Involvement of protein phosphatase-1-mediated MARCKS translocation in myogenic differentiation of embryonic muscle cells

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Summary

Myristoylated alanine-rich C kinase substrate (MARCKS) translocates from the cytosol to the plasma membrane while mononucleated myoblasts fuse to form multinucleated myotubes. Here, we show that protein phosphatase-1-mediated dephosphorylation of MARCKS largely influences its subcellular localization and the fusion process. Treatment with okadaic acid or tautomycin, which are potent inhibitors of protein phosphatases and cell fusion, was found to reversibly block the MARCKS translocation. Moreover, the dephosphorylating activity against MARCKS markedly increased during myogenesis, and this increase was closely correlated with the membrane fusion of the cells. In addition, protein phosphatase-1 was identified as a major enzyme that is responsible for dephosphorylation of MARCKS. Furthermore, a mutation preventing MARCKS phosphorylation and thus facilitating MARCKS translocation resulted in promotion of the cell fusion. In contrast, overexpression of MARCKS carrying a mutation that blocks myristoylation and thus prevents the MARCKS translocation impaired the myoblast fusion. Together with the fact that MARCKS regulates the cytoskeleton dynamics by crosslinking the actin filaments in the plasma membrane and that myoblast fusion accompanies massive cytoskeleton reorganization, these results suggest that protein phosphatase-1-mediated MARCKS localization at the membrane is required for the fusion of embryonic muscle cells.

Key words: Myoblast fusion, Dephosphorylation, Okadaic acid, Myristoylation, Filamentous actin

Introduction

A prominent event in the differentiation of skeletal muscle cells is the fusion of mononucleated myoblasts to form multinucleated myotubes (Bischoff and Holtzer, 1969; O'Neill and Stockdale, 1972). This process absolutely requires reorganization of cytoskeleton and accompanies various cellular events, including accumulation of muscle-specific proteins and redistribution of membrane components (Fulton et al., 1981; Nadal-Ginard, 1978; Pauw and David, 1979). Numerous studies using cultured myoblasts have suggested that phosphorylation and dephosphorylation of proteins play an important role in mediating differentiation of myoblasts (Toutant and Sobel, 1987; Rapuano et al., 1989; Adamo et al., 1989; Kim et al., 1992a; Kim et al., 1993; Baek et al., 1994). Okadaic acid, an inhibitor of protein phosphatases, has been shown to block membrane fusion of differentiating myoblast (Kim et al., 1991; Kim et al., 1992b). The activity of the protein phosphatases sensitive to okadaic acid has also been reported to increase progressively in differentiating myoblasts (Srinivasan and Begum, 1994).

The myristoylated alanine-rich C kinase substrate (MARCKS) is an abundant, high affinity cellular substrate for PKC. It has been implicated in various cellular events, such as cell mobility, neurosecretion and cell transformation (Aderem, 1992a; Blackshear, 1993). One of the striking features of MARCKS is its phosphorylation-dependent translocation between the cytosol and the plasma membrane. The activation of PKC in various systems leads to phosphorylation of MARCKS and its reversible translocation from the plasma membrane to the cytosolic compartment of the cells (Thelen et al., 1991; Wang et al., 1989). MARCKS is able to crosslink filamentous actins in the plasma membrane, and the phosphorylation of MARCKS dissociates the filamentous actin crosslinking and translocates it to the cytosol (Hartwig et al., 1992). Therefore, the subcellular localization of MARCKS is undoubtedly essential to its function in the regulation of the cytoskeleton dynamics, particularly in the interaction of actin filaments at the plasma membrane.

Ser/Thr-specific protein phosphatases are divided into two groups, type-1 and type-2 (Ingebritsen and Cohen, 1983a; Ingebritsen and Cohen, 1983b). The type-1 protein phosphatase (PP-1) preferentially dephosphorylates the β-subunit of phosphorylase kinase and is inhibited by nanomolar concentrations of two heat-stable proteins, termed protein phosphatase inhibitor-1 and -2 (Ingebritsen and Cohen, 1983a; Ingebritsen and Cohen, 1983b), as well as by micromolar concentrations of okadaic acid (Bialojan and Takai, 1988; Haystead et al., 1989; Cohen et al., 1990). By contrast, the type-2 protein phosphatases (PP-2), comprising PP-2A, PP-2B and PP-2C, specifically dephosphorylate the α-subunit of

phosphorylase kinase (Ingebritsen and Cohen, 1983a; Ingebritsen and Cohen, 1983b). Their activities are not affected by the inhibitor proteins, but PP-2A is specifically inhibited by another heat stable protein (I_1^{PP2A}) and extremely sensitive to nanomolar concentrations of okadaic acid (Cohen, 1989; Li et al., 1996). The bombesin- or vasopressin-stimulated phosphorylation of MARCKS is dynamically reduced upon removal of the PKC activator in Swiss 3T3, indicating that the phosphatase activity against MARCKS is present and could regulate the phosphorylation state of the protein (Rodriguez-Pena et al., 1986). Moreover, okadaic acid, which inhibits PP-1 and PP-2A, shows little or no effect on MARCKS phosphorylation on its own, but strongly inhibits the dephosphorylation of MARCKS, which were phosphorylated upon activation of PKC (Clarke et al., 1993).

We have recently shown that MARCKS translocates from the cytosol to the plasma membrane while mononucleated myoblasts fuse to form multinucleated myotubes (Kim et al., 2000). This MARCKS translocation was in part due to a decrease in the expression of PKCθ, which is the major enzyme responsible for MARCKS phosphorylation, during the myogenic process. We have also demonstrated that treatment with PMA, an activator of the protein kinase, blocks both MARCKS translocation and myoblast fusion. In order to clarify further whether the MARCKS shuttling is indeed involved in myogenesis, we examined the effect of okadaic acid on translocation of MARCKS during the differentiation process. In the present studies, we show that okadaic acid prevents the translocation of MARCKS from the cytosol to the plasma membrane and that the dephosphorylating activity against MARCKS markedly increases during the course of myogenesis. In addition, PP-1 was found to be the major enzyme responsible for the MARCKS dephosphorylation. Moreover, overexpression of MARCKS carrying a mutation that prevents myristoylation and thus blocks its membrane translocation impaired the fusion of cultured myoblasts. Therefore, we suggest that the protein phosphatase-1-mediated MARCKS localization at the membrane is involved in the fusion of embryonic muscle cells.

