Involvement of the θ -type protein kinase C in translocation of myristoylated alanine-rich C kinase substrate (MARCKS) during myogenesis of chick embryonic myoblasts

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The phosphorylation pattern of numerous proteins in the soluble extracts of chick embryonic muscle cells changes dramatically during myogenesis. One of these proteins, the 63 kDa protein, whose phosphorylation state declines during the differentiation process, was identified as the myristoylated alanine-rich C kinase substrate (MARCKS), a major, specific substrate of protein kinase C (PKC). This decrease in the phosphorylation state of MARCKS was due to a decrease in the level of protein in the cytosol with a simultaneous increase in its level in the membrane fraction. Immunostaining of the cultured myoblasts also revealed that MARCKS translocated from the cytosol to the plasma membrane and to the peripheral region of nuclei as the mononucleated myoblasts fused to form multinucleated myotubes. Immunoprecipitation with an anti-PKC- θ antibody, but not with the antibodies against the other PKC isoforms, such as conventional PKC- α , novel PKC- δ , and novel PKC- ϵ , inhibited

phosphorylation of MARCKS. Moreover, expression of PKC- θ was found to be down-regulated during the course of myogenic differentiation. In addition, treatment of the cells with PMA, which activates PKC- θ and hence increases the phosphorylation state of MARCKS, reversibly inhibited both MARCKS translocation and myoblast fusion. These results suggest that MARCKS is preferentially phosphorylated by PKC- θ in cultured myoblasts and that the down-regulation of PKC- θ is partly responsible for MARCKS translocation during myogenesis. These results also suggest that PKC- θ -controlled MARCKS translocation is associated with, or a requisite event for, myoblast fusion.

Key words: creatine kinase, myoblast fusion, PMA, protein phosphorylation.

INTRODUCTION

Development of skeletal muscle involves a series of processes in which a subset of mesodermal cells are determined to become myoblasts, which, in turn, fuse with each other to form myotubes and ultimately form functional mature muscle fibres [1,2]. This process accompanies various cellular events, such as the accumulation of muscle specific proteins, cytoskeleton reorganization, and redistribution of membrane components [3,4]. The myogenic differentiation of embryonic muscle cells largely depends on extracellular diffusible molecules from their environment, such as growth factors and hormones [5]. Many of these regulators render the generation of second messengers, like Ca^{2+} , cAMP, cGMP, or phospholipid metabolites, which are known to be generally involved in many cellular regulatory events [6–10]. A number of reports have suggested that protein phosphorylation plays an important role in the differentiation of myoblasts [11–16]. The activity of protein kinase C (PKC) gradually decreases as myogenic differentiation proceeds, and treatment with PMA, a powerful activator of PKC, prevents myoblast fusion [12,15,17].

The myristoylated alanine-rich C kinase substrate (MARCKS) is an abundant, major cellular substrate for PKC [18]. It has been implicated in various cellular events, such as cell mobility, neurosecretion, and cell transformation [19,20]. The activation of PKC in various systems leads to the phosphorylation of MARCKS and its reversible translocation from the plasma membrane to the cytoplasmic compartment of cells [21,22]. MARCKS binds to calmodulin only in the presence of Ca^{2+} , and

the phosphorylation of MARCKS prevents the binding of calmodulin [23,24]. MARCKS also binds to and cross-links actin filaments. This actin cross-linking activity is prevented by Ca^{2+} /calmodulin and by phosphorylation with PKC [25]. Therefore, it has been suggested that MARCKS plays a role in the regulation of the cytoskeleton dynamics, particularly in the interaction of actin filaments with the plasma membrane.

In an attempt to clarify the role of PKC-mediated protein phosphorylation in myogenesis, we first examined the changes in the pattern of protein phosphorylation in the soluble extracts of chick myoblasts cultured for various periods of time. In the present studies, we identified a 63 kDa protein as MARCKS and for the first time showed that myogenic differentiation accompanies the translocation of MARCKS from the cytosol to the plasma membrane. Moreover, we revealed that PKC- θ phosphorylates MARCKS in cultured myoblasts, and that its down-regulation during myogenesis is at least in part responsible for MARCKS translocation. In addition, PMA, which is an activator of PKC- θ , was found to inhibit both MARCKS translocation and myoblast fusion. Therefore, we suggest that PKC- θ -controlled MARCKS translocation is associated with, or a requisite event for, myoblast fusion.

