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14–3-3 Inhibits the *Dictyostelium* Myosin II Heavy-Chain-specific Protein Kinase C Activity by a Direct Interaction: Identification of the 14–3-3 Binding Domain

Meirav Matto-Yelin,* Alastair Aitken,† and Shoshana Ravid*‡

*Department of Biochemistry, Hadassah Medical School, The Hebrew University, Jerusalem 91120, Israel; and †Laboratory of Protein Structure, National Institute for Medical Research, London NW7 1AA, United Kingdom

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Myosin II heavy chain (MHC) specific protein kinase C (MHC-PKC), isolated from *Dictyostelium discoideum*, regulates myosin II assembly and localization in response to the chemoattractant cyclic AMP. Immunoprecipitation of MHC-PKC revealed that it resides as a complex with several proteins. We show herein that one of these proteins is a homologue of the 14–3-3 protein (Dd14–3-3). This protein has recently been implicated in the regulation of intracellular signaling pathways via its interaction with several signaling proteins, such as PKC and Raf-1 kinase. We demonstrate that the mammalian 14–3-3 ζ isoform inhibits the MHC-PKC activity in vitro and that this inhibition is carried out by a direct interaction between the two proteins. Furthermore, we found that the cytosolic MHC-PKC, which is inactive, formed a complex with Dd14–3-3 in the cytosol in a cyclic AMP-dependent manner, whereas the membrane-bound active MHC-PKC was not found in a complex with Dd14–3-3. This suggests that Dd14–3-3 inhibits the MHC-PKC in vivo. We further show that MHC-PKC binds Dd14–3-3 as well as 14–3-3ζ through its C1 domain, and the interaction between these two proteins does not involve a peptide containing phosphoserine as was found for Raf-1 kinase. Our experiments thus show an in vivo function for a member of the 14–3-3 family and demonstrate that MHC-PKC interacts directly with Dd14–3-3 and 14–3-3ζ through its C1 domain both in vitro and in vivo, resulting in the inhibition of the kinase.

INTRODUCTION

When cells of *Dictyostelium* are starved, they acquire the ability to bind cyclic AMP (cAMP) to specific receptors on the cell surface and to respond to this signal by chemotaxis, which requires phosphorylation and reorganization of myosin II (Rahmsdorf *et al.*, 1978; Malchow *et al.*, 1981; Berlot *et al.*, 1985, 1987; Yumura and Fukui, 1985). That is, the myosin II, which exists as thick filaments, translocates to the cortex in response to cAMP stimulation (Yumura and Fukui, 1985). This translocation is correlated with a transient increase in the rate of myosin heavy chain (MHC) and light chain phosphorylation (Malchow *et al.*, 1981; Berlot *et al.*, 1985, 1987). We have previously reported the isolation of a MHC-specific protein kinase C (PKC; MHC-PKC) from *Dictyostelium* that phosphorylates *Dictyostelium* MHC specifically and is homologous to α, β, and γ subtypes of mammalian PKC (Ravid and Spudich, 1989, 1992). In vitro phosphorylation of MHC by MHC-PKC results in inhibition of myosin II thick filament formation (Ravid and Spudich, 1989) 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 (Pasternak *et al.*, 1989).

‡ Corresponding author.

MHC-PKC, which is expressed during *Dictyostelium* development, regulates the myosin II reorganization in response to cAMP stimulation, by phosphorylating its MHC (Ravid and Spudich, 1992; Abu-Elneel *et al.*, 1996). MHC-PKC null cells exhibit a substantial myosin II overassembly in vivo and 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 (Abu-Elneel *et al.*, 1996). 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.

We have recently shown that cAMP exerts its effects on myosin II via the regulation of MHC-PKC (Dembinsky *et al.*, 1996). cAMP stimulation of *Dictyostelium* cells results in translocation of MHC-PKC from the cytosol to the membrane fraction and increasing MHC-PKC phosphorylation and its kinase activity (Dembinsky *et al.*, 1996). 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 (Dembinsky *et al.*, 1996).

In our search for the events that take place downstream of the cAMP receptor that affects the MHC-PKC behavior, we found that this kinase is subjected to several modes of regulation. cAMP stimulation of *Dictyostelium* generates a number of responses including cGMP accumulation (Mato *et al.*, 1977; Wurster *et al.*, 1977). We could show that cGMP accumulation is required for the activation of MHC-PKC. The cGMP does not affect the MHC-PKC directly but rather via the activation of cGMP-dependent protein kinase, which in turn phosphorylates the MHC-PKC leading to its activation (Dembinsky *et al.*, 1996). MHC-PKC also undergoes autophosphorylation in addition to its phosphorylation by cGMP-dependent protein kinase (Ravid and Spudich, 1989; Dembinsky *et al.*, 1997). The two kinds of phosphorylation involve different phosphorylation sites (Dembinsky *et al.* 1996, 1997). The MHC-PKC autophosphorylation domain contains a cluster of 21 serine and threonine residues; deletion of this domain abolishes both MHC-PKC autophosphorylation in vitro and cAMP-dependent MHC-PKC autophosphorylation in vivo (Dembinsky *et al.*, 1997). We have further shown that MHC-PKC autophosphorylation plays an important role in the kinase activation and subcellular localization. These studies indicate that the MHC-PKC activity is regulated by a number of different mechanisms.

A protein suggested to be involved in the regulation of PKC is 14–3-3 (for reviews see, Aitken, 1995, 1996; Aitken *et al.*, 1995b), which represents a highly conserved family of proteins found in a broad range of organisms and tissues (reviewed in Aitken, 1996). Numerous biological activities have been attributed to distinct members of this family, and 14–3-3 homologues in yeast have been implicated in cell cycle control (Ford *et al.*, 1994). Some members of the 14–3-3 family inhibit the activity of several PKC isoenzymes in vitro (Robinson *et al.*, 1994; Aitken *et al.*, 1995a). This inhibition can be overcome by the addition of diacylglycerol or phorbol ester (Robinson *et al.*, 1994; Aitken *et al.*, 1995a). It was therefore suggested that the interaction site of 14–3-3 on PKC is at or near the diacylglycerol/phorbol ester binding site, i.e., the cysteine-rich region (C1; Robinson *et al.*, 1994; Aitken *et al.*, 1995a). Recently, it has been shown that PKCθ interacts with 14–3-3τ and overexpression of later results in inhibition of PKCθ translocation and function, indicating that 14–3-3τ regulates the PKCθ activity (Meller *et al.*, 1996).

