mlcE- aggregates failed to progress beyond the mound stage much like MHC mutants and 7-11 cells. Analysis of single cell chemotaxis indicates that the cells exhibit reduced rates of chemotactic motility. However, as many as 50% of the mounds eventually completed development, forming aberrant looking fruiting bodies but with viable spores. Since myosin is required for *Dictyostelium* morphogenesis, we were interested in how a defective myosin lacking ELC affects myosin-dependent morphogenetic events. We have followed the behavior of two major cell types arising during *Dictyostelium* development, prestalk and prespore cells, using lacZ reporter genes expressed from cell type specific promoters (Dingermann et al., 1989; Jermyn and Williams, 1991). We have found that the absence of ELC had different impacts on the behavior of the two cell types: Prespore cells bearing myosin lacking ELC exhibited an apparent defect in morphogenetic localization during culmination while mlcE- prestalk A cells showed normal localization. However, when prestalk A mlcE- cells were mixed with wild-type cells to form chimeras, the mlcE- cells failed to localize as efficiently as wild-type cells. Our studies have revealed that during *Dictyostelium* development, prestalk and prespore cells exhibit different sensitivity to loss of myosin function caused by the absence of ELC. These observations suggest that distinct *Dictyostelium* morphogenetic events have different requirements for myosin-based motility.

### MATERIALS AND METHODS

#### Cells and growth condition

A thymidine auxotroph strain of *Dictyostelium discoideum*, JH10 (Hadwiger and Firtel, 1992) was grown in HL5 medium supplemented with 100 μg/ml thymidine.

#### General molecular biology

Standard molecular biological techniques were performed using common procedures (Sambrook et al., 1989). Molecular genetic techniques for working with *Dictyostelium* have also been described by Manstein et al. (1989) and Pollenz et al. (1992).

#### DNA constructs

The gene replacement vector, pmIcE-thyl, was generated by ligating a 3.2 kb BamHI-HindIII fragment containing the thy1 gene (Dynes and Firtel, 1989) with a 1.8 kb HindIII-EcoRI 3' genomic fragment of the mlcE gene (Pollenz and Chisholm, 1991) and cloned into blue- script vector pSK between the BamHI-EcoRI sites to create the vector pIthy-mlcE3'. A 1 kb ClaI-EcoRV 5' genomic fragment of the mlcE gene (Pollenz and Chisholm, 1991) was then treated with Klenow to fill the ClaI end and cloned into the vector pIthy-mlcE3' at the Klenow-filled BamHI site. Clones containing a properly oriented mlcE 5' fragment were identified by PCR and confirmed by DNA sequencing through the vector-insert junction.

*Dictyostelium* ELC cDNA tagged with a 30 base myc sequence at the 3' end was introduced into expression vector pBORP (Ostrow et al., 1994) to generate pmycELC. When over-expressed in wild-type AX3 cells, the mycELC competed efficiently with the endogenous ELC for binding to the MHC. Purified myc-tagged myosin exhibited wild-type biochemical properties and the cell lines expressing mycELC were phenotypically normal (Ho et al., unpublished). The pmycELC was introduced into mlcE- cell lines to test for functional complementation of phenotypic defects.

The cell type specific reporter constructs pmcemA-lacZ and ppspA-lacZ were generous gifts from Dr J. G. Williams (Imperial Cancer Research Fund, UK).

#### Transformation

*Dictyostelium* cells were transformed by electroperoration using a Bio-Rad Gene Pulser at 1.3 kV, 3 μF (Howard et al., 1988). For JH10 cells transformed by *thy1* selection, transformants were selected in HL5 medium without supplementing thymidine. For cells transformed with pBORP based vectors and cell-type marker constructs, 10 μg/ml G418 was added to HL5 medium as selection.

Western blot analysis. Protein samples from whole cell lysates or purified myosin were prepared and western blot analyses were performed as described (Pollenz et al., 1992) except that 15% SDS-PAGE was used to improve separation of the light chains. Immunoblots were visualized with two methods: 125I-Protein A or horseradish peroxidase-conjugated secondary antibody followed by chemiluminescent substrate (DuPont NEN, western blot chemiluminescence reagent).

