Published in final edited form as:

Cell Signal. 2010 July ; 22(7): 1097–1103. doi:10.1016/j.cellsig.2010.03.003.

PhosphoMARCKS Drives Motility of Mouse Melanoma Cells

Xiangyu Chen^{1,2} and Susan A. Rotenberg¹

¹Department of Chemistry & Biochemistry of Queens College

²Graduate Center of The City University of New York

Abstract

Phosphorylation of Myristoylated Alanine-rich C-Kinase Substrate (MARCKS) by protein kinase Cα (PKCα) is known to trigger its release from the plasma membrane/cytoskeleton into the cytoplasm, thereby promoting actin reorganization during migration. This study shows that once released into the cytoplasm, phosphoMARCKS directly promotes motility of melanoma cells. Aggressively motile B16 F10 mouse melanoma cells express high levels of phospho-MARCKS, whereas in weakly motile B16 F1 cells it is undetectable. Following treatment with okadaic acid (OA) (a protein phosphatase inhibitor), F1 cells exhibited a dramatic increase in phosphoMARCKS and was co-incident with a five-fold increase in motility. Both MARCKS phosphorylation and motility were substantially decreased when prior to OA addition, MARCKS expression was knocked out by a MARCKS-specific shRNA, thereby implicating MARCKS as a major component of the motility pathway. Decreased motility and phosphoMARCKS levels in OA-treated cells was observed with a PKC inhibitor (calphostin C), thus indicating that PKC actively phosphorylates MARCKS in F1 cells but that this reaction is efficiently suppressed by protein phosphatases. The mechanistic significance of phosphoMARCKS to motility was further established with a pseudo-phosphorylated mutant of MARCKS-GFP in which Asp residues replaced Ser residues known to be phosphorylated by PKCα. This mutant localized to the cytoplasm and engendered three-fold higher motility in F1 cells. Expression of an unmyristoylated, phosphorylation-resistant MARCKS mutant that localized to the cytoplasm, blocked motility by 40–50% of both OA-stimulated F1 cells and intrinsically motile F10 cells. These results demonstrate that phosphoMARCKS contributes to the metastatic potential of melanoma cells, and reveal a previously undocumented signaling role for this cytoplasmic phospho-protein.

Keywords

Protein kinase C substrate; mutant; cytoskeleton; phosphatase; metastasis

1. INTRODUCTION

Like many cancer cells such as breast, ovarian, and intestine, invasive behavior of highly metastatic B16 F10 melanoma cells is known to be dependent on PKCα activity (1–5). To

© 2010 Elsevier Inc. All rights reserved.

Corresponding Author: Susan A. Rotenberg, Department of Chemistry & Biochemistry, Queens College of CUNY, 65-30 Kissena Boulevard, Flushing, NY 11367. Tel. (718) 997-4133, Susan.Rotenberg@qc.cuny.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

begin to dissect the mechanism(s) through which PKC α activity promotes invasive activity, it is essential to identify its protein substrates that mediate the related cell surface phenotypes such as adhesion and motility. Some progress has been made in linking PKC α to adhesion by identifying relevant substrates that are associated with the plasma membrane or cytoskeleton. A prominent example is integrin $\beta 4$ which upon phosphorylation by PKC α impacts hemidesmosome formation and leads to the disruption of cell-cell interactions (6). Although their downstream effects are not completely characterized, other PKC(α) substrates include the cytoskeletal proteins MARCKS, adducin, GAP43, fascin, ERM (ezrin-radixin-moesin) proteins (7), and $\alpha 6$ -tubulin (8). Upon their phosphorylation by PKC(α), these protein substrates enact dynamic changes in the cytoskeleton that result in altered adhesion and migration behavior (9–12).

For the present studies, highly metastatic mouse B16 F10 melanoma cells were compared with B16 F1 cells, a related sub-line that has lower motility *in vitro* and very low metastatic potential in mice (13,14). However, metastatic activity of F1 cells can be induced by short-term treatment with phorbol ester (12-tetradecanoylphorbol-13-acetate) that binds the same site on conventional and novel PKC isoforms as the physiological activator diacylglycerol (DAG), thereby producing persistent activation of PKC and its association with the plasma membrane (13,15). At the cell membrane, PKC encounters its substrates that consequently mediate adhesion and migration. In both F1 and F10 cells, the most abundantly expressed isoform is PKC α (4,16). Our laboratory showed that engineered expression of kinase-dead PKC α in F10 cells inhibits cell adhesion and migration on collagen by 40–50% (4). Other studies have shown that PKC inhibitors, including small molecules such as calphostin C or anti-sense reagents, substantially inhibit metastasis of B16 melanoma cells *in vivo* (17,18).

