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Myosin light chain kinase A is activated by cGMP-dependent and cGMP-independent pathways

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Abstract Stimulation of *Dictyostelium* cells with the chemoattractant cAMP results in transient phosphorylation of the myosin regulatory light chain (RLC). We show that myosin light chain kinase A (MLCK-A) is responsible for RLC phosphorylation during chemotaxis, and that MLCK-A itself is transiently phosphorylated on threonine-166, dramatically increasing its catalytic activity. MLCK-A activation during chemotaxis is highly responsive to cellular cGMP levels and the cGMP-binding protein GbpC. MLCK-A[−] cells have a partial cytokinesis defect, and do not phosphorylate RLC in response to concanavalin A (conA), but cells lacking cGMP or GbpC divide normally and phosphorylate in response to conA. Thus MLCK-A is activated by a cGMP/GbpC-independent mechanism activated during cytokinesis or by conA, and a cGMP/GbpC-dependent pathway during chemotaxis.

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Keywords: *Dictyostelium*; Chemotaxis; cGMP; Myosin; Regulatory light chain; Phosphorylation

1. Introduction

Motile cells such as *Dictyostelium* respond to chemoattractant gradients by adopting a polarized morphology, allowing them to move up the gradient. Under starvation conditions, *Dictyostelium* cells both secrete and move toward cAMP, thereby using cAMP chemotaxis to form aggregates of cells. As a simple eukaryote with a highly developed chemotactic response, *Dictyostelium* is an ideal model system for elucidating the signaling pathways and cytoskeletal rearrangements responsible for chemotaxis [1].

The protrusive force for migration in eukaryotic cells comes from actin polymerization at the leading edge of the cell. Pioneering work in *Dictyostelium* has shown that myosin II, first described as the motor responsible for muscle contraction, also contributes to motility during chemotaxis [2]. Myosin is localized to the rear of polarized cells in a cAMP gradient [3], where it probably suppresses pseudopod formation and promotes retraction of the rear of the cell [4,5].

Both myosin heavy chain and the regulatory light chain (RLC) are transiently phosphorylated after stimulation with extracellular cAMP, and there is a transient increase in myosin association with the cytoskeleton [6,7]. RLC phosphorylation increases the motility and actin-activated ATPase activity of myosin [8]. Cells expressing a mutant RLC with alanine at the phosphorylation site (“S13A cells”) form fewer lateral pseudopods during chemotaxis, and do not lose their polarity at the peak concentration of a cAMP wave as wild-type cells do, indicating that RLC phosphorylation contributes to chemotaxis [9].

Chemotaxis is accompanied by a transient increase in intracellular cGMP levels [10,11], and cells unable to synthesize cGMP have a reduced RLC phosphorylation response to cAMP, as do cells lacking GbpC, a complex cGMP-binding protein containing ras, protein kinase, and rasGEF domains [12–14]. These studies indicate that cGMP and GbpC play major roles in RLC phosphorylation during chemotaxis. Cells lacking cGMP and GbpC have additional defects in myosin heavy chain phosphorylation and myosin assembly, and chemotax very poorly [13].

RLC is phosphorylated by myosin light chain kinase A (MLCK-A) and one or more other kinases that have not yet been identified [15]. MLCK-A is, respectively, activated approximately 7- and 200-fold by phosphorylation of threonine-289 near its C-terminus and threonine-166 in its activation loop ([16–18] and J.S., unpublished results). Unlike its well-known namesakes in the MLCK family of kinases, MLCK-A, a CAMK1 family member, is not regulated by Ca²⁺/calmodulin binding. MLCK-A is phosphorylated in response to cGMP addition in crude lysates, and by treatment of vegetative cells with conA [15,16]. MLCK-A knock-outs also have a partial defect in cytokinesis, suggesting that MLCK-A is activated during cytokinesis, as well [15]. Here we show that MLCK-A is solely responsible for RLC phosphorylation during chemotaxis, and that at least two signaling pathways are responsible for MLCK-A activation: a cGMP/GbpC-independent pathway that is activated by conA treatment and during cytokinesis, and a cGMP/GbpC-dependent pathway that predominates during chemotaxis.

