Autophosphorylation of Dictyostelium Myosin II Heavy Chain-specific Protein Kinase C Is Required for Its Activation and Membrane Dissociation*

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Myosin II heavy chain (MHC)-specific protein kinase C (MHC-PKC) isolated from the ameba, Dictyostelium discoideum, regulates myosin II assembly and localization in response to the chemoattractant cAMP. cAMP stimulation of Dictyostelium cells leads to translocation of MHC-PKC from the cytosol to the membrane fraction, as well as causing an increase in both MHC-PKC phosphorylation and its kinase activity. MHC-PKC undergoes autophosphorylation with each mole of kinase incorporating about 20 mol of phosphate. The MHC-PKC autophosphorylation sites are thought to be located within a domain at the COOH-terminal region of MHC-PKC that contains a cluster of 21 serine and threonine residues. Here we report that deletion of this domain abolished the ability of the enzyme to undergo autophosphorylation in vitro. Furthermore, after this deletion, cAMP-dependent autophosphorylation of MHC-PKC as well as cAMP-dependent increases in kinase activity and subcellular localization were also abolished. These results provide evidence for the role of autophosphorylation in the regulation of MHC-PKC and indicate that this MHC-PKC autophosphorylation is required for the kinase activation in response to cAMP and for subcellular localization.

We have previously reported the isolation of a MHC1-specific PKC (MHC-PKC) from the ameba, Dictyostelium that phosphorylates Dictyostelium MHC specifically and is homologous to α, β, and γ subtypes of mammalian PKC (1, 2). In vitro phosphorylation of MHC by MHC-PKC results in inhibition of myosin II thick filament formation (1) by inducing the formation of a bent monomer of myosin II, whose assembly domain is tied up in an intramolecular interaction that precludes the intermolecular interaction necessary for thick filament formation (3).

The MHC-PKC which is expressed during Dictyostelium development has been implicated in the increase in MHC phosphorylation observed in response to cAMP stimulation (1). We have recently found that elimination of MHC-PKC abolishes this cAMP-induced MHC phosphorylation, indicating that MHC-PKC is the enzyme which phosphorylates MHC in response to cAMP stimulation (4). MHC-PKC null cells exhibit a substantial myosin II overassembly in vitro, as well as aberrant cell polarization, chemotaxis, and morphological differentiation. Cells that overexpress MHC-PKC contain highly phosphorylated MHC. They show no apparent cell polarization and chemotaxis, and exhibit impaired myosin II localization (4). These findings establish that, in Dictyostelium, the MHC-PKC plays an important role in regulating the cAMP-induced myosin II localization required for cell polarization and, consequently, for efficient 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, which requires phosphorylation and reorganization of myosin II (5–9). That is, the myosin II, which exists as thick filaments, translocates to the cortex (9) in response to cAMP stimulation. This translocation is correlated with a transient increase in the rate of MHC as well as light chain phosphorylation (5, 6, 10). We have recently shown that cAMP exerts its effects on myosin II via the regulation of MHC-PKC (11). cAMP stimulation of Dictyostelium cells results in translocation of MHC-PKC from the cytosol to the membrane fraction, as well as increasing MHC-PKC phosphorylation and its kinase activity (11). We could also show that MHC is phosphorylated by MHC-PKC in the cell cortex, and this leads to myosin II dissociation from the cytoskeleton (11).