#### Morphological analyses

For DAPI staining and immunofluorescence, cells were prepared as previously described (Pollenz et al., 1992) except that cells were fixed with methanol for 5 minutes at -15°C. The monoclonal antibody specific for *Dictyostelium* MHC (mAb 396) was a generous gift from Dr Gerisch (Max Planck, Germany) (Pagh and Gerisch, 1986). Photomicrography was performed on a Zeiss Axioskop with χ40 or χ63 objectives.
Targeted disruption of the Dictyostelium ELC gene

For developmental studies, 5×10^6 cells in 0.2 ml DB (5 mM Na_2 HPO_4, 5 mM KH_2 PO_4, 1 mM CaCl_2, 2 mM MgCl_2) were placed on 2% agar in DB and monitored with a Nikon DIAPHOT inverted microscope for early stages. Development after the mound stage was monitored with a dissecting microscope. Development was also monitored on cells grown on bacterial lawns. Cells containing cell-type marker constructs were developed on nitrocellulose filters saturated with PDF (20 mM KCl, 9 mM K_2 HPO_4, 13 mM KH_2 PO_4, 1 mM CaCl_2, 2.5 mM MgSO_4) in a moist chamber. Cells at the desired developmental stages were fixed by placing a nitrocellulose filter on filter pads saturated with 1% glutaraldehyde in Z-buffer (60 mM Na_2 HPO_4, 40 mM NaH_2 PO_4, 10 mM KCl, 1 mM MgSO_4) for 1 hour and then sprayed with a mist of 1% glutaraldehyde. The fixed cells were then stained with X-gal following the protocol described by Dingermann et al. (1989).

Myosin purification and biochemical analysis

Cells harvested from 3-day suspension culture were used for myosin purification. Starting with 20 times more cells than JH10 culture, the mlcE^- cells produced enough cell mass for myosin purification after growing in suspension for 3 days although the total cell number did not increase significantly. Myosin was purified as described by Pollenz et al. (1992), except that the ammonium sulfate precipitation procedure was eliminated and a Superose 6 column (Pharmacia) was used instead of an S-500 column. ATPase assays were performed as described by Pollenz et al. (1992). Cytoskeletons were prepared following procedures of Kuczmarski et al. (1991).

Immunoprecipitation

Myosin was immunoprecipitated with the monoclonal antibody against MHC (mAb 396) provided by Dr Gerisch (Max Planck, Germany). Whole cell lysates and the immunoprecipitation were performed as described by Berlot et al. (1985).

Chemotaxis assay

Cells (5×10^6 to 1×10^7) were starved on 2% agar in phosphate buffer and were harvested for assay when aggregation initiated, as judged by the appearance of chemotactic territories. Chemotaxis was analyzed in a Zigmond gradient chamber (Neuro Probe, Inc., Cabin John, MD) with a cAMP gradient of 0-0.1 μM. After incubation in the chamber for 20 minutes following cAMP addition, the motility of cells was monitored on a Nikon Diaphot-TMD inverted microscope with a ×20 objective. The images of cells were collected through a Hamamatsu CCD camera 2400, captured every 10 seconds for a
period of 10 minutes using a Scion LG-3 frame grabber (Scion Corp., Frederick, MD). The images were analyzed on a Macintosh Centris 650 computer using the public domain NIH Image program (written by Wayne Rasband at the US National Institutes of Health and available from the Internet by anonymous ftp from zippy.nimh.nih.gov). The positions of cells in each frame were calculated as center of the best-fit ellipse.

RESULTS

Generation of ELC deficient mutants by gene targeting

A gene disruption construct was created by replacing two thirds of the coding sequence of the \textit{mlcE} gene with a selectable marker gene \textit{thy1} (Dynes and Firtel, 1989) (Fig. 1A). This construct was used to transform the \textit{Dictyostelium} thymidine auxotroph JH10 (Hadwiger and Firtel, 1992) and transformants selected for thymidine prototrophy. Whole cell lysates of the \textit{thy}+ transformants were analyzed by western blot to identify cell lines that failed to express ELC. ELC deficient cell lines were cloned by picking isolated plaques grown on bacterial lawns. In several independent transformations, we found 20% of the transformants to be ELC deficient.

