The major myristoylated PKC substrate (MARCKS) is involved in cell spreading, tyrosine phosphorylation of paxillin, and focal contact formation

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Abstract The expression of the myristoylated PKC substrate MARCKS is reduced in tumor-derived choroidal melanoma cells (OCM-1). We transfected the OCM-1 cells with MARCKS cDNA and we selected clones with stable overexpression of the protein. Tyrosine phosphorylation of paxillin, a biochemical marker of focal contact formation, was conserved upon serum starvation when MARCKS was overexpressed, while it was almost abolished in the control cells. Immunofluorescent labelling of paxillin and vinculin, another component of focal contact, revealed that these structures were conserved upon serum starvation when MARCKS was overexpressed but not in the control cells. Furthermore, the cell morphology was affected by the ectopic expression of MARCKS, leading to increased spreading and formation of membrane processes. These data suggest the involvement of MARCKS in cell spreading and focal contact formation.

Key words: Protein kinase C substrate; Cell spreading; Paxillin; Focal contact; Melanoma

1. Introduction

The myristoylated alanine-rich C kinase substrate (MARCKS) is an actin filament cross-linking protein also interacting with calmodulin through a calcium-dependent mechanism. Protein kinase C (PKC) phosphorylation of MARCKS negatively regulates these two interactions in vitro, and induces the translocation of MARCKS from the plasma membrane to the cytosol of various cell types (for reviews see [1,2]). PKC-dependent lysosomal localization of MARCKS was also described in fibroblasts [3], although the biological meaning of these observations remained obscure. The involvement of this protein in lipopolysaccharide (LPS) phagocytosis by macrophages was also proposed [4], and its co-localization with actin at the membrane of nascent phagosomes was described. Despite these data, no clear cellular function could be established for this protein until now.

The MRP/F52 protein, a MARCKS relative highly expressed in macrophages, was also characterized as involved in zymosan phagocytosis by macrophages [5]. Interestingly, a dominant negative mutant of this protein inhibits phagocytosis as well as cell spreading and tyrosine phosphorylation of paxillin [6], suggesting the involvement of MRP/F52 in cell spreading and focal contact formation. Similarly, a dominant negative mutant of MARCKS itself was recently shown to inhibit cell spreading and membrane ruffle formation in fibroblasts [7].

Underexpression of the MARCKS protein was observed in various cell lines transformed by chemicals or oncogenes [8–12]. Such a reduced expression was also observed in tumor-derived choroidal melanoma cells (OCM-1) in comparison with non-transformed choroidal melanocytes in culture (Manenti et al., submitted for publication). In this study, we transfected these melanoma cells with human MARCKS cDNA, and demonstrated that ectopic overexpression of the protein modified cell morphology, cell spreading, and focal contact formation. Paxillin and vinculin, two well established components of focal contacts, were used as markers of these structures by immunolabelling.

2. Materials and methods

2.1. Cell culture, cell transfection and clone selection

Melanoma cell line (spindle-shaped OCM-1) was kindly provided by Dr. M.A. Saornil (Valladolid Institute, Spain). These cells were cultured in RPMI 1640 medium supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml amphotericin B, 2 mM L-glutamine and 5% FCS. Cells were grown at 37°C under 5% CO_2 atmosphere. All culture reagents were from Gibco.

MARCKS cDNA fragment cloned into Pet-8c expression vector was kindly provided by Dr. P. Blackshear [13,14]. It was subcloned into the PJ7Ω eukaryotic expression vector into the *Sma*I restriction site after blunting of the insert, and this construct was used for the transfection into OCM-1 cells. The transfection experiments were performed by electroporation with a Gene Pulser II Apparatus (Bio-Rad) at 250 V and 950 μF. Concurrently, 5×10^6 subconfluent cells resuspended into 400 μl of culture medium devoid of FCS were transfected with 15 μg of the vector and 2 μg of the plasmid pMT-Neo carrying the resistance to geneticin. Transfected cells were plated in the culture medium supplemented with FCS in the absence of geneticin. Geneticin was added to the medium 24 h after plating.

