Dictyostelium Myosin II Is Regulated during Chemotaxis by a Novel Protein Kinase C*

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The myosin II heavy chain (MHC)-specific protein kinase C (MHC-PKC) isolated from Dictyostelium discoideum has been implicated in the regulation of myosin II assembly in response to the chemoattractant, cAMP (Ravid, S., and Spudich, J. A. (1989) J. Biol. Chem. 264, 15144–15150). Here we report that elimination of MHC-PKC results in the abolishment of MHC phosphorylation in response to cAMP. Cells devoid of MHC-PKC exhibit highly phosphorylated MHC and exhibit impaired myosin II localization and no apparent cell polarization and chemotaxis. The results presented here provide direct evidence that MHC-PKC phosphorylates MHC in response to cAMP and plays an important role in the regulation of myosin II localization during chemotaxis.

When cells of Dictyostelium are starved, they acquire the ability to bind cAMP to specific cell surface receptors and to respond to this signal by chemotaxis. The process of chemotaxis involves phosphorylation and reorganization of myosin II (1–5). In response to cAMP stimulation, myosin II, which exists as thick filaments, translocates to the cortex (5). This translocation is correlated with a transient increase in the rate of myosin II heavy chain (MHC) as well as light chain phosphorylation (3, 4, 6). In addition, in vitro studies strongly suggest that MHC phosphorylation plays an important role in the regulation of myosin II filament formation (7–10). The importance of MHC phosphorylation in the regulation of myosin II in vivo was demonstrated by Egelhoff et al. (11). They found that elimination of the MHC phosphorylation sites allows in vivo contractile activity, but this myosin II shows substantial overassembly. Mimicking the negative charge state of phosphorylated myosin II eliminates filament formation in vitro and renders the myosin II unable to drive any tested contractile event in vivo (11).

We previously reported the isolation of a MHC-specific PKC (MHC-PKC) from Dictyostelium that phosphorylates Dictyostelium MHC specifically and is homologous to α, β, and γ subtypes of mammalian PKC (9, 12). This kinase, which is membrane-associated and is expressed during Dictyostelium development, was implicated in the increase in MHC phosphorylation during chemotaxis in this species (9). In vitro phosphorylation of MHC by MHC-PKC results in inhibition of myosin II thick filament formation (9), by inducing the formation of a bent monomer of myosin II whose assembly domain is tied up in an intramolecular interaction that precludes intermolecular interaction, which is necessary for thick filament formation (10). The findings that MHC-PKC is a member of the PKC family and that it regulates myosin II assembly suggest a link between the extracellular chemotactic signal and subsequent intracellular events.

A considerable amount of information is now available regarding the regulation of myosin II by MHC phosphorylation, and a piecemeal is beginning to emerge in which the molecular changes in myosin II that are involved in MHC phosphorylation are related to cAMP-induced directed cell movement. Nevertheless, the molecular mechanism by which a cAMP signal is transmitted to myosin II, resulting in myosin II localization and chemotaxis, remains unclear. In an attempt to throw some light on this issue, we studied the role of MHC-PKC in vivo by eliminating and by overexpressing the MHC-PKC protein in Dictyostelium cells. Analysis of these cell lines allowed us to address directly the role of MHC-PKC in vivo and consequently the role of MHC phosphorylation in the regulation of myosin II. The results presented here indicate that MHC-PKC is a key modulator in the regulation of myosin II localization in response to the chemoattractant, cAMP.

EXPERIMENTAL PROCEDURES

Plasmid Constructs and Cell Lines—All DNA manipulations were carried out using standard methods (13). The vector pBS-MHCK (12) contains a 2.6-kb MHC-PKC cDNA clone. The MHC-PKC catalytic domain was deleted by digestion with Clal and filled in with Klenow enzyme. The vector pGEM26 containing the Thy1 gene, which complements the thymidine growth requirement of the Dictyostelium thymi- dine auxotroph strain J H10 (14), was restricted with BamHI and filled in with Klenow enzyme. The two fragments were ligated to produce the disruption vector pThy1-MHC-PKCαcat (Fig. 1A).

pDRE106 (see Fig. 2A) was produced by fusing an actin-5S promoter from pSc79 (15) to the cDNA clone encoding the MHC-PKC (12), and ligating this fused product into the extrachromosomal vector pDRE (16). The resulting plasmid pDRE106 contains the entire MHC-PKC coding region fused in phase to actin-5S promoter and in an antisense orientation relative to the neomycin gene (Fig. 2A). Full details of the construct are available upon request.