Materials and Methods

Materials

The cDNA for chick MARCKS and its mutant forms (N/S and A_2G_2: see below) and polyclonal anti-MARCKS antibody were kindly provided by P. J. Blackshear and D. J. Stumpo (NIEHS). Culture media, horse serum, antibiotic/antimycotics solution, and other culture reagents were from Gibco-BRL. [γ-^{32}P]ATP was obtained from Amersham; FuGENE 6 and complete protease inhibitor cocktail were from Boeringher Mannheim; okadaic acid, tautomycin, Ro-31-8220, protein phosphatase inhibitor-2, and protein phosphatase-2A inhibitor were from Calbiochem; rhodamine-phalloidin was from Molecular Probes. pcDNA3.1/*myc*-His and Vectashield were obtained from Invitrogen and Vector Laboratories, respectively. Antibodies against PP-1, c-Myc, and phospho-MARCKS (Ser152/156) were from Transduction Laboratories, Santa Cruz Biotechnology, and Cell Signaling Technologies, respectively. All secondary antibodies were from Jackson Immunoresearch. All other materials were purchased from Sigma, unless otherwise indicated.

Cell culture

Myoblasts from breast muscle of 12-day-old chick embryos were prepared as described previously (Ha et al., 1979; Kim et al., 1992a). The cells were plated on 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/antimycotics solution. One day after the cell seeding, the culture medium was changed with the same medium but containing 2% embryo extract for induction of differentiation. The cells were subjected to Giemsa staining, and the degree of myoblast fusion was determined as the percentage of the number of nuclei in fused cells to the total number of nuclei in 10 randomly chosen the fields under a microscope. Cells containing more than three nuclei were regarded as fused cells.

Preparation of subcellular fractions

Myoblasts cultured for appropriate periods were washed three times with ice-cold Earl's balanced salt solution (EBSS), harvested by centrifugation, and kept frozen at –70°C until use. The cells were resuspended in 50 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 1 mM dithiothreitol (DTT), and 1× complete protease inhibitor cocktail, and preparation of their subcellular fractions was performed as described previously (Kim et al., 2000). Protein concentration was determined by a previously described method (Bradford, 1976) or by using bicinchoninic acid when Triton X-100 was present (Smith et al., 1985). Bovine serum albumin was used as a standard.

Preparation of ^{32}P-labeled MARCKS and phosphatase assay

Phospho-MARCKS was prepared using purified MARCKS and partially purified PKCθ preparation, which was obtained by the heparin-agarose chromatography of the muscle extracts as described previously (Kim et al., 2000). Reaction mixtures contained 20 mM Hepes (pH 7.4), 0.25 mM EDTA, 0.25 mM EGTA, 1 mM $CaCl_2$, 1 mM DTT, 5 mM $MgCl_2$ and 0.15 mM [γ-^{32}P]ATP (0.05 mCi/ml). After incubating them for 3 minutes at 25°C, the samples were heated for 3 minutes at 95°C and centrifuged at 15,000 ***g*** for 30 minutes. The resulting ^{32}P-labeled MARCKS was used as the substrate for assaying the phosphatase activity. The enzyme activity was determined by incubation of ^{32}P-labeled MARCKS with the soluble extracts obtained from the cultured myoblasts in 50 mM Hepes (pH 7.4), 1 mM EDTA, 1 mM EGTA, 5 mM $MgCl_2$, 1 mM DTT, 1× complete protease inhibitor cocktail, and 100 nM Ro-31-8220 or staurosporine. Incubations were performed for 30 minutes at 30°C and stopped by addition of 2% SDS solution containing 1% 2-mercaptoethanol. The samples were heated for 3 minutes at 95°C and subjected to polyacrylamide gel electrophoresis in 10% slab gels under denaturing conditions (SDS-PAGE) (Laemmli, 1970), followed by autoradiography. When inhibitors were included, the extracts were incubated with them for 15 minutes prior to the addition of ^{32}P-labeled MARCKS.

Immunoprecipitation of PP-1

Myoblasts cultured for 48 hours were resuspended in 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM $MgCl_2$ and 1× complete protease inhibitor cocktail. The cells were disrupted by sonication on ice and centrifuged at 15,000 ***g*** for 30 minutes. The supernatants were incubated with 10 μg of anti-PP-1 IgG or preimmune IgG at 4°C for 2 hours in a rotating shaker. They were then added with 40 μl of 50% (v/v) protein A-Sepharose in the same buffer and incubated for 1 hour. After centrifugation, the supernatants were subjected to phosphatase assay. They were also subjected to SDS-PAGE as above, followed by autoradiography.

Plasmid construction

Since a stable cell line cannot be established with the primary culture of myoblasts and since these cells have a relatively low efficiency of

transfection, Myc-tagged MARCKS were prepared for identification of the transfected cells. The N/S mutant carries the Asn residue in the phosphorylation domain of MARCKS in place of Ser (Swierczynski and Blackshear, 1996). In the A_2G_2 mutant, the Gly residue next to the N-terminus of MARCKS was substituted with Ala. PCR reactions were performed using the cDNAs for the wild-type MARCKS and its mutant forms as the templates, and oligonucleotide primers carrying the *Hin*dIII and *Xba*I restriction sites. The sequences of the primers are as follows: primer 1, TGTTAAGCTTGCCACCATGGGTGCC, which includes a *Hin*dIII site; primer 2, TGTTAAGCTTGCCACCATGGCTGCC, which has an altered sequence that can complement with the A_2G_2 mutation site as well as the *Hin*dIII site; primer 3, GCGGTCTAGACTCCGCCGGCTCGGC, which has a *Xba*I site. The PCR products were cut off by treatment with *Hin*dIII and *Xba*I and ligated into the pcDNA3.1/*myc*-His plasmid that had been cut with the same enzymes. Thus, in the resulting plasmids, the cDNAs for MARCKS and its mutant forms are under control of the cytomegalovirus (CMV) promoter/enhancer. These constructs also have a polyadenylation signal of the bovine growth hormone (BGH) terminator at their 3′-termini.