The organization of the \textit{mlcE} gene in these ELC deficient cell lines was examined by southern analysis. Digested genomic DNA was probed with the \textit{Dictyostelium} ELC cDNA. A single 4.4 kb \textit{EcoRI} fragment from JH10 genomic DNA shifted to 7.5 kb in ELC deficient cells. One of the two \textit{HindIII} fragments (>9 kb) also increased in size. In the case of \textit{EcoRI} and \textit{HindIII} double digestion, a 5.6 kb and a 1.8 kb fragment were observed in genomic DNA from ELC deficient cells instead of 2.4 kb and 1.8 kb in wild type (Fig. 1B). The sizes of the \textit{EcoRI} fragment and the 5.6 kb \textit{EcoRI-HindIII} fragment are both consistent with a size shift expected from an insertion of a 3.2 kb \textit{thy1} gene into the wild-type \textit{mlcE} gene. No other inserted DNA sequences were found in these cells. No detectable ELC message was observed in ELC deficient cells using northern analysis (not shown). By western blot, ELC deficient cells did not contain detectable ELC while the levels of MHC and RLC were comparable to those of wild-type cells (Fig. 1C). These results indicate that these ELC deficient cell lines resulted from targeted disruption of the \textit{mlcE} gene.

The \textit{mlcE}–cells were then transformed with a vector capable of expressing wild-type ELC (ELC rescued \textit{mlcE}–) (Fig. 1C). The decreased mobility of ELC on SDS-PAGE was due to a 10 amino-acid myc epitope tag. When over-expressed in wild-type AX3 cells, the myc-tagged ELC replaced endogenous ELC in 98% of myosin and the presence of myc-tag did not affect the normal function of myosin (G. Ho et al., unpub-

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**Fig. 2.** The \textit{mlcE}– cells are defective in cytokinesis although myosin can be found in the cleavage furrow. The \textit{mlcE}– cells became multinucleate upon culture in suspension for more than two days (A), indicating a cytokinetic defect. The ELC rescued \textit{mlcE}– cells contained only 1-2 nuclei (B). Wild-type cells stained with antibody against myosin exhibited typical cortical staining (C). Suspension grown \textit{mlcE}– cells were found to contain long cytoplasmic bridges within 15 minutes upon attaching to a solid substrate, as if they were in the process of cytoplasmic division. Myosin was often seen in cleavage furrow-like structures during early stages of division (D), and in an intense spot at the center of a long, thin cytoplasmic bridge in cells toward the end of cytoplasmic division (E). Double immunolabelling with antibodies against myosin (F) and \textit{α}-tubulin (G) in the same cells showed that microtubule networks were generally less dense in regions exhibiting intense myosin staining. Bars, 20 μm.
lished). The phenotypic defects observed in the mlcE− cells were corrected in the ELC-rescued mlcE− cells (see below).

**Phenotypes of the mlcE− cells**

mlcE− cells became multinucleate in suspension culture. When cultivated in suspension for two days, the mlcE− cells increased significantly in size and became multinucleate (Fig. 2A). These giant cells eventually lysed after prolonged culture. Under the same culture conditions, the ELC-rescued mlcE− cells contained only 1-2 nuclei (Fig. 2B) and their sizes were indistinguishable from that of parental JH10 cells.

Myosin localized to cleavage furrow in mlcE− cells

When stained with a monoclonal antibody against MHC, myosin can be seen at the cortical region of mlcE− cells (Fig. 2D,E,F) as in wild-type cells (Fig. 2C) and in an intense spot at the center of a long, thin cytoplasmic bridge in cells toward the end of cytoplasmic division (Fig. 2E). Double immuno-labelling using antibodies against myosin and tubulin showed that microtubules seemed to be less abundant in myosin rich regions (Fig. 2F,G).

mlcE− cells polarized toward chemoattractant but showed decreased rates of chemotactic motility

When analyzed in a gradient of cAMP (0-0.1 µM), the roundness and chemotactic index exhibited by the mlcE− cells were comparable to that of wild-type and ELC-rescued mlcE− cells (Table 1), indicating that these cells were capable of polarizing toward chemoattractant and moving preferentially up a chemotactic gradient. However, the velocity of chemotactic movement was about 40-50% that exhibited by wild-type or ELC rescued mlcE− cells (Table 1).

mlcE− cells exhibited abnormal development

Following starvation on buffered agar, the mlcE− cells took 7-10 hours to reach the loose aggregate stage. The parental strain JH10 required 5-7 hours to reach a comparable developmental stage. While 50% of the mlcE− aggregates never progressed beyond the aggregation stage, the remainder eventually formed fruiting bodies. In JH10 and ELC rescued mlcE− cells, more than 90% of the aggregates developed into fruiting bodies. The fruiting bodies formed by mlcE− cell usually had abnormally short and thick stalks (Fig. 3).