For MHC-PKC disruption experiments, we used the Dictyostelium J H10 strain that is an auxotroph for thymidine. Cells were grown in H 5 medium and 50 µg/ml thymidine (Sigma). To express the MHC-PKC we used the Dictyostelium AX2 strain, that were grown in H 5 medium. To express the MHC-PKC along with 3X ALA myosin II (11), we co-transformed the Dictyostelium myosin II null cells (HS1 strain) with the pDRE160 and pBIG-ALA (11) expression vectors. Transformation was carried out using the calcium phosphate procedure (17). Transformants for the disruption of MHC-PKC were selected on the basis of their ability to grow in the absence of thymidine. Transformants for MHC-PKC and 3X ALA expression were selected on the basis of their resistance to the antibiotic G418 (Boehringer Mannheim), and their ability to grow in suspension since HS1 cells are unable to grow in suspension (11). Clones were initially screened with G418 at a concentration of 10 µg/ml, which was increased to 50 µg/ml. Because MHC-
PKC- cells were found to express the maximum amount of MHC-PKC in the presence of 50 μg/mL G418, all subsequent experiments were performed using this concentration. G418-resistant clones were screened by Western blot analysis using MHC-PKC polyclonal antibodies.

Dictyostelium cell lines were developed on MES plates (20 mM MES (pH 7.5), 0.2 mM CaCl₂, 2 mM MgSO₄, 2% agar) as described by Berlot et al. (3).

Southern and Western blot analysis—Dictyostelium DNA blotting was performed as described by Sambrook et al. (13). DNA probes were generated by random primer probe synthesis using a Random Prime DNA labeling kit (Boehringer Mannheim) according to the manufacturer's instructions.

For Western blot analysis, cells were developed for 4 h in shaking flasks as described (3). Samples were prepared from whole cell lysates (18) or from the insoluble fraction (9). Protein was determined by the method of Peterson (19), and lysates were electrophoresed on 10% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) (20). The Western blots were probed with affinity-purified MHC-PKC polyclonal antibody (12). This antibody recognizes the full-length MHC-PKC as well as the R domain.

MHC-PKC Activity Assay—MHC-PKC activity was assayed by two methods. In the first method, MHC-PKC activity was assayed directly on the kinase extracted from the insoluble cell fraction. Following resuspension of the insoluble cell fraction, cells were incubated at 22°C on a rotator. Reaction was initiated by the addition of ATP and stopped by the addition of 5% trichloroacetic acid to the supernatant containing sonication buffer, (10 mM Tris (pH 7.5), 50 mM KCl, 2 mM phenylmethylsulfonyl fluoride, 200 μM leupeptin, 200 μM pepstatin), they were lysed by sonication using an ultrasonic cell disruptor (Microson) model XL with a small-sized tip at 50% output power, and the extract was spun for 20 min at 4°C. MHC-PKC was extracted from the insoluble fraction using sonication buffer containing 0.5 mM KCl, as described (9). For kinase assay, 50 μg of solubilized MHC-PKC was incubated with LMM58 (0.5–1 mg/ml), 6 mM MgCl₂, 0.2 mM [γ-32P]ATP (500 cpn/ml), 1 mM dithiothreitol for 10 min at 22°C on a rotator. Reaction was initiated by the addition of ATP and stopped by the addition of 5% trichloroacetic acid. The precipitated LMM58 were pelleted in a microcentrifuge after incubation for 15 min on ice, washed twice with 5% trichloroacetate-buffer, and suspended in 20 μl of SDS-PAGE sample buffer, and electrophoresed on 7% SDS-PAGE gels. To determine incorporation of 32P into LMM58, the gels were stained and destained and the bands were cut out of the gels and counted in a scintillation counter in 5 ml of scintillation fluid.

In the second method, MHC-PKC was immunoprecipitated from the soluble and insoluble fractions described above. The supernatants of these fractions were added to preadsorbed MHC-PKC antibody-Staphylococcus A cell mixture prepared as described by Berlot et al. (3) and incubated for at least 1 h at 4°C with rotation. The immunoprecipitated MHC-PKC from the soluble and insoluble fractions was assayed for kinase activity as described above, except that the reaction was stopped by running the Staphylococcus A cell mixture containing the MHC-PKC and adding of 5% trichloroacetic acid to the supernatant to precipitate the LMM58.