Transient transfection of myoblasts

The cells were seeded on 100 mm plates at a density of 1.0×10^5 cells/ml and cultured for 24 hours. To prepare DNA for transfection, 12 μl of FuGENE 6 was diluted with 0.4 ml of MEM and incubated for 5 minutes at room temperature. The diluted solution was slowly added to the pcDNA derivatives (4 μg), mixed gently, and incubated for 15 minutes. The cultured myoblasts were rinsed once with MEM and slowly added with the FuGENE 6-treated DNA solution. After incubation for 4 hours, the transfected cells were rinsed with and cultured in the differentiation medium for 48 hours.

FACS analysis

Myoblasts that had been cultured for 24 hours were transfected with the pcDNA derivatives and incubated for 24 hours. The cells were washed twice in PBS and fixed in 2% paraformaldehyde for 15 minutes. They were again washed twice in PBS and incubated in PBS containing 0.5% saponin and 2% horse serum to permeabilize the cells and saturate nonspecific binding sites. The cells were incubated with the monoclonal antibody raised against c-Myc (9E10) and a polyclonal antibody raised against creatine kinase for 1 hour, and then with fluorescein isothiocyanate (FITC)-conjugated donkey anti-mouse IgG and phycoerythrin (PE)-conjugated goat anti-rabbit IgG, respectively, for the next 1 hour. They were then washed twice in PBS, separated by collagenase, and resuspended in PBS. The samples were analyzed with a FACStarplus (Becton Dickinson), and data were analyzed with CellQuest software.

Immunocytochemistry

The cells plated on collagen-coated glass coverslips were fixed with 3.7% paraformaldehyde in PBS for 10 minutes, followed by permeabilization with 0.5% Triton X-100 in PBS. All subsequent dilutions and washes were carried out with PBS containing 0.1% Triton X-100 (PBST). Nonspecific binding sites were saturated by incubation with 3% horse serum and 10% gelatin in PBST for 1 hour. The cells were incubated with the antibody raised against c-Myc for 1 hour and then with FITC-conjugated donkey anti-mouse IgG for the next 1 hour. For detection of filamentous actin, the cells were incubated with rhodamine-phalloidin. The coverslips were mounted in Vectashield and viewed in a laser scanning confocal microscope (Carl Zeiss LSM 510). FITC and rhodamine were excised by 488 nm argon and 543 nm HeNe laser, and the images were filtered by bandpass 505-530 nm and longpass 585 nm filters, respectively. The acquired images were processed with Adobe PhotoShop and printed with Tektronix phaser 450. No fluorescence was detected if primary antibody was omitted.

Results

Effects of protein phosphatase inhibitors on MARCKS translocation during myogenesis

In order to determine whether dephosphorylation of MARCKS is involved in translocation of the protein from the cytosol to the plasma membrane, the primary culture of chick myoblasts was treated with okadaic acid or tautomycin, potent inhibitors of protein phosphatases, at the time of medium change. Also, the cells that had been treated with okadaic acid or tautomycin for 24 hours were freed of the drug by changing the medium. These cells were further cultured for the next 48 and 24 hours, respectively, and subjected to analysis for subcellular distribution of MARCKS. Total cell lysates and membrane fractions were prepared from the cells and subjected to SDS-PAGE, followed by immunoblot analysis using an anti-MARCKS antibody. Fig. 1A shows that treatment with okadaic acid (lane O) or tautomycin (lane T) and subsequent removal of the drug from the medium (lanes F_O and F_T) show little or no effect on the total level of MARCKS. However, the drug treatment strongly inhibited the translocation of MARCKS to the membrane and this inhibitory effect could be reversed upon removal of the drug from the medium (Fig. 1B). We also examined the effect of the drug on membrane fusion of the cells that had been cultured under the same conditions. As shown in Fig. 1C, both okadaic acid and tautomycin reversibly blocked the cell fusion. These results suggest that

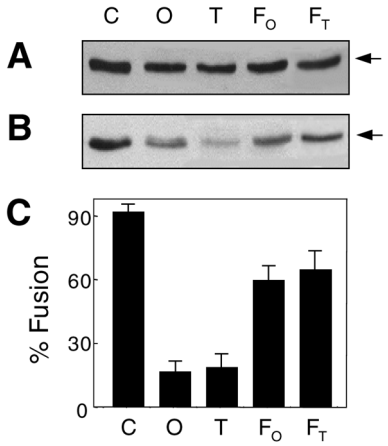

Fig. 1. Effects of protein phosphatase inhibitors on MARCKS translocation and myoblast fusion. Myoblasts were treated with 25 nM okadaic acid (lane O) or 0.5 μM tautomycin (lane T) at the time of medium change, as described in Materials and Methods. The cells that had been cultured for 24 hours in the presence of okadaic acid or tautomycin were freed of the drug by changing the medium (lanes F_O and F_T, respectively). These cells were further cultured for the next 48 and 24 hours, respectively. Lane C indicates the cells cultured for 72 hours without any treatment. Total cell lysates (A) and membrane fractions (B) were prepared and subjected to SDS-PAGE followed by immunoblot analysis using an anti-MARCKS antibody. The arrows indicate MARCKS. The cells cultured as above were subjected to Giemsa staining to determine the degree of cell fusion (C). Cells containing more than three nuclei were regarded as fused cells.