MARCKS (myristoylated alanine-rich C-kinase substrate) is one of the few intracellular substrates that is preferentially phosphorylated by PKC α , although other isoforms such as δ and ϵ (but not ζ) can perform this reaction (19). The non-phosphorylated protein crosslinks actin filaments and associates with the plasma membrane via its myristoyl tail. This association with the plasma membrane was shown to inhibit adhesion of human embryonic kidney 293 cells (20). Phosphorylation by PKC α at serine residues located in the phosphorylation site domain (21), specifically at Ser 159, Ser163, and Ser170 in human MARCKS, masks positive charges on the protein, whereupon MARCKS disengages from the membrane via the so-called ‘myristoyl/electrostatic switch’. This release of MARCKS into the cytoplasm allows for greater plasticity of the actin cytoskeleton thereby promoting adhesion and cell spreading via cytoskeletal rearrangement (12,22–24). During its transient interactions with the cytoskeleton, MARCKS is thought to regulate F-actin dynamics that in turn impact cell surface activities such as cell migration, exocytosis and endocytosis (1), and events controlling proliferation (25,26). The release of MARCKS into the cytoplasm can also occur through an interaction with calmodulin in the presence of increased Ca^{2+} concentrations. PKC signaling is independent of Ca^{2+} signaling since phosphorylation of MARCKS disrupts its complex with Ca^{2+} /calmodulin in the same effector domain (21). By controlling the binding of MARCKS to actin (21), PKC-mediated phosphorylation and Ca^{2+} /calmodulin binding provide a dual mechanism for regulating cytoskeletal dynamics. The finding that phosphorylation of MARCKS protects it from proteolysis (27), implies that the protein undergoes a conformational change that ensures its stability as a phospho-protein. However, upon its release into the cytoplasm, phosphoMARCKS is not known to enact additional functions. Although previous studies showed that the MARCKS protein associates with the microtubule organizing center of the centrosome (28), no biological role for cytoplasmic phosphoMARCKS has been reported. In the present work, phosphoMARCKS is shown to be a cytoplasmic component of the motility signaling pathway in melanoma cells, thereby contributing to the metastatic phenotype of melanoma by a mechanism that is distinct from its role at the plasma membrane.

2. MATERIALS AND METHODS

2.1 Materials

All cell culture reagents were purchased from Invitrogen (Carlsbad, CA). Rabbit monoclonal antibodies for phosphoMARCKS and MARCKS were obtained from Epitomics (Burlingame, CA). Mouse monoclonal GFP antibody and secondary antisera were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and the shRNA-encoding plasmid for mouse MARCKS was from Origene (Rockville, MD). Fugene 6 transfection reagent was obtained from Roche Applied Science (Indianapolis, IN), and PolyExpress DNA transfection reagent was purchased from InnoVita, Inc. (Gaithersburg, MD). Protein dye reagent and protein markers were from Bio-Rad (Hercules, CA). The QuikChange mutagenesis kit was purchased from Stratagene (La Jolla, CA), and chemiluminescence reagents (Supersignal West Pico) were acquired from Pierce Co. (Rockford, IL). Calphostin C was obtained from EMD Biosciences Inc., (La Jolla, CA), and DABCO was from Acros Organics (Morris Plains, NJ). The DAG-lactone reagent (JH-131E-153) was a gift from Dr. V. Marquez (NCI-Frederick, NIH) and was the pure *R*-enantiomer contained in the previously reported racemic mixture (HK654 in ref. 29).

2.2 Construction of cDNA plasmids

GFP-fusion proteins of bovine MARCKS (D/S-MARCKS, N/S-MARCKS) or the unmyristoylated constructs (wildtype (WT) and D/S) were expressed from EGFP-N1 plasmids (Clontech) that were provided by Dr. P. Blackshear, NIEHS (Research Triangle Park, NC). These constructs were fused at the C-terminus with GFP and were previously described (20). For the present work, a second mutation (Gly → Ala) was introduced into N/S-MARCKS at the site of myristoylation (Gly-2) in order to prevent acylation. The resulting double mutant (A2G_N/S-MARCKS) was constructed by a standard PCR approach (QuikChange) using a primer in which the mutated bases are underlined: 5'-CTTTGTTGAAGACCAGCATGGCCGCCAGTTCTCCAAGACCGC-3'. The sequence was confirmed by DNA sequencing of the entire open reading frame (Macrogen Inc., Rockville, MD).

2.3 Cell culture and transfection

B16 F1 or F10 cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 1% penicillin/streptomycin and 0.1% fungizone. Cells were grown at 37 °C in a 5% CO₂ atmosphere and split twice per week. One day prior to each experiment, cells were replated on plates so that they were at 60 – 70% density at the time of transfection. For transfection, cells were washed with PBS and cultured in serum-free medium prior to addition of the plasmid. For 60-mm plates (motility assay), 4 µg plasmid DNA was combined with Fugene 6 transfection reagent in a ratio of 1:2 (µg DNA/µl reagent) and added to cells in serum-free medium, followed by incubation for 6h at 37 °C, after which fetal bovine serum was restored to each plate and incubation was continued for 48–72 h. For cells transfected with Fugene 6, the efficiency was 50–60%. The shRNA-encoding plasmid (5 µg for 60 mm plate) was prepared with PolyExpress DNA transfection reagent in a ratio of 1:3 (µg shRNA/µl reagent) in serum-free medium. The reagent was added to cells in 2.8 ml complete medium and the cells were incubated for 72 h at 37 °C (5% CO₂). The bi-cistronic shRNA-encoding plasmid (Origene) co-expresses the shRNA product and red fluorescent protein (RFP) thereby providing a means by which to assess transfection efficiency. For F1 cells transfected with the PolyExpress reagent, efficiency was judged to be 80% or higher.