In Vivo Phosphorylation of MHC—To measure in vivo phosphorylation of MHC, the 32P-labeling and cAMP stimulation of PKC activity in the membrane fraction, having shown previously that intact MHC-PKC is required for the expression of R domain, but we could not detect it in MHC-PKC. In contrast, we found that 30% of the MHC-PKC would be expressed. We found that 30% of the MHC-PKC was inactivated by direct activation of the R domain and not the regenerated copy. We constructed a disruption vector that lacks the C domain but contains the R domain and the thy1 promoter. The Dictyostelium J H10 cell line (14) was transformed with the plasmid pThy1-MHC-PKC Δcat (Fig. 1A), and transformants were screened for the deletion by genomic Southern blotting. Fig. 1A shows the expected changes in the map of the MHC-PKC locus, and Fig. 1B shows a genomic Southern blot confirming the deletion. According to the map, the R domain has at its starting site the MHC-PKC native promoter; in contrast, the regenerated intact copy of MHC-PKC has no promoter at its starting site since this promoter is missing from the pThy1-MHC-PKC Δcat. We therefore expected that the R domain and not the regenerated copy of MHC-PKC would be expressed. We found that 30% of the transformants exhibited a pattern consistent with gene disruption events.

Several independent disrupted MHC-PKC clones (MHC-PKC- cells) and Dictyostelium J H10 cells were subjected to Western blot analyses (Fig. 1C). J H10 cells did not express the MHC-PKC protein during their vegetative stage (data not shown), as was reported for Ax3 cells (12), but did express it after 4 h of development (Fig. 1C). We had expected that the MHC-PKC disruption construct would result in the expression of R domain, but we could not detect it in MHC-PKC- cells either in the vegetative stage (data not shown) or in the developmental stage (Fig. 1C). Our results may indicate either that the R domain is not expressed at all or that it is expressed but is unstable and degraded.

Western and Southern analyses confirmed that the MHC-PKC gene was disrupted. To ensure that disruption of the MHC-PKC gene also results in elimination of its activity, we assayed J H10 and MHC-PKC- cells for the presence of MHC-PKC activity in the membrane fraction, having shown previ-
The JH10 cells were found to contain MHC-PKC activity (20 pmol/min/mg) that is comparable to the activity published previously for Dictyostelium Ax3 cells (9). In contrast, the MHC-PKC2 cells displayed negligible amounts of kinase activity (0.01 pmol/min/mg) associated with the membrane fraction. These results suggest that disruption of the MHC-PKC gene resulted in elimination of its activity and that MHC-PKC is the only MHC kinase (MHCK) activity in the membrane of Dictyostelium.

Overexpression of the MHC-PKC Protein—To overexpress the MHC-PKC protein, we constructed the expression vector pDRE106 (Fig. 2A), which was used to transform Dictyostelium.

**Fig. 1. Disruption of the MHC-PKC gene.** A, integration of pThy1-MHC-PKCcat into the MHC-PKC gene. The circle represents the transformation plasmid pThy1-MHC-PKCcat, which contains the MHC-PKC regulatory domain coding region (R), the Thy3 gene, and the plasmid sequence of the Bluescript vector. In the center of the diagram is the chromosomal region of the MHC-PKC gene (12). Integration of one copy of pThy1-MHC-PKCcat into the MHC-PKC locus would result in the map illustrated. The horizontal arrows indicate the size in kilobases of the restriction fragments that can be detected with a 1.0-kb probe (thick line) directed against the catalytic domain coding portion. B, Southern analysis of the MHC-PKC gene in Dictyostelium JH10 (wild type) and MHC-PKC2 cell lines. Restriction maps were generated using a probe that hybridized only to the C domain of MHC-PKC. This region is not represented in the transformation plasmid pThy1-MHC-PKCcat, and thus serves as a specific probe for the MHC-PKC locus. The map obtained by hybridizing the C domain probe to the restriction digest of DNA of wild type cells revealed a 10-kb BglII and a BglII/PstI fragment (lanes 1 and 3), as predicted in Fig. 1A. This restriction pattern was altered in MHC-PKC- cells, in that the BglII fragment had shifted to a fragment of about 18 kb, and the BglII/PstI digest revealed a 4.5-kb fragment (lanes 2 and 4). Total DNA from the different cell lines was digested with the enzymes indicated above, separated on 0.6% agarose gel, transferred to a nylon membrane, and hybridized with the probe indicated above. C, immunoblot analysis of Dictyostelium JH10 (wild type) and MHC-PKC- cells. Cells were developed for 4 h in suspension, and whole cell lysates were prepared as described under "Experimental Procedures." Samples (100 μg of total protein) were subjected to 10% SDS-PAGE and blotted on nitrocellulose. The immunoblots were stained with affinity-purified anti-MHC-PKC antibodies (12).

