Overexpression of the Myristoylated Alanine-rich C Kinase Substrate in Human Choroidal Melanoma Cells Affects Cell Proliferation

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ABSTRACT

Reduced expression of the myristoylated alanine-rich C kinase substrate (MARCKS) has been described in various cell lines after oncogenic or chemical transformation, leading to the question of whether this protein may be involved in cell proliferation. Here we compare the expression of MARCKS in human tumor-derived choroidal melanoma cells (OCM-1) and in primary cultures of normal choroidal melanocytes. We found an important down-regulation of the protein in the melanoma cell line. Stable transfection of these cells with the cDNA coding for MARCKS led to the selection of several clones expressing variable levels of the protein. Proliferation experiments performed with four of these clones revealed that cell growth was reduced by 35–40% when compared with control cells. Upon serum starvation, cell proliferation was almost abolished when the expression level of MARCKS was high, whereas it was only partially reduced in the controls. MARCKS overexpression induced a higher percentage of cells in the G0-G1 phase of the cell cycle upon serum starvation, as well as the inhibition of colony formation in soft agar. Finally, the expression of the CDK inhibitor p27 was increased in the cells presenting a high level of MARCKS protein. Altogether, these data suggest that the expression of this protein kinase C substrate affects the proliferation and partially reverts the transformed phenotype of the OCM-1 cells.

INTRODUCTION

Choroidal melanomas are the most common primary malignancy of the eye in adults (1). The cellular events leading to malignant transformation of normal choroidal melanocytes are still unknown. In vitro studies on growth regulation of skin melanocytes and comparison with cutaneous melanoma cells have brought some light on how the normal cells differ from their transformed counterparts (2). Recent technical advances have permitted the set up of choroidal melanocyte cultures (3), making in vitro investigations into the mechanisms leading to malignant transformation of choroidal melanocytes now possible.

The MARCKS (1) is a ubiquitous heat-stable protein that was often used as a marker of PKC activation in various cell types (reviewed in Refs. 4 and 5). This protein interacts with calmodulin through a calcium-dependent mechanism (6, 7) and cross-links the actin filaments in vitro (8). PKC phosphorylation of MARCKS inhibits the interaction with actin and calmodulin. The major part of the protein is bound to the plasma membrane of the cells, and PKC activation induces a reversible cytoplasmic translocation (9, 10), although this process was not always observed (11). The same conserved domain of the protein contains the PKC phosphorylation sites and is involved in the interaction with actin, calmodulin, and the acidic phospholipids of the membrane (12), explaining why PKC phosphorylation regulates these interactions. Both this PKC phosphorylation site domain and the conserved myristoylated N-terminal region of the protein (13–16) regulate membrane binding and cellular localization of MARCKS through the so-called myristoyl-electrostatic switch (17, 18). Immunolocalization experiments performed on macrophages revealed a punctate distribution of the protein at the substrate adherent surface of filopodia and pseudopodia and a disappearance of the immunolabeling subsequently to PKC activation (19). More recent developments on MARCKS cellular localization during lipopolysaccharide phagocytosis in macrophages revealed a recruitment of the protein at the membrane of forming phagosomes and the involvement of PKC phosphorylation for this process, suggesting a possible function of MARCKS and PKC in this type of phagocytosis (20). Lysosomal localization of MARCKS was also described in fibroblasts (21).