2.4 Cell lysis

Cells were lysed in 0.5 ml lysis buffer (50 mM Tris-HCl, pH 7.4, 1.25 mM EDTA, 1.25 mM EGTA, 1% Triton X-100, 0.1% 2-mercaptoethanol) containing protease inhibitors [1 mM phenylmethanesulfonyl fluoride, 10 ng/ml leupeptin, and 10 ng/ml soybean trypsin inhibitor] and serine/threonine phosphatase inhibitors (Sigma-Aldrich, St. Louis, MO). The cells were sonicated for 3×10 sec and centrifuged at $5700 \times g$ for 10 min to remove insoluble material. The resulting lysate was analyzed for protein content using the Bio-Rad protein reagent with bovine serum albumin as standard.

2.5 Western blot analysis

Cell lysates were denatured in sample buffer, followed by heating at 95 °C for 5 min. Samples were resolved by 7.5% SDS-PAGE gel and transferred to a PVDF membrane (Millipore Corp, Billerica, MA). Antibodies that were specific for phosphoMARCKS or MARCKS were applied to the blot and incubated with shaking at room temperature for 2 h. After washing the membrane three times with TBST (150 mM NaCl, 20 mM Tris pH 7.4, 0.1% Tween 20 v/v), the appropriate horseradish peroxidase-conjugated secondary antibody (1:5000) was applied and incubated for 1 h. After washing three times with TBST, the membrane was developed with chemiluminescence reagents.

2.6 Motility Assay

This assay was carried out with a 10-well glass slide and a cell sedimentation manifold (CSM, Phoenix, AZ). Cells (2×10^6 per ml) were pipetted into the manifold in triplicate thereby enabling their sedimentation onto the slide as a small concentric circle. After incubation for approximately 16 h at 37 °C and 5% CO₂, the manifold was removed ($t = 0$) allowing the cells to radiate outwardly. Both at $t=0$ and following incubation for 6h at 37 °C, bright field images of the cells were recorded and analyzed using Motic Image software. To analyze motility of cells that were successfully transfected with the shRNA-encoding plasmid, fluorescence imaging was used to detect only those cells that co-expressed RFP. The motility of each sample was judged by quantifying the increase in area occupied by the cells after 6h and averaging the results for triplicate samples.

2.7 Confocal microscopy

Forty-eight hours following transfection with the GFP construct, melanoma cells were plated onto a cover slip in each well of a 6-well plate, and incubated overnight at 37 °C. Cells were fixed with 4% paraformaldehyde, washed three times with $1 \times$ PBS for 5 min, and mounted with buffer consisting of 90% glycerol, 10% TBS, and 1,4-diazabicyclo[2.2.2]octane (DABCO) (100 mg/ml), and sealed. Fluorescent images were acquired on a Leica confocal microscope (True Confocal Point Scanning SP5) equipped with an argon ion laser, and a GFP filter set. All images were collected using an oil immersion 63 $\times$ objective lens.

3. RESULTS

3.1 Phosphorylated MARCKS is elevated in highly metastatic B16 melanoma cells

The initial inquiry addressed whether the level of MARCKS phosphorylation correlates with the motility phenotype of F1 and F10 cells. It was found that F10 cells, which are highly motile and metastatic in a syngeneic animal model (13), express a high level of phosphoMARCKS (80 kDa band), while the less motile and non-metastatic F1 cells do not express detectable levels of this phospho-protein. Both cell lines however express equivalent levels of unphosphorylated MARCKS (Figure 1).

3.2 Okadaic acid dramatically elevates phosphorylated MARCKS and motility in F1 cells

Because F1 cells express abundant PKC α (16), it was somewhat surprising that they did not exhibit detectable phosphoMARCKS either following engineered expression of constitutively active PKC α (Chen and Rotenberg, unpublished results), or following either brief (5 min) (not shown) or extended (6 h) treatment (Figure 2A) with diacylglycerol (DAG)-lactone, a membrane-permeable DAG analogue and PKC activator (29). A plausible explanation of these observations is that a significant fraction of PKC is already activated to produce phosphoMARCKS but that this phosphoprotein is being rapidly dephosphorylated. The possibility that endogenous phosphatase activity is exceedingly high relative to PKC activity was therefore explored as a means by which F1 cells maintain low levels of phosphoMARCKS and motility. Previous studies established that phosphoMARCKS undergoes dephosphorylation by PP1 and PP2A (30) each of which can be inhibited by okadaic acid (OA). It was observed that treatment of F1 cells with 1 μ M OA produced a dramatic elevation of phosphoMARCKS that was detectable within 1 h and remained high for 6 h (Figure 2A). It was further noted that if both DAG-lactone and OA were present continuously for 6 h, there was no further increase in phosphoMARCKS levels over that observed for OA treatment alone.