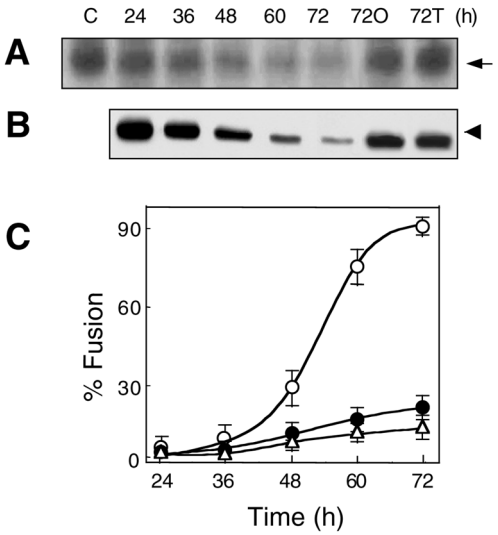

Fig. 2. Changes in the dephosphorylating activity against ^{32}P-labeled MARCKS during myogenic differentiation. (A) Soluble extracts were prepared from myoblasts that had been cultured for the indicated periods, and aliquots of them (30 μg/lane) were incubated with ^{32}P-labeled MARCKS (0.3 μg) for 30 minutes at 30°C. The extracts from the 72 hour cultured cells were also incubated as above but in the presence of 100 nM okadaic acid (lane 72O) or 5 nM tautomycin (lane 72T). As a control (lane C), ^{32}P-labeled MARCKS was incubated as above but without any addition. The samples were then subjected to SDS-PAGE followed by autoradiography. Note that the rate of dephosphorylation reaction was linear up to 30 minutes when incubated with the extracts of the cells cultured for 48 hours in the absence of okadaic acid. The arrows indicate ^{32}P-labeled MARCKS. (B) The same extracts were subjected to immunoblot analysis using an anti-phospho-MARCKS antibody. The arrowhead shows phospho-MARCKS. (C) The cells were cultured in the absence (○) and presence of 25 nM okadaic acid (△) or 0.5 μM tautomycin (●) for the indicated period, and the degree of cell fusion was determined as in Fig. 1.

dephosphorylation of MARCKS leads to translocation of the protein to the plasma membrane in cultured myoblasts and that the cell fusion is closely correlated with the membrane translocation of MARCKS.

To determine whether the dephosphorylating activity against MARCKS might change during myogenesis, soluble extracts were obtained from myoblasts that had been cultured for various periods and assayed for their dephosphorylation activity by incubation with ^{32}P-labeled MARCKS. To prevent the endogenous activity of PKCs, the extracts were pretreated with Ro-31-8220, a potent inhibitor of the enzymes. The samples were then subjected to SDS-PAGE followed by autoradiography. The ability of the soluble extracts to dephosphorylate ^{32}P-labeled MARCKS markedly increases (Fig. 2A) as the cells fuse to form myotubes (Fig. 2C). Moreover, this increase in the dephosphorylating activity was particularly evident in the extracts obtained from cells that are competent or committed for membrane fusion (i.e. cultured for 36-48 hours). However, treatment with okadaic acid or tautomycin strongly inhibited both dephosphorylating activity and membrane fusion (Fig. 2A and C, respectively). To determine whether the increase in the dephosphorylating activity indeed correlates with the phosphorylation state of MARCKS in the cells, the same extracts were subjected to immunoblot analysis using an antibody that reacts only with the phosphorylated form of MARCKS. As shown in Fig. 2B, the level of the phospho-MARCKS proteins decreased in parallel with the increase in the dephosphorylating activity. Moreover, treatment with okadaic acid or tautomycin blocked the decrease in the level of phospho-MARCKS. These results suggest that the MARCKS-specific phosphatase activity is upregulated just before or during the onset of myoblast fusion and may be required for myoblast fusion.

Identification of protein phosphatase responsible for MARCKS dephosphorylation

MARCKS that was phosphorylated can be almost completely dephosphorylated by the purified catalytic subunit of PP-2A (Clarke et al., 1993). The purified catalytic subunit of PP-1 (PP-1C) can also dephosphorylate MARCKS, although somewhat less efficiently than the catalytic subunit of PP-2A (Clarke et al., 1993). To identify the protein phosphatase(s) that is responsible for dephosphorylation of MARCKS during myogenesis, the soluble extracts obtained from the cells that had been cultured for 48 hours were incubated with ^{32}P-labeled MARCKS in the presence of increasing concentrations of tautomycin or okadaic acid. As shown in Fig. 3A, both tautomycin (open circle) and okadaic acid (closed circle) inhibited the phosphatase activity in a dose-dependent fashion. The concentrations required for a half-maximum inhibition (IC_{50}) by tautomycin and okadaic acid were about 1 nM and 60 nM, respectively. Since PP-2C is insensitive to both inhibitors and since the inhibition of PP-2B by tautomycin needs a more than 5000-fold higher concentration of the agents (Mackintosh and Klumpp, 1990), it appears unlikely that PP-2B and PP-2C act on phosphorylated MARCKS in differentiating myoblasts. In addition, it has been reported that PP-2A requires levels of okadaic acid at least 100-fold lower than those of PP-1 for complete inhibition, although the drug can inhibit both PP-1 and PP-2A (Bialojan and Takai, 1988). These results suggest that PP-1 is responsible for dephosphorylation of MARCKS.

To clarify further the involvement of PP-1 in MARCKS dephosphorylation, the same cell extracts were subjected to immunoprecipitation by treatment with an antibody raised against PP-1C. The resulting supernatants were then incubated with ^{32}P-labeled MARCKS, followed by SDS-PAGE and autoradiography. As shown in Fig. 3B, treatment with the anti-PP-1C IgG completely prevented the dephosphorylating activity (lane c), unlike treatment with preimmune IgG (lane b). We also examined the effects of phosphatase inhibitors on dephosphorylation of ^{32}P-labeled MARCKS. Fig. 3C shows that the dephosphorylating activity is strongly inhibited by protein phosphatase inhibitor-2 (lane b), which is a specific inhibitor of PP-1, but not by protein phosphatase-2A inhibitor (I_1^{PP2A}) (lane c). Moreover, the protein phosphatase activity was inhibited by treatment with both inhibitor proteins to an extent similar to that seen with protein phosphatase inhibitor-2 alone (lane d). Thus, it is likely that PP-1 is the major enzyme responsible for dephosphorylation of MARCKS during the myogenic differentiation.