Another characteristic of MARCKS is its regulated expression during cell proliferation and transformation. A preliminary study in Swiss 3T3 fibroblasts showed a maximal expression of the protein when the cells were blocked at the G0-G1 stage of the cell cycle by serum deprivation and a rapid and drastic posttranscriptional down-regulation of both MARCKS and its mRNA after G0-G1 exit induced by serum addition (22). Interestingly, similar down-regulations were reported in various cell lines transformed with oncogenes or chemical agents (23–27). However, the molecular mechanisms of these down-regulations seem to be different, depending on the cell lines and transforming agents used. Interestingly, MARCKS purified from bovine brain was recently characterized as a physiological and in vitro substrate for proline-directed protein kinases that may include MAP kinases and/or cyclin-dependent kinases (28). Altogether, these data opened the intriguing question whether MARCKS may be directly or indirectly involved in cell mitogenesis. Transfection experiments of cutaneous melanoma cells with MARCKS cDNA have been reported recently, which led to the establishment of clones with reduced cell proliferation (29). However, the authors failed to detect any overexpression of the protein in these clones, impeding any possible correlation between the protein level and the phenotype observed. In an other work, Wojtaszek et al. (30) demonstrated that reversion of ras-transformed fibroblasts by various chemical agents through a ras-independent pathway led to increased down-regulation of MARCKS. Furthermore, overexpression of MARCKS into these ras-revertant cell lines did not modify the phenotype with regard to phorbol ester-induced mitogenicity. The authors concluded from their data that MARCKS was not involved in mitogenesis.

The present work was designed to clarify the effect of MARCKS expression on cell proliferation and to bring some light on the possible cellular function of this protein. We demonstrate that forced overexpression of MARCKS in tumor-derived choroidal melanoma cells, where the amount of the endogenous protein was found to be very low, leads to decreased cell proliferation. The possible molecular mechanism of this effect will be discussed.

MATERIALS AND METHODS

Cell Culture. Primary cultures of human choroidal melanocytes were established essentially as described previously (3), and their purity was assessed by various techniques, including immunocytochemistry, using specific markers, fluorescence-activated cell sorting analysis, transforming growth factor β responsiveness, and cell proliferation behavior (Mouriaux et al.,

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3 The abbreviations used are: MARCKS, myristoylated alanine-rich C kinase substrate; PKC, protein kinase C.
and actin were from Santa Cruz Biotechnology and from Sigma, respectively. The antibodies against p27 with a specific polyclonal antibody directed against the COOH-terminal 20 amino acids were raised in rabbits. Proteins were separated by electrophoresis on 7.5% polyacrylamide gels (SDS/PAGE) as described by Laemmli (34) with a Bio-Rad mini protean apparatus.

The concentration of geneticin was reduced to 250 ng/ml. When enough cells were obtained, all of them overexpressing MARCKS at different degrees.

Subcloning of MARCKS cDNA into P780K Expression Vector and Cell Transfections. MARCKS cDNA fragment cloned into Pet-8c expression vector was kindly provided by Dr. P. Blackshear (Howard Hughes Medical Institute, Durham, NC; Refs. 32, 33). It was subcloned into the P780K eucaryotic expression vector into the Smal restriction site after blunting of the insert, and this construct was used for the transfection into COS and OCM cells. The transfection experiments were performed by electroporation with a Gene Pulser II Apparatus (Bio-Rad) at 250 V and 950 μF. Currently, 5 × 10⁶ 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.

Clone Selection. 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 a 24-well culture dish, and maintained in the presence of geneticin for 10 additional days. When they reached the confluency, cells were replated into 8.8-cm² dishes until they reached the confluency. Additional replating was then performed, and the number of colonies was scored. The antibiotic selection, nine clones transfected with P780K and three clones transfected with pMT-Neo were used in these experiments.

Cell Proliferation Experiments and Flow Cytometric Analysis. For proliferation experiments, cells were plated at a density of 15,000 cells per 8.8-cm² culture dish (Nunc) in RPMI 1640 supplemented with 2% or 5% FCS. The medium was changed each day during the proliferation experiments. After various times, cells were dissociated with 200 μl of 0.05% trypsin-0.02% EDTA for 5 min, and the reaction was stopped with RPMI 1640 containing 5% FCS. Cells were counted in a Coulter counter (Coultronics).

For flow cytometric analysis, cells were washed in PBS, harvested by trypsinization, and fixed in 70% ethanol. After one additional PBS wash, cells were treated with 0.1 mg/ml RNase A (Sigma) in 50 mM Tris (pH 7.6), 0.1% NP40, and 0.05% sodium citrate. Cellular DNA was stained with 200 μg/ml propidium iodide and quantified by flow cytometry with a Coulter Elite apparatus.