EXPERIMENTAL

Cell culture

Myoblasts from breast muscle of 12-day-old chick embryos were prepared as described previously [14,26]. The cells were plated on

Abbreviations used: MARCKS, myristoylated alanine-rich C kinase substrate; PKC, protein kinase C.

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collagen-coated culture dishes at a concentration of 5×10^5 cells/ml in minimum essential medium (MEM) containing 10% (v/v) horse serum, 10% (v/v) chick embryo extract, and 1% (v/v) antibiotic/antimycotic solution (Gibco BRL). One day after the cell seeding, the culture medium was changed to MEM containing 10% horse serum, 2% embryo extract, and 1% antibiotic/antimycotic solution for induction of differentiation (i.e. by reducing the concentration of embryo extract). When needed, PMA was added to the culture medium at the time of medium change. The cultured cells were subjected to Giemsa staining, and the extent of membrane fusion was expressed as the number of nuclei in fused cells as a percentage of the total number of nuclei in ten randomly chosen fields under a microscope. Cells containing more than three nuclei were regarded as fused cells.

Preparation of cell extract

Myoblasts cultured for appropriate periods were washed three times with ice-cold Earl's balanced salt solution (EBSS), harvested using a cell scraper, then centrifuged at 5000 *g* for 5 min and kept frozen at -70°C until use. The cells were homogenized in buffer A consisting of 50 mM Tris/HCl (pH 7.5), 5 mM MgCl_2 , 1 mM dithiothreitol, and 10% (v/v) glycerol. Buffer A also contained $1 \times$ protease inhibitor cocktail (Boehringer Mannheim) when the cell fractions were used for immunoblot analysis (see below), but not for phosphorylation assays because, for an unknown reason, the protease inhibitor cocktail strongly inhibited the phosphorylating activity. After centrifugation at 15000 *g* for 30 min, the supernatants were collected and referred to as soluble extracts. The precipitates were washed twice and resuspended in buffer A containing 1% (v/v) Triton X-100. The pellets were sonicated on ice for 30 s, and the resulting supernatants were referred to as the membrane fractions. For preparation of total cell lysates, myoblasts were directly sonicated in buffer A containing 1% Triton X-100. Protein concentrations were determined by the method of Bradford [27] or using bicinchoninic acid when Triton X-100 was present [28]. BSA was used as a standard.

Phosphorylation assay

Phosphorylation assays were performed by incubation of the reaction mixtures (in a final volume of 50 μl) containing 1.5 μCi of [γ - ^{32}P]ATP (6000 Ci/mmol; Amersham), 0.1 mM ATP, 5 mM MgCl_2 , and 30 μg of the soluble extracts or membrane fractions obtained from cultured myoblasts or the muscle extracts (see below) [14]. To prevent the effects of protein phosphatases, all of the phosphorylation assays were performed in the presence of 100 nM of okadaic acid. After incubation for 3 min at 25°C , the reaction was stopped by adding 50 μl of 4% (w/v) SDS. The samples were then electrophoresed on 10% (w/v) polyacrylamide slab gels containing SDS and 2-mercaptoethanol [29]. The gels were stained with Coomassie Blue R-250, dried, and exposed to X-ray film (Fuji) at -70°C .

Purification of the 63 kDa protein

The pectoralis muscles (60 g) from 15-day-old chick embryos were minced and homogenized in a Waring blender in 300 ml of buffer A. The homogenates were centrifuged at 100000 *g* for 2 h, and the resulting supernatants were referred to as muscle extracts. The muscle extracts were heated for 15 min at 95°C . After centrifugation, the supernatants (105 mg) were loaded onto a DEAE-Sepharose column (1.5×6 cm) equilibrated with buffer A. After washing the column with the same buffer, proteins were

eluted with a linear gradient of 0–0.3 M NaCl. The fractions containing the 63 kDa protein (27 mg) were pooled, and a saturated $(\text{NH}_4)_2\text{SO}_4$ solution (pH 7.5) was added to achieve a final concentration of 25%. The sample was then loaded onto a phenyl-Toyopearl column (1×5 cm) equilibrated with buffer A containing 25% $(\text{NH}_4)_2\text{SO}_4$. Proteins bound to the column were eluted with a reverse gradient of 25–0% $(\text{NH}_4)_2\text{SO}_4$. The fractions containing the 63 kDa protein were pooled (2.8 mg), dialysed against buffer A, and loaded onto a heparin-agarose column (1×4 cm) equilibrated with the same buffer. After washing the column with buffer A containing 0.2 M NaCl, the bound proteins were eluted with a linear gradient of 0.2–0.4 M NaCl. Elution of the 63 kDa protein was monitored by gel electrophoresis of the column fractions under denaturing conditions and by phosphorylation assay upon incubation with the partially purified PKC (see the Results section). The fractions containing only the 63 kDa protein were pooled and subjected to Edman degradation as described in the Results section.