**Fig. 2. Expression of MHC-PKC.** A, expression vector pDRE106 (see "Experimental Procedures" for details). B, Western blot analysis of whole cell lysates and of membrane fractions from wild type and from MHC-PKC- cells at the vegetative stage and after 4 h of development. Samples (40 μg of total protein) were subjected to 10% SDS-PAGE and blotted on nitrocellulose. Immunoblots were stained with affinity-purified anti-MHC-PKC antibodies (12). C, membrane-associated MHC-PKC specific activity of wild type and MHC-PKC- cells at the vegetative stage and after 4 h of development. MHC-PKC activity was assayed by incubating purified LMM58 with the MHC-PKC samples in 10 mM Tris (pH 7.6), 6 mM MgCl2, 0.2 mM [γ-32P]ATP, 1 mM dithiothreitol for 10 min at 22 °C. The results are mean values of five different determinations.

It is interesting that MHC-PKC is a membrane-associated protein (9). The JH10 cells were found to contain MHC-PKC activity (20 pmol/min/mg) that is comparable to the activity published previously for Dictyostelium Ax3 cells (9). In contrast, the MHC-PKC- cells displayed negligible amounts of kinase activity (0.01 pmol/min/mg) associated with the membrane fraction. These results suggest that disruption of the MHC-PKC gene resulted in elimination of its activity and that MHC-PKC is the only MHC kinase (MHCK) activity in the membrane of Dictyostelium.
Ax2 cells (MHC-PKC<sup>+</sup> cells). Fig. 2B shows a Western blot analysis of Ax2 and MHC-PKC<sup>+</sup> whole cell lysates during the vegetative stage and after 4 h of development, using MHC-PKC polyclonal antibodies (12). In Ax2 cells the MHC-PKC was detectable only after 4 h of development, as in A<sup>+</sup> cells (12). In MHC-PKC<sup>+</sup> cells the MHC-PKC protein was already strongly expressed in the vegetative stage (Fig. 2B), and the level of expression remained the same after 4 h of development (Fig. 2B).

The expressed MHC-PKC protein was active. Fig. 2C shows the MHC-PKC activity associated with membrane fractions of Ax2 and MHC-PKC<sup>+</sup> cells, in the vegetative stage as well as in cells developed for 4 h. We detected negligible amounts of MHC-PKC activity associated with the membrane fraction in vegetative Ax2 cells (Fig. 2C). This activity appeared upon starvation of the cells. In contrast, vegetative MHC-PKC<sup>+</sup> cells contained MHC-PKC activity comparable to that detected in Ax2 cells developed for 4 h. Upon cell starvation this activity was increased 5-fold (Fig. 2C).

The MHC-PKC<sup>+</sup> cells expressed comparable amounts of MHC-PKC protein during the vegetative and development stages (Fig. 2B), but the activity of MHC-PKC in MHC-PKC<sup>+</sup> vegetative cells was 5-fold lower than in MHC-PKC<sup>+</sup> cells developed for 4 h. To resolve this apparent discrepancy, we quantified the amount of MHC-PKC protein associated with cell membranes in MHC-PKC<sup>+</sup> cells at both stages. As shown in Fig. 2B, the amount of MHC-PKC protein associated with the membrane fraction in vegetative MHC-PKC<sup>+</sup> cells was lower than in developed MHC-PKC<sup>+</sup> cells. These results may indicate that a much smaller part of the MHC-PKC protein translocates to the membrane in the former case than in the latter. We found that translocation of MHC-PKC to membranes is in response to the extracellular signal, cAMP. Since in vegetative MHC-PKC<sup>+</sup> cells the cAMP signal is missing, it is likely that most of the expressed MHC-PKC resides in the cell soluble fraction in inactive state. To test this hypothesis, we compared the activity of the cytosolic and membrane-associated MHC-PKC. Since Dictyostelium contains several soluble MHCks (for review see Ref. 26), we first immunoprecipitated the MHC-PKC from the soluble and insoluble fractions and assayed it for kinase activity as described under "Experimental Procedures." We compared the 32P incorporated into LMM58 by soluble and by insoluble MHC-PKC. The soluble MHC-PKC possessed only 0.5% of the kinase activity present in the membrane-associated MHC-PKC. These results suggested that the MHC-PKC that resided in the cytosol is inactive and MHC-PKC translocation to the cortex resulted in activation of the enzyme.