Prespore cells were mislocalized during morphogenesis of mlcE− cells

To investigate the basis of the observed morphological abnormality, we have used mlcE− cell lines as hosts to express lacZ from the cell type specific promoters ecma (expressed in prestalk A cells) or pspA (expressed in prespore cells). As described by Jermyn and Williams (1991), wild-type cells expressing ecma-lacZ (prestalk cells) appeared randomly throughout the mounds, then sorted to the apex to form the morphogenetic organizing tip (also see Fig. 4A). The ecmA-lacZ cells later localized to the anterior of the slug (also see Fig. 4B) and during culmination, entered the stalk tube that extends through the spore mass elevating the spore case from the surface (also see Fig. 4C). Wild-type prespore cells, marked by pspA-lacZ, occupied most of the mounds under the tip and the posterior 4/5 of slugs (Harwood et al., 1989; Dingermann et al., 1989; Fig. 5A,B). During culmination, they formed the spores located within the spore case (Dingermann et al., 1989; Fig. 5C). The mlcE− cells bearing ecmA-lacZ or pspA-lacZ were expressed at stages comparable to those in wild type.

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**Table 1. Chemotaxis of mlcE− cells is less efficient**

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<tr>
<th></th>
<th>JH10</th>
<th>mlcE− (C1)</th>
<th>mlcE− (A2)</th>
<th>ELC rescued mlcE−</th>
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<tr>
<td>Chemotactic Index</td>
<td>0.85±0.08</td>
<td>0.78±0.17</td>
<td>0.64±0.36</td>
<td>0.71±0.16</td>
</tr>
<tr>
<td>Velocity (µm/min)</td>
<td>14.65±5.39</td>
<td>6.16±2.73</td>
<td>5.24±2.70</td>
<td>15.34±4.32</td>
</tr>
<tr>
<td>% Roundness</td>
<td>52.34±16.04</td>
<td>55.40±15.69</td>
<td>47.72±14.87</td>
<td>54.43±16.71</td>
</tr>
<tr>
<td>n</td>
<td>35</td>
<td>18</td>
<td>26</td>
<td>11</td>
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The chemotactic index was calculated as net displacement in the direction of gradient/total distance traveled. The percentage cell roundness was calculated as 100*4π*cell area/cell perimeter^2. A circle would produce a roundness of 100%, while a straight line would produce a roundness of 0%. The instantaneous velocity was calculated as the displacement of cells between two video frames captured at a 10 second interval/10 sec*60 sec/min. Average of instantaneous velocity between each frame collected in 10 minutes yielded velocity. n, number of cells analyzed.

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**Fig. 3.** The mlcE− cells form fruiting bodies with shorter and thicker stalks. Fruiting bodies formed by wild-type cells (A) and mlcE− cells (B) after 6 days of growth on a lawn of *Klebsiella aerogenes*. Bar, 0.5 mm.
Their distribution resembled wild type throughout early developmental stages prior to culmination (Figs 4D,E, 5D,E). However, mlcE- fruiting bodies displayed a morphology distinct from wild-type fruiting bodies (compare Figs 4C and F, 5C and F). The bases of mlcE- stalks were always surrounded by a population of cells that were pspA-lacZ positive (Fig. 5F), suggesting that these mlcE- prespore cells failed to localize in the spore case and remained in their original, more posterior position.

Both mlcE- prestalk and prespore cells mislocalized when mixed with wild-type cells during morphogenesis

