Myristoylated alanine-rich C kinase substrate (MARCKS) is involved in myoblast fusion through its regulation by protein kinase C α and calpain proteolytic cleavage

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MARCKS (myristoylated alanine-rich C kinase substrate) is a major cytoskeletal protein substrate of PKC (protein kinase C) whose cellular functions are still unclear. However numerous studies have implicated MARCKS in the stabilization of cytoskeletal structures during cell differentiation. The present study was performed to investigate the potential role of Ca²⁺-dependent proteinases (calpains) during myogenesis via proteolysis of MARCKS. It was first demonstrated that MARCKS is a calpain substrate *in vitro*. Then, the subcellular expression of MARCKS was examined during the myogenesis process. Under such conditions, there was a significant decrease in MARCKS expression associated with the appearance of a 55 kDa proteolytic fragment at the time of intense fusion. The addition of calpastatin

peptide, a specific calpain inhibitor, induced a significant decrease in the appearance of this fragment. Interestingly, MARCKS proteolysis was dependent of its phosphorylation by the conventional PKC α . Finally, ectopic expression of MARCKS significantly decreased the myoblast fusion process, while reduced expression of the protein with antisense oligonucleotides increased the fusion. Altogether, these data demonstrate that MARCKS proteolysis is necessary for the fusion of myoblasts and that cleavage of the protein by calpains is involved in this regulation.

Key words: actin cytoskeleton, Ca²⁺, calpain, myristoylated alanine-rich C kinase substrate (MARCKS), myogenesis, protein kinase C α (PKC α).

INTRODUCTION

During muscle cell differentiation, mononucleated myoblasts fuse to form multinucleated myotubes [1]. Myoblasts withdraw from the cell cycle, undergo terminal differentiation and express muscle-specific proteins [2]. The fusion process requires the reorganization of the cytoskeleton and redistribution of membrane components [3]. Major events, including inter-myoblast recognition, alignment and fusion itself, depend on cell–cell as well as on cell–extracellular matrix interactions, which are regulated at least by transmembrane molecules such as integrin and cadherin [4]. Phosphorylation of focal adhesions [5] and proteolysis [6] are also well-known mechanisms for regulating transmembrane signalling and the subsequent muscular differentiation.

Calpains are intracellular cysteine proteases that require Ca²⁺ ions for activity. They are composed of two families of ubiquitous and tissue-specific isoforms (for review, see [7]). The major ubiquitous isoforms [μ - and m-calpains] are heterodimeric enzymes composed of a specific 80 kDa catalytic subunit associated with a common 30 kDa regulatory subunit. It has been recently shown that calpain activity could be regulated by phosphorylation [8]. These proteases are also inhibited tightly by a specific intracellular inhibitor, calpastatin [9]. Proteolysis via the calcium-dependent protease calpains is thought to be involved in housekeeping functions, including cytoskeletal protein interactions, receptor processing and regulation of numerous transducing

enzymes [e.g. PKC (protein kinase C), Rho and Src kinase], and in numerous pathologies that include muscular dystrophy [10], cancer, cataract, diabetes and Alzheimer's disease [11].

Calpains have been implicated in the fusion process [12,13], during which they cleave many cytoskeletal components. In a previous study [14], activating limited proteolytic cleavage of PKC α by calpains during myogenesis was also reported, which provided evidence for an important role of the two enzymes in a common pathway during this process.

The MARCKS (myristoylated alanine-rich C kinase substrate) is a high-affinity cellular substrate for PKC. MARCKS phosphorylation by PKC regulates its translocation between the plasma membrane and the cytosol [15]. This phosphorylation event has been suggested to mediate destabilization of the cytoskeletal structure because MARCKS is able to cross-link actin filaments at the plasma membrane [16]. Phosphorylation of MARCKS has been also used to demonstrate PKC activation in response to various agonists, underlying an important function mediated by this protein, such as cellular adhesion, cell spreading or vesicle trafficking. In a previous study, we have demonstrated the existence of a MARCKS–PKC α complex in skeletal muscle [17], suggesting that PKC α is the major enzyme involved in MARCKS phosphorylation in muscle cells. Specific proteolytic cleavage of MARCKS by a cellular protease has been described in several tissues [18]. This event may be a ubiquitous mechanism regulating cellular levels of MARCKS. In addition, it has been demonstrated

Abbreviations used: BCIP, 5-bromo-4-chloroindol-3-yl phosphate; CS peptide, calpastatin peptide; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; FBS, foetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HS, horse serum; LB, Luria-Bertani; MARCKS, myristoylated alanine-rich C kinase substrate; NBT, Nitro Blue Tetrazolium; PKC, protein kinase C; PSD, phosphorylation site domain; RT, reverse transcriptase; TBS, Tris-buffered saline.

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that the phosphorylation rate of MARCKS changes during myogenesis and that MARCKS is involved in fusion of embryonic chicken muscle cells [19].