After 3 weeks of culture of the transfected cells in the presence of 500 μg/ml of geneticin, isolated resistant clones were trypsinized with 2–5 μl of the trypsin solution, replated in 24-well culture dishes and maintained in the presence of geneticin for 10 additional days. When they reached confluence, cells were replated into 8.8 cm^2 dishes until they reached confluence. Additional replating was then performed, and the concentration of geneticin was reduced to 250 μg/ml. When enough cells were available from each clone, the expression of MARCKS was tested by immunoblotting on a crude Triton-soluble heat-stable fraction. Nine clones were obtained, all of them overexpressing MARCKS to different degrees.

2.2. Immunoprecipitation, SDS gels and Western blots

For immunoprecipitation experiments, cells were lysed in RIPA buffer containing 13 mM Tris, pH 7.3, 33 mM NaCl, 3 mM EDTA, 1 mM dithiothreitol, 33 mM NaF, 2 mM sodium vanadate, 13 mM Na_4PO_7, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 1 mM PMSF, 0.05% deoxycholate, 1% NP-40, and 0.1% SDS. The lysate was cleared by centrifugation at 4°C for 10 min at 14000 rpm. Paxillin was immunoprecipitated using protein G-Sepharose CL-4B beads pre-

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coated with 4 μg of monoclonal anti-paxillin antibody (Transduction Laboratories, clone 349). The immune complexes obtained were heated in 50 μl of Laemmli buffer. Proteins were separated by electrophoresis on 10% polyacrylamide gels (SDS-PAGE) as described by Laemmli [15] with a Bio-Rad mini protean gel system. Electroblotting on nitrocellulose membranes was performed with a semi-dry transfer apparatus, and immunoblotting was performed with a specific monoclonal anti-phosphotyrosine antibody (Upstate Biotechnology, clone 4G10).

2.3. Immunofluorescence

For immunofluorescence experiments, cells were cultured in four-well chamber glass slides (Lab-Tek) and processed as follows. Cells were first extensively washed with PBS, and then fixed with 3% formaldehyde in PBS for 15 min. After three additional washes of 5 min in PBS, permeabilization was performed with 70% ethanol for 10 min, followed by three additional PBS washes. Saturation of non-specific sites was done with 1% BSA in PBS for 60 min, and followed by incubation with primary antibodies for 45 min in PBS-BSA. Monoclonal anti-vinculin (Sigma, clone hVin-1) and anti-paxillin were used at 1:400 and 1:500 dilutions respectively. After four washes in PBS-BSA, cells were incubated with secondary TRITC-coupled anti-mouse antibody (Sigma) in PBS-BSA for 30 min and extensively washed with PBS before mounting. Fluorescence was recorded with a Zeiss Axioskop microscope using a 68× oil planapo lens on a Kodak T-Max 400 ASA film.

3. Results

Two clones (OCM-K1 and OCM-K4) of OCM-1 cells overexpressing the MARCKS protein were used in this study, and compared to control cells transfected with the PJ7 empty vector (OCM-PJ7). Western blots were performed on cellular fractions from these three cell lines using a specific antibody against MARCKS. The results presented in Fig. 1 show a 4–8-fold overexpression of MARCKS in OCM-K1 and OCM-K4 cells by comparison with OCM-PJ7 cells.

First we compared the kinetics of cell adhesion on a matrix of fibronectin for the three cell lines. 50–60% of the cells were bound to the fibronectin layer 1 h after seeding, but no significant difference could be observed between the three cell types (not shown). These data suggest that MARCKS is not directly involved in the early mechanisms of cell adhesion.

Tyrosine phosphorylation of paxillin is often used as a biochemical marker of focal contact formation. Since the MRP/F52 protein seems to be involved in paxillin phosphorylation in macrophages, we addressed the question whether MARCKS expression could also affect tyrosine phosphorylation of this focal contact component in our cells. Immunoprecipitation of paxillin was performed from crude cellular fractions, and tyrosine phosphorylation was quantified by Western blot with an anti-phosphotyrosine antibody. The experiment was performed in parallel on exponentially growing

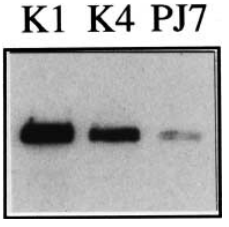

Fig. 1. Forced expression of MARCKS in melanoma cells. Western blots were performed with a polyclonal antibody against MARCKS on crude Triton-soluble fractions from OCM-K1 (K1) and OCM-K4 (K4) clones as well as from OCM-PJ7 (PJ7) control cells. Note the overexpression of MARCKS in the two K1 and K4 clones.