The ability to form chimeric aggregates provides an opportunity to compare the behavior of wild-type and mlcE- cells in the same developing tissue. In the chimeric mounds and early fingers, both prestalk and prespore mlcE- cells behaved in a manner similar to wild-type cells i.e. ecmA-lacZ cells were found in the tip while pspA-lacZ cells occupied the posterior area (Figs 6A,B, 7A,B). The cell type localizations in the chimeric mounds were indistinguishable from mounds formed by either wild-type or mlcE- cells (compare Fig. 6A,B with Fig. 4A,B). By the time tipped mounds elongated into fingers, however, the mlcE- prestalk A cells were found to be absent from the tip (Fig. 6C) and the mlcE- prespore cells were localized preferentially to the posterior portion of the prespore zone (Fig. 7C). In the chimeric fruiting bodies, very few mlcE- prestalk A cells were seen in the stalks and the majority of them were found in the upper and lower cup of spore case (Fig. 6D). These ecmA-lacZ positive areas in the spore case are derived from ‘anterior like cells’ that originate from prespore zone in the slug (Jermyn and Williams, 1991) as well as a subpopulation of prestalk cells situated in the rear portion of prestalk zone (Pst0 cells) (Early et al., 1993). The mlcE- prespore cells accumulated preferentially in the lower part of the spore case as well as along the stalk of the chimeric fruiting bodies (Fig. 7D). These abnormal localizations were exclusively associated with chimeric structures formed by mixtures of marked mlcE- and wild-type cells, but were not found in chimeras formed by mixing marked and unmarked wild-type cells (Figs 6E, 7E,F).

Fig. 4. The localization of mlcE- prestalk cells appears normal throughout developmental stages. Localization of mlcE- prestalk cells (D,E,F), as revealed by X-gal staining pattern of ecmA-lacZ cells, appeared to be indistinguishable from that of wild type (A,B,C). The mlcE- prestalk cells localized to the anterior tip in mounds (D) and finger stages (E), and can be found in the stalk and areas surrounding the spore case (upper and lower cup) (F). The population of unstained cells at the base of the mlcE- stalk correspond to the localization of the pspA-lacZ cells. Bar, 0.5 mm.
Targeted disruption of the *Dictyostelium* ELC gene

ELC<sup>-</sup> myosin exhibited abnormal biochemical properties

One advantage of mlc<sup>-</sup> cells over the previously described 7-11 antisense derived ELC mutants (Pollenz et al., 1992) is the complete absence of ELC. This provides a cleaner system for the analysis of the biochemical properties of ELC deficient myosin than did 7-11 cells. We have examined the association of RLC with ELC<sup>-</sup> myosin. Myosin immunoprecipitated from mlc<sup>-</sup> cell extracts using a monoclonal antibody against MHC had 56±10% of the wild-type RLC level (Table 2). Similar levels of RLC were found in myosin released by ATP from Triton cytoskeletons prepared from mlc<sup>-</sup> cells. In contrast, myosin purified from mlc<sup>-</sup> cells carried only 10±2.2% of the wild-type level of RLC (Table 2). These observations suggest that the absence of ELC reduces the stability of RLC binding to MHC.

During purification by the protocol of Clark and Spudich (1974), ELC<sup>-</sup> myosin behaved like wild type until the step where actin and myosin were separated by gel filtration chromatography. Following removal of actin, salt-dependent assembly was used to concentrate the myosin. In the absence of actin, the ELC<sup>-</sup> myosin assembled normally, but failed to efficiently solubilize in buffer containing 250 mM KCl. Following centrifugation at 16,000 g, large white pellets were observed which contained more than 70% of the ELC<sup>-</sup> myosin (Fig. 8A), suggesting that ELC<sup>-</sup> myosin formed abnormal aggregates at this stage. In contrast, more than 90% of wild-type myosin was found in the supernatant (Fig. 8A) and a

### Table 2. RLC level of ELC<sup>-</sup> myosin is reduced after purified from mlc<sup>-</sup> cells

<table>
<thead>
<tr>
<th>Method</th>
<th>RLC/MHC (%)</th>
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<tbody>
<tr>
<td>Immunoprecipitation</td>
<td>56±10</td>
</tr>
<tr>
<td>ATP-released cytoskeletons</td>
<td>70±29</td>
</tr>
<tr>
<td>Biochemical purification</td>
<td>10±2.2</td>
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Western blots of myosin prepared by different methods were probed with a polyclonal antibody against myosin and visualized by 125I-Protein A. Relative intensities of RLC/MHC were established by densitometry of the autoradiograph and the ratio obtained from ELC<sup>-</sup> myosin was normalized to that of wild-type myosin from the same autoradiograph. Each ratio was the average of results from at least two independent experiments.