In the present study, we sought to expand our understanding of the mechanisms involved in myoblast fusion to better define the role of calpains in this process. Together with the facts that MARCKS regulates cytoskeleton dynamics and that myoblast fusion requires important cytoskeletal reorganization, the present results demonstrate, for the first time, direct cleavage of MARCKS by calpains, and that a required step during myoblast fusion is calpain-mediated proteolysis of phosphorylated MARCKS.

MATERIALS AND METHODS

Materials

Chemicals and materials were obtained from the following sources. DMEM (Dulbecco's modified Eagle's medium), FBS (foetal bovine serum) and HS (horse serum) were from Gibco-BRL, and culture dishes were from Fischer Scientific. The DNA-extraction kit and the Effectene Transfection Reagent were from Qiagen. The RNA-extraction kit was from Qbiogene, RT (reverse transcriptase), DNase, RNasin and primers for RT-PCR were from Invitrogen. LightCycler-FastStart DNA Master SYBR Green I kit was from Roche Diagnostics. The NBT (Nitro Blue Tetrazolium)/BCIP (5-bromo-4-chloroindol-3-yl phosphate) was purchased from Promega. PMA was from Sigma, CS peptide (calpastatin peptide) was purchased from Calbiochem, Immobilon-P membrane was from Millipore and the BCA (bicinchoninic acid) protein assay kit was purchased from Pierce. Primary antibodies were from Santa Cruz Biotechnologies, and secondary antibodies were from Sigma. Purified modified antisense oligonucleotides were from Eurogentec.

Expression and purification of recombinant MARCKS

Bacterial expression of recombinant MARCKS

Escherichia coli strain BL-21 was transformed with the pET-3d plasmid containing MARCKS cDNA [20]. The colonies containing the plasmid were selected with 100 µg/ml ampicillin. Frozen stocks of transformed bacteria were then grown overnight at 37 °C in LB (Luria-Bertani) medium with 100 µg/ml ampicillin.

A 2-litre volume of LB medium containing 100 µg/ml of ampicillin was inoculated with the overnight culture. The cells were grown to a D_{600} of approx. 1.2, and then were grown further in the presence of 0.4 mM IPTG (isopropyl β-D-thiogalactoside) for 4 h.

Protein extraction

Bacteria were collected by centrifugation for 10 min at 3000 g at 4 °C, resuspended in 180 ml of ice-cold lysis buffer A [10 mM Tris/HCl, pH 7.5, 1 mM EDTA, 1 % Triton X-100, 1 mM DTT (dithiothreitol), 10 mM benzamidine, 6 µg/ml leupeptin and 0.5 mM NaN₃] and sonicated four times for 2 min. After a 10 000 g centrifugation for 15 min, the supernatant was recovered and heated for 10 min at 90 °C. The precipitated proteins were then eliminated by a 15 min centrifugation at 10 000 g. The NaCl concentration of the supernatant was adjusted to 2.5 M.

Phenyl-Sepharose chromatography

Extracted proteins (30 mg) were loaded on to 60 ml of phenyl-Sepharose resin. MARCKS was eluted during loading and rinsing of the column with buffer B (20 mM Tris/HCl, pH 7.5, 2 mM

EGTA, 2 mM EDTA, 0.5 mM DTT and 1 mM NaN₃) containing NaCl (2.5 M) and PMSF (1 mM).

DEAE-EMD chromatography

Fractions containing MARCKS were dialysed three times against 5 litres of buffer B without NaCl and loaded on to a 20 ml DEAE-EMD column (Merck). The adsorbed proteins were eluted with a six bed volume of a 0–0.5 M NaCl linear gradient in buffer B.

Cell culture

C2C12 myoblasts were grown in DMEM with 10 % (v/v) FBS during proliferation and in DMEM with 2 % (v/v) HS during differentiation in gelatin-coated dishes (1 %) under a 5 % CO₂ atmosphere at 37 °C.

For the evaluation of the percentage of fusion, cells were rinsed and fixed at room temperature (22 °C) in 4 % (w/v) paraformaldehyde for 15 min. After staining with Hansen's haemalum (8 min), nuclei were counted and the percentage of fusion was calculated as follows: fusion (%) = (number of nuclei in myotubes/total number of nuclei in single myoblasts and in myotubes) × 100. Fusion was estimated on four wells per culture and for each well, 12 randomly selected fields were counted. Fusion inhibition (%) was calculated as follows: fusion inhibition (%) = {1 – [fusion (%) in treated cultures/fusion (%) in control cultures]} × 100.

Treatments

Phorbol ester

PMA dissolved in DMSO was added to the medium (DMEM and 2 % HS) after 4 days of differentiation, at a concentration of 150 nM for 1 h. The cells were then processed for Western blot analysis.

Gö 6976

Gö 6976 was added at a final concentration of 100 nM for 1 h. The cells were then processed for Western blot analysis.