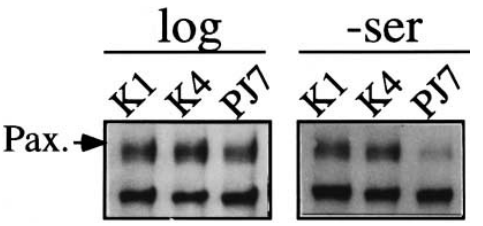

Fig. 2. MARCKS-dependent tyrosine phosphorylation of paxillin. Immunoprecipitation of paxillin was performed and followed by Western blotting of the resulting immunoprecipitates with an antibody against phosphotyrosine. This was performed in parallel for exponentially growing cells (log; left panel) and with cells subjected to serum deprivation (−ser; right panel). Similar amounts of paxillin were applied on each lane (not shown).

cells or after 24 h of serum starvation. The results are shown in Fig. 2. In the presence of serum, tyrosine phosphorylation of paxillin is slightly higher in OCM-K1 and OCM-K4 cells than in the control. After serum deprivation, a dramatic decrease of paxillin phosphorylation was observed in the control cells (OCM-PJ7), while it was not significantly affected in the cells presenting a high level of MARCKS expression (OCM-K1 and OCM-K4). These data suggest that MARCKS could be involved in the serum-independent phosphorylation of paxillin in melanoma cells.

To further argue for a role of MARCKS in focal contact formation, we performed immunofluorescence experiments with antibodies against paxillin and vinculin, another component of focal contacts (Fig. 3). Again, the experiment was performed in parallel with exponentially growing (log) and serum-deprived (−ser) cells. In exponentially growing cells, both paxillin and vinculin were detected in focal contact structures in all three cell types. However, the punctate structures observed appeared slightly less abundant in the PJ7 control cells than in the K1 and K4 clones overexpressing the MARCKS protein. This difference became evident when cells were grown in the absence of serum for 24 h. In these conditions, focal contact labelling almost disappeared from the PJ7 control cells, while it remained stable in the two clones. Interestingly, the morphology of K1 and K4 cells appeared significantly different from the PJ7 control cells. The cells appeared more spread on the substratum, with frequent membrane processes, while the control cells presented a general spindle-shaped morphology identical to the non-transfected OCM-1 cells (not shown). These data suggest that MARCKS expression improves the OCM-1 cells' capacity to spread on the substrate and to subsequently assemble focal contact structures, especially in the absence of serum-induced signalling.

4. Discussion

In this work, we describe for the first time the positive effect of MARCKS expression on cell spreading and focal contact formation in transformed cells. In particular, we demonstrate that serum-independent formation of focal contacts was restored by MARCKS overexpression in the choroidal melanoma cells. These observations are in good correlation with the sustained tyrosine phosphorylation of paxillin observed in the same conditions. Indeed, the phosphorylation of this protein has been often correlated with its association to focal adhesion structures (for a review, see [16]). Our results are remi-