To determine whether the increase in the dephosphorylating activity against ^{32}P-labeled MARCKS during myogenesis (Fig. 2A) might be due to a change in the expression of PP-1C, the

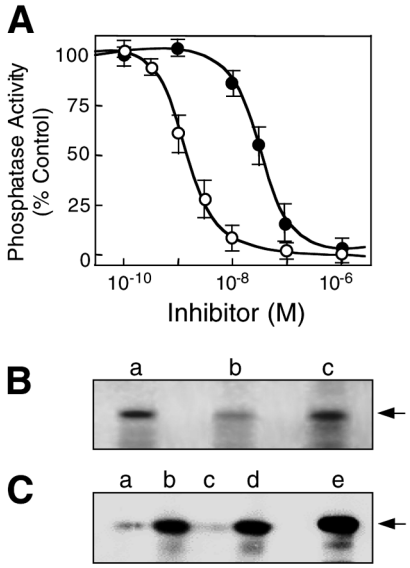

Fig. 3. Identification of protein phosphatase responsible for dephosphorylation of MARCKS in cultured myoblasts. (A) Effects of increasing concentrations of tautomycin and okadaic acid on dephosphorylation of ^{32}P-labeled MARCKS. Soluble extracts were prepared from the cells cultured for 48 hours and subjected to the phosphatase assay in the presence of increasing concentrations of tautomycin (○) and okadaic acid (●) as described in Materials and Methods. The resulting samples were subjected to SDS-PAGE followed by autoradiography. The bands corresponding to ^{32}P-labeled MARCKS were quantified using a densitometer. The intensity of the band seen without any treatment was expressed as 100% and the others were expressed as relative values. Each of the data points represents mean±s.e. of triplicate determinations. (B) Effect of immunoprecipitation with anti-PP-1C IgG on dephosphorylation of ^{32}P-labeled MARCKS. Soluble extracts were prepared from the cells cultured for 48 hours and incubated with 10 μg of preimmune IgG (lane b) or anti-PP-1C IgG (lane c) at 4°C for 2 hours. After incubation, the samples were treated with protein A-Sepharose and centrifuged. The resulting supernatants were then subjected to the phosphatase activity assay followed by SDS-PAGE and autoradiography. Lane a represents ^{32}P-labeled MARCKS incubated alone. (C) Effects of protein phosphatase inhibitors on dephosphorylation of ^{32}P-labeled MARCKS. Soluble extracts were prepared and assayed for their phosphatase activity as above but in the absence (lane a) or presence of 20 nM protein phosphatase inhibitor-2 (lane b), 100 nM protein phosphatase-2A inhibitor (lane c), or both (lane d). Lane e represents ^{32}P-labeled MARCKS incubated alone. The arrows indicate the phosphorylated MARCKS.

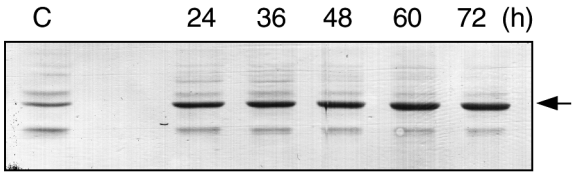

Fig. 4. Changes in the protein level of PP-1C during myogenic differentiation. Soluble extracts were prepared from the cells that had been cultured for the indicated periods. They (30 μg each) were then subjected to immunoblot analysis using an anti-PP-1C antibody. Extracts of A431 cells were used as a control (lane C). The arrow indicates PP-1C.

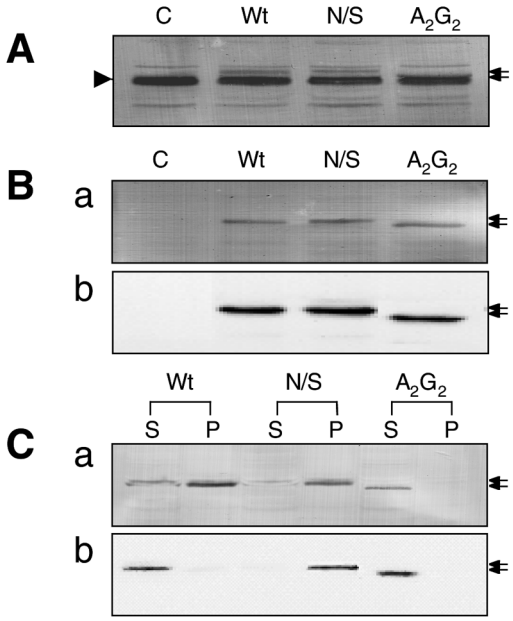

Fig. 5. Transient overexpression and subcellular localization of MARCKS and its mutant forms in cultured myoblasts. Myoblasts that had been cultured for 24 hours were transfected with the cDNAs for MARCKS (Wt) and its mutant forms (N/S and A_2G_2) and further incubated for 48 hours in the absence (a) or presence of 100 nM okadaic acid (b). After incubation, total cell lysates were prepared and subjected to immunoblot analysis using anti-MARCKS (A) or anti-Myc antibody (B). Lanes C represent the mock transfected with empty vector. Total cell lysates were also separated into soluble and membranous fractions, which were then subjected to immunoblot analysis using anti-Myc antibody (C). Letters S and P represent the supernatant (soluble) and pelleted (membrane) fractions, respectively. The arrowhead indicates the endogenous MARCKS, and the arrows show the Myc-tagged MARCKS proteins.

same extracts were subjected to immunoblot analysis using the anti-PP-1C IgG. However, the protein level of PP-1C remained nearly constant during the entire culture periods (Fig. 4). PP-1 is known to consist of multiple subunits, including catalytic, targeting, and regulatory subunits. Thus, it is possible that the expression of either targeting or regulatory subunit or both of them may change during myogenesis and result in an increase in the capacity of dephosphorylating MARCKS.