CS peptide

CS peptide was added at a final concentration of 10 µM, supplemented or not with PMA (150 nM) or Gö 6976 (100 nM). After 1 h of treatment, the cells were processed for Western blot analysis.

Oligonucleotide antisense and plasmid transient transfection assays

Sequences of phosphorothioate oligonucleotides against MARCKS mRNA (antisense, 5'-CAGGGGATAGTTCGGC-3' and random, 5'-GCAGGGATTTCGGCGTA-3') were selected from the published sequence [21]. Random sequences were analysed and did not hybridize with any gene reported in the database (GenBank®). Antisense oligonucleotides were added to the medium (DMEM and 2 % HS) at a final concentration of 2.5 µM.

The cDNAs encoding MARCKS wild-type or PSD⁻ [lacking the PSD (phosphorylation site domain)] [21] were inserted into pCMV using *Eco*RI and *Bgl*II enzymes. Cells were transfected using Effectene Transfection Reagent at 0.2 µg/cm² with a DNA/Effectene Reagent ratio of 1:20, as described previously [22].

Protein extraction

After different times of culture, proteins were extracted with extraction buffer (20 mM Tris/HCl, pH 7.5, containing 2 mM

EGTA, 0.5 mM DTT, 1 mM NaN_3 , 10 mM benzamidine, 10 $\mu\text{g}/\text{ml}$ leupeptin and 1 mM PMSF), sonicated and centrifuged or not for 30 min at 100 000 g . The cytosolic fraction (supernatant), the particulate fraction (resuspended pellet) and the non-centrifuged extract, corresponding to the total fraction, were separated by SDS/PAGE.

SDS/PAGE

SDS/PAGE was carried out on 10 % polyacrylamide separating gels according to Laemmli [23]. Samples were run at 25 mA for 150 min.

Immunoblotting

Proteins obtained after SDS/PAGE were electrotransferred (0.8 mA/cm²) on to an Immobilon-P membrane. Membranes were incubated at room temperature for 2 h with 5 % (w/v) fat-free dried milk in TBS (Tris-buffered saline: 50 mM Tris/HCl, pH 8.0, 138 mM NaCl and 2.7 mM KCl). Primary antibodies in TBS containing 1 % (w/v) dried milk were incubated with the membranes for 2 h at room temperature. The antibody dilutions were as follow: anti-[MARCKS (N-terminal)], 1:500; anti-[MARCKS (C-terminal)], 1:100 and anti-PKC α , 1:1000. After three washes (10 min) with TBS, the secondary antibody (alkaline-phosphatase-conjugated anti-goat IgG, 1:10 000) was added and incubated for 1 h at room temperature. Following this incubation, the membrane was washed three times (10 min) with TBS. Specific binding of antibodies was detected using alkaline phosphatase detection and the NBT/BCIP substrate. A video densitometer (Geldoc, Bio-Rad) was used for quantification of proteins.

MARCKS proteolysis by calpain

μ -Calpain was purified according to Garret et al. [24]. Myoblast extracts or purified MARCKS were incubated at 30 °C with or without purified μ -calpain with a ratio of 1:500. The reaction was enhanced by addition of CaCl_2 (final concentration, 1 mM). At different times, the reaction was stopped by the addition of Tris/HCl, pH 6.8, containing 1 % (w/v) SDS, 30 % (v/v) glycerol, 0.01 % (w/v) Bromophenol Blue R and 0.02 % (w/v) 2-mercaptoethanol, and incubation during 2 min at 100 °C. The fractions were then separated by SDS/PAGE.

Immunoprecipitation

Protein A beads (100 mg) were washed three times with PBS, incubated with anti-PKC α antibody (2 μg) or non-immune IgG for 2 h at room temperature, and washed five times with extraction buffer containing 0.1 % (w/v) BSA.

Proteins extracted at different states of differentiation were incubated with Protein A coupled with anti-PKC α antibody or non-immune IgG for 4 h at 4 °C. After washing five times with extraction buffer, SDS sample buffer was added to the Protein A beads and the cleared fractions were separated by SDS/PAGE.

Immunohistochemistry

At different states of differentiation, cultured cells were rinsed with PBS and fixed for 15 min with paraformaldehyde (4 %) at room temperature.

Cells were then permeabilized for 5 min with PBS containing Triton X-100 (1 %), and blocked for 15 min with PBS supplemented with BSA (3 %). After three washes, anti-[MARCKS (N-terminal)] antibody (1:100) in PBS containing BSA (1 %) was added for 4 h at room temperature. After three washes (10 min) with PBS, the secondary antibody (FITC-conjugated anti-goat

IgG, 1:200) was added and incubated for 1 h at room temperature. Cells were then stained with Evans Blue (0.5 %) for 3 min. The samples were then mounted with glycerol and observed under a fluorescence microscope (Hund 500).

Quantification of MARCKS mRNA by real-time PCR

Total RNA was extracted from C2C12 myoblasts using an RNA-extraction kit derived from the guanidinium isothiocyanate/phenol/chloroform method [25].