OA-induced elevation of phosphoMARCKS in F1 cells was paralleled by a three-fold increase in motile behavior (Figure 2C). In contrast, brief DAG-lactone exposure (5 min) had no effect on motility (Figure 2C), consistent with its inability to induce phosphoMARCKS formation (Figure 2A). However, while continuous treatment (6 h) with DAG-lactone failed to produce phosphoMARCKS (Figure 2A), it nonetheless produced a 2-fold increase in motility, as compared with a 3-fold higher motility engendered by OA alone; motility was not further increased by their combined treatment (Figure 2D). Because continuous treatment with DAG-lactone alone was capable of stimulating motility without increasing phosphoMARCKS, it is possible that this PKC activator induced the phosphorylation of additional PKC substrates that promote motility and that were relatively stable to endogenous phosphatases.

To establish that PKC was the major source of the phosphoMARCKS levels produced with OA, F1 cells were pre-treated with calphostin C, a PKC inhibitor that requires photoactivation by fluorescent light (31). This inhibitor blocked both OA-induced accumulation of phosphoMARCKS (Figure 2B) and motility (Figure 2C). It is noted that other PKC inhibitors were tested at 1 μ M (*bis*-indoleylmaleimide and Go6976) but unlike calphostin C, they did not decrease phosphoMARCKS levels. This outcome may be due to a preference by calphostin C for plasma membranes where activated PKC and MARCKS interact (32). Overall, these findings imply that endogenous PKC activity is high in F1 cells but that signaling through PKC is weak due to the efficient action of protein phosphatases that rapidly restore phosphoMARCKS to the unphosphorylated state. When inhibited by OA, these phosphatases can no longer mute the PKC activity in these cells.

To investigate the specific contribution of phosphoMARCKS to F1 motility under conditions of OA treatment, a mouse MARCKS-specific shRNA vector was transfected into F1 cells prior to addition of OA. This bi-cistronic vector simultaneously expresses the shRNA product and red fluorescent protein (RFP). By tracking the movement of fluorescent cells, the impact of down-regulation of MARCKS on motility could be directly assessed in those cells that expressed the shRNA reagent. In Figure 3, the effect of shRNA on MARCKS expression and motility was compared with that of a scrambled control shRNA (Control) and the empty vector (VC). A nearly complete loss of MARCKS expression was evident in cells treated with the MARCKS shRNA whereas in the control samples MARCKS levels were unaffected (Figure 3A). Importantly, knock-down of MARCKS correlated with a substantial decrease in motile behavior. After correcting for background

motility (signified by the VC minus OA condition), the degree of inhibition (relative to the VC plus OA condition) was calculated as 66%, whereas with the scrambled control shRNA, inhibition was only 10%. This finding implies that phosphoMARCKS activity contributes two-thirds of the total motility of F1 cells under conditions of OA treatment.

3.3 Engineered expression of MARCKS-GFP mutants results in characteristic sub-cellular localization and motility effects on F1 and F10 cells

The four PKC α -mediated phosphorylation sites that comprise the phosphorylation site domain in MARCKS are well defined for this PKC α substrate (22), and comply with the consensus sequence recognized by PKC (32). Plasmids encoding mutant forms of MARCKS-GFP were used (generously provided by P. Blackshear, NIEHS). For the pseudo-phosphorylated mutant, all four serine residues had been mutated to Asp (D) residues (S $\rightarrow$ D) to simulate the presence of a negatively charged phosphate. Similarly, the phosphorylation-resistant mutant was produced by mutating all four sites to Asn (N) residues (S $\rightarrow$ N).

The sub-cellular localization of each mutant was visualized by confocal microscopy (Figure 4). As predicted from the model for the interaction of MARCKS and the membrane (34), the pseudo-phosphorylated mutant was detected in the cytoplasmic compartment, whereas the phosphorylation-resistant mutant was found exclusively at the cell periphery. The wildtype MARCKS-GFP (WT-MARCKS-GFP) was detected primarily at the plasma membrane of F1 cells, which was consistent with weak signaling through PKC in untreated F1 cells due to protein phosphatase activity. However, treatment with 1 μ M OA for 1 h led to the appearance of WT-MARCKS-GFP in the cytoplasm. This outcome correlated with the detected increase in endogenous phosphoMARCKS by OA (Figure 2A) and would be expected to occupy the cytoplasm. Expression of an unmyristoylated WT-MARCKS-GFP (produced by Gly2 $\rightarrow$ Ala mutation) resulted in GFP signals distributed both at the membrane and in the intracellular space. This distribution suggests that for the unmyristoylated wildtype protein (A2G_WT) that is unphosphorylated, membrane association is still possible, while WT-MARCKS that has undergone phosphorylation consequently appears in the cytoplasm, in agreement with a previous study (20). Thus, removal of the acyl group is not sufficient to dissociate the WT protein completely from the membrane.

Following transient transfection of these plasmids into parental (untreated) F1 cells, each GFP-fusion protein was stably expressed as a 100 kDa protein at equivalently high levels, as detected by Western blot with GFP or MARCKS antibodies (Figure 5A). It is noted that the level of expression of each MARCKS-GFP construct far exceeded the levels of endogenous MARCKS protein, since in order to detect the endogenous MARCKS by Western blot, at least a 10-fold higher protein sample amount was required (not shown).