Immunocytochemistry

Cells were plated on collagen-coated glass coverslips and fixed in 50% methanol/50% acetone at room temperature for 5 min, followed by washing three times with PBS. Non-specific binding sites were saturated by incubation with 3% (w/v) BSA in PBS for 60 min. The cells were incubated with the chicken MARCKS antibody (diluted 1:500) [30] for 1 h and then with FITC-conjugated donkey anti-rabbit IgG (diluted 1:250) (Jackson ImmunoResearch Laboratories, West Grove, PA, U.S.A.) for the next 1 h. The coverslips were mounted in Vectashield (Vector Laboratories, Burlingame, CA, U.S.A.) and viewed in a laser-scanning confocal microscope (Carl Zeiss LSM 410; Carl Zeiss, Jena, Germany). Samples were excited by a 488 nm argon laser, and the images were filtered by a longpass 515 nm filter. No fluorescence was detected if primary antibody was omitted.

In vivo labelling of MARCKS

Myoblasts cultured for appropriate periods were washed with phosphate-free Dulbecco's modified Eagle's medium, and starved for phosphate by incubation in the same medium for 30 min. Cellular phosphoproteins, including MARCKS, were metabolically labelled for 2 h by treating with 0.5 mCi of $^{32}\text{P}_i$ (9000 Ci/mmol, New England Nuclear) per ml. The cells were then washed three times with ice-cold EBSS and lysed in 50 mM Tris/HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, and $1 \times$ protease inhibitor cocktail. The lysate was immunoprecipitated by incubation with 10 μg of the anti-MARCKS IgG for 1 h and then with protein-A-Sepharose. The anti-IgG was purified from the antiserum raised against purified chicken brain MARCKS using a protein-A-Sepharose column. The resulting samples were subjected to gel electrophoresis under denaturing conditions as above, followed by exposure to X-ray film.

RESULTS

Phosphorylation of proteins in chick embryonic myoblasts

In order to determine whether the pattern of protein phosphorylation in cultured myoblasts changes during myogenic differentiation, soluble extracts were prepared from cells that had been cultured for various periods of time and then incubated with [γ - ^{32}P]ATP. As shown in Figure 1(A), the phosphorylation states of several proteins markedly changed in the extracts of differentiating myoblasts, in agreement with our previous results [14]. In particular, phosphorylation of the 63 kDa protein was

Figure 1 Changes in the pattern of protein phosphorylation during myogenic differentiation of cultured myoblasts and development of embryonic muscle

(A) Soluble extracts were prepared from the cells cultured for the indicated periods of time (h). (B) Soluble muscle extracts were also prepared from skeletal muscle tissue of 9–21-day embryos or adult chicks. Each 30 μ g extract was subjected to an *in vitro* phosphorylation assay by incubation with [γ - 32 P]ATP as described in the Experimental section. Arrow indicates the 63 kDa phosphoprotein; the letter A shows the adult chick muscle.

most prominent during proliferation (i.e. cultured for 24–36 h) but gradually declined as the cells committed to membrane fusion (i.e. after 48 h of the culture). The extents of the fusion for the cells cultured for 24, 36, 48, 60, and 72 h were 3.4 ± 2.4 , 8.4 ± 3.9 , 24.5 ± 5.2 , 78.6 ± 3.8 , and 87.4 ± 3.2 % (mean $\pm$ S.E.M.) respectively. Further studies were initiated to study the possible involvement of this 63 kDa protein in myogenic differentiation.

Since the amount of the 63 kDa protein in the cultured cells was not expected to be enough for identification of the protein, we prepared soluble muscle extracts from 9 to 21-day embryos as well as from adult chicks. We compared the phosphorylation pattern of the 63 kDa protein in the muscle extracts with that of the cultured myoblasts. Figure 1(B) shows that the phosphorylation states of several proteins were markedly changed during muscle development. Significantly, phosphorylation of the 63 kDa protein increased from day 9, reached a maximal level at day 15, and then declined rapidly from day 18. In adult muscle, phosphorylation of the 63 kDa protein could not be detected. Thus, it appeared that the phosphorylation of the 63 kDa protein might be under developmental regulation.