Members of the PKC family are composed of a single polypeptide consisting of an NH2-terminal lipid binding regulatory domain and an ATP-binding catalytic domain located in the COOH-terminal region of the protein (12, 13). PKC is phosphorylated on multiple serine and threonine residues located in both domains (14–17). Three clusters of autophosphorylation sites have been mapped in vitro in the PKC βII isoenzyme at the NH2 terminus, the COOH-terminal tail, and the hinge region between the regulatory and catalytic domains (14). However, in vivo studies have indicated that the autophosphorylation sites are all localized at the COOH-terminal tail of PKC (18, 19). Bond and co-workers (20) have replaced the three clusters of the autophosphorylation sites found in vitro by alanine residues. Altering the autophosphorylation sites in this way at the NH2-terminal region or at the hinge region did not affect the activity or subcellular localization of the kinase. However, replacing the autophosphorylation sites at the COOH-terminal tail with alanine residues resulted in inactive, Triton X-100-insoluble enzyme (20). Similar results have been reported by Su et al. (21), who found that deletion of 23 amino acids from the COOH terminus of PKCa, which includes the autophosphorylation sites, caused total loss of catalytic activity. These results indicate that the PKC autophosphorylation sites located at the COOH-terminal tail play an important role in the regulation of the kinase in vivo.

MHC-PKC also undergoes phosphorylation in vivo, which consists of both autophosphorylation and phosphorylation by another kinase, presumably cGMP-dependent protein kinase.

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1 The abbreviations used are: MHC, myosin II heavy chain; PKC, protein kinase C; MES, 4-morpholineethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; ST, serine-threonine.
These two kinds of phosphorylation involve different phosphorylation sites (11). Here we report that deletion of 33 amino acids from the COOH-terminal tail of MHC-PKC, which includes a cluster of 21 serine and threonine residues, abolished both MHC-PKC autophosphorylation in vitro and the cAMP-dependent MHC-PKC autophosphorylation. This indicates that this portion of the protein contains the in vitro and in vivo MHC-PKC autophosphorylation sites. We further present data showing that MHC-PKC autophosphorylation plays an important role in the kinase activation and subcellular localization.

EXPERIMENTAL PROCEDURES

Cell Culture and Development—Dictyostelium discoideum strain amebas were grown in HL-5 medium (22), harvested at a density of 2 × 106 cells/ml, washed twice in MES buffer (20 mM MES, pH 6.8, 0.2 mM CaCl2, 2 mM MgSO4), and resuspended at a density of 5 × 106 cells/ml to initiate development. Cells were shaken at 100 rpm at 22°C for 3.5 h. 5 mM caffeine was added to the suspension 30 min prior to the addition of cAMP.

Expression of MHC-PKCAST—All DNA manipulations were carried out using standard methods (23). We used the expression vector pDXA-HT which contains the actin-15 promoter and allows the expression of foreign proteins carrying a NH2-terminal His tag (24). pDXA-MHC-PKCAST was constructed as follows. The vector pBS-MHCK (25), containing a 2.6-kilobase pair MHC-PKC cDNA clone, was digested with SmaI and ScaI, which deleted a fragment of 398 base pairs from the 3′ of the MHC-PKC clone. The deleted fragment contains 99 base pairs of coding region and 210 base pairs of noncoding region. The deleted coding region is 33 amino acids in length and contains a cluster of 21 serine and threonine (ST) residues which are thought to be the MHC-PKC autophosphorylation domain (Fig. 1, ST domain). The resulting MHC-PKC fragment was named MHC-PKCAST. This MHC-PKCAST fragment was sequenced to confirm the deletion of the ST domain. It was cloned into pDXA-HY digested with SmaI. pDXA-MHC-PKCAST was used for the transformation of MHC-PKC cells (4) using calcium phosphate precipitate (25). Clones were selected on the basis of their resistance to G418 (Boehringer Mannheim) and screened using Western blot analysis (see below).

Purification of His-tagged MHC-PKCAST—50 ml of 2 × 106 cells/ml expressing MHC-PKCAST were washed twice in 20 mM phosphate buffer (pH 6.5), and the cells were lysed in 1 ml of lysis buffer containing 20 mM HEPES (pH 7.5), 1% Triton X-100, 0.2% Nonidet P-40, 200 mM NaCl, 5 mM β-mercaptoethanol, and protease inhibitor mix (50 mM imidazole, 200 μM leupeptin, and 200 μM pepstatin). The extracts were centrifuged in a microcentrifuge at 15 min for 4°C, and the supernatant was incubated with 50 μl of a slurry of Ni+-agarose beads (Qiagen) in 20 mM phosphate buffer, pH 6.5, and 200 mM KCl for 1 h at 4°C. The bead-protein complex was washed three times with lysis buffer, twice with lysis buffer containing 20 mM imidazole, and twice with 5°C lysis buffer containing 50 mM imidazole. The MHC-PKCAST was eluted with 100 μl of lysozyme buffer containing 150 mM imidazole and then eluted with 100 μl of lysis buffer containing 250 mM imidazole.