Cell Fractionation, SDS Gels, and Immunoblotting. For cell fractionation, 2–3 × 10⁶ cells were resuspended in 200 μl of 50 mM Tris-HCl buffer (pH 7.3), containing 100 mM NaCl, 2 mM EDTA, 2 mM EGTA, 0.5% Triton X-100, and probe inhibitors leupeptin, pepstatin, and aprotinin, kept on ice for 15 min, and centrifuged in an Eppendorf centrifuge for 15 min at 12,000 rpm. Because MARCKS is a heat-stable protein, the supernatant was heated at 80°C for 8 min, cooled on ice, and centrifuged at 12,000 rpm for 10 min. The heat-stable supernatant containing MARCKS was used for additional experiments. Proteins were separated by electrophoresis on 7.5% polyacrylamide gels (SDS/PAGE) as described by Laemmli (34) with a Bio-Rad mini protein gel system. Electroblotting on nitrocellulose membranes was performed with a semidry transfer apparatus. Immunoblotting against MARCKS was performed with a specific polyclonal antibody directed against the COOH-terminal 20 amino acids of the human MARCKS sequence. This antibody was kindly provided by Dr. H. Taniguchi (Toyko, Japan). The antibodies against p27 and actin were from Santa Cruz Biotechnology and from Sigma, respectively.

RESULTS

Differential Expression of MARCKS in Choroidal Melanoma Cells and Melanocytes. Because down-regulation of MARCKS was reported in various transformed cell lines, we investigated the expression of the protein in tumor-derived choroidal cell line OCM-1 and in primary cultures of human choroidal melanocytes. Immunoblotting analysis was performed on heat-stable, detergent-soluble fractions from both cell types. As shown in Fig. 1, a clear underexpression of the protein was observed in the transformed OCM cells by comparison with the melanocytes. Similar results were obtained when a second melanoma cell line (MKT-Br) was compared with primary cultures of melanocytes obtained from three different donors (data not shown). These data demonstrate that MARCKS expression is down-regulated in transformed choroidal melanoma cells, in good correlation with previous studies performed on various transformed cell types.

Forced Expression of MARCKS in OCM-1 Cells. The coding region of human MARCKS cDNA was cloned into the P780K eucaryotic expression vector as described in "Materials and Methods," and the corresponding construct (P780K) was transfected into the cells by electroporation. Transient high expression of MARCKS was detected by immunoblotting in COS-7 cells 72 h after the transfection, confirming the validity of the P780K expression construct (data not shown).

Transfection of the OCM-1 cells was then performed with this construct as well as with the empty P780K vector as a control. After antibiotic selection, nine clones transfected with P780K and three clones transfected with P780K were tested by immunoblotting for the expression of MARCKS and compared with the OCM-1 cell line. All of the clones transfected with MARCKS cDNA overexpressed the specific proteins.
protein to different degrees, whereas the clones derived from PJ7-O transfection presented levels of MARCKS expression similar to the OCM-1 parental cells (data not shown). The correct cellular localization of the protein was verified by immunoblotting on crude cytoplasmic and membrane preparations from the various clones, confirming that 70–80% of the protein was present in the membrane fraction (data not shown).

The expression of MARCKS in clones K1, K2, K4, and K5 was investigated by Western blot detection (Fig. 2A, top panel) and compared with melanocytes (Mel) and PJ7 control cells. The analysis was performed on fractions from exponentially growing cells or after 48 h of serum starvation. A quantification of MARCKS protein in the various fractions was performed (Fig. 2B) and expressed as nanograms of MARCKS per 200,000 cells. These data demonstrate that: (a) MARCKS expression is dramatically reduced in PJ7 (and OCM-1) cells by comparison with the melanocytes; and (b) MARCKS level in the transfected clones growing in the presence of serum was 1.8-fold (K5) to 9-fold (K2) higher than the endogenous protein content observed in PJ7 cells. Immunoblotting of these fractions with an antibody against actin was also performed (Fig. 2A, middle panel) and showed similar levels of this protein in the various fractions (with maximal variations of 30–40% between certain fractions), except in the melanocytes, where the actin content was found very high.