Identification of the 63 kDa protein as MARCKS

To determine the identity of the 63 kDa protein, we purified the protein to apparent homogeneity from muscle extracts obtained from the pectoralis muscles of 15-day-old chick embryos as described in the Experimental section. The purified 63 kDa protein was digested with trypsin and the tryptic peptides were separated by reverse-phase HPLC. Two major peptide fragments were sequenced by automated Edman degradation. The sequences obtained for the two peptides were identical to two internal sequences of chicken brain MARCKS, EAGEGAEESEGGAAAAEAGGK and AAEEASAAAAGSREAAK, corre-

Figure 2 Changes in the level of MARCKS protein during differentiation of cultured myoblasts

Total cell lysates (A), soluble extracts (B), and membrane fractions (C) were prepared from the cells cultured for the indicated periods of time (h) as described in the Experimental section. The same amounts of protein (30 μ g/lane) were subjected to electrophoresis on 10% polyacrylamide slab gels containing SDS and 2-mercaptoethanol, followed by immunoblot analysis using an antibody raised against chicken MARCKS (diluted 1:500). Arrows indicate the 63 kDa MARCKS protein.

sponding to residues 142–161 and 179–196, respectively [31]. These results indicate that the purified 63 kDa protein is identical to the MARCKS protein.

To determine whether the 63 kDa phosphoprotein in cultured myoblasts is also identical to MARCKS, soluble extracts of the cells prepared as above were subjected to immunoprecipitation analysis using the antibody raised against chick MARCKS. After centrifugation, the supernatants were subjected to assay for protein phosphorylation by incubation with [γ - 32 P]ATP. Phosphorylation of the 63 kDa protein, but not of others, was completely inhibited by treatment with the MARCKS antibody but not by non-immune IgG (results not shown). These results indicate that the 63 kDa protein in cultured myoblasts is MARCKS.

Localization of MARCKS in differentiating myoblasts

MARCKS is known to shuttle between the cytosol and the plasma membrane upon phosphorylation and dephosphorylation. Therefore, we postulated that the decrease in the level of MARCKS phosphorylation observed during the course of myogenic differentiation might be due to the translocation of the protein from the cytosol to the plasma membrane. To test this possibility, total cell lysates, soluble extracts, and membrane fractions were prepared from cells that had been cultured for various periods of time. These samples were subjected to immunoblot analysis using the chicken MARCKS antibody. Figure 2(A) shows that the protein level of MARCKS remained nearly constant during the entire period of culture, indicating that the expression of MARCKS does not change during the differen-

Figure 3 Localization of MARCKS in cultured myoblasts

Cells that had been cultured for 24 h (**A**) or 72 h (**B** and **C**) were subjected to immunostaining with the chicken MARCKS antibody as described in the Experimental section. Bar, 10 μ m.

Figure 4 Identification of PKC isotype responsible for MARCKS phosphorylation

The muscle extracts prepared from 15-day embryos were dialysed against buffer A containing 0.1 M NaCl. (**A**) The resulting sample was subjected to chromatography on heparin-agarose followed by gel filtration on a Superose-12 column as described in the text. Proteins were eluted by collecting 0.5 ml fractions at a flow rate of 10 ml/h. Aliquots of the fractions indicated (30 μ l) were subjected to an *in vitro* phosphorylation assay by incubation with purified MARCKS (0.3 μ g) and [γ - 32 P]ATP. (**B**) The same fractions were also subjected to immunoblot analysis using a polyclonal antibody to PKC- θ (Santa Cruz Biotechnology).

tiation of myoblasts. Instead, the protein level of MARCKS in the soluble extracts decreased (Figure 2B), while the protein level in the membrane fraction gradually increased, during the time of culture (Figure 2C). These results strongly suggest that MARCKS translocates from the cytosol to the plasma membrane during the process of myogenic differentiation.

To clarify further the translocation of MARCKS, cultured myoblasts were immunostained using the chicken MARCKS

antibody followed by confocal microscopy. As shown in Figure 3(A), MARCKS was diffusely stained in the cytosol of unfused, bipolar myoblasts. No sign of the staining was observed in the plasma membrane or the nuclear membrane in the mono-nucleated myoblast. In contrast, MARCKS was predominantly distributed along the plasma membranes in myotubes (Figure 3B). Interestingly, MARCKS was also intensely stained in the peripheral region of clustered nuclei of the fused myotubes (Figure 3C). It has been reported that approx. 20 % of MARCKS is localized in the nuclear fraction in bovine cerebral cortex [32]. These results are consistent with the finding with immunoblot analysis that the protein level of MARCKS in the soluble fraction decreased with increasing time of culture, with a concomitant increase in its level in the membrane fraction. Thus it appears clear that MARCKS translocates from the cytosol to the membrane during myogenic differentiation.