Total RNA (2 μg) were used to synthesize specific first-strand cDNA. RNAs were incubated for 15 min in buffer containing 50 mM Tris/HCl, pH 8.3, 75 mM KCl, 3 mM MgCl_2 , 20 mM DTT, 20 units of RNasin and 14 units of DNase I. After this time, dNTPs (250 μM each), 3'-primers (2 μM) and 100 units of RT (M-MuLVRT) were added, and incubation was extended to 1 h at 37 °C.

The real-time PCR assay involving LightCycler™ technology associates thermocycling with online fluorescence detection of the PCR products. PCR reactions were performed in a volume of 20 μl containing oligonucleotide primers (5 μM each), MgCl_2 (5 mM) and DNA Master SYBR green containing *Taq* DNA polymerase, reaction buffer, dNTPs and double-stranded DNA-specific fluorescent dye SYBR green I and cDNA of MARCKS or GAPDH (glyceraldehyde-3-phosphate dehydrogenase) (5 μl). Amplification occurred in a two-step procedure: denaturation at 95 °C for 8 min, and 35 cycles with denaturation at 95 °C for 8 s, annealing at 68 °C for 6 s and elongation at 72 °C for 10 s. After each elongation phase, the fluorescence of SYBR green I was measured and increasing amounts of PCR products were monitored from cycle to cycle. For each primer pair used, melting-curve analysis showed a single melting peak after amplification, indicating specific product. Moreover, PCR products were subjected to analysis by restriction enzyme digestion and electrophoresis on 10 % polyacrylamide gels (results not shown). Quantification data were analysed using the LightCycler analysis software, version 3.5 (Roche Diagnostics). GAPDH cDNA was used as constant internal standard. Primers used for amplification were: MARCKS 3'-primer, 5'-CTCCTCCTTGTCGGCGGCCGG-3'; MARCKS 5'-primer, 5'-GGCCACGTAAAAGTGAACGGG-G-3'; GAPDH 3'-primer, 5'-ACAACCTGGTCTCAGTGTAG-CC-3'; and GAPDH 5'-primer, 5'-AAGGTCATCCCAGAGC-TGAACGG-3'.

Statistical analysis

Results are means $\pm$ S.E.M. Student's *t* test was used to determine the significance of differences: *P* < 0.05 being significant.

RESULTS

Human recombinant MARCKS proteolysis by purified calpain

The results presented in Figure 1 show that MARCKS was almost completely proteolysed by μ -calpain after 20 min of incubation. A high-molecular-mass degradation fragment of 55 kDa was generated under these conditions. This fragment, observed after 10 and 20 min of incubation (Figure 1A), was recognized by the antibody directed against the C-terminal sequence of MARCKS (Figure 1C). The antibody directed against the N-terminal sequence of MARCKS did not recognize this fragment (results not shown), indicating that proteolysis occurred in the N-terminal region of the protein. As for the controls, no proteolytic products were observed in the presence of EGTA or leupeptin and in the absence of calpain. The C-terminal 55 kDa fragment of MARCKS contains the PSD region, since this region was found in the 40 kDa

Figure 1 Human recombinant MARCKS proteolysis by purified calpain

In vitro proteolysis of purified human recombinant MARCKS (50 μ g) was performed with 0.1 μ g of μ -calpain in the presence of Ca^{2+} (1 mM) for 0, 1, 5 and 10 min at 30 °C. Three controls were used: 1, MARCKS + Ca^{2+} ; 2, MARCKS + μ -calpain + EGTA (10 mM); and 3, MARCKS + μ -calpain + Ca^{2+} + leupeptin (10 μ g/ml). Reactions were stopped by the addition of SDS as described in the Material and methods section; after heating, samples were analysed by SDS/PAGE. (A) Coomassie Blue staining. (B and C) Immunoblots with N-terminal and C-terminal anti-MARCKS antibodies respectively.

C-terminal fragment generated by cathepsin-mediated cleavage [18]. Furthermore, since recombinant MARCKS is not myristoylated, this post-translational modification was not necessary for *in vitro* calpain-mediated degradation of MARCKS.

Cellular MARCKS proteolysis by added purified calpain: regulation by PKC phosphorylation

To confirm *in vitro* data, experiments were performed by fusing myoblast cell extracts and exogenous purified calpain. Under such conditions, intracellular MARCKS protein levels were reduced by 25 % within 10 min of incubation, while a 55 kDa fragment was generated (Figures 2A, 2C and 2D).

Cells were then treated for 1 h with PMA, in order to activate PKC, or with Gö 6976, a specific inhibitor of conventional PKCs. In the presence of PMA, both MARCKS degradation and the appearance of the degradation fragment increased by 45 and 65 % respectively. In contrast, incubation of cells with Gö 6976 totally inhibited calpain-mediated MARCKS proteolysis. It is important to underline that calpain activity on casein is not affected either by PMA or Gö 6976 (results not shown).