Western Blot Analysis—Cells were developed for 4 h in shaking flasks as described above. Cells were washed in 10 mM Tris-HCl, pH 8.0, and 150 mM KCl and lysed in 50 mM Tris-HCl, pH 8.0, 20 mM sodium pyrophosphate, pH 6.8, 5 mM EDTA, 5 mM EGTA, 0.5% Triton X-100, and protease inhibitor mix. Protein was determined by the method of Peterson (26), and lysates were electrophoresed on sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) (27). Western blots were probed with MHC-PKC polyclonal antibody (2), and the blots were developed using a horseradish peroxidase-coupled secondary antibody (Bio-Rad Laboratories). ECL was performed using a kit from Amer sham Corp.

Autophosphorylation—Developed Ax2 cell suspensions containing 5 × 106 cells/ml were added to an equal volume of ice-cold 2 × lysis buffer (40 mM Tris-Cl, pH 7.5, 0.2% Nonidet P-40, 2 mM dithiothreitol, 10 mM EDTA, and protease inhibitor mix) and centrifuged for 5 min in a microcentrifuge at 4°C. The supernatant was preincubated by incubation with 30 μl of rabbit preimmune serum at 4°C for 1 h followed by incubation with Staphylococcus A cells at 4°C for 30 min. Staphylococcus A cells were centrifuged, and 10 μl of MHC-PKC antibody were added to the supernatant and incubated at 4°C for 1 h, followed by incubation with 50 μl of Protein A-conjugated agarose (100 μg/ml) at 4°C for 1 h and then centrifuged for 1 min. Pellets were washed twice in 1 × lysis buffer containing 1 mg/ml bovine serum albumin and once with 1 × lysis buffer only. The protein A-MHC-PKC complex was resuspended in 200 μl of phosphate mix (20 mM Tris-HCl, pH 7.5, 200 μM MnCl2, 1 mM dithiothreitol, 0.5 mM CaCl2, 0.04 mM EDTA, 300 mM KCl, and 5 units of alkaline phosphatase (Boehringer Mannheim)) and incubated at 37°C for 30 min. The phosphatase-treated protein α-MHC-PKC complex was washed twice in 1 × lysis buffer and incubated in 200 μl of phosphate inhibitor mix (100 mM NaF, 200 μM Na3VO4, 10 mM Tris-HCl, pH 7.5, and 0.2 μM [γ-32P]ATP) and incubated at 22°C for 20 min and then centrifuged for 1 min. The pellets were washed in 1 × lysis buffer and resuspended in SDS sample buffer and boiled for 5 min. The supernatants from a microcentrifuge spin were immunoprecipitated using anti-PKC antiserum (1:2000 dilution), and the immunoprecipitates were electrophoresed on a 7% SDS-PAGE gel. Autoradiography of the MHC-PKC complexes was performed using autoradiography film and PhosphorImaging. Autoradiography of MHC-PKCAST was performed as described above using MHC-PKCAST purified with Ni+-agarose beads as described above.