Expression of MARCKS in Tumor Melanoma Cells

**Table 1** Percentage of cells in G0-G1

<table>
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<th></th>
<th>K1</th>
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<th>K4</th>
<th>K5</th>
<th>PJ7</th>
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<td>48.4</td>
<td>44.1</td>
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<tr>
<td>+ser</td>
<td>44.6</td>
<td>52.7</td>
<td>53.3</td>
<td>35.6</td>
<td>46.5</td>
<td>42.4</td>
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Expression of the CDK Inhibitor p27 Is Increased in the Cells Overexpressing MARCKS. To test the effect of MARCKS expression on cell proliferation, we investigated the effect of serum deprivation on four different clones (K1, K2, K4, and K5) by comparison with the control (PJ7) and the nontransfected OCM cells (OCM). Cells were seeded at the same density. After 24 h, the cells were either counted or submitted to serum deprivation for 48 additional h and then counted. Fig. 3 shows the effect of serum starvation on cell proliferation. A striking inhibition of K1, K2, K4, and K5 cells was observed, whereas the proliferation of PJ7 and OCM was partially reduced but not blocked. These data suggest that MARCKS overexpression partially restored the cellular expression of the CDK inhibitor p27.

MARCKS Expression Affects Cell Proliferation. To test the effect of MARCKS expression on cell proliferation, we investigated the effect of serum deprivation on four different clones (K1, K2, K4, and K5) by comparison with the control (PJ7) and the nontransfected OCM cells (OCM). Cells were seeded at the same density. After 24 h, the cells were either counted or submitted to serum deprivation for 48 additional h and then counted. Fig. 3 shows the effect of serum starvation on cell proliferation. A striking inhibition of K1, K2, K4, and K5 cells was observed, whereas the proliferation of PJ7 and OCM was partially reduced but not blocked. These data suggest that the serum-independent growth capacity of the OCM-1 cells was inhibited by ectopic MARCKS overexpression.

To clarify the influence of MARCKS expression on cell cycle progression, fluorescence-activated cell sorting analysis was performed on exponentially growing cultures from various clones presenting stable MARCKS expression. In A, Western blot analysis of MARCKS (upper panel), actin (middle panel), and p27 (bottom panel) was performed from Triton-soluble fractions of the various cell types. The experiment was performed from exponentially growing cells (+ Serum) or after 48 h of serum starvation (− Serum). Amounts of protein corresponding to 2 × 10^6 cells were loaded on the gel for each fraction, after previous verification of balanced loading by Coomassie Blue staining. In B, densitometric quantification of MARCKS protein was described in "Materials and Methods." The amount of MARCKS was expressed as nanograms of protein.

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formed on K1, K2, K4, K5, PJ7, and OCM-1 cells. In this experiment, exponentially growing cells were submitted to serum deprivation for 24 h and stimulated for 15 additional h with 5% serum. The percentage of cells in G0-G1 in the different conditions is presented in Table 1. The cell cycle distribution in exponentially growing conditions (log) appeared similar for the various cell types, with 30–45% of the cells in G0-G1. After 24 h of serum starvation, a clear accumulation in G0-G1 was observed for K1, K2, K4, and K5 (61, 60, 64, and 55%, respectively), whereas the effect was weak for PJ7 cells (48%) and even absent for the OCM cells (44%). Serum stimulation performed for 15 h indicates that exit from the G1 “block” was similar for the clones and the controls.

The proliferation of the various cellular clones was then compared in the presence of serum. Cells were seeded at the same low density and counted after 1, 3, and 5 days of culture. The results of these experiments are shown in Fig. 4, where the number of cells was plotted versus the number of days of culture. They confirm the reduced proliferative capacity of K1, K2, and K4 cells that represent a 35–40% inhibition of the PJ7 and OCM cells proliferation. By contrast, K5 cells present a proliferation rate almost identical to PJ7 and OCM, in good correlation with the low level of MARCKS expression observed under exponentially growing conditions in these cells (Fig. 2).