In the following experiments, we compared MHC-PKC<sup>−</sup> cells to JH10 cells and MHC-PKC<sup>+</sup> cells to Ax2 cells that were transformed with the vector pnDel (16); we refer to both cell lines as wild type cells.

In Vivo Phosphorylation of MHC in Response to cAMP—Wild type cells but not MHC-PKC mutant cells responded to cAMP stimulation by an increase in MHC phosphorylation (Fig. 3), as was reported for Dictyostelium A<sup>+</sup> cells (3). Removal of MHC-PKC resulted in elimination of the cAMP-dependent MHC phosphorylation. These results indicate that MHC-PKC is the kinase that phosphorylates MHC in response to cAMP. These findings are consistent with previous observations that cAMP stimulation of Dictyostelium cells causes transient increases in phosphorylation of MHC occurring primarily on threonine (4) and that MHC-PKC phosphorylates MHC on threonine residues only (9). Unstimulated developed wild type and MHC-PKC<sup>−</sup> cells exhibited the same basal levels of MHC phosphorylation (Fig. 3).

Overexpression of the MHC-PKC resulted in highly phosphorylated MHC. cAMP stimulation of these cells resulted in a slight increase in MHC phosphorylation. To find out whether the expressed MHC-PKC in MHC-PKC<sup>−</sup> cells phosphorylates the same sites on MHC as the native MHC-PKC in wild type cells, we compared the phosphorylation levels of MHC after cAMP stimulation of cells expressing the 3X ALA MHC (17) and in cells that co-expressed MHC-PKC and the 3X ALA MHC. In the 3X ALA MHC mutant, the phosphorylation sites previously shown to be a target of several MHCks (27, 28) were converted to alanine residues, thus eliminating phosphorylation at these positions. These phosphorylation sites are localized within the region of MHC that is phosphorylated by MHC-PKC. The rationale for this experiment was that if the highly phosphorylated MHC in developed MHC-PKC<sup>−</sup> cells (Fig. 3) reflects additional phosphorylation sites on MHC phosphorylated by the expressed MHC-PKC, then overexpression of MHC-PKC...

3 A. Dumbinsky, H. Rubin, and S. Ravid, submitted for publication.

4 S. Ravid, T. T. Egelhoff, and J. A. Spudich, unpublished data.
PKC with 3X ALA MHC should result in phosphorylated MHC, while cells expressing 3X ALA MHC alone will contain non-phosphorylated MHC. We found that cAMP stimulation of 3X ALA cells or cells co-expressing MHC-PKC and 3X ALA MHC do not respond to this stimulation by increasing the phosphorylation level of MHC. The MHC from both cell lines contained the same basal level of phosphorylation as was found for wild type cells (data not shown). These results indicate that MHC-PKC overexpression did not result in phosphorylation of new sites on MHC.

To find out whether the expressed MHC-PKC in vegetative MHC-PKC cells also resulted in highly phosphorylated MHC as was found for developed MHC-PKC cells (Fig. 3), we compared the phosphorylation level of MHC isolated from vegetative MHC-PKC and wild type cells. We found that the phosphorylation levels of MHC in both cell lines were similar (data not shown). These results indicate that although the MHC-PKC in vegetative MHC-PKC cell is active, it was incapable of phosphorylating MHC in vivo.

Isolation of Triton-resistant Cytoskeletons—In order to study the effect of the aberrant MHC phosphorylation on the localization properties of myosin II in the MHC-PKC mutant cells, we isolated cAMP-stimulated actin-enriched Triton-insoluble cytoskeletons (21). In unstimulated developed wild type cells, 28% of the myosin II was insoluble. Addition of cAMP resulted in a rapid accumulation of myosin II associated with the Triton-insoluble cytoskeleton (up to 46%), followed by an increase in myosin II solubility (Fig. 4). In unstimulated developed MHC-PKC cells, 46% of the myosin II was already insoluble. Addition of cAMP resulted in a gradual increase in myosin II insolubility (up to 71%). In contrast to wild type cells, the increase in myosin II insolubility was not followed by increase in myosin II solubility. In MHC-PKC cells 19% of myosin II was associated with the Triton-insoluble cytoskeleton, this proportion decreased gradually upon addition of cAMP. These results indicate that elimination of MHC-PKC leads to excess of Triton-insoluble myosin II, and that overexpression of the protein results in an excess of Triton-soluble myosin II.