The mechanistic significance of phosphoMARCKS to motile behavior was established by transfecting F1 and F10 cells with the appropriate gain-of-function or loss-of-function MARCKS mutant. As shown in Figure 5B, the pseudo-phosphorylated mutant of MARCKS [(D/S)] significantly enhanced movement of each parental cell line, but a more pronounced effect was observed in F1 cells. Expression of the phosphorylation-resistant mutant [N/S], which only localizes to the plasma membrane region (Figure 4), produced a nearly complete dominant-negative effect on motility of F10 cells in which there was detectable phosphoMARCKS (Figure 1). Although this mutant had no effect on weakly motile F1 cells (Figure 5), it had a substantial dominant negative effect on motility of OA-treated F1 cells (Figure 6A), similar to its extent of inhibition of the intrinsic motility of F10 cells (Figure 5).

A second mutation was introduced into N/S-MARCKS-GFP, namely (Gly-2→Ala), that prevented myristoylation and consequently inhibited the ability of this mutant to associate with the membrane. In contrast to the myristoylated phosphorylation-resistant mutant (N/S-MARCKS) that associates with the plasma membrane region (Figure 4), this double mutant, A2G_N/S-MARCKS, could be expressed at levels comparable to the WT-MARCKS (Figure 5A) but was entirely localized to the cytoplasm, similar to the pseudo-phosphorylated mutant D/S-MARCKS-GFP (Figure 4). Acting within the cytoplasmic compartment, this unmyristoylated phosphorylation-resistant MARCKS mutant had a dominant negative effect on motility of F1 cells treated with OA (Figure 6A). OA-stimulated cells expressing this double mutant exhibited a 40–50% loss of motility as compared with cells transfected with the analogous unmyristoylated mutant for WT-MARCKS (A2G_WT) or the empty vector (VC). The double mutant was also tested on highly motile F10 cells that express a high level of endogenous phosphoMARCKS (Figure 1). As shown in Figure 6B, F10 cells expressing A2G_N/S-MARCKS exhibited a 40–50% decrease in motile behavior. These dominant-negative effects on motility by the cytoplasmic double mutant lend further support to a cytoplasmic mechanism for MARCKS that contributes to the motility phenotype and is distinct from the inhibitory role performed by myristoylated MARCKS that is bound at the cell periphery.

4. DISCUSSION

In the conventional model for MARCKS regulation, phosphorylation of this protein by PKC promotes its release from the cytoskeleton/plasma membrane that in turn enables rearrangement of the actin cytoskeleton (23,34). Although phosphorylation of MARCKS not only disposes it to the cytoplasm but also ensures its structural stabilization (27), the possibility that phosphoMARCKS has an additional role in the cytoplasm had not been previously addressed. A new insight emerging from the present study is that phosphoMARCKS promotes motility, presumably by engendering protein-protein interactions in the cytoplasmic compartment. Characterization of this cytoplasmic function of phosphoMARCKS provides an avenue for further study.

These studies show that MARCKS is a major determinant in the motility phenotype of metastatic mouse melanoma cells. An important observation was that the low level expression of phosphoMARCKS in non-metastatic F1 cells and the attendant weak cell motility are apparently determined by the relative balance of PKC and protein phosphatase activities at the cytoskeleton/plasma membrane region, which is the site of encounter between activated PKC and MARCKS. From earlier studies by Gopalakrishna and Barsky (13), it was noted that the ability to promote PKC binding to the plasma membrane was the key distinction between the actions of the phorbol ester TPA (induces PKC binding to membranes and subsequent metastatic activity) and membrane permeable DAG analogues (do not induce membrane association of PKC or metastasis). This observation would explain the inability of DAG-lactone (following a 6-h treatment) to induce detectable accumulation of phosphoMARCKS in the present study (Figure 2A). It is also notable that in the same early study (13) the basal activity of membrane-bound PKC in untreated parental F1 cells was approximately 10% of total PKC activity, whereas in F10 cell membranes that value was almost 30%. Our findings support a model in which the low level of membrane-bound PKC in membranes of untreated F1 cells can be countered effectively by protein phosphatases, as given by the absence of detectable phosphoMARCKS by Western blot (Figure 1), and the low level of WT-MARCKS-GFP in the cytoplasmic space (Figure 4). Since treatment with OA produced a dramatic increase in phosphoMARCKS and motility (Figure 2A, 2C), as well as the appearance of WT-MARCKS-GFP in the cytoplasm (Figure 4), it is likely that in untreated F1 cells, PP1 and PP2A protein phosphatases block the accumulation of phosphoMARCKS and thereby suppress motile behavior. These findings

provide a plausible explanation for the non-metastatic activity of F1 cells (13). By contrast, the high level of PKC in the F10 plasma membrane is sufficient to override the effect of phosphatases, resulting in highly motile (and metastatic) behavior. Because knockdown of MARCKS in OA-treated F1 cells to a very low level resulted in two-thirds loss of motile behavior of transfectant cells (Figure 3), it is likely that the cycling of MARCKS between its phospho-dephospho states plays a substantial role in a phenotype that undoubtedly involves other substrates of PKC. Our additional observation that only a membrane-selective inhibitor calphostin C (32), was competent in abolishing both phosphoMARCKS accumulation in OA-treated F1 cells (Figure 2B) and their motility (Figure 2C), gave a strong indication that membrane-associated PKC was the primary source of kinase activity. Consequently, anti-cancer therapies that target membrane-associated PKC may prove to be beneficial. In this regard, a previous report demonstrated the anti-metastatic effect of calphostin C on B16 melanoma cells in a mouse model (17).