Effects of N/S and A_2G_2 mutations on MARCKS translocation

To clarify whether MARCKS translocation is involved in myoblast fusion, we first examined the effects of the mutations that could influence membrane translocation of MARCKS. The cDNAs for two different mutant forms of MARCKS, N/S and A_2G_2, were used for cell transfection (Swierczynski and Blackshear, 1996). In N/S, the Ser phosphorylation site is replaced by Asn and therefore cannot be phosphorylated by protein kinases. In A_2G_2, the Gly residue, which is located next to the N-terminus of MARCKS and serves as the myristoylation site, is substituted with Ala. Thus, N/S should be targeted to the plasma membrane, while A_2G_2 should remain in the cytosol. The two forms of MARCKS were also

Myc-tagged for their identification upon immunoblot analysis using anti-Myc antibodies.

Myoblasts that had been cultured for 24 hours were transfected with the cDNAs and further cultured for 48 hours in the absence or presence of okadaic acid (Fig. 5B,C, panels a and b, respectively). Total cell lysates were prepared from the cells and subjected to immunoblot analysis. While Fig. 5A shows the endogenous MARCKS proteins that were detected with an anti-MARCKS antibody, Fig. 5B represents the Myc-tagged, wild-type MARCKS (Wt) and its mutant forms (N/S and A_2G_2) that were expressed from the transfected cDNAs and detected with an anti-Myc antibody. Of note is that A_2G_2, which should lack the myristoyl group, migrated faster than the Myc-tagged, wild-type MARCKS or N/S in the gel. These results show that the transfected cells could overexpress the Myc-tagged MARCKS proteins. We then examined the effects of the mutations in the phosphorylation and myristoylation sites on MARCKS translocation in vivo. The soluble and membrane fractions were obtained from the cultured myoblasts that had been transfected with the cDNAs for the Myc-tagged wild-type MARCKS, N/S and A_2G_2. These fractions were then subjected to immunoblot analysis using an anti-Myc antibody as above. When the transfected cells were cultured in the absence of okadaic acid, the majority of the wild-type MARCKS (Wt) and N/S were recovered in the membrane fraction (Fig. 5Ca). Upon treatment with the drug, the wild-type MARCKS was recovered in the soluble fraction while the membrane translocation of N/S was not affected (Fig. 5Cb). In contrast, A_2G_2 remained exclusively in the soluble fraction, whether the transfected cells were cultured in the absence or presence of okadaic acid. These results indicate that dephosphorylation and myristoylation of MARCKS are essential for its translocation in vivo from the cytosol to the plasma membrane of the cultured myoblasts.

Involvement of MARCKS translocation in myoblast fusion

To determine whether translocation of MARCKS is indeed involved in membrane fusion of cultured myoblasts, the cells were transfected with the cDNAs for the Myc-tagged, wild-type MARCKS, N/S and A_2G_2 as above, cultured for 48 hours, and subjected to immunostaining using an anti-Myc antibody. They were then observed under a confocal microscope. The cells expressing the wild-type MARCKS (Wt) (Fig. 6A) and N/S (Fig. 6D) readily fused to form multinucleated myotubes with intense staining of the protein along the plasma membrane. In contrast, the cells expressing A_2G_2 had an unfused, bipolar shape with diffuse staining of MARCKS in the cytosol (Fig. 6G). Thus, it appears that translocation of MARKS from the cytosol to the plasma membrane is associated with or a requisite step for membrane fusion of cultured myoblasts.

To determine whether the MARCKS translocation during myogenesis is correlated with its capability of crosslinking filamentous actin, the same cells were also stained using pallotoxin, a selective ligand of filamentous actin but not of the globular form. The cytoskeletal networks of filamentous actin in cells expressing the wild-type MARCKS and N/S were co-localized with the membrane-translocated MARCKS (Fig. 6A-C and D-F, respectively). However, construction of the filamentous actin network was impaired in the cells expressing A_2G_2 (Fig. 6G-I), although unfused myoblasts showed longitudinally penetrated actin filaments (data not shown). These results suggest that MARCKS translocation to the plasma membrane is tightly associated with its ability to crosslink filamentous actin, which may positively influence the membrane fusion of cultured myoblasts.

Effects of N/S and A_2G_2 mutations on expression of muscle specific proteins

During myogenesis, the membrane fusion of myoblasts accompanies the accumulation of muscle-specific proteins, such as creatine kinase and myosin heavy chain. To examine whether the prevention of MARCKS translocation and hence blocking the myoblast fusion might influence the expression of