2. Materials and methods

2.1. In vivo phosphorylation of RLC and MLCK-A

Starved cells were pulsed with cAMP, treated with caffeine, labeled with ³²P[P_i], and stimulated with cAMP and DTT as described [13], except that typically 60 nM cAMP pulses were used. RLC and MLCK-A

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were immunoprecipitated as described [17], and ^{32}P incorporation was quantitated using a BioRad GS-505 Molecular Imager System. The myosin heavy chain was coimmunoprecipitated, and its intensity on coomassie stained gels was used to verify equal loading over the time courses. RLC phosphorylation after conA treatment was determined using urea glycerol gels as described previously [16].

2.2. Cyclic GMP levels during conA treatment

Cells were harvested and washed in 20 mM 2-(N-morpholino) ethanesulfonic acid-KOH, pH 6.8, 2 mM MgCl_2 , and resuspended at 2×10^8 cells/ml. Air was bubbled through the cell suspension for 10 min, conA was added to 75 $\mu\text{g}/\text{ml}$, and cGMP levels were measured as described [13].

2.3. *gbc* gene disruption

A genomic PCR product spanning codons 1855–2048 of *gbc* (579 bp) was inserted into the *Xba*I/*Bam*HI sites of pBsr Δ BamHI (kindly provided by Dr. Kazoh Sutoh), and a second genomic PCR product corresponding to codons 749–1002 (759 bp) was inserted into the *Hind*III site. In the resulting plasmid, 2.6 kb of the *gbc* gene are replaced with the 1.3 kb Bsr cassette, with the Bsr and *gbc* open reading frames on opposite strands. The insert of this plasmid (pJS84) was amplified by PCR, gel purified, and 3 μg was electroporated into JH10 cells [19]. Colonies were selected using 5 $\mu\text{g}/\text{ml}$ blasticidin (Invitrogen). Genomic DNA was isolated from resistant colonies using DNAzol (Invitrogen), and used to screen candidates by PCR. Additional DNA for Southern blotting was isolated as described [20].

2.4. Dictyostelium plasmids and cell lines

Extrachromosomal plasmids expressing MLCK-A^{T166A} and MLCK-A^{T289A} have been described [17]. The *mlkA*[−] line in JH10 cells, and the *gca*[−]/*sgc*[−], *gbcA*[−]/*gbcB*[−] and *gbcC*[−] lines in DH1 cells have also been described [13,15,21].

3. Results

3.1. MLCK-A is responsible for RLC phosphorylation during chemotaxis

We used metabolic labeling to examine RLC phosphorylation after chemotactic stimulation in wild-type and MLCK-A null (*mlkA*[−]) cells to determine whether MLCK-A is the kinase responsible for RLC phosphorylation during chemotaxis. In the JH10 parental strain, RLC phosphorylation increased dramatically after stimulation with cAMP, with a maximum at approximately 60 s, whereas RLC phosphorylation in MLCK-A nulls did not (Fig. 1A and B). Thus MLCK-A is the only kinase responsible for the chemoattractant-elicited increase in RLC phosphorylation. The basal level of RLC phosphorylation seen in the MLCK-A nulls is due to an additional MLCK activity that has not been identified.

MLCK-A is itself transiently phosphorylated during chemotaxis, with a maximum ~20 s after stimulation (Fig. 1C). We expressed MLCK-A^{T166A} and MLCK-A^{T289A} in *mlkA*[−] cells in order to determine which site is phosphorylated in response to cAMP. Phosphorylation of MLCK-A^{T289A} increased dramatically, while phosphorylation of MLCK-A^{T166A} increased only slightly (Fig. 1C), indicating that chemotactic stimulation results in phosphorylation primarily of threonine-166. RLC phosphorylation levels in cells expressing MLCK-A^{T166A} are greatly reduced (Fig. 1A and B), indicating that phosphorylation at threonine-166 is necessary for robust RLC phosphorylation. The fold increase in RLC phosphorylation in MLCK-A^{T289A} cells is indistinguishable from wild-type, within experimental error (Fig. 1B). The higher basal level of RLC phosphorylation in these cells is probably due