A second major finding of the present work revealed that upon formation by PKC, phosphoMARCKS transmits the motility signal from the cytoplasm, as shown by the increased motility that resulted from expression of a cytoplasmic pseudo-phosphorylated mutant of MARCKS (D/S-MARCKS-GFP) (Figure 4 and Figure 5). This function was further supported with a phosphorylation-resistant mutant (A2G_N/S) that was also cytoplasmic due to the absence of the myristoyl group (Figure 4). This double mutant, whose cytoplasmic site of action was distinguished from that of the membrane-bound, myristoylated mutant (N/S-MARCKS-GFP), effectively blocked up to 50% of motility of OA-stimulated F1 cells and intrinsically motile F10 cells (Figure 6). Similar inhibitory effects on motility by this double mutant were observed with human cancer cell lines derived from various tissues, namely A375 melanoma cells (55% inhibition), MDA-MB-231 breast cells (55% inhibition), and LNCaP prostate cells (30% inhibition) (unpublished data), thereby implicating a widespread significance for cytoplasmic phospho-MARCKS function. In view of the dominant negative effect of the A2G_N/S double mutant, the presence of phosphate groups on MARCKS is likely to be a major structural determinant for productive interaction of MARCKS with its cytoplasmic target(s) and may therefore be independent of Ca^{2+} /calmodulin-dependent pathways. However, the precise mechanism by which cytoplasmic phosphoMARCKS collaborates with its unidentified targets and other PKC substrates to produce the motility phenotype has yet to be defined.

5. CONCLUSIONS

This work revealed that phosphoMARCKS acts as a cytoplasmic determinant of the metastatic potential of weakly metastatic F1 cells (undetectable phosphoMARCKS) and aggressively metastatic F10 cells (abundant phosphoMARCKS). Marked increases in both phosphoMARCKS levels and motility of F1 cells could be achieved by treatment with OA, and reversed by pre-treatment with MARCKS shRNA. These findings suggest that dephosphorylation of phosphoMARCKS is part of a mechanism by which F1 cells maintain a low metastatic potential. Gain-of-function and loss-of-function mutations at the phosphorylation domain of MARCKS-GFP constructs established the significance of cytoplasmic phosphoMARCKS in the motility phenotype. This newly reported cytoplasmic function is distinct from the cell surface events mediated by MARCKS through its reversible binding to the plasma membrane/actin cytoskeleton.

Abbreviations

PKC α	protein kinase C α
GFP	green fluorescent protein

RFP	red fluorescent protein
D/S-MARCKS	pseudo-phosphorylated MARCKS
N/S-MARCKS	phosphorylation-resistant MARCKS
A2G_WT-MARCKS	unmyristoylated wildtype MARCKS
A2G_N/S-MARCKS	unmyristoylated and phosphorylation-resistant MARCKS
OA	okadaic acid
DAG	diacylglycerol

Acknowledgments

We thank Dr. Perry Blackshear (NIEHS, Research Triangle Park, NC) for contributing several of the MARCKS-GFP constructs used here, Dr. Areti Tsiola for providing expert training on the confocal microscope, and Ms. Danae Fonseca (Hunter College – C.U.N.Y.) for technical assistance. This work was conducted in part with equipment in the Core Facility for Imaging, Cellular and Molecular Biology at Queens College. Funding (to SAR) for this research was provided by the National Institutes of Health (CA 125632) and The Professional Staff Congress of The City University of New York.