Phosphorylation—Dictyostelium Ax2 and MHC-PKCAST cells were developed and treated with caffeine as described above. Before and after the application of cAMP stimulus, 100 μl of developed cells were added to 200 μl of reaction mixture containing 0.2% Triton X-100, 8 mM MgCl2, 200 mM Tris-HCl (pH 7.5), and 0.2 μM [γ-32P]ATP and incubated for 30 s at 22°C. The MHC-PKC and MHC-PKCAST were immunoprecipitated and analyzed using SDS-PAGE. Densitometric scanning of the Coomassie Blue-stained gels was used to determine the relative amounts of immunoprecipitated MHC-PKC and MHC-PKCAST. The amounts of 32P incorporated into the proteins were determined using the PhosphorImager. Relative phosphorylation of MHC-PKC and MHC-PKCAST was determined by dividing the values obtained with the PhosphorImager by the values obtained by scanning of the Coomassie Blue-stained gels. In vivo phosphorylation of MHC was carried out as described previously (5, 6).

MHC-PKC Activity—This was assayed directly using the kinase extract from the insoluble cell fraction. Following resuspension of 1 × 106 developed Ax2 and MHC-PKCAST cells in 1 ml of sonication buffer (10 mM Tris-HCl, pH 7.5, 50 mM KCl, and protease inhibitor mix), they were incubated with 1 μM CAMP and lysed using an ultrasonic cell disruptor (Microson) model XL with a small sized tip at 50% output power. The extract was spun in a microcentrifuge for 20 min at 4°C. MHC-PKC and MHC-PKCAST were extracted from the insoluble fraction using sonication buffer containing 1% Triton X-100 and 0.5 mM KCl. For kinase assay, the solubilized MHC-PKC or MHC-PKCAST were incubated with LMM58 (0.5–1 mg/ml), 6 mM MgCl2, 0.2 mM [γ-32P]ATP (500 cpm/pmol), 1 mM DTT for 10 min at 22°C on a rotator. Reaction was initiated by the addition of 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% trichloroacetic acid, resuspended 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. The amounts of MHC-PKC and MHC-PKCAST in the cell extracts were determined using densitometric scanning of Western blots and normalized to the total amount of protein determined as described previously (26).

Biochemical Analysis of MHC-PKC and MHC-PKCAST Distribution—Following resuspension of 1 × 107 developed Ax2 and MHC-PKCAST cells in 1 ml of sonication buffer (10 mM Tris, pH 7.5, 50 mM KCl, and protease inhibitor mix), they were lysed by sonication as described above, and the extract was spun in a microcentrifuge for 20 min at 4°C. The soluble fraction was immunoprecipitated with MHC-PKC antibody as described above. MHC-PKC and MHC-PKCAST were extracted from the insoluble fraction using sonication buffer containing 1% Triton X-100 and 0.5 mM KCl. The extract was spun in a microcentrifuge for 10 min at 4°C, and the solubilized MHC-PKC and MHC-PKCAST were immunoprecipitated as described above. To quantify the amounts of MHC-PKC and MHC-PKCAST in the soluble and insoluble fractions, the immunoprecipitated MHC-PKC and MHC-PKCAST from both fractions were electrophoresed on 7% SDS-PAGE gels and the Coomassie Blue-stained gels were analyzed as described above.

Triton-resistant Cytoskeleton Analysis—Triton-insoluble cytoskeleton analysis was performed as described previously (28). Supernatant and cytoskeletal pellet fractions were resuspended in SDS-PAGE sample buffer, boiled for 5 min, and electrophoresed on 7% SDS-PAGE gels. The relative amounts of myosin II were determined by SDS-PAGE gel analysis as described above.
RESULTS

Expression of MHC-PKCAST Protein in MHC-PKC Cell Line—Phosphorylation of sites located at the COOH-terminal tail of several PKC is important for the regulation of the kinase activity and subcellular localization (18, 20, 21, 29). MHC-PKC undergoes autophosphorylation in vitro and phosphorylation in vivo in response to cAMP stimulation, and this phosphorylation coincides with the activation of the kinase (11). The MHC-PKC autophosphorylation sites are thought to be located within the COOH-terminal tail of MHC-PKC, which contains a cluster of 21 serine and threonine residues (Fig. 1, ST domain) (1, 2). To study the in vivo role of MHC-PKC autophosphorylation, we engineered an MHC-PKC in which the putative autophosphorylation domain was deleted (MHC-PKCAST). The MHC-PKCAST was expressed in Dictyostelium cells that were previously engineered to lack expression of MHC-PKC (so-called MHC-PKC- cells) (4).