All these findings demonstrate further the importance of cellular MARCKS phosphorylation by a conventional PKC for its cleavage by calpain. To verify if calpain activity acts only on cytosolic phosphorylated MARCKS, a similar experiment was performed after cell fractionation (Figure 2A). Under such conditions, no significant variation was observed in membrane MARCKS. Because recombinant MARCKS has no post-translational modification, it is possible that the association of the non-phosphorylated MARCKS with other components protects it from proteolysis by calpain (results not shown).

MARCKS subcellular expression during myogenesis: evidence for its cleavage by intracellular calpain activity at the time of fusion

There was no significant variation in MARCKS mRNA levels measured by RT-PCR during myoblast differentiation.

Figure 2 Proteolysis of MARCKS from cellular extracts by purified calpain: effect of PKC activation or inhibition

Cells were treated after 4 days of differentiation during 1 h with DMSO (control), PMA (150 nM) or Gö 6976 (100 nM). Total proteins or those obtained after fractionation from treated cells were extracted and incubated with 0.1 μ g of purified μ -calpain and 1 mM of Ca^{2+} for 0, 5, 10 and 20 min. Protein (100 μ g) was then separated by SDS/PAGE and immunoblotted with the C-terminal anti-MARCKS antibody. (A) Immunoblot of total or membrane and cytosolic MARCKS proteolysis kinetic; (B) controls; (C and D) quantification of the decrease in MARCKS and of the appearance of the 55 kDa generated fragment in the total fraction ($n = 3$).

Immunoblots with anti-C-terminal antibodies showed that MARCKS was a 80 kDa protein in myoblasts. The protein is present in both the cytosolic and membrane fractions during all differentiation steps, fusion being characterized by an important decreased expression between days 3 and 6 (Figures 3A and 3B). Concomitantly, the appearance of a 55 kDa fragment was observed in the cytosolic fraction only (Figure 3A). Similar results were obtained with rat primary myoblast cultures (results not shown). Our previous work demonstrating activation of calpains during fusion [12, 13] and the present data strongly suggest that the reduced expression of MARCKS, which was not due to a decrease in mRNA levels, and the appearance of the 55 kDa fragment resulted from calpain cleavage after PKC phosphorylation. However, a decreased level of membrane MARCKS was also observed

Figure 3 Subcellular expression of MARCKS during fusion

Proteins obtained from myoblasts at different differentiation days (d) were fractionated as described in the Materials and methods section. Each fraction (100 μ g) was then separated by SDS/PAGE and immunoblotted with antibody directed against the C-terminal region of MARCKS. (A) Cytosolic fraction; (B) particulate fraction. The panels show results from one experiment, similar results were obtained in two other experiments.

during fusion (days 3–6), and we cannot rule out the involvement of other proteolytic systems, namely cathepsins. Alternatively, a partial translocation of membrane MARCKS towards the cytosol after PKC phosphorylation may also account for our observations.

To confirm the intracellular calpain activity on MARCKS and the effect of *in situ* PKC phosphorylation on this phenomenon, additional experiments were performed using cytosol and particulate fractions of fusing myoblasts. In such experiments, a cell-permeant specific calpain inhibitor was used, the CS peptide, corresponding to the inhibitory domains of calpastatin.

Figures 4(A) and 4(B) show that MARCKS was mainly present in the particulate fraction as reported previously [26]. However, the presence of PMA induced a translocation from the particulate fraction to the cytosolic one (40%). In contrast, Gö 6976 induced an increased amount of MARCKS in the particulate fraction (30%), without any significant variation in the cytosolic fraction. These observations are in accordance with the fact that PKC is involved in the cytosolic relocation of MARCKS. Moreover, they indicate the involvement of conventional PKC under our experimental conditions.

In the presence of CS peptide, a 10% increase in MARCKS was observed in the cytosolic fraction of control and Gö 6976-treated cells. This increase reached 20% in the presence of PMA. No significant variation was observed in the particulate fraction for either condition.

The presence of CS peptide induced a significant decrease in the amount of the 55 kDa cytosolic degradation fragment under all the conditions tested (Figures 4A and 4C). However, the decrease was less marked in Gö 6976-treated cells. Altogether, these observations indicate that phosphorylation of MARCKS by a conventional PKC induced its translocation to the cytosol and suggested that its calpain-mediated breakdown occurred in this fraction.