Altogether, these data indicate that high levels of MARCKS expression in the OCM cells significantly reduce their proliferation rate and their serum-independent proliferation capacity.

**Colony Formation in Soft Agar.** The capacity to grow and form colonies in soft agar conditions was often used as a tool to study the degree of cell transformation. We used this technique to compare the adhesion-independent growth capacity of K1, K2, K4, K5, and PJ7 cells (Fig. 5). We found that the colony number and their size was directly dependent on the level of MARCKS cellular expression. For PJ7 cells, we counted 1380 colonies from 2000 cells initially seeded into the culture dish. The colony number was reduced by 40% for K1 cells (840 colonies) and by more than 75% for K2 cells (300 colonies). By contrast, the total number of colonies was not significantly affected for K4 and K5 (1200 and 1500, respectively), although in that case, the average size of these colonies was significantly reduced. Note that for all five cell lines under study, the expression level of MARCKS nicely correlates with the degree of colony size reduction, and that modifications of colony morphology can also be observed (compare PJ7 and K5 for example). These data, together with the previous ones, further reinforced the idea that ectopic expression of the MARCKS protein partially reverts the transformed phenotype of the OCM-1 cells.

**DISCUSSION**

In this report, we show that MARCKS, a physiological PKC substrate, is down-regulated in the OCM-1 choroidal melanoma cells and that forced expression of the protein reduces the proliferation rate of these cells.

Down-regulation of the MARCKS protein in various transformed cell lines has been already described by others, but a certain heterogeneity appeared from the literature concerning both the level of down-regulation and the mechanism involved (transcriptional or post-transcriptional). Our study demonstrates for the first time that MARCKS down-regulation also occurs in tumor-derived cell lines when compared with their nontransformed counterparts (choroidal melanocytes).

The transfection of the OCM-1 cells with the cDNA of MARCKS and the selection of various clones overexpressing the protein clearly demonstrate that increased levels of MARCKS do not suppress cell growth and, in this manner, do not impede clone selection. The hypothesis that MARCKS expression could hinder clone selection was proposed recently (29) in a work where the authors failed to detect any protein overexpression into clones of cutaneous melanoma cells transfected with MARCKS cDNA. The discrepancy with our results may be due to the difference of cell system and perhaps to the expression vector used and to the method of transfection. However, in a recent work, cytoarchitectural distortions due to very high expression of MARCKS were observed in Ltk- cells (18), suggesting that this situation may induce toxic or lethal effects to the cells.

One important topic of this work concerns the inhibitory effect of MARCKS on cell proliferation. Thirty to 40% inhibition was observed after 3 days of culture. Interestingly, the proliferation rate of K5 cells was not significantly affected, in good correlation with the

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**Fig. 4.** Effect of MARCKS expression on cell proliferation. Proliferation experiments were performed as described in “Materials and Methods” in the presence of 5% serum in the culture medium. Cells were counted 1 day (D1), 3 days (D3), and 5 days (D5) after seeding. Note the high cellular density after 5 days of culture, which may explain the limited growth inhibition at this time. This is one representative experiment from three experiments performed in triplicate; bars, SD.

**Fig. 5.** Colony formation in soft agar. The cells from clones K1, K2, K4, K5, and PJ7 (control) were grown in soft agar conditions as described in “Materials and Methods” for 3 weeks. The colony number after that time was counted as described in “Materials and Methods.” The minimal size taken into account was 0.2 mm, corresponding to ~30–50 cells. A representative photograph of colony morphology is shown for each cell type, and the corresponding quantification of colony number is indicated.
low level of MARCKS overexpression observed in these cells. The fact that the percentage of inhibition did not increase after 5 days might be attributed to the high cellular density of the controls (P17 and OCM) at this time. In these experiments, we could not correlate the growth inhibition observed with clear variations of the cell cycle distribution. This argues for a general slowing down of the cell proliferation rate rather than an effect at a specific point of the cell cycle.