Immunolocalization of Myosin II—Myosin II distribution in chemotactically competent wild type and MHC-PKC mutant cells was examined by immunofluorescent microscopy, in the presence and in the absence of cAMP stimulation (Fig. 5). Staining of myosin II in wild type cells without cAMP stimulation revealed that most of the cells are round and that myosin II is evenly distributed in the cell as a loose fibrous network (Fig. 5). cAMP stimulation of these cells resulted in cell polarization and localization of myosin II to the cell cortex leaving the anterior part of the cell free of myosin II (Fig. 5), as was reported earlier for Dictyostelium AX2 cells (5). In contrast, myosin II distribution in MHC-PKC cells was highly abnormal. The majority of unstimulated developed MHC-PKC cells contained a dense cortical ring of myosin II (Fig. 5). cAMP stimulation of these cells resulted in abnormal cell polarization and myosin II localization (Fig. 5). Myosin II was localized in several areas along the cell cortex in a discontinuous manner, and the proportion of polarized cells was much lower than that in stimulated developed wild type cells (data not shown). These results indicate that, in the absence of MHC-PKC, the cAMP-dependent mechanism of myosin II localization is severely impaired, resulting in abnormal cell polarization.

In unstimulated developed MHC-PKC cells, as in wild type cells, myosin II was scattered throughout the cytoplasm and the cells were round (Fig. 5). Strikingly, upon cAMP stimulation MHC-PKC cells did not undergo myosin II localization or shape changes (Fig. 5). These results suggest that MHC-PKC overexpression that leads to highly phosphorylated MHC (Fig. 3) caused elimination of myosin II localization and cell polarization.

Chemotaxis Properties—To study the effect of the aberrant
myosin II localization and cell polarization on the ability of MHC-PKC mutant cells to perform chemotaxis, we used the Zigmond chamber (23). We found that these mutant cells responded much more slowly and to a lesser extent to the cAMP gradient as compared to the wild type cells (Fig. 6). Whereas 90% of the wild type cells accumulated along the side of the bridge with the higher concentration of cAMP 10 min after cAMP gradient formation, only 30% of the MHC-PKC mutant cells accumulated. Furthermore, MHC-PKC mutant cells are rounder and less polar than wild type cell line (data not shown).

Developmental Properties—To determine whether the impaired mechanisms of myosin II phosphorylation, localization and chemotaxis in MHC-PKC mutants affect cell differentiation, we compared the cell differentiation process in wild type and MHC-PKC mutants (Fig. 7). Wild type cells formed fruiting bodies after 24–26 h of starvation, as compared to 44–48 h for MHC-PKC mutant cells. In addition, MHC-PKC mutant cells exhibited abnormal differentiation morphology; MHC-PKC mutant cells were larger than wild type cells (Fig. 7A), and they appeared as multiple slugs that were derived from multitipped aggregates (Fig. 7B). These results may indicate that tip formation in MHC-PKC mutant cells is disrupted. Although MHC-PKC mutant cells form fruiting bodies, their morphology was distorted (Fig. 7D), exhibiting different shape and sizes in contrast to the oval shape fruiting bodies formed by wild type cells (Fig. 7C). Fig. 7D represents one example of MHC-PKC mutant fruiting bodies. They have two or more upward extensions ending in small fruiting body-like forms; these forms do not appear to contain spores.

To determine whether the distorted MHC-PKC mutant cells contain viable spores, we collected spores from both wild type cells and MHC-PKC mutant cells from fruiting bodies on bacterial lawns and examined them using the spore viability assay (described under "Experimental Procedures"). The viability of spores generated by MHC-PKC mutant cells developed on bacterial lawns was about 50% of that of wild type spores.