MHC-PKC- cells transformed with the pDXA-MHC-PKCAST construct (see “Experimental Procedures”) expressed MHC-PKCAST at 125–150% of the level of MHC-PKC in Ax2 cells (Fig. 2). The expressed MHC-PKCAST migrated on SDS-PAGE with an apparent molecular mass of about 80 kDa (Fig. 2), fitting well with the predicted molecular mass of 80 kDa and indicating that the protein was not phosphorylated. In contrast, MHC-PKC migrated on SDS-PAGE of cell extracts of Ax2 with an apparent molecular mass of about 94 kDa (Fig. 2), although the predicted molecular mass of MHC-PKC is 84 kDa (2). These results are consistent with a migration of the autophosphorylated form of MHC-PKC on SDS-PAGE (1), indicating that the MHC-PKC was in its phosphorylated form, under these experimental conditions. In addition to the 94-kDa band of MHC-PKC, another band with an apparent molecular mass of 90 kDa was found (Fig. 2). This protein could be either partially phosphorylated form of MHC-PKC or a degradation product of MHC-PKC.

MHC-PKC but Not MHC-PKCAST Underwent Autophosphorylation in Vitro—To find out whether the ST domain of MHC-PKC is indeed the kinase autophosphorylation domain, we studied the ability of MHC-PKCAST to undergo autophosphorylation in vitro. To do this, we developed Ax2 and MHC-PKCAST cells. The MHC-PKC was immunoprecipitated and the MHC-PKCAST purified using Ni+-agarose beads as described under “Experimental Procedures.” To increase autophosphorylation, the proteins were treated with phosphatase prior to the autophosphorylation reaction as described under “Experimental Procedures.” The phosphatase-treated MHC-PKC immunocomplexed to protein A-Sepharose and the MHC-PKCAST-Ni+-agarose complex were incubated with [γ-32P]ATP and MgCl2, and the extent of the autophosphorylation was analyzed using autoradiography as described under “Experimental Procedures.”

On addition of ATP and MgCl2 to MHC-PKC, it migrated with a molecular mass of 94 kDa (Fig. 3A) which is consistent with a molecular mass of the autophosphorylated form of MHC-PKC (1). Autoradiography revealed that MHC-PKC indeed underwent autophosphorylation (Fig. 3B), and similar results have been previously reported (1). MHC-PKC appeared as a doublet on SDS-PAGE (Fig. 3A), which may represent different extents of autophosphorylation. In contrast, addition of ATP and MgCl2 to MHC-PKCAST did not alter the migration pattern of the protein, and it migrated with a molecular mass predicted for nonphosphorylated truncated MHC-PKC protein (Fig. 3C). Autoradiography of MHC-PKCAST revealed that the protein was unable to undergo autophosphorylation (Fig. 3D). These results indicate that the ST domain is the in vitro MHC-PKC autophosphorylation domain.

MHC-PKC and Not MHC-PKCAST Is Phosphorylated in Response to cAMP Stimulation—We have previously reported that, in response to cAMP stimulation, MHC-PKC undergoes a phosphorylation which is composed of both autophosphorylation and phosphorylation in a cGMP-dependent manner (11). To address whether MHC-PKCAST is able to undergo in vivo phosphorylation in response to cAMP stimulation, we stimulated Ax2 and MHC-PKCAST cells with cAMP and a total lysate of amoeba labeled with [γ-32P]ATP. MHC-PKC and MHC-PKCAST were immunoprecipitated and the levels of their phosphorylation were determined as described under “Experimental Procedures.”