Identification of the PKC isotype that phosphorylates MARCKS

In order to identify which PKC isotype phosphorylates MARCKS, muscle extracts, obtained as described above, were dialysed against buffer A. The dialysed proteins were then loaded on a heparin-agarose column (1 $\times$ 10 cm) equilibrated with buffer A containing 0.1 M NaCl. After collecting the flow-through fractions, the column was washed with the same buffer. The proteins bound to the column were eluted with a linear gradient of 0.1–0.4 M NaCl. The resulting fractions were assayed for kinase activity by incubation with [γ - 32 P]ATP and purified MARCKS. A single peak of the phosphorylation activity was eluted at approx. 0.2 M NaCl (results not shown). MARCKS itself was eluted in the fractions corresponding to approx. 0.3 M NaCl, based on immunoblot analysis.

Fractions with high activity were pooled and concentrated by ultrafiltration using YM10 membrane (Amicon). The resulting proteins were subjected to gel filtration on a Superose-12 column (1 $\times$ 30 cm) equilibrated with buffer A containing 0.1 M NaCl. Aliquots of the column fractions were assayed for their ability to phosphorylate purified MARCKS, as described above. The same

Table 1 Effect of Ca^{2+} or EGTA on MARCKS phosphorylation by partially purified PKC- θ in the absence or presence of PMA

Partially purified PKC- θ from the Superose-12 column (1 μg) was incubated with the indicated reagents at 4 °C for 15 min. After incubation, samples underwent addition of purified MARCKS (0.3 μg) and [γ - ^{32}P]ATP, and further incubated at 25 °C for 3 min, followed by the phosphorylation assay as described in the Experimental section. The resulting X-ray films were scanned using a densitometer, and the extent of phosphorylation seen without any treatment was expressed as 100%, and the others expressed as relative values. Each value represents the mean $\pm$ S.D. of data obtained from three separate experiments.

Additions	Relative MARCKS phosphorylation (%)
None	100
Ca^{2+} (0.5 mM)	93 $\pm$ 3
EGTA (2 mM)	86 $\pm$ 9
PMA (10 nM) and phosphatidylserine (10 μg /ml)	188 $\pm$ 12
Ca^{2+} , PMA and phosphatidylserine	204 $\pm$ 21
EGTA, PMA and phosphatidylserine	216 $\pm$ 16

Figure 5 Immunoprecipitation of the protein kinase responsible for the MARCKS phosphorylation using antibodies against various PKC isoforms

Soluble extracts from myoblasts cultured for 24 h were incubated with increasing amounts of non-immune rabbit IgGs (○) or polyclonal antibodies against PKC- θ (●), PKC- ϵ (▲), PKC- δ (△) or PKC- α (■) (Santa Cruz Biotechnology) at 4 °C for 1 h and then with protein A-Sepharose for 1 h. After incubation, samples were centrifuged and the resulting supernatants were subjected to an *in vitro* phosphorylation assay by incubation with purified MARCKS and [γ - ^{32}P]ATP. The intensity of the MARCKS band obtained in the absence of the antibodies was determined using PhosphorImager (Fuji) and expressed as 100%; other values were determined relative to this. Each of the data points represents mean $\pm$ S.E.M. of triplicate determinations.

fractions were also subjected to immunoblot analysis using an antibody against PKC- θ , based on the report that PKC- θ is predominantly present in skeletal muscle [33]. Figure 4 shows that elution of the kinase activity, as assessed by MARCKS phosphorylation, from the gel filtration column (Figure 4A) closely overlaps with the elution of an 82 kDa protein that interacts with the antibody (Figure 4B). The molecular mass of PKC- θ isolated from mammalian sources has been estimated to

Figure 6 Changes in the protein levels of PKC- θ (A) and creatine phosphokinase (B) and *in vivo* phosphorylation of MARCKS (C) during differentiation of cultured myoblasts