REFERENCES

1. Wiederkehr A, Staple J, Caroni P. *Exp. Cell Res* 1997;236:103–116. [PubMed: 9344590]
2. Batlle E, Verdu J, Dominguez D, del Mont Llosas M, Diaz V, Loukili N, Paciucci R, Alameda F, Garcia de Herreros A. *J. Biol. Chem* 1998;273:15091–15098. [PubMed: 9614119]
3. La Porta CA, Comolli R. *Clin. Exp. Metastasis* 1997;15:568–579. [PubMed: 9344041]
4. Sullivan RM, Stone M, Marshall J, Uberall F, Rotenberg SA. *Mol. Pharmacol* 2000;58:729–737. [PubMed: 10999942]
5. Lahn M, Kohler G, Sundell K, Su C, Li S, Paterson BM, Bumol TF. *Oncology* 2004;67:1–10. [PubMed: 15459489]
6. Rabinowitz I, Tsomo L, Mercurio AM. *Mol. Cell Bio* 2004;24:4351–4360. [PubMed: 15121854]
7. Jaken S, Parker PJ. *BioEssays* 2000;22:245–254. [PubMed: 10684584]
8. Abeyweera TP, Chen X, Rotenberg SA. *J. Biol. Chem* 2009;284:17648–17656. [PubMed: 19406749]
9. Ballestrem C, Wehrle-Haller B, Hinz B, Imhof RA. *Mol. Biol. Cell* 2000;11:2999–3012. [PubMed: 10982396]
10. Korb T, Schluter K, Enns A, Spiegel HU, Senninger N, Nicolson GL, Haier J. *Expt. Cell Res* 2004;299:236–247.
11. Qian F, Zhang ZC, Wu XF, Li YP, Xu Q. *Biochem. Biophys. Res. Commun* 2005;333:1269–1275. [PubMed: 15979576]
12. Larsson C. *Cell. Signalling* 2006;18:276–284. [PubMed: 16109477]
13. Gopalakrishna R, Barsky SH. *Proc. Natl. Acad. Sci. USA* 1988;85:612–616. [PubMed: 3422445]
14. Prezioso JA, Wang N, Duty L, Bloomer WD, Gorelik E. *Clin. Exp. Metastasis* 1993;11:263–274. [PubMed: 8097141]
15. Newton AC. *Chem. Rev* 2001;101:2353–2364. [PubMed: 11749377]
16. Sanz-Navares E, Fernandez N, Kazanietz MG, Rotenberg SA. *Cell Growth & Diff* 2001;12:517–524.
17. Liu B, Renaud C, Nelson KK, Chen YQ, Bazaz R, Kowynia J, Timar J, Diglio CA, Honn KV. *Int. J. Cancer* 1992;52:147–152. [PubMed: 1379995]
18. Hanauske AR, Sundell K, Lahn M M. *Curr. Pharm. Des* 2004;10:1923–1936. [PubMed: 15180529]
19. Fujise A, Mizuno K, Ueda Y, Osada S, Hirai S, Takayanagi A, Shimizu N, Owada MK, Nakajima H, Ohno S. *J. Biol. Chem* 1994;269:31642–31648. [PubMed: 7989336]

20. Spizz G, Blackshear PJ. *J. Biol. Chem* 2001;276:32264–32273. [PubMed: 11413143]
21. Verghese GM, Johnson JD, Vasulka C, Haupt DM, Stumpo DJ, Blackshear PJ. *J. Biol. Chem* 1994;269:9361–9371. [PubMed: 8132675]
22. Graff JM, Stumpo DJ, Blackshear PJ. *J. Biol. Chem* 1989;264:11912–11919. [PubMed: 2473066]
23. Thelen M, Rosen A, Nairn AC, Aderem A. *Nature* 1991;351:320–322. [PubMed: 2034276]
24. Blackshear PJ. *J. Biol. Chem* 1993;268:1501–1504. [PubMed: 8420923]
25. Manenti S, Malecaze F, Chap H, Darbon JM. *Cancer Res* 1998;58:1429–1434. [PubMed: 9537244]
26. Brooks G, Brooks SF, Goss MW. *Carcinogenesis* 1996;17:683–689. [PubMed: 8625478]
27. Spizz, G G, Blackshear PJ. *J. Biol. Chem* 1996;271:553–562. [PubMed: 8550618]
28. Michaut MA, Williams CJ, Schultz RM. *Dev Biol* 2005;280:26–37. [PubMed: 15766745]
29. Garcia-Bermejo ML, Leskow FC, Fuji T, Wang Q, Blumberg PM, Ohba M, Kurok T, Han K-C, Lee J, Marquez VE, Kazanietz MG. *J. Biol. Chem* 2002;277:645–655. *erratum J. Biol. Chem.* 279 (2004) 23846. [PubMed: 11584014]
30. Clarke PR, Siddhanti SR, Cohen P, Blackshear PJ. *FEBS Lett* 1993;336:37–42. [PubMed: 8262213]
31. Bruns RF, Miller FD, Merriman RL, Howbert JJ, Heath WF, Kobayashi E, Takahashi I, Tamaoki T, Nakano H. *Biochem. Biophys. Res. Comm* 1991;176:288–293. [PubMed: 1708246]
32. Rotenberg SA, Huang MH, Zhu J, Su L, Riedel H. *Mol. Carcinogenesis* 1995;12:42–49.
33. Kennelly, PJ PJ, Krebs EG. *J. Biol. Chem* 1991;266:15555–15558. [PubMed: 1651913]
34. Arbuzova A, Schmitz AAP, Vergeres G. *Biochem. J* 2002;362:1–12. [PubMed: 11829734]

A. **B.** **Figure 1.**

PhosphoMARCKS is elevated in murine melanoma cells having high motility. (A) Western blot analysis of B16 F1 and F10 cells was performed to determine endogenous expression of MARCKS and phosphoMARCKS. Cell lysates were prepared and resolved by SDS-PAGE (50 μg protein per lane) in duplicate gels, followed by transfer to PVDF membranes. The blots were developed with either anti-phosphoMARCKS (1:4000) or anti-MARCKS (1:1000). (B) Comparison of motility behavior of parental F1 and F10 cells (as described in Methods section 2.6). Each cell line was assayed in triplicate and the results were averaged. The results are representative of three independent experiments.