Although 14–3-3 proteins are thought to play important roles generally in signal transduction and in the regulation of PKC, it is not clear whether these two proteins interact in vivo and what the nature of this interaction is. In this report we investigate the role of Dd14–3-3 and the mammalian 14–3-3 ζ isoform in the regulation of MHC-PKC in vivo. Our finding indicate that the 14–3-3ζ inhibits the MHC-PKC activity in vitro and that MHC-PKC forms a complex with *Dd14–3-3* in vivo only in the cytosol, which may account for the lack of MHC-PKC activity in the cytosol. We were also able to map the interaction site of Dd14–3-3 and 14–3-3ζ on MHC-PKC to the C1 region. Our results therefore indicate that the interaction between MHC-PKC and Dd14–3-3 has a physiological function.

MATERIALS AND METHODS

Cell Culture and Development

Growth and development in suspensions of *Dictyostelium discoideum* cell lines were as described previously (Berlot *et al.*, 1985). Cells were washed in 20 mM phosphate buffer, pH 6.0, and resuspended at a density of 2×10^7 cells/ml to initiate development. Cells were shaken at 100 rpm at 22°C for 3.5 h prior to use.

Construction of Expression Vector Encoding MHC-PKC-C1

All DNA manipulations were carried out using standard methods (Sambrook *et al.*, 1989). We used the expression vector pDXA-HY, which contains the actin-15 promoter and allows the expression of proteins carrying a N-terminal His-tag (Manstein *et al.*, 1995). pDXA-MHC-PKC-C1 was constructed as follows: the vector pBS-MHCK (Ravid and Spudich, 1992) containing a 2.6-kb MHC-PKC cDNA clone was digested with *Sma*I and *Bsa*BI to yield a 417-bp fragment encoding 139 amino acids that are the C1 domain (MHC-PKC-C1). The MHC-PKC-C1 fragment was cloned into pDXA-HY (Manstein *et al.*, 1995) digested with *Sma*I. The MHC-PKC-C1 fragments were sequenced to confirm the presence of the desired deletions. The pDXA-MHC-PKC-C1 was used for the transformation of MHC-PKC null cells (Abu-Elneel *et al.*, 1996) using a calcium phosphate precipitate (Egelhoff *et al.* 1991). To achieve autonomous replication of the expression vectors, pDXA-MHC-PKC-C1 was co-transformed with a plasmid bearing a copy of the open reading

frame (Manstein *et al.*, 1995). Clones were selected on the basis of their resistance to G418 (Boehringer Mannheim, Indianapolis, IN) and screened using Western blot analysis (see below).

Purification of His-tagged MHC-PKC-C1

Approximately 2×10^6 cells expressing MHC-PKC-C1 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 KCl, 5 mM 2-mercaptoethanol, and a protease inhibitor mixture [200 μg/ml phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml leupeptin, and 1 μg/ml pepstatin]. The extracts were centrifuged in a microcentrifuge for 15 min at 4°C and the supernatant was incubated with 50 μl of a slurry of Ni^+-agarose beads (Qiagen, Chatsworth, CA) 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 lysis buffer containing 50 mM imidazole. The protein was eluted with 100 μl of lysis 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 3.5 h in shaking flasks as described (Berlot *et al.*, 1985). Samples were prepared from whole cell lysates (De Lozanne and Spudich, 1987) or from the insoluble fraction (Dembinsky *et al.*, 1996). Protein was determined according to the method of Peterson (1977), and lysates were electrophoresed on SDS-polyacrylamide gels (SDS-PAGE; Laemmli, 1970). Western blots were blocked with 5% milk-TBS containing 1% normal goat serum and probed with affinity-purified MHC-PKC polyclonal antibodies (Ravid and Spudich, 1992) or polyclonal antibodies against 14–3-3 that recognize conserved regions in a wide variety of eukaryotic 14–3-3 (Martin *et al.*, 1993). The blots were developed using a horseradish peroxidase-coupled secondary antibody (Bio-Rad Laboratories, Richmond, CA). ECL was performed with a kit from Amersham (Arlington Heights, IL).

Preparation of 259-Raf Peptide

The phosphorylated peptide from the region around Ser-259 of Raf-1 was synthesized using *N*-9-fluorenylmethoxycarbonyl (Fmoc) chemistry on an Applied Biosystems 430A peptide synthesizer and reagents supplied by the manufacturer. The phosphoserine residue was synthesized by incorporation of diisopropyl phosphoramidite (Novabiochem, Switzerland) after synthesis using unprotected Fmoc-serine in the desired position or by direct synthesis using Fmoc-phosphoserine (Novabiochem). Peptides were purified by reverse-phase high-performance liquid chromatography. Their structures were verified by mass spectrometry and peptide concentrations were verified using amino acid analysis.

Preparation of Immunoprecipitation Assays

After resuspension of 1×10^7 developed cells in 1 ml of sonication buffer (10 mM Tris-HCl, pH 7.5, 50 mM KCl, 2 mM PMSF, 100 μM leupeptin, 100 μM pepstatin), cells were lysed by sonication with an ultrasonic cell disruptor (Microson, Farmingdale, NY) model XL with a small-sized tip at 50% output power, and the extract was centrifuged in a microcentrifuge for 20 min at 4°C. The lysates were precleared by incubation with 30 μl of rabbit preimmune serum at 4°C for 1 h, followed by incubation with *Staphylococcus aureus* cells at 4°C for 30 min. *S. aureus* cells were centrifuged and the supernatants were subjected to immunoprecipitation. Immunoprecipitation assays were performed by incubating the precleared cell lysates with the appropriate antibody for 1 h at 4°C in the presence or the absence of 10 or 100 μM 259-Raf peptide. The antigen–antibody complexes were collected with protein A-conjugated agarose (100 mg/ml) at 4°C for 1 h. The immunoprecipitates were then washed twice in IP buffer (20 mM Tris-HCl, pH 7.5, 1% Triton X-100, 1 mM dithiothreitol, 25 mM KCl, and the protease inhibitor mixture) containing 1% bovine serum albumin and twice in IP buffer without bovine serum albumin before analysis by Western blot. Densitometric scanning of the Western blots was used to determine the relative amounts of immunoprecipitated proteins.

Association of 14–3-3ζ with MHC-PCK and MHC-PKC-C1

The different cell lines were developed as described above, lysed in 2× lysis buffer (40 mM Tris-HCl, pH 7.5, 2% Triton X-100, 2 mM dithiothreitol, 50 mM KCl, and the protease inhibitor mixture), and cleared by centrifugation. 14–3-3ζ at 10 μM (Jones *et al.*, 1995) was added to the extracts in the presence or in the absence of 10 or 100 μM 259-Raf peptide and incubated for 30 min at room temperature. The appropriate proteins were immunoprecipitated as described above. Immobilized proteins were washed three times in 1× lysis buffer and analyzed on Western blots.