(A) Soluble (panel a) and membrane fractions (panel b) were prepared from the cells cultured for various periods of time. Aliquots (50 μg) were subjected to immunoblot analysis as described in Figure 4. The numerals shown on top of the gels show the length of time (h) the cells were in culture. Arrows indicate the 82 kDa PKC- θ . (B) As a control, the same sample was immunoblotted using a polyclonal antibody to creatine phosphokinase (indicated by the arrow). (C) Cells cultured for the indicated periods of time were subjected to *in vivo* labelling in the presence of ^{32}P , followed by immunoprecipitation analysis as described in the Experimental section. The arrow indicates the ^{32}P -labelled MARCKS.

be in the range of 80–85 kDa [34,35]. In addition, little or no signal was obtained for the other PKC isoforms, including PKC- α , PKC- δ , and PKC- ϵ , when the active fractions from the Superose-12 column were pooled and subjected to immunoblot analysis (results not shown). Thus, it appears likely that MARCKS in chick embryonic myoblasts is preferentially phosphorylated by PKC- θ .

To characterize the biochemical properties of the partially purified PKC- θ from chick muscle, the pooled fractions were assayed for MARCKS phosphorylation in the presence and absence of Ca^{2+} , EGTA, and/or PMA. As shown in Table 1, the enzyme was insensitive to both Ca^{2+} and its chelating agent. On the other hand, PMA in the presence of phosphatidylserine stimulated the enzyme activity whether or not Ca^{2+} or EGTA was present. These results again suggest that the protein kinase phosphorylating MARCKS is the Ca^{2+} -insensitive isoform of PKC.

In order to clarify whether PKC- θ present in cultured myoblasts can indeed phosphorylate MARCKS, soluble extracts prepared from myoblasts cultured for 24 h were immunoprecipitated with the PKC- θ antibody. After centrifugation, the supernatants were assayed for their ability to phosphorylate purified MARCKS in the presence of [γ - ^{32}P]ATP. MARCKS protein phosphorylation was inhibited by treatment with the PKC- θ antibody in a dose-dependent manner, but not by treatment with antibodies against the other PKC isoforms, including conventional PKC- α , novel PKC- δ , or novel PKC- ϵ ,

Figure 7 Phosphorylation of the membrane-bound MARCKS in the absence and presence of partially purified PKC- θ

Membrane fractions were prepared from cells cultured for the indicated periods of time (h) as described in the Experimental section and their ability to phosphorylate membrane-bound MARCKS was assayed in the absence (A) and presence (B) of partially purified PKC- θ . The arrow indicates the phosphorylated MARCKS protein band. This band, but not others, could be eliminated when immunoprecipitation was performed using the MARCKS antibody prior to the gel electrophoresis (results not shown). Lane K indicates the partially purified PKC- θ incubated alone.

or with non-immune IgG (Figure 5). These results indicate that MARCKS in cultured myoblasts is preferentially phosphorylated by PKC- θ .

Expression of PKC- θ during myogenic differentiation

The decrease in the phosphorylation state of MARCKS in soluble extracts of cultured myoblasts (see Figure 1A) appeared to be more dramatic than the decrease in protein level (see Figure 2B). To examine whether or not the expression of PKC- θ changes during myogenic differentiation, soluble extracts were prepared from cells cultured for various periods of time and subjected to immunoblot analysis. As shown in Figure 6(A, panel a), the protein level of PKC- θ gradually decreased during the time of culture. Furthermore, the decrease in the protein level of PKC- θ in the soluble fractions was closely correlated with the decrease in the phosphorylation of MARCKS *in vivo* (Figure 6C). On the other hand, creatine kinase, which is one of the muscle-specific proteins, markedly accumulated during the same culture period (Figure 6B). Thus the expression of PKC- θ responsible for *in vivo* phosphorylation of MARCKS appears to be down-regulated during myogenesis.

PKCs are also known to shuttle between the cytosol and the plasma membrane upon treatment with various stimuli [36]. It is possible that the observed decrease in the protein level of PKC- θ may be due to translocation of the protein from the cytosol to the plasma membrane during the time of culture. To test this