![Fig. 5. The mlc<sup>-</sup> prespore cells fail to localize properly during culmination. Prespore cells, labeled by pspA-lacZ, occupied posterior region in mounds and fingers formed by both wild-type (A,B,) and mlc<sup>-</sup> cells (D,E). All the pspA-lacZ cells localized to the spore case in wild-type fruiting bodies (C). In mlc<sup>-</sup> fruiting bodies, however, a population of prespore cells was found surrounding the stalk (F) as if they had failed to reach the spore case during culmination. Bar, 0.5 mm.](image-url)
Fig. 6. mlcE<sup>-</sup> prestalk cells mislocalize in chimeric fingers and fruiting bodies formed by mixing with wild-type cells. In chimeric mounds formed by mixing mlcE<sup>-</sup> cells carrying the ecmA-lacZ marker with an equal number of unmarked wild-type cells, the mlcE<sup>-</sup> prestalk cells were able to localize to the tip, the expected position for prestalk cells (A,B). However, in chimeric fingers, the mlcE<sup>-</sup> prestalk cells were found to be absent from the anterior most region (C). Most of the mlcE<sup>-</sup> prestalk cells then localized to the upper and lower cup of the spore case in chimeric fruiting bodies (D). When wild-type cells carrying the ecmA-lacZ marker were mixed with unmarked wild-type cells for development, labeled cells localized normally (E). Bar, 0.25 mm.

Fig. 7. mlcE<sup>-</sup> prespore cells mislocalize in chimeric fingers and fruiting bodies formed by mixing with wild-type cells. When mlcE<sup>-</sup> cells carrying the pspA-lacZ marker were mixed with unmarked wild-type cells, the mlcE<sup>-</sup> prespore cells were found to localize properly in chimeric mounds and early fingers (A,B). In the chimeric slug, the mlcE<sup>-</sup> prespore cells were found predominantly toward the posterior area of the prespore zone (C). (Compare the staining of chimeras formed between wild-type marked and unmarked cells in E.) Besides the population of mlcE<sup>-</sup> cells accumulating around the stalk, the mlcE<sup>-</sup> prespore cells that reached the spore case in chimeric fruiting bodies localized predominantly to the lower portion of the spore case (D). The chimeric fruiting bodies formed by mixing wild-type marked and unmarked cells were indistinguishable from the structure formed by a single cell line (F). Bar, 0.25 mm.
Targeted disruption of the Dictyostelium ELC gene

visible pellet was rarely observed. Consistent with its insolubility in high salt buffer, we were unable to purify ELC− myosin using the protocol described by Kubalek et al. (1992), which used multiple cycles of salt-dependent assembly-disassembly to achieve rapid purification. Following each assembly cycle, we observed increased loss of ELC− myosin due to reduced solubility in high salt buffer.

The ELC− myosin in the 16,000 g high salt pellet exhibited significant high salt calcium ATPase activity with a Vmax of 515 nmole/min/mg (63% of wild-type myosin) and actin activated ATPase less than 10% of wild type (Fig. 8B).

DISCUSSION

We have disrupted the gene encoding the Dictyostelium myosin essential light chain. In the absence of ELC, the mutant cells were unable to undergo cytokinesis in suspension, a process that has been demonstrated to be myosin dependent (Chen et al., 1994; DeLozanne and Spudich, 1987; Knecht and Loomis, 1987; Manstein et al., 1989; Pollenz et al., 1992). Myosin purified from mlcE− cells lacked actin activated ATPase activity that powers the motor function of myosin. We have previously reported similar results from the ELC deficient mutant generated by antisense RNA overexpression (Pollenz et al., 1992). Our observations from the mlcE− mutants further support the conclusion based on the antisense cell line 7-11 that ELC is required for myosin function in vivo.

Like the ELC deficient 7-11 cells (Pollenz et al., 1992) and mlcR− cells (Chen et al., 1994), mlcE− cells also exhibited abnormal development. In all of these myosin light chain mutants, aggregation was delayed relative to wild type.
However, unlike 7-11 and mlcR− cells, a subpopulation of mlcE− mounds were capable of completing the developmental program, forming aberrant looking fruiting bodies with viable spores. The more severe developmental defect of 7-11 cells might result from pleiotropic effect(s) of the antisense inhibition or inactivation of gene(s) required for development through multiple insertion of the antisense expression construct.