Figure 2.

Okadaic acid-treated F1 cells exhibit dramatically higher levels of phospho-MARCKS and increased cell motility. (A) F1 cells were treated continuously with 5 μM DAG-lactone (DAG) and/or 1 μM okadaic acid (OA), or DMSO (0.1% v/v) for 6 h. Lysates were prepared for Western blotting (100 $\mu\text{g}/\text{lane}$). Parallel blots were developed with antibodies that specifically detected either phosphoMARCKS (1:2000) or MARCKS protein (1:1000). (B) A 10-cm culture plate containing F1 cells were treated with 1 μM calphostin C or DMSO (0.1% v/v) and placed on a fluorescent light box for 15 min, followed by 1 h incubation 37°C (5% CO_2). The cells were washed twice with 5 ml PBS and 5 ml complete medium was restored, followed by addition of 1 μM OA or DMSO (0.1% v/v) for 1 h or 6 h, as

indicated. Lysates were prepared and duplicate Western blots (75 μ g/lane) were developed with phosphoMARCKS (1:2000) or MARCKS antibodies (1:1000 dilution). For both (A) and (B) the bands corresponding to MARCKS and phosphoMARCKS were at 85 kDa. (C) Parental F1 cells were plated in triplicate onto 10-well slides. Cells were treated with 5 μ M DAG-lactone for 5 min (by wash-out with complete medium) and/or continuous 6-h treatment with 1 μ M OA (or DMSO (0.1% v/v). Calphostin C (1 μ M) or DMSO were added as indicated, as described in (B). The extent of cell movement was analyzed for triplicate samples over a 6-h period and averaged. (D) Cell motility was measured as in (C) except that DAG-lactone was present continuously during the 6-h assay. All results are representative of three independent experiments.

Figure 3.

Knock-down of MARCKS in OA-treated F1 cells leads to decreased motility. Cells were transfected with plasmid DNA (5 μg) encoding mouse MARCKS shRNA, a scrambled shRNA control (SC), or the empty vector (VC), as described in the 'Methods'. Expression of MARCKS shRNA was coupled with co-expression of red fluorescent protein (RFP). At 55 h post-transfection, cells were re-plated in triplicate onto 10-well slides and incubated overnight at 37 °C (5% CO₂). At 72 h post-transfection (t = 0 for motility assay), cells were treated with 1 μM OA or DMSO (0.1% v/v) and incubated for 6 h at 37 °C (5% CO₂). For measuring motility of fluorescent cells, images were recorded at t = 0 and t = 6 h (A). Alternatively, cells were lysed at 6 h post-treatment with 1 μM OA or DMSO (0.1% v/v),

and lysates were analyzed by western blot (75 μ g/lane) (B). The blots were developed with anti-MARCKS (1:1000) and anti- β -actin (1:2000). All results are representative of three independent experiments.

Figure 4.

Confocal microscopy of F1 cells transfected with MARCKS-GFP mutants. Cells were transiently transfected with 4 μ g plasmid DNA encoding pseudo-phosphorylated MARCKS-GFP (D/S), phosphorylation-resistant MARCKS-GFP (N/S), WT-MARCKS-GFP (WT), or unmyristoylated GFP constructs (A2G_N/S and A2G-WT). Cells expressing WT-MARCKS were treated with either 1 μ M OA or DMSO (0.1% v/v), followed by fixation in 4% paraformaldehyde, as described in the 'Methods' (section 2.7) and viewed under a Leica confocal microscope. The results are representative of a minimum of two independent experiments.

Figure 5.

Motility behavior of B16 melanoma cells correlates with gain-of-function and loss-of-function MARCKS mutants. (A) Western blot analyzed with anti-GFP (1:500) or anti-MARCKS (1:1000) of whole cell lysates from F1 cells transfected with GFP-MARCKS constructs (20 μg/lane). The experiment is representative of two independent experiments. (B) Motility was measured following transient transfection of F1 cells (left panel), and F10 cells with the control vector (VC) or one of the following myristoylated MARCKS-GFP constructs: wildtype (WT), pseudo-phosphorylated MARCKS (D/S); phosphorylation-resistant MARCKS (N/S).

Figure 6.

Motility of F1 and F10 cells transfected with phosphorylation-resistant mutants of MARCKS-GFP. (A) F1 cells were transfected with the unmyristoylated analogue A2G_N/S-MARCKS-GFP, N/S-MARCKS-GFP, WT-MARCKS-GFP, or the vector control (VC), followed by treatment with 1 μ M OA for the entire experimental period (6 h). (B) Motility of F10 cells was measured following transfection with A2G_WT-MARCKS or A2G_S/N-MARCKS-GFP, or the vector control (VC). The extent of movement was analyzed after 6 h. All motility assays were carried out in triplicate, as described in the 'Methods'. The results are representative of three independent experiments.