Expression of a MARCKS–PKC α complex during fusion

To identify more precisely the conventional PKC isotype [α , β or γ] involved in MARCKS regulation, we performed immunoprecipitations and immunoblots during fusion. A co-precipitation of MARCKS with PKC α was observed after 4 days of differentiation, in the presence of an antibody raised against PKC α (Figures 5A and 5B). These results demonstrate the existence of a MARCKS–PKC α complex during the fusion process. This observation corroborates previous results obtained *in vitro* after purification of MARCKS from muscle [17] and underlines the close relationship between the two partners. Moreover, PKC α was the only conventional (Ca²⁺-dependent PKC) expressed during fusion (Figure 5C). Thus in our model, MARCKS translocation

Figure 4 Calpain activity from fusing myoblasts on cytosolic and particulate MARCKS

Treatments were realized during 1 h after 4 days of differentiation with DMSO (control; C), PMA (150 nM) or Gö 6976 (100 nM) supplemented or not with CS peptide (10 μ M). Protein obtained after cell fractionation (100 μ g) was separated by SDS/PAGE and immunoblotted with the anti-[MARCKS (C-terminal)] antibody. (A) Immunoblot; (B) relative quantification (percentage of MARCKS levels against control) of cytosolic and particulate MARCKS observed in (A); (C) relative quantification of the cytosolic 55 kDa degradation fragment observed in (A) ($n = 3$).

and phosphorylation involved the PKC α isoenzyme. However, we cannot prove that only PKC α was involved in MARCKS regulation. Indeed, it has been recently demonstrated in C2C12 cells that PKC ϵ (a Ca²⁺-independent novel PKC) also induced MARCKS cytosolic translocation [27].

Effect of MARCKS expression levels on fusion

To investigate the effects of MARCKS expression levels on the fusion process, we next used an oligonucleotide antisense strategy and transfection experiments with MARCKS cDNAs.

Oligonucleotide antisense induced a significant decrease in both MARCKS content (Figure 6A) and a 38 $\pm$ 6.9% increase in fusion ($n = 4$).

When C2C12 cells were transfected with MARCKS cDNA vectors, Western blot analysis showed an increase in MARCKS expression (Figure 6B). Overexpression of wild-type MARCKS induced a decrease of approx. 42 $\pm$ 14.2% ($n = 4$). These results demonstrate without ambiguity that decreased MARCKS levels are mandatory during fusion, in accordance with the previously

Figure 5 Identification of a MARCKS-PKC α complex and PKC α , β and γ expression during fusion

Immunoprecipitations from total cellular extracts (4 days of differentiation) were realized in the absence (–) or in the presence (+) of anti-PKC α antibody as described in the Materials and methods section. Immunoprecipitated proteins were then run on SDS/PAGE and immunoblotted with the anti-[MARCKS (C-terminal)] antibody (A) and anti-PKC α antibody (B). (C) Total cellular extracts (50 μ g of protein) were separated directly by SDS/PAGE and immunoblotted with antibodies directed against PKC α , β and γ .

Figure 6 Effects of MARCKS expression level on fusion

(A) Oligonucleotide antisense treatments against MARCKS were carried out at 2.5 days of differentiation, in differentiation medium with PBS (Con), random sequence (2.5 μ M) (Ran) or antisense sequence (2.5 μ M) (AS) as described in the Materials and methods section. Total proteins were extracted after 4 days of differentiation and 100 μ g were separated by SDS/PAGE and immunoblotted with the anti-[MARCKS (C-terminal)] antibody. The panel shows results from one experiment, similar results were obtained in two other experiments. (B) The cells were transfected at 2 days of differentiation as described in the Materials and methods section with pCMV-vectors: empty (MCS) or containing MARCKS cDNA (WT or PSD[–]). A control was realized in the absence of vector (C). Total proteins were extracted from the transfected cells at day 4 of differentiation. Protein (100 μ g) was separated by SDS/PAGE and immunoblotted with the anti-[MARCKS (C-terminal)] antibody. The panel shows results from one experiment, similar results were obtained in two other experiments. In these conditions, the extent of fusion (%) was estimated as described in the Materials and methods section.

observed MARCKS calpain cleavage during this process (Figures 3 and 4). In contrast, when the mutant MARCKS that cannot be phosphorylated by PKC (PSD[–]) was overexpressed, the fusion process decreased by approx. $62 \pm 8.9\%$ ($n = 5$). This indicates that phosphorylation of MARCKS by PKC is essential for myoblast fusion. Therefore a partial decrease in membrane MARCKS induced by PKC phosphorylation (translocation to the cytosolic fraction and subsequent cytosolic breakdown of MARCKS by calpains) occurred during fusion.

Immunolocalization of MARCKS during myogenesis

Figure 7 shows MARCKS localization in proliferating (Figure 7A) and fusing cells (Figure 7B) (4 days of differentiation). The antibody against N-terminal MARCKS was used. A diffuse

Figure 7 Immunolocalization of MARCKS in myoblasts

Myoblasts cultured in proliferation medium (A) or cultured for 4 days in differentiation medium (B) were immunostained with the anti-[MARCKS (N-terminal)] antibody as described in the Materials and methods section and then with FITC-conjugated anti-IgG antibody. Magnification, $\times 1000$. The arrow indicates contact between two fusing cells.

cytosolic pattern clearly appeared during proliferation. In contrast, when the cells fused intensively, MARCKS appeared as punctate structures, particularly in the vicinity of fusion sites (arrow), and diffusely in the cytosol. This observation corroborates previous immunoblot data (Figure 3), which indicated that MARCKS was present in either particulate or cytosolic fractions during fusion. It indicates further that the presence of MARCKS at the membrane-fusion sites does not disrupt the fusion process.