Association of 14–3-3ζ with the Membrane-bound MHC-PKC

Approximately 1×10^7 developed Ax2 cells were resuspended in 1 ml of sonication buffer (10 mM Tris-HCl, pH 7.5, 50 mM KCl, 2 mM PMSF, 100 μM leupeptin, 100 μM pepstatin) and then lysed by sonication. MHC-PKC was extracted from the insoluble fraction with sonication buffer containing 0.5 M KCl, the extract was centrifuged in a microcentrifuge for 10 min at 4°C, and the solubilized MHC-PKC was incubated with 1 μM and 10 μM 14–3-3ζ. MHC-PKC was immunoprecipitated and examined by Western blot analysis with 14–3-3 antibody as described above.

Association of 14–3-3ζ with 259-Raf Peptide

14–3-3ζ was coupled to protein A-Sepharose by incubation with 14–3-3 antibody (Martin *et al.*, 1993), followed by incubation with protein A-Sepharose. The 14–3-3ζ immunocomplex was washed in 1× lysis buffer before incubating with 10 or 100 μM 259-Raf peptide for 30 min at room temperature. The immobilized proteins were washed three times in 1× lysis buffer and analyzed by SDS-PAGE.

Biochemical Analysis of MHC-PKC-C1 Distribution

The different cell lines were developed and stimulated with 1 μM cAMP, and at the indicated time points, 1×10^7 cells were lysed by using sonication as described above. MHC-PKC and MHC-PKC-C1 were immunoprecipitated from the soluble fraction with MHC-PKC antibody as described above. MHC-PKC and MHC-PKC-C1 were extracted from the insoluble fraction in sonication buffer containing 0.5 M KCl, the extract was centrifuged in a microcentrifuge for 10 min at 4°C, and the solubilized MHC-PKC and MHC-PKC-C1 were immunoprecipitated as described above. To quantify the amounts of MHC-PKC and MHC-PKC-C1 in the soluble and insoluble fractions, the immunoprecipitated MHC-PKC and MHC-PKC-C1 from both fractions were electrophoresed on 7% and 12% SDS-PAGE gels, respectively, and the Coomassie-blue-stained gels were analyzed as described above.

Biochemical Analysis of Dd14–3-3 Distribution

Ax2 cells were developed, and at the indicated time points, 1 μM cAMP was added to 1×10^7 cells that had been lysed by sonication as described above. The extracts were centrifuged in a microcentrifuge for 15 min at 4°C, and the soluble and insoluble fractions were subjected to Western blot analysis with antibody against 14–3-3.

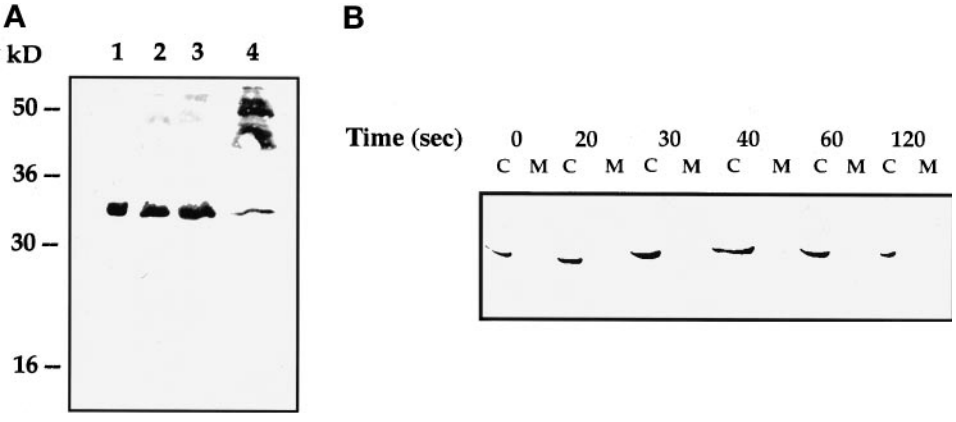

Figure 1. *Dictyostelium* expresses Dd14–3-3, a protein homologous to the 14–3-3. (A) Immunoblot analysis and biochemical localization of Dd14–3-3. Lane 1, vegetative *Dictyostelium* Ax2 cells; lane 2, 4-h developed *Dictyostelium* Ax2 cells; lane 3, recombinant 14–3-3ζ; lane 4, immunoprecipitation of Dd14–3-3 from developed *Dictyostelium* cells. (B) Biochemical localization of Dd14–3-3. Ax2 cells were developed and at the indicated times, 1 μM cAMP was added to 1×10^7 cells, which were lysed and fractionated into cytosol (C) and membrane (M) fractions as described in MATERIALS AND METHODS. Samples were subjected to 12% SDS-PAGE and blotted onto nitrocellulose. Immunoblots were stained with anti-14–3-3 antibodies.

MHC-PKC Inhibitor Assay

MHC-PKC activity was assayed directly on the kinase extracted from the insoluble cell fraction of developed Ax2 cells as described previously (Abu-Elneel *et al.*, 1996). To measure the inhibitory effect of the 14–3-3ζ on MHC-PKC activity, the required concentration of inhibitor (0–1 μM) was added to a total kinase assay volume of 25 μl. The inhibition was expressed as the percentage of kinase activity with the inhibitor as compared with the total kinase activity without the inhibitor.

RESULTS

Identification of a Dictyostelium Protein Homologue to the Mammalian 14–3-3 (Dd14–3-3)

Immunoprecipitation of MHC-PKC has revealed that it resides as a complex with several proteins, one of these proteins being myosin II (Dembinsky *et al.*, 1996). Recent studies have indicated that a family of proteins named 14–3-3 interact with PKCs in vitro, thereby inhibiting their activity (Toker *et al.*, 1990; Robinson *et al.*, 1994; Aitken *et al.*, 1995a,b). It was therefore of interest to find out whether a member of the 14–3-3 family is expressed by *Dictyostelium* and whether this protein interacts with MHC-PKC and regulates its activity.

A polyclonal antiserum that recognizes conserved regions in a wide variety of eukaryotic 14–3-3 (Martin *et al.*, 1993) recognized a band with an apparent molecular mass of 31 kDa in homogenates of vegetative (Figure 1A, lane 1) and 4-h developed (Figure 1A, lane 2) *Dictyostelium* cells separated by SDS-PAGE. The apparent molecular mass of the *Dictyostelium* 14–3-3 protein (Dd14–3-3) is consistent with previous reports for 14–3-3 proteins such as mammalian 14–3-3ζ (Figure 1A, lane 3; Toker *et al.*, 1990; Jones *et al.*, 1995). A 31-kDa band was also immunoprecipitated from 4-h developed (Figure 1A, lane 4) and vegetative (our unpublished results) *Dictyostelium* cells. These results indicate that *Dictyostelium* expresses a protein that is specifically recognized by antibodies against 14–3-3.