It has been well established that myosin II is required for Dictyostelium development (DeLozanne and Spudich, 1987; Knecht and Loomis, 1987). The fact that some mlcE− cells were able to develop suggested to us that the motile events required for Dictyostelium development may be less sensitive to reduction of myosin function than is cytokinesis. Analyses of the Dictyostelium MHC mutants have established a series of myosin II-dependent motile events during development. During aggregation, the lack of polarized cell shape and directed movement of MHC mutants in response to the chemoattractant cAMP suggested a role of myosin II in chemotaxis (Wessels et al., 1988). Once in aggregates, myosin II is required to support the spiraling motion persisting into culmination (Elliott et al., 1993). Tip formation, which involves sorting of pstA cells to form the morphogenetic organizing center (Williams et al., 1989), also requires myosin II (Elliott et al., 1993; Traynor et al., 1994). In addition, immunolocalization of myosin II to the cortex of peripheral and anterior cells in slugs also suggests a role for myosin II in slug movement (Elliott et al., 1991). Using a Dictyostelium mutant expressing a cold sensitive myosin heavy chain, Springer et al. (1994) have identified two distinct developmental stages that require functional myosin. In addition to the dependence of tip formation stage on myosin, they demonstrated that myosin is also essential during culmination for formation of a normal stalk and raising of the spore head. Our results also suggest that myosin is required for culmination. In addition, we have observed different sensitivities among morphogenetic events to the loss of myosin function caused by the absence of ELC. We have found that the localization of prespore cells during culmination is most susceptible to the functional defect of myosin. Despite the apparently normal behavior of mlcE− prestalk cells, many mlcE− prespore cells failed to become localized to the spore case and remained at the base of the fruiting bodies. One possible explanation for this phenomenon is that the absence of ELC reduced the efficiency of myosin-based motility required for the movement of prespore cells into the spore case. This is supported by the abnormal localization of both cell types in the developing chimeras formed from a mixture of wild-type and mlcE− cells. The conventional models for culmination suggest that the major motive force during culmination is provided by prestalk cells. Through their continual entry into stalk tube, the stalk tube extends, lifting the spore mass off the substratum (Durston et al., 1976). Our observations suggest that the prespore cells may play a more active role during culmination by contributing myosin dependent motive force. Alternatively, since myosin II has been shown to be required for cell shape changes during Drosophila embryo development (Young et al., 1993), it is possible that the absence of ELC has contributed to a failure in generating normal cell shape changes that are crucial for localizing the spores atop the stalk. We have shown that mlcE− cells were able to polarize efficiently in single cell chemotaxis assay. Preliminary observations of labeled mlcE− cells within the wild-type aggregation stream suggest that the mlcE− cells were able to maintain regular cell shape without the significant distortion observed in MHC mutants (Shelden and Knecht, 1995). Their efficiency for cell shape change during later developmental stages remains to be tested.

Our analysis has also revealed other myosin-dependent motile events during mlcE− development that were affected to a lesser extent by the absence of ELC and resulted in less efficient motility. While the chemotactic directionality of mlcE− cells was similar to wild-type cells, their rate of movement in a chemotactic gradient was reduced. This reduced chemotactic motility is consistent with the delay of aggregation in mlcE− cells. To our surprise, the mlcE− prestalk A cells seemed as competent as wild type to sort into the tip, a process shown to be myosin dependent (Elliott et al., 1993; Traynor et al., 1994). The mlcE− prestalk A cells were less efficient at completing the transition from tipped mounds to fingers although in about half the mounds, the reduced motility appeared sufficient to support the formation of aberrant fruiting bodies. Our studies have therefore revealed variable degrees of defects among myosin-based morphogenetic motilities during development of mlcE− cells and have provided evidence suggesting that these motile events have different threshold requirements for myosin function.