Fig. 1. MLCK-A is required for RLC phosphorylation during chemotaxis, and is activated by threonine-166 phosphorylation. Cells were labeled with [^{32}P]orthophosphate, and RLC and MLCK-A were immunoprecipitated from aliquots at the indicated times (in seconds) before and after stimulation with cAMP. (A) RLC phosphorylation in the wild-type strain (JH10), MLCK-A null cells (*mlkA*[−]), and *mlkA*[−] cells overexpressing MLCK-A^{T289A} and MLCK-A^{T166A}. Gels for JH10 and *mlkA*[−] are representative of four independent experiments, and gels for MLCK-A^{T289A} and MLCK-A^{T166A} are representative of two independent experiments. (B) RLC phosphorylation was quantitated by phosphorimaging gels such as those presented in Part A, and the ratio of ^{32}P incorporation at 60 and −15 s is plotted. Error bars indicate the S.D. obtained from averaging two or more data sets. The statistical significance that the maximal response in the mutant strains differs from wild-type was tested with Student's *t* test. For *mlkA*[−], $P = 0.05$; T289A, $P = 0.49$ (not significant); T166A, $P = 0.13$. (C) MLCK-A phosphorylation in JH10, and *mlkA*[−] cells overexpressing MLCK-A^{T166A} or MLCK-A^{T289A}. For JH10, data is representative of four independent experiments, and for the MLCK-A^{T166A} and MLCK-A^{T289A} overexpressors, similar results were obtained in two independent experiments. All exposures for panels A and B were for 24 h, using BioMax MS film (Kodak) and one intensifying screen.

to the fact that the mutant MLCK-A proteins are overexpressed ~10-fold (data not shown). Overexpression probably also serves to magnify the small increase in RLC phosphorylation that is observed in MLCK-A^{T166A} cells. We also examined whether recruitment of MLCK-A to the cell cortex might provide an additional activation mechanism. MLCK-A remained diffusely localized in the cytosol after cAMP stimulation, suggesting that a change in localization does not contribute to MLCK-A's activation (data not shown).

3.2. MLCK-A is activated by cGMP and GbpC during chemotaxis

In *Dictyostelium* cGMP is synthesized by two guanylyl cyclases, encoded by *gca* and *sgc* genes, and in *gca*[−]/*sgc*[−] cells, no detectable cGMP is present [21–23]. In these cells, a smaller in-

crease in MLCK-A phosphorylation is observed after stimulation with cAMP compared with the DH1 parental strain (Fig. 2). Thus MLCK-A phosphorylation is mostly, but not exclusively, dependent on cGMP. Cells lacking the phosphodiesterases GbpA and GbpB have elevated basal and stimulated levels of cGMP, and stimulated levels do not return to baseline until 3–4 min compared with 30 s for wild-type cells [13]. In *gbpA⁻gbpB⁻* cells, MLCK-A phosphorylation is sustained and slightly elevated compared to wild-type cells, suggesting that sustained cGMP levels results in sustained MLCK-A activation (Fig. 2).

GbpC is implicated in mediating the effects of cGMP in response to chemoattractants, and experiments on a *gbpC* gene disruption strain engineered in DH1 cells point to a key role for this protein in myosin regulation during chemotaxis [12,13]. In order to evaluate the role of GbpC in MLCK-A activation, we disrupted the genomic *gbpC* locus in JH10 cells, since this is the genetic background used for our other studies on MLCK-A. The *gbpC* gene was disrupted using the homologous recombination strategy summarized in Fig. 3. In a PCR screen, 15/15 transformants had a disrupted *gbpC* locus. Seven gave a PCR product of the expected size, but the rest gave a product that was ~0.3 kb smaller than expected, and were not further characterized. Southern blotting was used to confirm that the disrupted *gbpC* genomic locus had the predicted structure, and that the construct had not integrated into a secondary site (Fig. 3B), and cGMP-binding assays on lysates

from the *gbpC⁻* cells confirmed that the protein is gone (data not shown). During development, these cells formed aggregates and fruiting bodies that were approximately half the size of wild-type (Fig. 3C). This is consistent with a severe defect in chemotaxis to cAMP, as has been characterized in detail for *gbpC⁻* cells isolated in the DH1 parental strain [13].

In metabolic labeling experiments using JH10 cells, cAMP stimulation resulted in MLCK-A phosphorylation levels that were 10.2-fold higher than those before stimulation, compared with 3.1-fold higher in *gbpC⁻* cells isolated in the JH10 background (Fig. 2). In parallel experiments in the DH1 background, a comparable effect of a *gbpC* gene disruption was observed (Fig. 2), although for unknown reasons the MLCK-A phosphorylation response was much more dramatic in JH10 than in DH1 cells. The decreases in MLCK-A phosphorylation that we observe in cell lines lacking cGMP and GbpC correlate with decreases in RLC phosphorylation in these strains ([13]; JLS, unpublished results). Thus GbpC is needed for the full MLCK-A and RLC phosphorylation responses.