The Dd14–3-3 resided permanently in the cytosol of 4-h developed Ax2 cells regardless of cAMP stimulation. Figure 1B shows subcellular fractionation experiments performed with 4-h developed cells to assess the expression of Dd14–3-3 protein in the cytosol. Ax2 cells developed for 4 h and stimulated with cAMP and cytosolic and particulate fractions, prepared as described in MATERIALS AND METHODS, were analyzed by Western blotting using 14–3-3 antibodies. As shown in Figure 1B, expression of the Dd14–3-3 protein was exhibited in the cytosolic fractions regardless of cAMP stimulation. These results indicate that Dd14–3-3 is a cytosolic protein whose localization properties are unaffected by cAMP stimulation.

Recombinant 14–3-3ζ Inhibits MHC-PKC Activity

It has recently been shown that different isoforms of 14–3-3 inhibit the activity of PKC in vitro (Robinson *et al.*, 1994; Aitken *et al.*, 1995a). We therefore tested whether the recombinant 14–3-3ζ protein (Robinson *et al.*, 1994) also inhibits the activity of MHC-PKC. MHC-PKC was extracted from membranes of developed Ax2 cells and subjected to kinase assay in the presence of increasing concentrations of 14–3-3ζ as described in MATERIALS AND METHODS. Figure 2 shows that addition of increasing amounts of recombinant 14–3-3ζ resulted in inhibition of MHC-PKC activity with inhibition coefficient $(IC)_{50}$ of 0.62 μM. These results are consistent with previous reports using PKC purified from sheep brain and 14–3-3ζ (Robinson *et al.*, 1994; Aitken *et al.*, 1995a) and indicate that, similar to mammalian PKC, MHC-PKC activity is inhibited by 14–3-3ζ. That *Dictyostelium* expresses a protein homologue to the mammalian 14–3-3 protein and that the recombinant 14–3-3ζ inhibits its activity may indicate

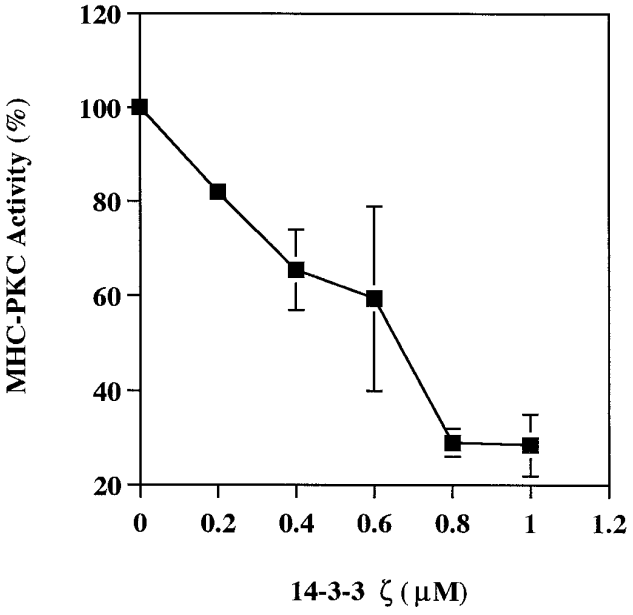

Figure 2. Inhibition of MHC-PKC activity by 14–3-3ζ. Various concentrations of recombinant 14–3-3ζ were incubated with MHC-PKC in a kinase assay as described in MATERIALS AND METHODS. The inhibition was expressed as a percentage of the kinase activity with the inhibitor as compared with the total kinase activity without the inhibitor. Data are the mean ± SEM (n = 4).

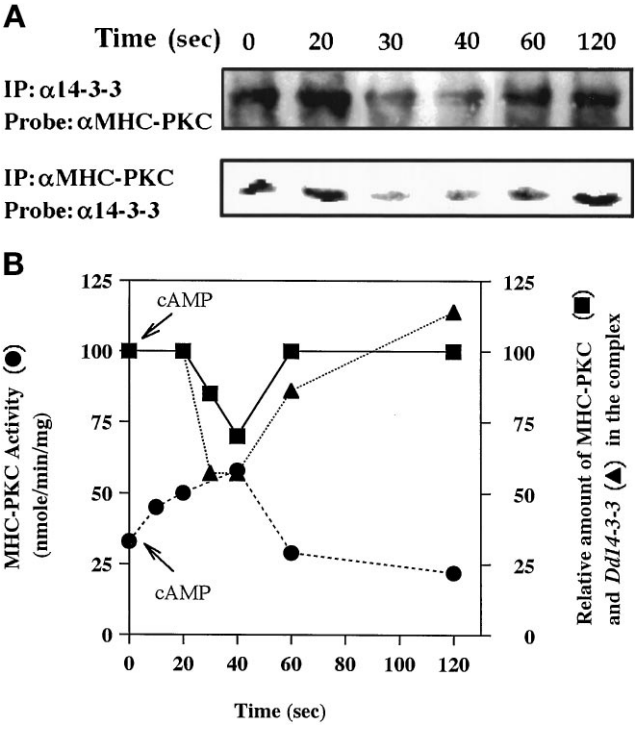

Figure 3. MHC-PKC forms a complex with Dd14–3-3 that is cAMP-dependent. (A) Ax2 cells were developed and stimulated with cAMP, and cell lysates were prepared as described in MATERIALS AND METHODS. Top, the cell lysates were immunoprecipitated with 14–3-3 antibodies and subjected to Western blot analysis using MHC-PKC antibodies as described in MATERIALS AND METHODS. Bottom, the cell lysates were immunoprecipitated with MHC-PKC antibodies and subjected to Western blot analysis using 14–3-3 antibodies as described in MATERIALS AND METHODS. (B) Quantification of the amounts of MHC-PKC and Dd14–3-3 in the complex shown in A and the activity of MHC-PKC that was measured as described in MATERIALS AND METHODS.

that Dd14–3-3 also has an inhibitory role on the MHC-PKC in vivo.