Figure 8 Effect of PMA on MARCKS translocation and membrane fusion of myoblasts

(A) The cells cultured for 24 h (panel a) and 72 h (panel b) were incubated in the absence (lane C) or presence of 10 nM PMA (lane P) for 30 min. After incubation, membrane fractions were prepared from the cells and subjected to electrophoresis under denaturing conditions followed by immunoblot analysis using the MARCKS antibody, as in Figure 2 (A). (B) Cells were treated with 10 nM PMA at the time of medium change (lane P). Also, cells that had been cultured for 24 h in the presence of PMA were freed of the drug by changing the medium (lane F). These cells were further cultured for 48 and 24 h respectively. Total cell lysates (panel a) and membrane fractions (panel b) were prepared and subjected to the electrophoretic analysis as above. Lane C indicates the cells cultured for 72 h without any treatment. The cells cultured under the same conditions were stained and examined under a microscope to determine the degree of fusion, as described in the Experimental section (C).

possibility, the membrane fractions were prepared from the same cultured cells and subjected to immunoblot analysis. Little or no PKC- θ was detected in the membrane fractions obtained at any time during the culture period (Figure 6A, panel b). These results suggest that the localization of PKC- θ responsible for MARCKS phosphorylation may be restricted to the cytosolic fraction (unlike most typical PKCs).

To test whether other PKC isotypes are present in the plasma membrane which can phosphorylate MARCKS, the membrane fractions were incubated with [γ - 32 P]ATP. We could not detect any protein(s) in the membrane fractions that were phosphorylated to a significant extent (Figure 7A). However, a number of proteins, including the 63 kDa MARCKS, could be phosphorylated when the membrane fractions were incubated with partially purified PKC- θ (Figure 7B). These results further suggest that PKC- θ is responsible for the phosphorylation of MARCKS and that it is localized to the cytosol, but probably not in the membrane fraction, of cultured myoblasts.

Effects of PMA on MARCKS translocation

PMA can alter the phosphorylation state of MARCKS by activating PKC- θ . In fact, it has been reported that PMA treatment increases *in vivo* phosphorylation of MARCKS [37]. Therefore, treatment of cultured myoblasts with the drug is likely to influence the shuttling of MARCKS by changing its phosphorylation state. In addition, PMA has been shown to block myoblast fusion [38]. To determine whether PMA can indeed block MARCKS translocation, the cells cultured for 24 or 72 h were incubated with the drug for 30 min. After incubation, membrane fractions were prepared from the cells and subjected to immunoblot analysis using the MARCKS antibody. As shown in Figure 8(A, panel a), the protein level of MARCKS in membrane fractions of the cells treated with PMA was markedly reduced as compared to that in the untreated control cells. On the other hand, the MARCKS level remained unchanged in the cells cultured for 72 h, which already formed myotubes, on treatment with PMA (Figure 8A, panel b). This result is consistent with our finding that the expression of PKC- θ is down-regulated during the course of myogenesis.

In an attempt to elucidate the link between MARCKS translocation and myoblast fusion, cells were treated with PMA at the time of medium change. Also, the cells that had been cultured for 24 h in the presence of PMA were freed of the drug by changing the medium. These cells were further cultured for 48 and 24 h respectively. They were then subjected to immunoblot analysis for determining the level of MARCKS in total cell lysates and membrane fractions (Figure 8B, panels a and b respectively). The cells cultured under the same conditions were also stained and examined under a microscope to determine the degree of fusion (Figure 8C). PMA inhibited both MARCKS translocation and myoblast fusion in parallel. Furthermore, its inhibitory effect on both processes could be reversed upon removal of the drug from the culture medium. These results suggest that MARCKS translocation, which is negatively controlled by PKC- θ -dependent phosphorylation, may be associated with or a requisite step for myogenic differentiation.

DISCUSSION

One of the most prominent events in the differentiation of skeletal muscle cells is the membrane fusion of mononucleated myoblasts into multinucleated myotubes. This process absolutely requires the influx of Ca^{2+} and accompanies various cellular events, including massive reorganization of the cytoskeleton and redistribution of membrane components. Since MARCKS binds to Ca^{2+} /calmodulin and has been proposed to regulate cytoskeleton dynamics by cross-linking actin filaments and the plasma membrane, the PKC-mediated phosphorylation of MARCKS may play an important role in the regulation of the myogenic process. In proliferating and fusion-competent cells, the phosphorylation of MARCKS would lead to retention of the protein in the cytosol by inhibiting its actin cross-linking activity and hence provide a potentially less 'rigid' environment [39]. On the other hand, in the cells committed to fusion, dephosphorylation would result in translocation of MARCKS to the plasma membrane, where it could cross-link actin filaments, providing a more 'rigid' environment, such as is needed for positioning and movement of contractile elements.