3.3. *GbpC* and cGMP do not play a role in cytokinesis

Since GbpC is implicated in myosin II regulation during chemotaxis it might also be involved in the regulation of myosin during cytokinesis. Myosin II is essential for cytokinesis in suspension cultures; in cells lacking functional myosin, an accumulation of large, multinucleate cells, rather than an increase in cell number, is observed [24,25]. We found that growth rates in axenic media for the two *gbpC⁻* cell lines were indistinguishable from their parental strains, indicating that *gbpC⁻* cells are able to divide in suspension (data not shown). Using DAPI staining to visualize the nuclei, no increase in multinucleate cells was observed in either *gbpC⁻* strain, or in *scg⁻/gca⁻* cells (Fig. 3D). Thus, cGMP and GbpC appear to be dispensable for cytokinesis.

3.4. *ConA*-activation of MLCK-A is not mediated by cGMP or *GbpC*

In light of our findings that MLCK-A is activated by cGMP and GbpC during chemotaxis, we investigated whether this same pathway activates MLCK-A after conA treatment. We found that cGMP levels remain essentially unchanged after conA treatment of cells (Fig. 4A). We used *gca⁻/sgc⁻* cells to evaluate whether basal levels of cGMP might be needed for RLC phosphorylation after conA treatment. For wild-type cells approximately half of the RLC is phosphorylated prior to conA treatment, and increases to 100% or nearly 100% after treatment (Fig. 4B, see also [15,16]). In *gca⁻/sgc⁻* cells, quantitative phosphorylation of RLC is observed after conA treatment indicating that cGMP is not required for the response, and GbpC was also found to not be required (Fig. 4B).

4. Discussion

In order to understand signaling pathways and myosin regulation during chemotaxis, it is important to know whether RLC phosphorylation involves MLCK-A, another MLCK (which is present but has not been identified), or a combination of enzymes. Here we show that MLCK-A is solely responsible for RLC phosphorylation during chemotaxis, and that

Fig. 2. Cyclic GMP and GbpC promote MLCK-A activation in vivo. (A) MLCK-A was immunoprecipitated from aliquots of ³²P-labeled cultures at the indicated times before and after stimulation with cAMP. Incorporation of ³²P into MLCK-A was visualized by autoradiography of SDS gels. All gels were exposed to BioMax MS film (Kodak) for 24 h, using one intensifying screen. (B) MLCK-A phosphorylation was quantitated by phosphorimaging gels such as those presented in Part A. The ratio of ³²P incorporation at the maximum time point (20–30 s for all cell lines except *gbpA⁻/gbpB⁻*) and at -15 s is plotted. Error bars indicate the S.D. obtained from averaging at least two data sets. The statistical significance that the maximal response in the mutant strains differs from wild-type was tested with Student's *t* test. For *gca⁻*, *P* = 0.07; A/B⁻, *P* = 0.34 (not significant); C⁻ (DH1 background), *P* = 0.20; C⁻ (JH10 background), *P* = 0.04.

Fig. 3. Gene disruption of *gbpC*. (A) The strategy for disrupting *gbpC* in the parental strain JH10 using a construct that replaces an internal portion of *gbpC* with a cassette encoding blasticidin resistance (Bsr). The primers used in the PCR screen are indicated as convergent arrows. The domains in GbpC are abbreviated as follows: LRR, leucine rich repeats; Ras, raslike domain; kinase, protein kinase domain; RasGEF, N-terminal Ras guanine nucleotide exchange factor domain; DEP, domain found in dishevelled, Egl-10, and pleckstrin; cG, cGMP-binding domain; GRAM, domain found in glucosyltransferases, Rab-like GTPase activators, and myotubularins. (B) Southern blot confirming the structure of the disrupted *gbpC* locus, and demonstrating that the construct did not insert into secondary sites. DNA from the parental strain (lanes 1 and 3) and the gene disruption strain (lanes 2 and 4) was digested with *HpaII* and probed with Bsr (probe #1, lanes 1 and 2) or a portion of *gbpC* which is retained in the disrupted locus (probe #2, lanes 3 and 4). These probe fragments are indicated in Part A. (C) Wild-type (JH10) and *gbpC*[−] cells were developed on black HA filters as described [28], and photographed at identical magnification. (D) The number of nuclei per cell was determined by DAPI staining of cells that had been grown and fixed in suspension. White bars, JH10 cells; dark grey bars, *gbpC*[−] in JH10 background; striped bars, DH1; black bars, *gbpC*[−] in DH1 background; light grey bars, *gca*[−]*lsgc*[−] in DH1 background.