Intracellular myosin localization in mlcE− cells appeared normal. Myosin can be seen at the cell cortex as well as in the cleavage furrow. The amount of myosin found in the Triton-insoluble cytoskeleton of mlcE− cells was comparable to that of wild type (data not shown). This is in contrast to mlcR− cells in which myosin accumulates in a more endoplasmic location forming dense patches (Chen et al., 1994). Therefore, unlike mlcR− cells in which mislocalization of myosin may be a major cause of the cytokinesis defect, the similar phenotype in mlcE− cells seems likely caused by a functional defect of myosin. The myosin-containing cytoplasmic bridges observed in suspension grown mlcE− cells following attachment to a surface suggest that the ‘cleavage furrow’ was not capable of completing contraction. The mlcE− cells appear to divide by a process described by Fukui et al. (1990) as ‘traction-mediated cytokinesis’. It has been recently postulated that ‘traction-mediated cytofission’ may be part of the regular mechanism of cytokinesis for cells grown on a solid surface (DeBiasio et al., 1994). This idea is consistent with our observations that mlcE− cells were able to grow at a normal rate on culture plates in spite of the cytokinesis defect in suspension culture. Most of the microtubules seemed to distribute away from myosin-rich regions. Fukui et al. (1990) have observed in MHC mutants that microtubules penetrate into the cortical cytoskeleton and they attribute this phenomenon to the weakened cortical cytoskeletal architecture resulting from the absence of myosin. Our observation would then suggest that this role of myosin in strengthening cytoskeletal architecture requires only the presence of myosin and a functional defective myosin such as ELC− myosin was able to perform the task.

The mlcE− cells were able to respond to chemoattractant by polarizing and moving toward the source but exhibited a lower rate of movement. This is different from the chemotactic behavior of MHC mutants in which both chemotactic index and movement rate were defective (Wessels et al., 1988). Since mlcE− cells contain myosin that can localize properly but
exhibits an enzymatic defect, the difference in chemotactic directionality between mlcE- cells and MHC mutants suggests that proper orientation of cells toward chemoattractant requires the proper localization of myosin. Consistent with this notion, MHC mutants containing assembly incompetent myosin (HMM) also exhibited defective chemotactic directionality (Wessels et al., 1988). The rate of chemotactic motility, however, appears to depend on the contractile function of myosin. As a result, the mlcE- cells which contain functionally defective myosin exhibited less efficient movement in a chemotactic gradient.

We have observed reduced association of RLC to MHC in ELC- myosin in vitro. This result is consistent with the structural models of chicken skeletal muscle myosin S1 (Rayment et al., 1993) and scallop myosin regulatory domain (Xie et al., 1994). Extensive side chain interactions between the light chains and with the head-rod junction of the heavy chain suggest that the light chains support the alpha-helical head-rod junction. The absence of ELC appears to disrupt the interactions in this region weakening the association of RLC with MHC, although immunoprecipitation studies and the presence of normal levels of RLC in cytoskeletal preparations suggest that in vivo RLC is associated with most of the myosin.

In considering the mechanism for the phenotypic defects observed in mlcE- cells, it seems likely that the abnormal aggregation of purified ELC- myosin does not reflect its in vivo state since myosin localization in mlcE- cells and the level of bound RLC in vivo were similar to wild type. Since current models suggest that the light chains provide support for the head-rod junction to function as a lever in amplifying conformational changes during a power stroke (Rayment et al., 1993; Uyeda and Spudich, 1993; Vibert and Cohen, 1988), it is likely that the absence of ELC reduces its function as a lever and results in less efficient motor function. It is also possible that the absence of ELC contributes to a defect in enzymatic function, specifically actin activated ATPase. Thus it seems likely that the phenotypes of mlcE- cells resulted from a combination of both mechanical and enzymatic defects in ELC- myosin. Cytokinesis would appear to be the process most susceptible to defective myosin function. During the chemotactic response, the ability to orient and rates of motility showed different sensitivities to the reduction of myosin function. The transition from mound stage to finger stage may represent a threshold range that challenges the functional limit of the defective ELC- myosin. The threshold may be lower in some mounds so that the reduced myosin function was able to support further development. Similar arguments may apply to the process of prespore cell localization later during development. One potentially important difference between the mhcA- (MHC null mutant), mlcR- and mlcE- cells is the localization of myosin. In the mhcA- cells, the absence of myosin heavy chain eliminates thick filaments completely. In the mlcR- cells, much of the myosin appears to be abnormally localized (Chen et al., 1994). In contrast, the mlcE- cells showed normal localization of a myosin with reduced enzymatic activity. Thus the differences in phenotype between mhcA-, mlcR- and mlcE- cells may reflect the consequences of no myosin, abnormally localized myosin and normally localized less active myosin, respectively. If this is true, it suggests a potentially important structural role for non-muscle myosin in addition to its enzymatic role in contractile processes. The phenotypes of mlcE- cells therefore most likely result from an interplay between the functional defects of myosin and different threshold requirements for myosin function among myosin-dependent processes.

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