

Atypical Protein Kinase C ζ Suppresses Migration of Mouse Melanoma Cells¹

Eduardo Sanz-Navares, Nieves Fernandez, Marcelo G. Kazanietz, and Susan A. Rotenberg²

Department of Chemistry and Biochemistry [E. S.-N., S. A. R.] and Graduate Center [E. S.-N.], Queens College of the City University of New York, Flushing, New York 11367, and Center for Experimental Therapeutics and Department of Pharmacology, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania [N. F., M. G. K.]

Abstract

Mouse melanoma B16 F1 cells cultured in RPMI 1640 supplemented with the melanin precursors tyrosine and phenylalanine display increased melanin levels and elevated migration while down-regulating protein kinase C (PKC) ζ to low levels. Although control experiments rule out a direct role by melanin, PKC ζ down-regulation is shown to be a critical determinant of cell migration. Transfection of high-motility cells with either wild-type PKC ζ or its regulatory domain suppresses migration. Known to bind to the regulatory domain of PKC ζ , the proapoptotic protein prostate apoptosis response-4 (Par-4) coimmunoprecipitates with PKC ζ as a 47-kDa protein. Transfection of Par-4 (or its leucine zipper element) further suppresses migration of low-motility cells (which express high levels of PKC ζ), whereas high-motility cells (which express low levels of PKC ζ) are unaffected by Par-4 overexpression. It is proposed that in nonmetastatic cells, the PKC ζ Par-4 complex provides a brake on migration that is released by melanin precursors that initiate PKC ζ down-regulation. Elevation of PKC ζ in melanoma cells, or preventing its down-regulation through the dietary restriction of tyrosine and phenylalanine, may therefore control metastatic behavior.

Introduction

PKC³ is a family of structurally related isoforms that participate in signaling pathways governing proliferation and dif-

ferentiation in normal cells. In cancer cells, the roles of individual isoforms have been associated with tumor growth and with adhesion and motility, two aspects of metastatic behavior. The conventional PKC isoforms (α , β , and γ) are stimulated by Ca²⁺, phospholipid (typically phosphatidylserine), and diacylglycerol or phorbol esters. In addition to the conventional isoforms, there are two distinctive groups: the Ca²⁺-independent novel isoforms (δ , ϵ , η , θ) and the atypical isoforms which consist of PKC ζ and PKC λ/ι (where λ and ι are murine and human variants, respectively, of the same isoform). The latter grouping is designated atypical because their activity is not regulated either by Ca²⁺, diacylglycerol, or phorbol esters. Although unresponsive to activation by phorbol esters, the regulatory domain of PKC ζ is distinctive in its structure and provides a crucial binding site for proteins that participate in diverse signaling pathways (reviewed in Ref. 1). Examples of PKC ζ -binding proteins include the proapoptotic protein Par-4 (2), the pleckstrin homology domain of the RAC protein kinase (3), and the transcription factor RBCK1 (RBCC protein interacting with PKC; Ref. 4).

The role of PKC isoforms in the metastasis of melanoma cells is an area of active study. The B16 F1 cell line, developed by Fidler (5) in 1973, is a well-established murine melanoma cell line that can be used to analyze metastasis in a syngeneic animal model (C57BL/6 mice). An interesting property of B16 F1 cells is that the presence of tyrosine and phenylalanine in the culture medium influences their metastatic activity in the animal (6). B16 F1 cells that are cultured in DMEM medium, which contains high levels of these aa, are rendered highly metastatic in mice. On the other hand, cells that are cultured in RPMI 1640, which contains low levels of these aas, are only weakly metastatic. In a recent study, the lower metastatic activity produced by aa deprivation was attributed to the increased release of inhibitors of plasminogen activator (7). As precursors to melanin, tyrosine and phenylalanine trigger both melanogenesis (by activation of tyrosinase) and metastasis in a manner that may involve the collaboration of more than one PKC isoform.