MHC-PKC was transiently phosphorylated in response to cAMP stimulation (Fig. 4, A and B), with peak phosphorylation at about 40 s (Fig. 4B) (see also Dembinsky et al. (11)). In
contrast, addition of cAMP to cells expressing the MHC-PKCAST protein resulted in very low phosphorylation levels (Fig. 4, A and B), whose magnitude was similar to the basal level of MHC-PKC phosphorylation determined 120 s after cAMP stimulation (Fig. 4B). This low level of phosphorylation in the MHC-PKCAST may be due to the phosphorylation of the protein by another kinase possibly cGMP-dependent protein kinase, as we recently suggested (11). The inability of MHC-PKC to undergo both autophosphorylation in vitro (Fig. 3) and cAMP-dependent phosphorylation (Fig. 4), indicate that the in vitro and in vivo autophosphorylation sites of MHC-PKC are the same and are located within the ST domain. The finding of relatively high phosphorylation levels of MHC-PKC prior to cAMP stimulation (Fig. 4, A and B) is consistent with the results presented in Fig. 2, in which MHC-PKC from Ax2 extract migrated on SDS-PAGE with an apparent molecular mass consistent with migration of the kinase in its autophosphorylated form (1).

**MHC-PKCAST Protein Remains in the Cell Membrane Regardless of cAMP Stimulation**—We reported previously that, on cAMP stimulation, MHC-PKC translocates to the membrane, presumably as part of the kinase activation mechanism. This translocation coincides with the kinase phosphorylation (11). It was therefore of interest to study the localization properties of MHC-PKCAST on cAMP stimulation.

Ax2 and MHC-PKCAST cells were developed and treated with caffeine, stimulated with cAMP, and lysed using sonication, and the MHC-PKC and MHC-PKCAST were immunoprecipitated from the soluble and the insoluble fractions using specific MHC-PKC polyclonal antibody (see “Experimental Procedures”). Fig. 5 shows that, prior to cAMP stimulation, about 30% of the MHC-PKC resided in the insoluble fraction, whereas cAMP stimulation was followed by a rapid transient association of up to about 60% of the MHC-PKC with the membrane fraction, as reported previously (11). In contrast, about 70% of MHC-PKCAST remained in the membrane regardless of cAMP stimulation (Fig. 5). These results indicate that the MHC-PKC autophosphorylation mechanism is involved in the dissociation of the kinase from the membrane.

**MHC-PKC, but Not MHC-PKCAST, Responds to cAMP Stimulation by Increasing Its Kinase Activity**—To examine the possible role of MHC-PKC autophosphorylation in the activation of the kinase, we studied the specific activity of MHC-PKC and MHC-PKCAST in response to cAMP stimulation. Ax2 and MHC-PKCAST cells were stimulated with cAMP, and the kinase was solubilized from cell membranes and assayed for kinase activity as described under “Experimental Procedures.”

Although Dictyostelium contains several myosin heavy chain kinases, all except MHC-PKC are located in the cytosol (for review, see Tan et al. (30)). Furthermore, cells in which the MHC-PKC was eliminated do not show MHC phosphorylation activity in their membranes (4). Accordingly, all subsequent kinase assays were performed on MHC-PKC or MHC-PKCAST that were solubilized from the cell membrane fraction.

Fig. 6 shows that cAMP stimulation of Ax2 cells resulted in a transient increase in membrane-associated MHC-PKC kinase activity as reported previously (11). These cAMP-stimulated increases in MHC-PKC activity coincided with the cAMP-stimulated membrane association (Fig. 5) and phosphorylation (Fig. 4) of MHC-PKC, suggesting that these processes are linked to each other and may be required for the activation of MHC-PKC, as was proposed previously (11). In contrast, cAMP stimulation of MHC-PKCAST did not increase the kinase activity (Fig. 6). MHC-PKCAST showed a basal level of MHC kinase activity with magnitude similar to that shown by MHC-PKC isolated from nonstimulated Ax2 cells (Fig. 6). These results indicate that MHC-PKC autophosphorylation plays a role in the cAMP-dependent activation of the kinase. Interestingly, MHC-PKCAST activity decreased 60 s after cAMP stim-
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Fig. 6. MHC-PKCAST is a function-defective protein. MHC-PKC specific activity was determined by lysing cAMP-stimulated cells by sonication, extracting the MHC-PKC from the membrane fraction, and subjecting to kinase assay as described under “Experimental Procedures.” (Error bars = ± S.E.; n = 6.)