The presence of membrane punctate structures and the cytosolic diffuse pattern of MARCKS during fusion presumably indicates that MARCKS exists in association with different components in the two compartments. This may in part contribute to explain the different affinity of calpains against membrane and cytosolic MARCKS. In contrast, the uniform MARCKS localization in proliferating cells suggests a differential regulation of MARCKS during fusion and proliferation.

DISCUSSION

Previous studies have emphasized that calpains promote cell fusion of cultured myoblasts [12,13,28] by acting on PKC α [14]. Evidence for a MARCKS-PKC α complex in skeletal muscle has also been provided by *in vitro* experiments [17]. MARCKS is a PKC substrate that cycles on and off membranes by a mechanism termed the myristoyl-electrostatic switch [29,30]. MARCKS regulates cytoskeletal structures by interacting with actin filaments [16] and PtdIns(4,5) P_2 [31], especially after integrin activation [27].

In the present study, we demonstrate that MARCKS is partially down-regulated by calpains during the fusion of cultured C2C12 cells, the appearance of a cytosolic 55 kDa degradation fragment being associated with this phenomenon. The stability of MARCKS mRNAs during differentiation of C2C12 cells is in accordance with a proteolytic event. Using purified human recombinant MARCKS, we also demonstrate that purified calpain induced a similar cleavage of MARCKS and generated a 55 kDa degradation product. This fragment corresponds to the C-terminal sequence of the protein containing the PSD region and lacking the myristoylated N-terminal domain. These results are in accordance with the cytosolic localization of P55 in differentiating cells because P55 lacks the myristoylation site and cannot be associated to the plasma membrane [32]. The involvement of calpains in MARCKS cleavage was confirmed at the cellular level by using a very specific calpain inhibitor (CS peptide) and by demonstrating that myoblast extracts have a Ca^{2+} -dependent degradation activity on MARCKS. This proteolytic process is dependent on the phosphorylation status and/or localization of MARCKS: cytosolic phosphorylated MARCKS is a good substrate for calpains, whereas non-phosphorylated MARCKS is a poor substrate. On the

other hand, an accumulation of MARCKS in migrating muscle cells that stably overexpress the calpastatin inhibitor has been reported recently [22]. In the present study, we provide evidence for phosphorylated MARCKS cleavage by calpains during myoblast fusion, adding a new regulatory mechanism for MARCKS biological function that remains to be identified.

Spizz and Blackshear [18] have reported that cathepsin B is involved in non-phosphorylated MARCKS proteolysis in fibroblasts. Possibly, cathepsins could promote membrane MARCKS turnover during fusion, in contrast with calpains, which could be involved in a still unknown regulated process. However, Braun et al. [33] have shown that an unknown protease specifically cleaved the myristoylated N-terminus of MARCKS in macrophage extracts.

The 55 kDa cytosolic degradation product observed under our experimental conditions may have a specific function because it was observed in living cells during the fusion process. The generated myristoylated N-terminal peptide could also have a specific function, since this part of the molecule was reported to interact with calmodulin [34]. In addition to controlling the Ca^{2+} level, calmodulin is also well known to regulate the actin cytoskeleton via regulation of $\text{PtdIns}(4,5)\text{P}_2$ [35]. Altogether, these data suggest that MARCKS cleavage may be an important and complex regulatory mechanism for its function, which depends on the cellular model and the external stimuli.

We also demonstrated that $\text{PKC}\alpha$ is involved in phosphorylation and translocation of MARCKS from the membrane to the cytosol in C2C12 cells, and that this activity promotes MARCKS proteolysis by calpains. $\text{PKC}\alpha$ is present at the time of intense fusion, whereas other conventional PKCs [β and γ] are not. Additionally, cellular MARCKS immunoprecipitates with $\text{PKC}\alpha$. These findings are in accordance with previous observations *in vitro* [17] and other experiments [36]. Kim et al. [19] have reported that the novel $\text{PKC}\theta$ isotype is responsible for MARCKS phosphorylation in differentiating chicken myoblasts. Such a difference could be explained by the different species origin of the cells. Accordingly, Disatnik et al. [27] have reported that $\text{PKC}\theta$ is only expressed in the late differentiation steps of C2C12 mouse myotubes.