MHC-PKC Formed a Complex with Dd14–3-3 That Is Dependent on cAMP Stimulation

The cytosolic MHC-PKC has very low kinase activity that is increased upon cAMP-mediated MHC-PKC translocation to the membrane (Dembinsky *et al.*, 1996). It was, therefore, of interest to determine 1) whether MHC-PKC forms a complex with Dd14–3-3 in vivo that inhibits the cytosolic MHC-PKC and can account for the low activity of the cytosolic kinase and 2) whether cAMP stimulation affects the interaction between the two proteins. The following series of experiments were performed to determine whether the Dd14–3-3 and MHC-PKC formed a complex. The cytosolic fractions of developed cAMP-stimulated Ax2 cells were immunoprecipitated with MHC-PKC antibodies. The immunoprecipitates were subjected to Western blot analysis using 14–3-3 antibodies as described in MATERIALS AND METHODS. Dd14–3-3 was detected in MHC-PKC immunoprecipitates (Figure 3A), indicating that the two proteins interact with each other. A reciprocal experiment was performed to provide additional evidence for this MHC-PKC-Dd14–3-3 association. After the addition of cAMP to Ax2 cells, Dd14–3-3 was immunoprecipitated from the cytosol and subjected to Western blot analysis using MHC-PKC antibody as described in MATERIALS AND METHODS. As shown in Figure 3A, MHC-PKC was detected in Dd14–3-3 immunoprecipitates and further indicate that the two proteins formed a complex in the cytosol of *Dictyostelium* cells. Quantification of the amounts of the MHC-PKC and Dd14–3-3 in the complex revealed that addition of cAMP resulted in a transient decreased amounts of MHC-PKC and *Dd14–3-3* in the complex (Figure 3B).

To find out whether there is a correlation between the cAMP-dependent MHC-PKC-Dd14–3-3 dissociation and the appearance of membrane-bound active MHC-PKC after cAMP stimulation, we compared the amounts of MHC-PKC and Dd14–3-3 in the complex to the MHC-PKC activity that appeared in the membrane. It is apparent from Figure 3B that the cAMP-dependent association of MHC-PKC and Dd14–3-3 is inversely proportional to the MHC-PKC activation. The finding that the cytosolic MHC-PKC is inactive is consistent with the finding that MHC-PKC forms a

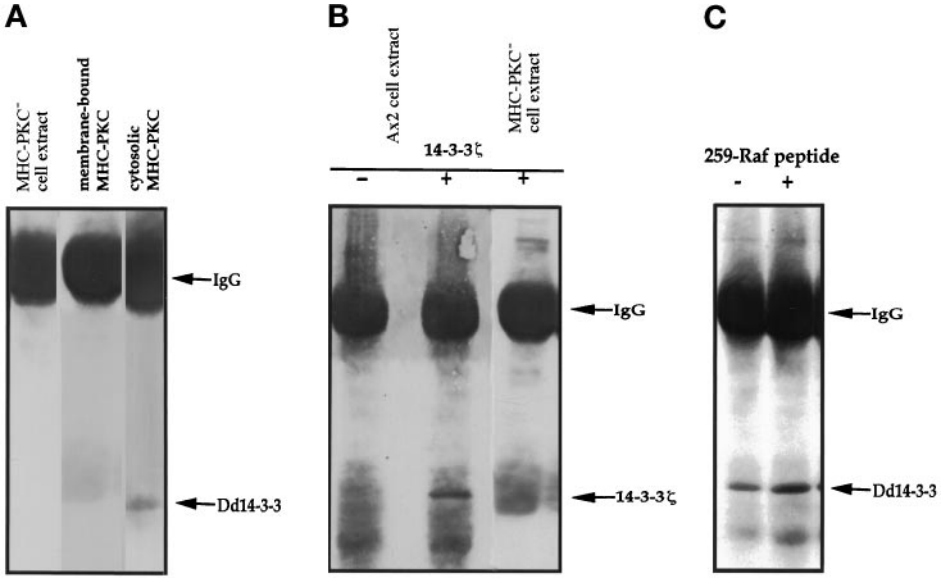

Figure 4. Analysis of the MHC-PKC interactions with Dd14–3-3 and 14–3-3ζ. (A) Ax2 cells were developed and lysed by sonication, and the soluble and insoluble fractions were separated. MHC-PKC null cells were developed, and a whole cell extracts were prepared. The MHC-PKC was immunoprecipitated from these fractions and subjected to Western blot analysis using 14–3-3 antibodies as described in MATERIALS AND METHODS. (B) Ax2 and MHC-PKC null cells were developed and lysed by sonication, and the MHC-PKC was extracted from the insoluble fraction as described in MATERIALS AND METHODS. The solubilized MHC-PKC was incubated with 10 μM 14–3-3ζ and the mixture was immunoprecipitated with MHC-PKC antibodies and analyzed by Western blot analysis with 14–3-3 antibody as described in MATERIALS AND METHODS. (C) Ax2 cell lysates were incubated with 100 μM 259-Raf peptide, immunoprecipitated with MHC-PKC antibodies, and analyzed using Western blot analysis with 14–3-3 antibody as described in MATERIALS AND METHODS.

complex with Dd14–3-3 in the cytosol and with the result that 14–3-3ζ inhibits the MHC-PKC activity in vitro (Figure 2). These results indicate that, in vivo, MHC-PKC forms a complex specifically with *Dd14–3-3* that inhibits its activity and the interaction between the two proteins is in a cAMP-dependent manner.

Membrane-bound MHC-PKC Was Not Found in a Complex with Dd14–3-3

As mentioned above the cytosolic MHC-PKC forms a complex with Dd14–3-3 that may result in inactivation of the kinase. Because the Dd14–3-3 is found only in the cytsol (Figure 1B), it is conceivable that the active membrane-bound MHC-PKC would not be found in a complex with Dd14–3-3. We performed the following tests to find out whether this is indeed the case. Ax2 cells were developed and stimulated with cAMP. The MHC-PKC was immunoprecipitated from the membrane and the cytosolic fractions as described in MATERIALS AND METHODS. The MHC-PKC immunoprecipitates were subjected to Western blot analysis using 14–3-3 antibodies. For a control we prepared cell extracts from MHC-PKC null cells (Abu-Elneel *et al.*, 1996) and subjected them to immunoprecipitation with MHC-PKC antibodies, followed by Western blot analysis using 14–3-3 antibodies. As shown in Figure 4A, the 14–3-3 antibodies did not detect any protein in the immunoprecipitates derived from MHC-PKC null cell extracts, indicating that the interaction between MHC-PKC and Dd14–3-3 is specific. It is evident from Figure 4A that the cytosolic MHC-PKC formed a complex with Dd14–3-3 and the membrane-bound MHC-PKC did not. These results are consistent with the findings that 14–3-3ζ inhibits the activity of MHC-PKC (Figure 2) and further indicate that the two proteins form a complex in the cytosol that inactivates MHC-PKC, on cAMP stimulation, the MHC-PKC dissociates from the Dd14–3-3 and translocates to the membrane. This releases the inhibition of Dd14–3-3 and activates the kinase.