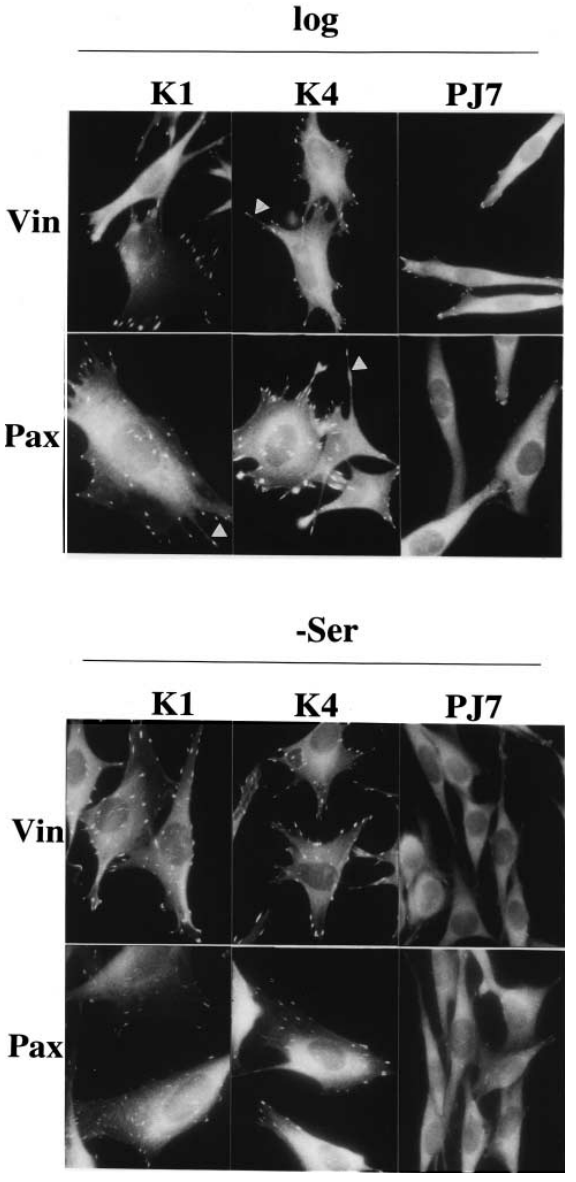

Fig. 3. Immunofluorescent labelling of paxillin and vinculin. Immunolabelling of paxillin (Pax) and vinculin (Vin) was performed on the three cell lines (K1, K4, and PJ7 control). Cells either in exponential growing phase (log; upper panel) or grown without serum for 24 h (−ser; lower panel) were processed in parallel. Arrowheads point to cell processes observed in MARCKS expressing cells.

niscent of those obtained by Li et al. [6] with the MRP/F52 protein in macrophages. These authors observed the dominant negative effect of an MRP mutant on PKC-dependent cell spreading and tyrosine phosphorylation of paxillin, suggesting a possible function of MRP in these two cellular events. The same type of experiment was recently performed with a dominant negative mutant of MARCKS itself in fibroblasts, and a negative effect on cell spreading and membrane ruffling formation was also observed [7]. The authors also described an inhibitory effect of the mutant on cell adhesion itself, in opposition with our own data. However, this discrepancy may be explained by the experimental protocols used (the culture dishes were rocked in their experiment but not in our protocol) and by the completely different cell systems and transfected material used (inhibitory effect of a dominant negative mutant in one case and positive effect of the wild type protein in our system). Despite this contradictory point, these data converge for a cellular function of MARCKS in cell spreading and focal contact formation.

The molecular mechanisms of this function remain unclear. It is now well established that both integrin and growth factor signalling modulate focal contact assembly (for a review see [17]). As a main feature, we observed that the MARCKS-dependent increase of focal contacts was maintained in the absence of serum, suggesting that the integrin-dependent pathway is concerned. Interestingly, various studies described the direct involvement of PKC in the integrin-mediated formation of focal contacts and paxillin phosphorylation [18,19]. However, whether the effect of MARCKS expression on these cellular events is PKC-dependent in our system remains to be established.

The down-regulation of MARCKS has been described in various transformed cell types [8–12], and the possible implication of the protein in cell proliferation and mitogenesis was questioned. The new data presented here and by others may suggest that this down-regulation could be linked to the loss of adhesion-dependent proliferation linked to transformation rather than to mitogenic signal transduction pathways linked to growth factors. The down-regulation of cytoskeletal and focal adhesion proteins like vinculin or α-actinin was described in cancer cells [20,21], and the down-regulation of MARCKS could reflect the same type of cellular regulation. Interestingly, we observed a partial reversion of the transformed phenotype of the OCM-1 cells overexpressing MARCKS (including in the two clones presented in this study) leading to the inhibition of colony formation in soft agar and to the loss of serum-independent proliferation capacity (Manenti et al., manuscript submitted).

Since MARCKS is an actin cross-linking protein [22] negatively regulated by PKC phosphorylation and because actin cytoskeleton regulation and focal adhesion formation are interdependent cellular events, it would be of interest to further clarify the molecular function of MARCKS and how the PKC pathway regulates this function. In particular, whether MARCKS is directly implicated in early actin-dependent events like cell spreading and membrane ruffle formation and/or in later focal contact formation remains to be clarified.

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