Fig. 4. Activation of MLCK-A by conA treatment is GbpC/cGMP-independent. (A) Cellular cGMP levels were measured in the wild-type strain Ax3. Data points are the average of three independent measurements, and the error bars indicate the S.D. (B) In vivo changes in RLC phosphorylation in vegetatively growing cells after mock-treatment with buffer or after conA treatment (lanes marked “−” and “+”, respectively) were determined by running whole cell lysates on urea-glycerol gels. The RLC was visualized by immunoblotting, and the positions of unphosphorylated and phosphorylated forms are indicated.

MLCK-A activation after cAMP stimulation is associated with an increase in phosphorylation of the activation-loop residue threonine-166, which dramatically increases the enzymatic activity of MLCK-A [17].

We also show that maximal phosphorylation (and hence activation) of MLCK-A depends on GbpC, and is highly

responsive to cellular cGMP levels. The role of GbpC and cGMP in MLCK-A activation essentially parallels our previous findings that cGMP and GbpC promote RLC phosphorylation during chemotaxis [13], and allows us to formulate a relatively complete model of the signaling pathway resulting in RLC phosphorylation during chemotaxis (Fig. 5). Chemoattractants lead to the activation of guanylyl cyclases, resulting in an increase in intracellular cGMP levels. GbpC, which binds cGMP with high affinity [12], is activated by this increase in cGMP levels. GbpC, in turn, either phosphorylates MLCK-A on threonine-166, or activates another protein kinase that does so, and the resulting increase in MLCK-A activity leads to a robust increase in RLC phosphorylation [16].

By contrast, we show here that MLCK-A activation in response to conA is not the result of cGMP signaling and GbpC. We also found that a small amount of MLCK-A activation during chemotaxis is cGMP/GbpC-independent. An economical model consistent with our data is that the cGMP/GbpC-independent pathway responsible for the conA response is the same as the cGMP/GbpC-independent pathway that plays a minor role during chemotaxis (Fig. 5).

An increase in RLC phosphorylation during cytokinesis has been observed in several studies using mammalian tissue culture cells [26], supporting a role for myosin II as a molecular motor in the contractile ring during this process. MLCK-A nulls have a partial defect in cytokinesis [15], suggesting it is activating during cytokinesis. This would result in an increase in RLC phosphorylation during cytokinesis, as in mammalian cells. Surprisingly, the cytokinesis defect observed in MLCK-A nulls is not shared by S13A cells, which harbor a non-phos-

Fig. 5. Model for the signaling pathways controlling RLC phosphorylation. RLC phosphorylation in *Dictyostelium* is carried out by MLCK-A and one or more additional kinases, represented as “other(s)” in the cartoon. According to the model, the other kinase(s) only contributes to constitutive RLC phosphorylation. Weakly activated pathways are indicated by a dotted arrow, and stronger pathways by a solid arrow. ConA and cAMP binding to receptors on the cell surface lead to MLCK-A activation and RLC phosphorylation by pathways indicated. Cytokinesis may also lead to MLCK-A activation by the cGMP/GbpC-independent pathway, but since the evidence for this pathway is indirect, it is marked with a question mark.

phorylatable RLC [27], indicating that MLCK-A may have additional important substrates during cytokinesis. Since *gbpC⁻* and *gca⁻lsgc⁻* cells do not appear to have a cytokinesis defect, the pathway activating MLCK-A during cytokinesis is most likely cGMP/GbpC-independent. Indeed, *gbpC* mRNA levels increase dramatically by 8 h of development [12], providing additional support for our model that GbpC plays a major role in cAMP chemotaxis, but little or no role during cytokinesis or in response to conA treatment, both processes of the vegetative cell.

The present study has demonstrated a decisive link between cGMP, GbpC, MLCK-A activation, and RLC phosphorylation during chemotaxis. It also provides evidence for a cGMP/GbpC-independent pathway that is involved in conA capping, plays a minor role during chemotaxis, and may also be activated during cytokinesis. Further studies aimed at identifying the components of this cGMP/GbpC-independent pathway may provide valuable insights into each of these processes.

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