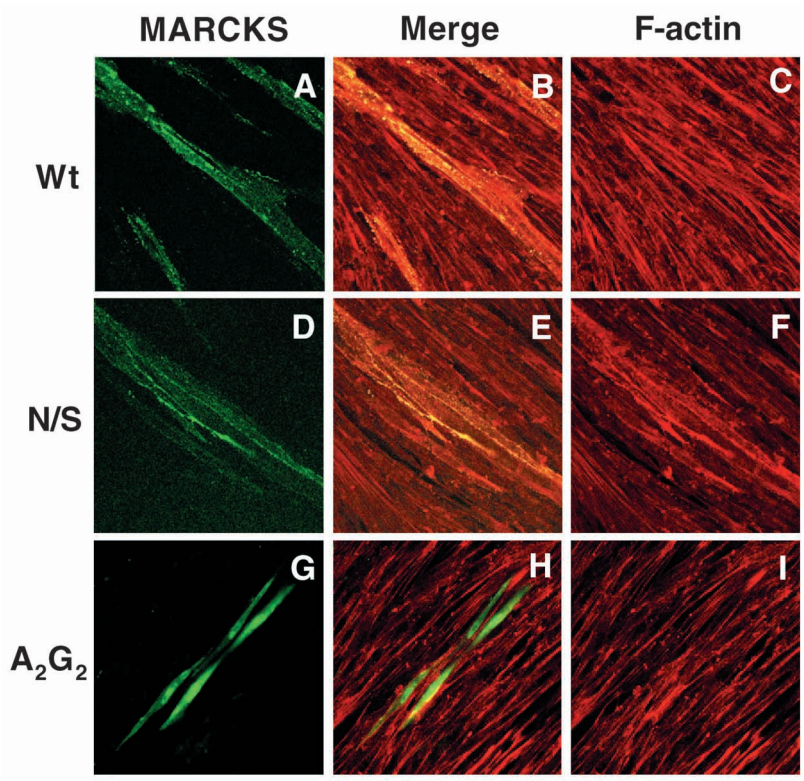

Fig. 6. Effect of overexpression of N/S and A_2G_2 on myoblast fusion and colocalization of MARCKS with filamentous actin. Myoblasts cultured for 24 hours were transfected with the pcDNAs for Myc-tagged, wild-type MARCKS (A-C), N/S (D-F) and A_2G_2 (G-I). These cells were further cultured for the next 48 hours, followed by immunostaining with anti-Myc antibody and then with FITC-conjugated anti-IgG antibody, as described in Materials and Methods. They were also subjected to rhodamine-phalloidin staining (C,F,I). The middle panels (B,E,H) show the overlapped views of the left and right panels for colocalization of MARCKS and F-actin. The extents of membrane fusion were 82%, 93% and 27% for the cells transfected with the pcDNAs for Myc-tagged wild-type MARCKS, N/S and A_2G_2, respectively. Magnification, 400× (A-F); 500× (G-I).

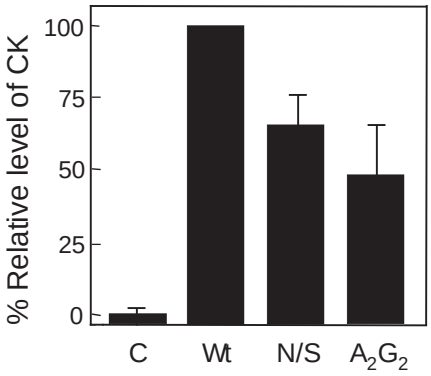

Fig. 7. Effects of the N/S and A_2G_2 mutations on accumulation of creatine kinase. Myoblasts that had been cultured for 24 hours were transfected with recombinant pcDNAs for Myc-tagged wild-type MARCKS, N/S and A_2G_2. After incubation for 48 hours, the cells were stained with anti-Myc antibody (FITC) and anti-creatine kinase antibody (PE) followed by FACS analysis, as described in Materials and Methods. The level of creatine kinase (CK) in the cells transfected with pcDNA for the wild-type MARCKS was expressed as 100% and the others were expressed as relative values. Each of the data represent the mean±s.e. of triplicate determinations.

muscle-specific proteins, the cells were transfected with the cDNAs for the Myc-tagged wild-type MARCKS, N/S and A_2G_2, as above. After culturing the cells for 48 hours, they were stained with both anti-Myc and anti-creatine kinase antibodies, followed by FACS analysis to measure the expression levels of creatine kinase in the transfected cells only. Fig. 7 shows that accumulation of creatine kinase could be detected not only in cells expressing the wild-type MARCKS and N/S but also in cells expressing A_2G_2 (although its level in cells expressing A_2G_2 is significantly less than that seen in those expressing the wild-type MARCKS and N/S), despite the fact that the latter cannot fuse to form myotubes, unlike the others. Similar results were obtained for the expression of myosin heavy chain (data not shown). These results strongly suggest that the expression of muscle-specific proteins is independent of the membrane translocation of MARCKS as well as myoblast fusion.

Discussion

We have previously shown that MARCKS translocates from the cytosol to the plasma membrane during the course of myogenic differentiation of cultured myoblasts and this translocation is mediated in part by the decrease in the expression of PKCθ, which is the major enzyme responsible for the phosphorylaion of MARCKS (Kim et al., 2000). In the studies described here, we demonstrated that the shuttling of MARCKS between the cytosol and the plasma membrane of cultured myoblasts could also be controlled by the protein phosphatase-mediated dephosphorylation of MARCKS. Treatment of the cultured myoblasts with okadaic acid or tautomycin was found to reversibly block MARCKS translocation, similar to its effect on the membrane fusion of the cells (Kim et al., 1991; Kim et al., 1992b). Moreover, the dephosphorylating activity against MARCKS was found to markedly increase in the cells that are competent or committed for membrane fusion. Thus, it is clear that dephosphorylation as well as phosphorylation of MARCKS is involved in the regulation of its subcellular localization in the embryonic muscle cells.

In a cell-free system, PP-1, PP-2A and PP-2C could dephosphorylate recombinant MARCKS or a synthetic peptide containing the phosphorylation domain of MARCKS. In intact Swiss 3T3 cells, okadaic acid, added at concentrations for inhibiting PP-1 and PP-2A but not PP-2C, exerts little effect on MARCKS phosphorylation on its own, but largely prevents the dephosphorylation of MARCKS that occurred following the activation of PKC by bombesin with subsequent receptor blockade (Clarke et al., 1993). Therefore, it has been suggested that, although dephosphorylation of MARCKS can be mediated by PP-2C in vitro, MARCKS might be dephosphorylated by okadaic-acid-sensitive PP-1 and/or PP-2A in Swiss 3T3.