MHC-PKC Isolated from the Membrane Fraction Formed a Complex with Recombinant 14–3-3ζ

The membrane-bound MHC-PKC was not found in a complex with Dd14–3-3 in vivo (Figure 4A). This is consistent with the finding that Dd14–3-3 is cytosolic, regardless of cAMP stimulation (Figure 1). However, the addition of 14–3-3ζ inhibits the activity of MHC-PKC isolated from membranes (Figure 2). We hypothesize that the membrane-bound MHC-PKC does not form a complex with Dd14–3-3 in vivo; however, addition of a large excess (1000-fold) of 14–3-3ζ to membrane-bound MHC-PKC in vitro will result in binding of the two proteins leading to the inhibition of the kinase. The following series of experiments were performed to determine whether MHC-PKC isolated from *Dictyostelium* membrane extract formed a com-

plex with 14–3-3ζ. *Dictyostelium* Ax2 cells and MHC-PKC null cells (Abu-Elneel *et al.*, 1996) were developed and lysed by sonication. MHC-PKC was extracted from the insoluble fraction and the solubilized MHC-PKC was incubated with 1 μM and 10 μM 14–3-3ζ. MHC-PKC was immunoprecipitated and subjected to Western blot analysis using 14–3-3 antibodies as described in MATERIALS AND METHODS. As shown in Figure 4, A and B, although the membrane-bound MHC-PKC did not form a complex with Dd14–3-3, the addition of 1000-fold excess of 14–3-3ζ to MHC-PKC, isolated from the membrane fraction, resulted in the binding of the two proteins. These results suggest that 14–3-3ζ inhibits the activity of MHC-PKC by a direct interaction. The absence of recombinant 14–3-3ζ in immunoprecipitates derived from MHC-PKC null cells indicate that the interaction between MHC-PKC and 14–3-3ζ is specific.

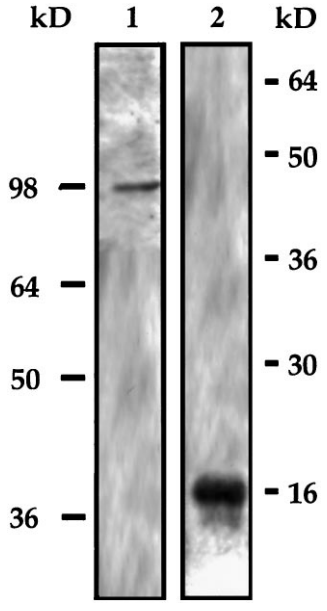

Figure 5. Expression of MHC-PKC-C1 in MHC-PKC null cells. Western blot analysis of Ax2 (lane 1) and MHC-PKC-C1 cells (lane 2) is shown. The cell lines were developed and cell extracts were subjected to Western blot analysis using MHC-PKC antibodies as described in MATERIALS AND METHODS.

The Interaction of MHC-PKC, Dd14–3-3, and 14–3-3ζ Is Not Mediated by Sequences Found in 259-Raf Peptide

Several observations suggest that 14–3-3 interactions with other proteins involve binding to sequences containing phosphoserine. First, the 14–3-3 activation of tyrosine hydroxylase requires prior phosphorylation of tyrosine hydroxylase with the serine/threonine kinase calmodulin kinase II (Furukawa *et al.*, 1993). Second, phosphatase treatment of Raf-1 and Bcr inhibits their association with 14–3-3 in vitro (Michaud *et al.*, 1995). Third, 14–3-3 binding to Raf-1 can block the ability of phosphatases to inhibit Raf-1 activity (Dent *et al.*, 1995). Fourth, phosphorylation of nitrate reductase is essential for its interaction with 14–3-3 (Moorhead *et al.*, 1996). Muslin *et al.* (1996) used a panel of phosphorylated peptides based on Raf-1 and defined the 14–3-3 binding motif that is included within the peptide named "259-Raf peptide." This peptide, which contains phosphoserine at position 259, inhibits the interaction between Raf-1 and 14–3-3 (Muslin *et al.*, 1996). Thus, these data suggest that binding to phosphoserine could account for a large number of reported 14–3-3 interactions. It was therefore of interest to test whether the interaction of MHC-PKC, Dd14–3-3, and 14–3-3ζ is also mediated by a similar mechanism. For this purpose we tested whether 259-Raf peptide inhibits the interaction of MHC-PKC, Dd14–3-3, and 14–3-3ζ. Phosphorylated 259-Raf peptide (10 and 100 μM) were added to extracts of developed Ax2 cells, and then the MHC-PKC was immunoprecipitated and subjected to Western blot analysis using 14–3-3ζ antibody. As shown in Figure 4C, the peptide did not interfere with the complex formation of MHC-PKC and Dd14–3-3. Similar results were also obtained with 14–3-3ζ (our unpublished results). In a control experiment, we found that the phosphorylated 259-Raf peptide formed a complex with 14–3-3ζ (our unpublished results). These results indicate that although the phosphorylated 259-Raf peptide binds 14–3-3ζ, 259-Raf did not inhibit the interaction of 14–3-3ζ with MHC-PKC. This suggests that the binding of Dd14–3-3 and 14–3-3ζ to MHC-PKC occurs by a different mechanism.

MHC-PKC Binds Dd14–3-3 and 14–3-3ζ through Its C1 Domain

To begin unraveling the molecular mechanism by which MHC-PKC interacts with Dd14–3-3 and 14–3-3ζ, we attempted to map the domain of MHC-PKC to which Dd14–3-3 and 14–3-3ζ bind. Because it was suggested that 14–3-3ζ binds to the cysteine-rich domain (Robinson *et al.*, 1994; Aitken *et al.*, 1995a) that is located at the C1 domain of PKC, we constructed a vector that expressed the C1 domain of MHC-PKC (MHC-PKC-C1; Figure 5). The MHC-PKC-C1 contains 139 amino acids from the N-terminal region of MHC-PKC that includes the two cysteine-rich domains (Ravid and Spudich, 1992). For the expression of the MHC-PKC-C1, we used an expression vector that adds a histidine tag to the N-terminal region of the expressed protein (Manstein *et al.*, 1995). The MHC-PKC-C1 protein was expressed in MHC-PKC null cells (Abu-Elneel *et al.*, 1996).