The most dramatic effect was observed during development of MHC-PKC mutant cells (Fig. 7E). These cells formed aggregates and failed to complete the developmental cycle by forming fruiting bodies. These findings, along with the failure of the MHC-PKC mutants to exhibit normal chemotaxis, suggest that MHC-PKC is required for proper development and may play an important role during the initial step of development, namely chemotaxis to cAMP.

Cytokinesis—MHC-PKC is expressed only during development in wild type cells (12), and we therefore anticipated that this kinase would not regulate myosin II during cytokinesis. Since MHC-PKC mutant cells in the vegetative stage expressed the MHC-PKC protein in an active state (see Fig. 2C), we examined whether MHC-PKC affects the growth of MHC-PKC mutant cells. We found that these cells exhibit growth pattern similar to that of wild type cells (data not shown). These results are consistent with the finding that MHC from vegetative wild type and MHC-PKC mutant cells contained the same levels of phosphorylation.

**DISCUSSION**

This study demonstrates that a member of the PKC family, MHC-PKC, is the enzyme that phosphorylates MHC in response to stimulation by the chemoattractant cAMP in Dictyostelium discoideum and is part of the chemotactic sensing mechanism in this species. Furthermore, MHC phosphorylation by MHC-PKC plays an important role in the regulation of myosin II localization and is required for proper chemotaxis and differentiation.

MHC from MHC-PKC mutant cells exhibits the same basal level of phosphorylation as MHC from wild type cells. This phosphorylation, which is cAMP-independent, may represent serine phosphorylation carried out by other MHCKs, since the cAMP-stimulated increases in phosphorylation occur primarily on threonine (4). In addition to MHC-PKC, several other MHCKs have been identified in Dictyostelium (8, 29, 30). Whether these...
kinases regulate myosin II during different kinds of contractile events or at different stages of the life cycle, or whether they function in a redundant and overlapping fashion, is unknown. Our results indicate that the different kinases respond to different signals, i.e., MHC-PKC responds to cAMP stimulation while the other MHCKs may not. In addition, MHC-PKC is expressed only during Dictyostelium development, while the other known Dictyostelium MHCKs are expressed during the vegetative stage only, or during the vegetative stage as well as the development stage (for review see Ref. 26). These findings indicate that the different Dictyostelium MHCKs may function at different stages of the life cycle of Dictyostelium and respond to different signals. It is also possible that the localization within the cell of the different MHCKs determines their role in the regulation of myosin II. Support for this idea comes from the findings that, although the MHC-PKC in vegetative MHC-PKC− cells is active, it did not phosphorylate the MHC (see below).

Overexpression of MHC-PKC resulted in highly phosphorylated MHC that does not represent new phosphorylation sites. MHC-PKC phosphorylates four sites on MHC in vitro (9); nevertheless, the in vivo levels of phosphate incorporation into MHC are very low (0.05 mol of phosphate/mol of MHC) (3, 31). It was proposed that incorporation of a single phosphate to a single myosin II filament is enough for filament disassembly (31). It is therefore conceivable that in vivo MHC-PKC phosphorylates small fraction of MHC and/or less then four sites on a single MHC, which is sufficient for filament disassembly. In MHC-PKC− cells the overexpression of MHC-PKC may result in phosphorylation of larger fraction of MHC and/or more than one site that results in the inability of myosin II to form filaments.

The Triton-resistant cytoskeleton analysis and the immunofluorescent microscopy study provide evidence that MHC-PKC plays a key role in controlling myosin II assembly into the cortical cytoskeleton. In MHC-PKC− cells, myosin II displayed substantial overassembly in these assays and cAMP stimulation resulted in a gradual increase in myosin II association with the cytoskeleton with no apparent dissociation. The simplest explanation for these results is that, in unstimulated MHC-PKC− cells, the absence of MHC-PKC drives myosin II molecules into filaments in vivo and that these filaments have high affinity for the cortical cytoskeleton and therefore accumulate as a cortical ring. Upon cAMP stimulation additional myosin II translocates to the cytoskeleton and, subsequently, cannot dissociate from it because of the absence of MHC-PKC. These results also suggest that myosin II association and dissociation from the cytoskeleton are two separate processes. Myosin II in MHC-PKC− cells exhibits a low level of association with the cytoskeleton; addition of cAMP results in a decrease in myosin II insolubility. The interpretation of these results is that the overexpression of MHC-PKC results in highly phosphorylated myosin II, which is unable to form filaments and to accumulate at the cortex. The myosin II observed in the cytoskeletal fraction in the Triton-resistant cytoskeleton assay may represent small filaments, large enough to associate with the cytoskeleton but not sufficient to drive cell polarity and chemotaxis. Stimulation by cAMP results in dissociation of myosin II from the cytoskeleton and increase in MHC phosphorylation, possibly as a result of cAMP-induced MHC-PKC translocation to the membrane and phosphorylation of the cortical myosin II. These results are consistent with the results reported by Yumura and Kitanishi-Yumura (31), which indicate that during contraction, the myosin II that have moved toward the foci are phosphorylated by a specific MHCK that is localized at the foci, with the resultant disassembly of filaments which are finally released from membrane cytoskeleton.