Fig. 7. In vivo phosphorylation of MHC and association of myosin II with Triton-insoluble cytoskeletal fraction. In A, the rate of MHC phosphorylation was determined by immunoprecipitation of MHC from 32P-labeled cells and quantifying it using the PhosphorImager as described under “Experimental Procedures.” The relative phosphorylation rate is the ratio of the rate of MHC phosphorylation measured in vivo at a given time after cAMP stimulation to the mean rate of MHC phosphorylation measured in vivo before cAMP stimulation. (Error bars = ± S.E.; n = 4.) In B, aliquots of a developed and caffeine-treated cell suspension were removed before and after stimulation with 1 μM cAMP, added to ice-cold Triton mixture, and centrifuged as described under “Experimental Procedures.” Percent of insoluble MHC was determined by quantifying the amounts of MHC in the pellets and in the supernatants using densitometric scanning of the Coomassie Blue-stained gels and calculating the ratio of the amount of MHC in the pellet to the total amount of MHC for each time point. (Error bars = ± S.E.; n = 4.)

Several PKC are important in the regulation of the kinase activity and subcellular localization (18, 20, 21, 29). Here we have examined the functional role of autophosphorylation of MHC-PKC using deletion and biochemical analyses. Our results indicate that (i) it is the ST domain which is autophosphorylated and (ii) that MHC-PKC autophosphorylation plays a role in its partition into the cytosol and its activation in vivo.

We have previously shown that cAMP stimulation of Dictyostelium cells resulted in several changes in MHC-PKC behavior (11). First, cAMP stimulation causes MHC-PKC to translocate to the membrane. Second, translocation coincides with increases in MHC-PKC phosphorylation. A third change is an increase in MHC-PKC activity. The association of MHC-PKC with the membrane is necessary for MHC-PKC activation since the cytosolic MHC-PKC has a very low kinase activity. There are two types of MHC-PKC phosphorylation, which are different in their extent and their sites; autophosphorylation, which may occur at the membrane and accounts for most of the MHC-PKC phosphorylation, and cGMP-dependent phosphorylation, possibly via cGMP-dependent protein kinase which may take place in the cytosol. The two different phosphorylations occur on different serine and/or threonine residues in MHC-PKC.
PKC has also been reported for PKC heterologous kinase and its involvement in the regulation of PKC consistent with our previous report that MHC-PKC and MHC-PKCAST are phosphorylated in vitro by cGMP-dependent protein kinase (11). A similar phosphorylation of PKC by a heterologous kinase and its involvement in the regulation of PKC has also been reported for PKCo (35), however the identity of this kinase is unknown. We suggested previously that a plausible candidate for a PKC kinase is a cGMP-dependent protein kinase (11). Phosphorylation of MHC-PKC by another kinase may be required for the kinase translocation to the membrane which is required for the kinase activation, whereas the MHC-PKC autophosphorylation may be required for its activation and membrane dissociation.

Previous studies with mammalian PKC have shown that elimination of the COOH-terminal autophosphorylation sites fully inactivates the kinase (21, 29). In contrast, upon deletion of the ST domain the MHC-PKC retained its basal kinase activity and lost the cAMP-dependent increases in its activity. These findings may indicate that the expressed protein is folded properly and the absence of cAMP-dependent increases in the kinase activity was a result of the ST domain deletion, it further suggests that this domain plays a key role in the cAMP-dependent kinase activation. It is noteworthy that the basal level of MHC-PKCAST activity decreased 60 s after cAMP stimulation. This may result from phosphatases removing the phosphates that were incorporated into the MHC-PKCAST by cGMP mechanism thereby decreasing the kinase activity.