Figure 5 shows a Western blot analysis of the full-length MHC-PKC expressed by developed Ax2 cells (Figure 5, lane 1) and the MHC-PKC-C1 expressed in MHC-PKC null cells (Figure 5, lane 2). MHC-PKC null cells transformed with the pDXA-MHC-PKC-C1 construct (see MATERIALS AND METHODS) expressed

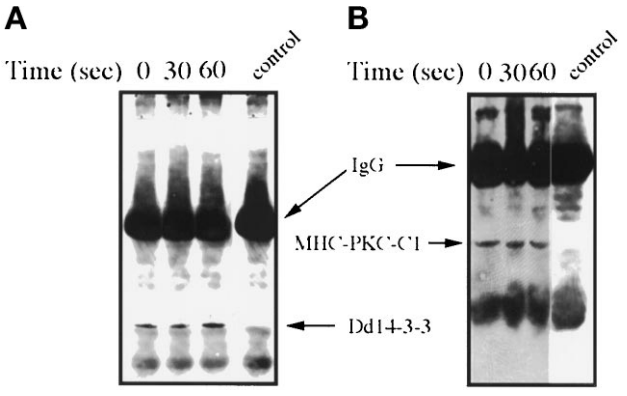

Figure 6. MHC-PKC-C1 forms a complex with Dd14–3-3 and 14–3-3ζ that is cAMP-independent. Extracts were prepared from developed cAMP-stimulated MHC-PKC-C1 and MHC-PKC null cells (control) as described in MATERIALS AND METHODS. (A) Cell lysates were immunoprecipitated with MHC-PKC antibodies and subjected to Western blot analysis using 14–3-3 antibodies as described in MATERIALS AND METHODS. (B) Cell lysates were immunoprecipitated with 14–3-3 antibodies and subjected to Western blot analysis using MHC-PKC antibodies as described in MATERIALS AND METHODS.

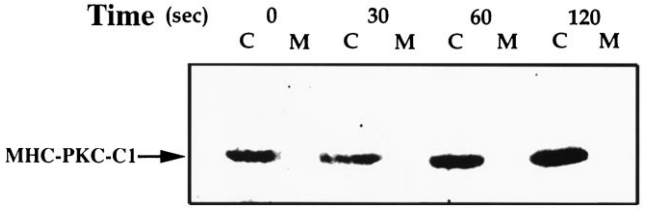

Figure 7. MHC-PKC-C1 localized to the cytosol regardless of cAMP stimulation. MHC-PKC-C1 cells were developed, and at the indicated time points, 1 μM cAMP was added to 1×10^7 cells that were lysed and fractionated into cytosol (C) and membrane (M) fractions as described in MATERIALS AND METHODS. Samples were subjected to SDS-PAGE on 12% gels and blotted onto nitrocellulose. Immunoblots were stained with anti-MHC-PKC antibodies.

MHC-PKC-C1 at 200–300% of the level of MHC-PKC in Ax2 cells (Figure 5). The expressed MHC-PKC-C1 migrated on SDS-PAGE with an apparent molecular weight of 16 kDa that is the molecular weight calculated for the sequence.

To find out whether MHC-PKC binds Dd14–3-3 and 14–3-3ζ through its C1 domain, we investigated whether the expressed MHC-PKC-C1 domain forms a complex with Dd14–3-3 and 14–3-3ζ. Furthermore, we investigated whether cAMP stimulation affects the interaction between the MHC-PKC-C1 and Dd14–3-3 as was found for MHC-PKC. To test whether MHC-PKC-C1 forms a complex with *Dd14–3-3,* we prepared extracts from developed cAMP-stimulated MHC-PKC-C1, immunoprecipitated the MHC-PKC-C1, and subjected it to Western blot analysis using 14–3-3 antibodies as described in MATERIALS AND METHODS; for a control we used MHC-PKC null cells. Figure 6A shows that Dd14–3-3 was detected in MHC-PKC-C1 immunoprecipitates, indicating that MHC-PKC-C1 forms a complex with Dd14–3-3 and that the MHC-PKC binds to Dd14–3-3 through its C1 domain. The amount of MHC-PKC-C1 and Dd14–3-3 in the complex was not affected by the addition of cAMP. As shown in Figure 6A, the 14–3-3 antibodies did not detect any protein in the immunoprecipitates derived from MHC-PKC null cell extracts, indicating that the interaction between MHC-PKC-C1 and Dd14–3-3 is specific.

A reciprocal experiment was performed to obtain additional evidence for this MHC-PKC-C1-Dd14–3-3 association. We prepared extract from developed cAMP-stimulated MHC-PKC-C1 cells and immunoprecipitated the Dd14–3-3, which was subjected to Western blot analysis using MHC-PKC antibodies as described in MATERIALS AND METHODS; for control we used MHC-PKC null cells. Figure 6B shows that MHC-PKC-C1 was detected in Dd14–3-3 immunoprecipitates, further supporting the idea that MHC-PKC interacts with Dd14–3-3 through its C1 domain. We also found that MHC-PKC-C1 formed a complex with 14–3-3ζ by a direct interaction (our unpublished results). MHC-PKC antibodies did not detect any protein in the immunoprecipitates derived from MHC-PKC null cell extracts. These results indicate that MHC-PKC binds specifically to Dd14–3-3 and 14–3-3ζ via its C1 domain and this interaction is not affected by cAMP stimulation.

MHC-PKC-C1 Protein Resides Permanently in the Cytosol Regardless of cAMP Stimulation

As indicated above, the interaction between MHC-PKC-C1 and Dd14–3-3 was unaffected by cAMP stimulation, in contrast to the interaction between MHC-PKC and Dd14–3-3. It was, therefore, of interest to investigate the localization properties of MHC-PKC-C1 in response to cAMP. MHC-PKC-C1 cells were developed and stimulated with cAMP, and the MHC-PKC-C1 was immunoprecipitated from the soluble and the insoluble fractions with specific MHC-PKC polyclonal antibody, as described in MATERIALS AND METHODS. As shown in Figure 7, in contrast to MHC-PKC in which cAMP stimulation results in translocation of the kinase to the membrane (Dembinsky *et al.*, 1996), stimulation of MHC-PKC-C1 cells with cAMP did not affect the localization properties of MHC-PKC-C1 and the protein resided permanently in the cytosol regardless of cAMP stimulation. Similar results were obtained by using Ni^+-agarose to isolate the MHC-PKC-C1 from the different cAMP-stimulated cell fractions (our unpublished results). These results may indicate that the C1

domain is not sufficient to drive the MHC-PKC to the cell membrane in response to cAMP stimulation.

DISCUSSION

We have examined the role of Dd14–3-3 in modulating the enzymatic activity and the biological function of MHC-PKC. The 14–3-3 proteins make up a well-conserved family of proteins present in mammalian cells and in flies, yeast, and plants (Aitken, 1996). Herein we present data that *Dictyostelium* expresses a protein that is recognized specifically by antibodies raised against eukaryotic 14–3-3 proteins (Martin *et al.*, 1993). This is the first evidence that *Dictyostelium* expresses a protein that is a member of the 14–3-3 protein family. Another indication for the presence of a member of the 14–3-3 family in *Dictyostelium* was provided by the isolation of a cDNA clone form *Dictyostelium* that exhibit high homology to several members of the 14–3-3 family (Knetsch, Ennis, van Heusden, Snaar-Jagalska, sequence GenBank accession number X95568).