A number of results obtained in the present studies suggest that PP-1 is the major protein phosphatase that is responsible for the dephosphorylating activity against MARCKS in cultured myoblasts. First, both tautomycin and okadaic acid blocked the dephosphorylating activity against MARCKS at concentrations that can inhibit PP-1 but not PP-2A. Second, immunoprecipitation by an antibody raised against the catalytic subunit of PP-1 (PP-1C), but not by preimmune IgG, prevented the MARCKS dephosphorylating activity. Third, the phosphatase activity was strongly inhibited by the protein phosphatase inhibitor-2, a specific protein inhibitor of PP-1, but not by the inhibitor protein specific to the protein phosphatase-2A. Thus, it appears clear that PP-1 participates in dephosphorylation of MARCKS during myogenesis.

However, it is noteworthy that the concentration of okadaic acid used to inhibit dephosphorylation effectively in vivo (Fig. 1, 25 nM) was much lower than the micromolar concentrations generally accepted to inhibit PP-1 in other cells (Bialojan and Takai, 1988; Haystead et al., 1989; Cohen et al., 1990), which suggests that PP-2A may also participate in MARCKS dephosphorylation in cultured myoblasts. Furthermore, the concentration of tautomycin required for prevention of the MARCKS dephosphorylation in vivo was much higher than that needed in vitro (Figs 1, 3). Favre et al. have systematically demonstrated using MCF7 cells that the phosphatase inhibitors, including okadaic acid and tautomycin, have distinct cell permeability properties (Favre et al., 1997). Thus, it appears possible that okadaic acid shows much higher permeability to myoblasts than to other cells, whereas tautomycin has the opposite property.

Also noteworthy is the observation that the protein level of PP-1C remained nearly constant during the entire time course of myogenesis, despite the fact that the dephosphorylating activity against MARCKS markedly increased during the same period. In vivo, PP-1C exists as a complex with other proteins that target it to particular subcellular localizations, modify its substrate specificity, and regulates its enzyme activity (Lavoinne et al., 1991). In the rabbit skeletal muscle, PP-1C has been shown to interact with glycogen particle (PP-1G), sarcoplasmic reticulum (PP-1SR), myofibrils (PP-1M), and the cytoplasmic inhibitor-2 protein (PP-1I) (Dent et al., 1990). In insulin-stimulated L6 rat myoblasts, the protein level of PP-1G dramatically increases in response to the agonist, while that of PP-1C remains constant (Srinivasan and Begum, 1994). Thus, it appears possible that the increase in the dephosphorylation

activity against MARCKS in cultured chick myoblasts may be regulated by the other subunits of PP-1, such as the targeting or regulatory subunits. Further studies are required to identify the subunit(s), whose expression may change and hence alter the activity of PP-1C during differentiation of chick myoblasts.

The membrane translocation of MARCKS appears to be essential or a requisite event in the membrane fusion of cultured myoblasts. Distribution of MARCKS changes from the cytosol to the plasma membrane of the cells and this translocation appears to be regulated by both the increase in the PP-1-mediated MARCKS dephosphorylation and the decrease in the PKCθ-mediated MARCKS phosphorylation during the course of myogenic differentiation (Kim et al., 2000). Furthermore, overexpression of A_2G_2, which cannot be targeted to the plasma membrane because it lacks a myristoylation site, impaired the cell fusion. By contrast, the cells expressing N/S, which lacks a phosphorylation site and thus readily translocates to the membrane, underwent fusion similarly to those expressing the wild-type MARCKS. In addition, formation of filamentous actin cytoskeleton is largely dependent on translocation of MARCKS to the plasma membrane and its distribution coincides with that of MARCKS in cells overexpressing the wild-type MARCKS or N/S. It has been reported that alterations in MARCKS distribution by PKC-mediated phosphorylation result in disintegration of the membrane skeleton, and cytochalasin D, an inhibitor of actin polymerization, enhances PKC-induced translocation of MARCKS to the cytosol (Douglas et al., 1997; Vaaraniemi et al., 1999). Together with the fact that MARCKS regulates the cytoskeleton dynamics by crosslinking the actin filaments in the plasma membrane and that myoblast fusion accompanies massive cytoskeleton reorganization, we suggest that the PP-1-mediated MARCKS localization at the membrane is required for the fusion of embryonic muscle cells.

Stumpo et al. have reported that MARCKS-deficient mice exhibit abnormal brain development but do not have any obvious defects in other major organs including muscle, questioning the involvement of MARCKS in myoblast differentiation (Stumpo et al., 1995). However, they also reported that MARCKS is highly expressed in many developing neuronal tissues in which phenotypic alterations were not seen in the MARCKS-deficient mouse, as well as in many extracranial tissues in the mouse. Therefore, they suggested that the MARCKS homologue MRP (MARCKS-related protein) (Aderem, 1992b; Blackshear, 1993) might also be highly expressed in these tissues during development and prevent them from exhibiting an abnormal phenotype. Similarly, MRP in embryonic muscle tissues may serve as a functional equivalent of MARCKS and prevent any defect in muscle development in MARCKS-deficient mice, although the most sensitive processes would be affected by the reduction in total amount of 'MARCKS equivalents' (Stumpo et al., 1995).

Of interest was the finding that expression of muscle-specific proteins, such as creatine kinase and myosin heavy chain, could occur in the fusion-arrested myoblasts upon overexpression of A_2G_2. This result is consistent with our previous finding that okadaic acid blocks the membrane fusion of chick myoblasts with little effect on induction of muscle-specific proteins (Kim et al., 1991). It has also been shown that prevention of cell fusion, such as by prolonged culture in a low-calcium medium or treatment with cytochalasin B, does not interfere with the synthesis of muscle-specific proteins and the other differentiative processes, including transport and release of calcium (Holtzer et al., 1975; Constantin et al., 1995). Thus, the synthesis of muscle-specific proteins occurring independently of the blockade of membrane fusion by A_2G_2 expression could be an additional example of the uncoupling of biochemical differentiation from morphological differentiation, despite the fact that both processes normally occur simultaneously during the myogenic differentiation of embryonic skeletal muscle cells.

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