In the present studies, we have demonstrated that the 63 kDa protein, which was identified as MARCKS, was translocated from the cytosol to the plasma membrane as the cultured myoblasts fused to form myotubes. Immunoblot analysis using an antibody raised against chicken MARCKS revealed that the protein level of MARCKS in the cytosolic fraction of rapidly

proliferating and fusion-competent cells was much higher than that observed in the membrane fraction. The level of MARCKS in the cytosol gradually decreased with a concomitant increase in its level in the plasma membrane as cell fusion proceeded. Furthermore, immunostaining of the cells with the MARCKS antibody unequivocally demonstrated that the diffusely stained MARCKS in the cytosol of proliferating myoblasts was almost entirely translocated to the plasma membrane of fused myotubes. Therefore, it appears that the translocation of MARCKS from the cytosol to plasma membrane is associated with, or a requisite event for, myogenic differentiation.

In the process of muscle differentiation, membrane fusion of myoblasts also brings about clustering of nuclei. Immunostaining of the cells with the MARCKS antibody has revealed that MARCKS translocated not only to the plasma membrane but also to the peripheral region of nuclei during myogenesis. Although the mechanism of nuclei clustering is not yet known, the specific localization of MARCKS in the nuclear membrane of myotubes among numerous subcellular organelles suggests that its cross-linking activity of actin filaments may be involved in the clustering of nuclei.

In general, MARCKS has been employed as an indicator for the activation of PKC regardless of isoform. However, *in vitro* studies have demonstrated that conventional PKC- α , novel PKC- δ , and novel PKC- ϵ , but not atypical PKC- ζ , phosphorylate partially purified recombinant MARCKS protein [40,41]. More recently, Überall et al. [42] have clearly shown that the θ -type isoform of PKC, but not atypical PKC- λ , also phosphorylates MARCKS in intact NIH 3T3 fibroblasts. In our *in vitro* studies, MARCKS was found to be preferentially phosphorylated by PKC- θ in cultured myoblasts. Firstly, only a single peak of the MARCKS phosphorylation activity was found upon chromatography of the soluble muscle extracts on both heparin-agarose and Superose-12 columns. Second, the size of the phosphorylating activity determined by the latter gel filtration column, which is approx. 82 kDa, was in good agreement with the mass of PKC- θ isolated from mammalian sources. Third, immunoprecipitation with the antibodies raised against PKC- θ , but not with those against other PKC isoforms, could eliminate the MARCKS phosphorylation activity from the soluble extracts of cultured myoblasts (see Figure 5) as well as from the embryonic muscle extracts (results not shown). Thus it appears likely that PKC- θ is partly responsible for the phosphorylation of MARCKS in cultured myoblasts and embryonic muscle tissue.

Of note is the finding that the membrane fractions prepared from cultured myoblasts were unable to phosphorylate MARCKS present in the same membrane fraction. The same membrane-bound MARCKS, however, could readily be phosphorylated upon incubation with PKC- θ , which was partially purified from the soluble muscle extracts. Moreover, little or no PKC- θ was detected in the membrane fractions obtained at any time of the culture. Thus it appears that the plasma membrane of cultured myoblasts lacks the protein kinase needed to phosphorylate MARCKS. However, the general mechanism accepted for the activation of PKC is that the enzyme associates with the membrane and becomes activated by phospholipids and diacylglycerol at the surface of membrane. Since our phosphorylation assays were performed *in vitro* using Triton-solubilized membrane fractions from cultured myoblasts, we cannot exclude a possibility of the presence of agonist-sensitive, membrane-bound PKC isoforms that are capable of phosphorylating MARCKS upon stimulation. Nevertheless, our finding that the kinase activity capable of phosphorylating MARCKS was absent from the membrane fraction of cultured myoblasts is not surprising. The myogenic differentiation process leading the

cells to fuse and to ultimately form myofibrils is an irreversible process that no longer requires dissolution of actin filaments. In support of this notion, the expression of PKC- θ responsible for the phosphorylation of MARCKS *in vivo* as well as *in vitro* was found to be down-regulated during differentiation of the muscle cells. On the other hand, PMA, which activates PKC- θ and MARCKS phosphorylation, could prevent both MARCKS translocation and myoblast fusion. Therefore, the PKC- θ -mediated phosphorylation of MARCKS in proliferating and fusion-competent myoblasts, and down-regulation of PKC- θ expression leading to dephosphorylation and translocation of MARCKS in the cells committed for membrane fusion, may represent essential events in the process of myogenic differentiation.

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