The results presented in this study indicate that MHC-PKC and Dd14–3-3 interact in vivo and that this interaction is affected by cAMP stimulation. cAMP stimulation transiently decreased the amounts of the two proteins in the complex. The cytosolic MHC-PKC has very low kinase activity; however, on cAMP stimulation, the kinase translocates to the membrane and is activated (Dembinsky *et al.*, 1996). The data presented herein are consistent with these previous results, and we hypothesize that the complex between MHC-PKC and Dd14–3-3 that is formed in the cytosol results in the inhibition of the kinase activity in vivo. However, on cAMP stimulation, the complex dissociates, and the kinase translocates to the membrane where it is activated. These results indicate that the Dd14–3-3 plays a role in maintaining the kinase in an inactive state by anchoring the MHC-PKC to the cytosol. These results are supported by the findings that overexpression of 14–3-3τ inhibits the translocation of PKCθ (Meller *et al.*, 1996).

Similar to PKC the MHC-PKC is inhibited in vitro by 14–3-3ζ protein. The mechanism underlying this inhibition is unknown. However, several lines of evidence from this and other studies may provide some clues. The 14–3-3 family has a motif that shows some similarities with the pseudosubstrate site on PKC (Maraganore, 1987). This motif may bind the active site for PKC, thus, blocking access of substrate. However, a second interaction site must be present to account for the noncompetitive inhibition (Toker *et al.*, 1990). This site may be the sequence in 14–3-3 similar to the C terminus of the annexin family of Ca^{2+}-phospholipid and membrane binding proteins (Aitken *et al.*, 1990). Annexin V is a potent inhibitor of PKC (Schlaepfer *et al.*, 1992). Mochly-Rosen *et al.* (1991) have investigated receptors for activated C kinase (RACKs) and the mechanism by which PKC is translocated to the plasma membrane when activated by calcium. They have shown that the C terminus of annexins can prevent PKC association with RACKs proteins. Since 14–3-3 does not inhibit PKM (Toker *et al.*, 1990) the second interaction site may involve the regulatory domain of PKC. This suggestion is supported by the diacylglycerol/phorbol ester reactivation that indicates involvement of the cysteine-rich C1 region of PKC (Robinson *et al.*, 1994; Aitken *et al.*, 1995a) and by our finding that MHC-PKC interacts with Dd14–3-3 and 14–3-3ζ through its C1 domain.

How does cAMP stimulation affect the interaction between MHC-PKC and Dd14–3-3? It has been shown that diacylglycerol and phorbol ester remove the inhibition of PKC by 14–3-3 (Robinson *et al.*, 1994; Aitken *et al.*, 1995a). These results indicate that diacylglycerol/phorbol ester and 14–3-3 share the same binding site. Diacylglycerol is known to be a PKC cofactor; on external stimulation in many cell systems, synthesis of diacylglycerol activates the PKC (Bell, 1986; Bell and Burns, 1991). How diacylglycerol activates PKC is unknown. It is possible that the role of diacylglycerol is to remove the inhibition of 14–3-3 on PKC by competing for the binding site. In *Dictyostelium,* cAMP stimulation also results in increase in intracellular diacylglycerol (Ginsburg and Kimmel, 1989; Cubitt *et al.*, 1993). It is, therefore, possible that the cAMP-mediated increases in diacylglycerol lead to competition between diacylglycerol and Dd14–3-3 on the binding to MHC-PKC thereby removing the inhibition from the kinase. The data presented herein provide an additional indication that this is indeed the case. We have shown herein that, in vivo, MHC-PKC binds Dd14–3-3 via its C1 domain, and it is known that diacylglycerol binding site on PKC is located within the C1 domain (Bell and Burns, 1991).

The mechanism by which MHC-PKC interacts with Dd14–3-3 is different from that found for the interaction between Raf-1 and 14–3-3. Several observations suggest that 14–3-3 interaction with signaling proteins involve binding to phosphoserine (Furukawa *et al.*, 1993; Dent *et al.*, 1995; Michaud *et al.*, 1995). Muslin *et al.* (1996) have shown that 14–3-3 is a sequence-specific phosphoserine-binding protein. The identified peptide (Raf-259) derived from the Raf-1 sequence that contains a phosphoserine residue at position 259 is required for the interaction between 14–3-3 and Raf-1 (Michaud *et al.* 1995; Muslin *et al.*, 1996). Our results indicate that a sequence similar to Raf-259 is not involved in the interaction between MHC-PKC and Dd14–3-3. In contrast to the suggestion of Muslin *et al.* (1996), Dd14–3-3 binds to MHC-PKC C1 domain and Raf-259 like sequences are not involved in that interaction. These results indicate that different signal-

ing proteins interact with 14–3-3 through different sequences/domains.

The role of Dd14–3-3 in the regulation of MHC-PKC requires further studies. However, our studies allow speculation on different roles for Dd14–3-3. The *Dd14–3-3* may stabilize the inactive form of MHC-PKC in the cytosol. Additionally, Dd14–3-3 may play a role in bringing two proteins together. For example it is possible that Dd14–3-3 brings the MHC-PKC and cGMP-dependent protein kinase together. We have provided evidence that MHC-PKC is phosphorylated by cGMP-dependent protein kinase in the cytosol and this phosphorylation is required for the translocation and activation of MHC-PKC (Dembinsky *et al.*, 1996). It is therefore plausible that in *Dictyostelium, Dd14–3-3* forms a complex with MHC-PKC and cGMP-dependent protein kinase that makes the MHC-PKC accessible to cGMP-dependent protein kinase, allowing it to phosphorylate and activate the MHC-PKC. We found herein that MHC-PKC-C1 forms a complex with Dd14–3-3 that is insensitive to cAMP stimulation. As we showed earlier (Dembinsky *et al.*, 1996), the putative cGMP-dependent protein kinase sites on MHC-PKC are located within the C2 and C4 domains. This may explain why the complex MHC-PKC-C1–*Dd14–3-3* is cytosolic and insensitive to cAMP stimulation; although the MHC-PKC-C1 and cGMP-dependent protein kinase are brought together by Dd14–3-3, the kinase cannot phosphorylate the MHC-PKC-C1 because its phosphorylation sites are missing and so the C1 is not phosphorylated and resides in a complex with Dd14–3-3 in the cytosol.

The results presented herein in combination with our pervious reports indicate that MHC-PKC is subjected to multiple modes of regulation (Dembinsky *et al.*, 1996, 1997). Recently, our laboratory (our unpublished observation) and other researchers (Thanos and Bowie, 1996) have observed a rather striking homology between the large central domain of MHC-PKC and the catalytic domain of diacylglycerol kinase. These findings suggest that MHC-PKC is a more complex enzyme than previously thought and may have multiple functionality regulated by multiple mechanisms.

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