Both MHC-PKC mutants failed to exhibit normal chemotaxis toward cAMP. Wessel et al. (32) suggested that myosin II is required for fine tune locomotion during chemotaxis, and therefore cells lacking myosin II fail to exhibit efficient chemotaxis. On the basis of these results, we propose the following model for Dictyostelium chemotaxis. An unstimulated cell is rounded because of a contractile shell formed by an actin-myosin II network in the cortex. This network presumably inhibits events necessary for pseudopodal projection. Stimulation of one edge of the cell with the chemoeattractant cAMP results in translocation of myosin II to the cortex and translocation of MHC-PKC to the site of stimulation, where it phosphorylates MHC and leads to local breakdown of myosin II thick filaments. Thus the barrier to pseudopodial extension is removed and the cell extends toward the source of the chemoeattractant. The disassembled myosin II reassembles in the posterior portion of the cell, strengthening the existing cortical actin-myosin II network there and providing further inhibition of membrane ruffling in that area. In this way, the interaction between myosin II and MHC-PKC may play a major role in the generation of cell polarity for efficiently directed migration. In the absence of MHC-PKC, the cAMP-dependent mechanisms of myosin II phosphorylation and localization are disrupted. Instead of local breakdown of myosin II due to MHC phosphorylation by MHC-PKC, the myosin II forms a ring around the cell. As a result the cells are not properly polarized and exhibit inefficient chemotaxis. Cells overexpressing the MHC-PKC are unable to polarize since their myosin II is unable to assemble into filaments and to associate with the cortical cytoskeleton for the generation of cell polarization.

Our findings are consistent with those of Egelhoff et al. (11), which suggested that MHC-PKC phosphorylates only myosin II. In that study the phosphorylation sites previously shown to be a target of two MHCKs (27, 28) were converted either to alanine residues (3X ALA), or to aspartate residues (3X ASP), which mimics the negatively charged state of the phosphorylated molecule. The 3X ALA mutant and the MHC-PKC− mutant exhibit similar behavior. In both cell lines, myosin II exhibits substantial overassembly in vivo. Myosin II in MHC-PKC− and 3X ASP mutants (11) is unable to form filaments, and this renders the myosin II unable to drive development. The similarity of the myosin II mutant cell lines and the MHC-PKC cell lines may indicate that MHC-PKC is specific for myosin II and probably does not phosphorylate other proteins in vivo.

MHC-PKC overexpression had a dramatic effect on Dictyostelium development but no effect on growth, even though the MHC-PKC was expressed to the same extent during the vegetative and developed stages. Since MHC-PKC is not normally expressed during the vegetative stage, we anticipated that its overexpression in that stage would result in highly phosphorylated myosin II, which would not be able to form filaments and to actuate cytokinesis, as was reported for Dictyostelium hmm cells (18). However, overexpression of MHC-PKC did not affect cell growth, because of the incapability of MHC-PKC to phosphorylate MHC in vegetative cells, possibly because of the different localization of myosin II and active MHC-PKC. In vegetative MHC-PKC− cells, most of the MHC-PKC is localized in the cytosol in inactive form; it is conceivable that the myosin II is not accessible to the active membrane-associated MHC-PKC. Another possible explanation is that the target sites of MHC-PKC phosphorylation on MHC differ from the sites that play a role in the regulation of myosin II during cytokinesis, and that the later sites may be phosphorylated by other MHCKs.
The results presented here provide a direct in vivo evidence that MHC-PKC plays a critical role in the regulation of myosin II localization within the cells in response to cAMP stimulation. Since PKC is a ubiquitous protein, and it has been shown to phosphorylate myosin II in other cell systems (for review see Ref. 26), it is possible that PKC in other systems plays a role similar to that played by MHC-PKC.

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