Such results provide evidence for a common signalling pathway of $\text{PKC}\alpha$ and MARCKS, and demonstrate that activation of $\text{PKC}\alpha$ for MARCKS phosphorylation is a required step for cytosolic translocation of MARCKS and its proteolysis by calpains during myoblast fusion. These results are also in agreement with the observation that short-term PMA treatments promote cell fusion [37] and are able to induce MARCKS down-regulation at the protein level [38]. Yokoyama et al. [39] also demonstrated that PMA induces MARCKS phosphorylation at least for 6 h of treatment. Moreover, in our experimental conditions an increased fusion was observed 6 h after the addition of PMA for 1 h. In contrast, long-term treatments (12 or 24 h), which strongly induced $\text{PKC}\alpha$ down-regulation [14] had a negative effect (results not shown). Numerous studies have demonstrated already that phosphorylation often induces a cytosolic localization of the substrate and modulates the rate of protein cleavage by calpains [40,41].

The interaction between MARCKS and $\text{PKC}\alpha$ is probably direct, since a complex has been isolated [17]. Similarly, a co-immunoprecipitation of $\text{PKC}\alpha$ and diacylglycerol kinase ζ which also contains a PSD domain, has recently been reported. This association is significantly attenuated by $\text{PKC}\alpha$ phosphorylation of PSD [42]. Thus it is also possible that the association of MARCKS with $\text{PKC}\alpha$ and probably other components protects non-phosphorylated membrane MARCKS from calpain-mediated proteolysis. Our observation that MARCKS myristoylation did not interfere with calpain activity *in vitro* (Figure 1) supports this hypothesis further.

In order to investigate the functional role of MARCKS during myoblast fusion, we manipulated its expression level. MARCKS underexpression as a result of an antisense oligonucleotide promoted C2C12 cell fusion, whereas wild-type MARCKS overexpressions had the opposite effect. These observations confirm that a reduction in MARCKS levels is necessary at the time of fusion. In addition, a reduction in MARCKS phosphorylation by PKC (overexpression of MARCKS PSD⁻) induced a more drastic effect on fusion. This supports further a critical role for cytosolic MARCKS and the importance of its regulation by calpains in myoblast fusion that remain to be clearly defined.

The recent data of Kim et al. [43] showed that myoblasts overexpressing wild-type MARCKS and non-phosphorylatable MARCKS fuse more extensively than those transfected with MARCKS lacking the myristoylation site. Such an observation suggests that membrane MARCKS was essential for myoblast fusion and that overexpression of cytosolic unmyristoylated MARCKS decreased fusion. In the same work, Kim et al. [43] also provided evidence for a decrease in intracellular phosphorylated MARCKS levels by using anti-phospho-MARCKS antibody. The authors assimilate this observation with a phosphatase activity on MARCKS, but unfortunately they did not measure total MARCKS levels during fusion. Thus their observations may also reflect increased proteolysis.

The immunolocalization of MARCKS at the time of fusion indicated a punctate pattern, which suggests an increased local concentration of the protein at the membrane, including at fusion sites. Such a punctate pattern is always observed for specialized rafts named caveolae, where $\text{PtdIns}(4,5)\text{P}_2$ and Ca^{2+} are concentrated [31]. Interestingly, caveolae are not present during myoblast proliferation and are essential for the fusion process [44]. In addition, MARCKS and $\text{PKC}\alpha$ have been found to be localized already in these vesicular structures in association with phospholipase D [45,46].

The importance of MARCKS phosphorylation by PKC, which induces its translocation to the cytosol and the resulting disintegration of actin-based cytoskeletal structures has been reported extensively [30,47]. In the present study, we have identified one of the kinases responsible as being a conventional $\text{PKC}\alpha$. Disatnik et al. [27] found that, in skeletal muscle, MARCKS is initially localized in adhesion sites and quickly translocates to the cytosol upon integrin activation. In C2C12 cells, only the conventional $\text{PKC}\alpha$ has been shown to be activated upon integrin activation [48]. Furthermore, $\text{PKC}\alpha$ has been shown to selectively target at cell–cell contacts upon physiological stimulation [49]. Interestingly, under the same conditions, MARCKS translocation to the cytosol has been observed [50]. At cell–cell contacts, signalling through PKC would mediate the co-ordinated organization of intra- and extra-cellular component molecules. The partial proteolysis of cytosolic MARCKS by calpain could be involved in the irreversible localization of phosphorylated MARCKS in the cytosol, since the 55 kDa fragment does not contain the myristoylated part of the protein. This process, which induces a partial decrease in MARCKS levels at the membrane, could be associated with a local increase in free $\text{PtdIns}(4,5)\text{P}_2$. This event that induces an improved mobility of membrane proteins [51] may participate in membrane fusion.

In conclusion, our data support that MARCKS present at the fusion site is a key signalling molecule downstream of the $\text{PKC}\alpha$ pathway. Possibly, MARCKS may mediate the massive cytoskeleton reorganization that accompanies myoblast fusion. Thus, in C2C12 cells, both calpains and $\text{PKC}\alpha$ activated during fusion are involved in MARCKS down-regulation. Cultured muscle cells appear to be a good model for elucidating the precise mechanisms of myoblast fusion and functions of MARCKS, which are still

unknown. Additional investigations using proteomic tools have been undertaken to this end.

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