The experiments of serum starvation demonstrate a clear effect of MARCKS expression on the cell capacity to proliferate in the absence of serum. The case of K5 cells is of special interest because the low MARCKS expression observed in exponentially growing cells was increased in the absence of serum, in good correlation with the low proliferation rate observed in these conditions. However, the molecular mechanism and biological meaning of this observation (also true for the clone K1) remains unclear, although it is reminiscent of results obtained by others in Swiss 3T3 fibroblasts (22). Because growth factor-independent proliferation is a characteristic of transformed cells, these data open the interesting possibility that MARCKS overexpression partially reverts the transformed phenotype of OCM cells.

This hypothesis was further reinforced by the experiments of colony formation in soft agar, where high levels of MARCKS expression (K1 and K2) led both to the inhibition of colony formation and to spectacular size reductions, whereas intermediate levels of MARCKS (K4 and K5) did not decrease the number of colonies but significantly reduced their size. Interestingly, similar data were recently obtained by others in Swiss 3T3 fibroblasts (22). Because growth factor-independent proliferation is a characteristic of transformed cells, these data open the interesting possibility that MARCKS overexpression partially reverts the transformed phenotype of OCM cells.

A possible effect of MARCKS expression on cell proliferation was already proposed by Brooks et al. (29). This was based on transfection experiments performed in cutaneous melanoma cells. However, these authors performed their study on clones that did not overexpress the protein, and it seems difficult in these conditions to establish a correlation between the observed cell phenotype and MARCKS function. In an other type of experiments, Wojtaszek et al. (30) investigated the possible function of MARCKS in mitogenic regulation in ras-transformed fibroblasts. The transformed phenotype of these cells (where MARCKS protein is poorly expressed) was reversed by various agents independently of ras expression, and an increased downregulation of MARCKS was observed in the revertants. Furthermore, re-expression of the protein after transfection did not modify the mitogenic properties of these cells upon phorbol ester treatment. Because overexpression of MARCKS was induced in cells that already reverted to the normal nontransformed phenotype, the eventual effect of MARCKS expression on cell proliferation is more difficult to detect than in our system. Furthermore, these authors addressed the question of the mitogenic stimulation by phorbol esters, whereas we were interested in the cell proliferation rate in normal culture conditions. Taken together, these data probably reflect the diversity of MARCKS regulation upon various cell transformation mechanisms.

The molecular mechanism by which MARCKS may influence cell proliferation remains unclear, but one may notice that this PKC substrate is also a calmodulin-binding protein and may act as a regulator of this molecule in the cell. Calmodulin is required for cell cycle progression during G1 and mitosis (36), and variations of free calmodulin in the cells affect the progression through these stages of the cell cycle. Interestingly, Herget et al. (37) performed transfection experiments of MARCKS in Rat-1 cells (where the endogenous protein was found poorly expressed), and they observed that overexpression of this protein modified the response to calmodulin antagonists.

Recently, various studies described the negative control of cell cycle progression by PKC isozymes. For example, PKC-mediated cell cycle arrest was observed in intestinal epithelial cells, with a concomitant induction of p21 and p27 CDK inhibitors (38). Whether the phosphorylation of MARCKS may be involved in such mechanisms remains to be established.

MARCKS is also an actin cross-linking protein that may regulate the actin network organization under certain conditions. The dynamic organization of the actin network is one of the key mechanisms involved in cell adhesion and spreading. Recently, various works described the importance of cell adhesion for progression through G1 and for the G1-to-S transition of the cell cycle. In particular, adhesion-dependent regulation of various cell cycle effectors, such as cyclin D1 and cyclin A, as well as p27, was observed (39). We cannot rule out from our experiments that the negative effect of MARCKS expression on cell proliferation may reflect modifications of the cell adhesion properties rather than a direct regulation of the cell cycle effectors. To this respect, the expression of a dominant-negative mutant of MARCKS was recently found to inhibit cell adhesion and spreading in fibroblasts (40). We also recently observed modifications of paxillin phosphorylation and of focal contact formation in K1 and K4, two of the cellular clones used in this study (41). Additional experiments are now needed to better understand the molecular links between the MARCKS-dependent modifications of cell proliferation and adhesion.

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REFERENCES

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