Deletion of the ST domain results in kinase that is unable to partition into the cytosol, indicating that the autophosphorylation of the ST domain plays a role in the dissociation of the kinase from the membrane. The seemingly contradictory results that autophosphorylation is required for both kinase activation and membrane dissociation may be explained as follows: the cluster of the 21 serine/threonine residues is autophosphorylated in two steps. In the first step only a fraction of the sites is autophosphorylated and this autophosphorylation is required for the activation of the kinase in response to cAMP. In the second step the remaining sites are autophosphorylated and this phosphorylation is required for dissociation of the kinase from the membrane. We are currently attempting to express MHC-PKC proteins in which the autophosphorylation sites are randomly converted to alanine residues. Experiments with these altered MHC-PKC proteins will enable us to explore the in vitro role of the different autophosphorylation sites.

The myosin II Triton-solubility and phosphorylation characterization in MHC-PKCAST are similar to that in the mutant from which the MHC-PKC was eliminated (4). The inability of MHC-PKCAST to increase its activity in response to cAMP stimulation is also reflected in the state of MHC phosphorylation and its Triton-solubility; MHC in MHC-PKCAST cells is not phosphorylated in response to cAMP stimulation. These results are consistent with the findings that the cAMP-dependent MHC phosphorylation is carried out by MHC-PKC (4). If the enzyme does not respond catalytically to cAMP stimulation, the cell cannot respond to cAMP stimulation by phosphorylating the MHC.

The aberrant cAMP-dependent myosin II Triton-solubility in MHC-PKCAST cells is consistent with the finding that it is through MHC phosphorylation that myosin II distributed throughout the cell in response to cAMP stimulation. MHC-PKCAST and MHC-PKC cells (4) contain highly Triton-insoluble myosin II. This contrasts with wild type cells in which cAMP stimulation resulted in translocation of myosin II to the cortex, followed by dissociation of myosin II from the membrane fraction (5, 6, 11). In the two mutant cell lines, 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-PKCAST cells, the absence of MHC-PKC drives myosin II molecules into filaments in vitro and that these filaments have high affinity for the cortical cytoskeleton. cAMP stimulation, additional myosin II translocates to the cytoskeleton and, subsequently, cannot dissociate from it because of the presence of catalytically inactive MHC-PKCAST. The observed phenotype of MHC-PKCAST cells described here is not a result of the expression of MHC-PKCAST per se but rather results from the absence of the ST domain; this is indicated by previous experiments in which we engineered cells expressing MHC-PKC under the same actin promoter, and the resulting cells had a wild type phenotype.

The detailed mechanism by which autophosphorylation could control MHC-PKC activity remains to be elucidated. One possibility is that the autophosphorylation triggers a conformational change that is important in relieving inhibition of MHC-PKC pseudosubstrate proteotpe similar to PKC (12). Supporting this hypothesis, the putative substrate (and pseudosubstrate) binding site of PKC is thought to be located near the COOH-terminal autophosphorylation sites (36, 37). This model is further supported by the observation that the catalytic fragment of PKC retains histone kinase activity, even though it can no longer autophosphorylate (16). This suggests that removal of the regulatory domain eliminates the requirement for COOH-terminal autophosphorylation. Alternatively, this autophosphorylation may be critical for enzyme-substrate recognition or for interaction of MHC-PKC with regulatory factors.

A model consistent with both our data and previous data is that MHC-PKC is first synthesized as an inactive precursor that is cytosolic (11). The kinase is then recognized by a cGMP-dependent protein kinase, which phosphorylates it, possibly at the C-2 and C-4 domains. This phosphorylation may be required for the kinase translocation to the cell membrane. The membrane-bound enzyme is then stimulated by autophosphorylation at the ST domain. The first step of autophosphorylation may activate the MHC-PKC causing MHC phosphorylation, followed by a second step of autophosphorylation resulting in a decrease in the enzyme’s membrane affinity so that it partitions into the cytosol. This returns the enzyme to its basal state.

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