Evidence that demonstrates a causal relationship between melanogenesis and metastasis remains elusive. Certain conventional and novel PKC isoforms are known to have an active role in either or both phenomena. In an early study, it was found that treatment of B16 F1 cells with phorbol 12-myristate 13-acetate, a potent activator of many PKC isoforms (except the atypical isoforms), was sufficient to convert weakly metastatic cells to highly metastatic cells in a mouse model (8). Since then, biochemical, recombinant DNA technology, and antisense techniques have established that PKC α , a conventional isoform that is 12-O-tetradecanoylphorbol-13-acetate (TPA)-responsive, promotes cell migration and metastasis of melanoma (9–11). Evidence that associates specific isoforms with melanogenesis is limited at present, although subcellular localization of individual iso-

Received 5/8/01; revised 8/2/01; accepted 8/6/01.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ This work was funded by NIH Grant CA60618 and the Professional Staff Congress of the City University of New York Research Foundation (to S. A. R.) and by NIH CA74197 and the American Cancer Society (to M. G. K.).

² To whom requests for reprints should be addressed, at Department of Chemistry and Biochemistry, Queens College of the City University of New York, 65-30 Kissena Boulevard, Flushing, NY 11367. Phone: (718) 997-4133; Fax: (718) 997-5531; E-mail: susan_rotenberg@qc.edu.

³ The abbreviations used are: PKC, protein kinase C; Ca²⁺, ionized cytosolic calcium; Par-4, prostate apoptosis response-4; aa, amino acid(s); RPMI*, RPMI 1640 supplemented with tyrosine and phenylalanine; Reg, regulatory domain fragment; WT, wild type; GFP, green fluorescent protein; IP, immunoprecipitation.

Table 1 Culture conditions of B16 F1 cells determine melanin levels and cell behavior

Medium	Melanin content ($\mu\text{g}/10^6$ cells) ^a	Migration (no. of cells) ^a	Adhesion (no. of cells) ^a
DMEM	16.7 \pm 0.3	26 \pm 1	13 \pm 2
RPMI 1640	3.3 \pm 0.5	10 \pm 1	22 \pm 2
RPMI*	17.9 \pm 0.4	29 \pm 2	12 \pm 1
Transfected cells:			
RPMI* + control	3.6 \pm 0.1	24 \pm 2	
RPMI* + WT PKC ζ	3.9 \pm 0.2	8 \pm 1	

^a Values are representative of three or more experiments.

forms may provide additional clues. In this regard, subcellular fractionation of mouse melanoma cells has shown that the conventional isoform PKC α is associated primarily with plasma and endosomal membranes, the perinuclear region, and the cytoskeleton (12, 13). By contrast, the conventional isoform PKC β (but not PKC α) is physically associated with melanosomes, and it was shown to activate tyrosinase (the rate-limiting step of melanin synthesis) by direct phosphorylation (14). However, up-regulation of tyrosinase protein can also accompany the engineered overexpression of PKC α in B16 cells (15). The novel isoform PKC δ was recently demonstrated to be instrumental in eliciting both metastatic potential and melanogenesis in BL6 murine melanoma cells (16). Taken together, these studies suggest that the conventional (α , β) and novel isoforms (exemplified by δ) carry out overlapping functions that promote both melanogenesis and metastasis.

In the present work, a comparison of B16 F1 cells that are cultured in either standard RPMI 1640 or RPMI 1640 media supplemented with tyrosine and phenylalanine (RPMI*) provides a context in which to examine a previously unrecognized role for PKC ζ in cell movement. Our findings indicate that in weakly metastatic cells, the expression of this atypical isoform suppresses cell migration. Whereas other PKC isoforms promote metastasis through their elevated expression, PKC ζ contributes to this phenotype through its down-regulation. This contribution by an atypical isoform broadens the range of control that PKC isoforms collectively exert to produce the metastatic phenotype of melanoma cells.

Results

Expression of PKC ζ Is Decreased in B16 F1 Melanoma Cells Cultured in RPMI* Cells. In the initial stage of this study, we investigated migration and adhesion behavior of B16 F1 cells that had been cultured in either RPMI 1640 or RPMI 1640 media containing phenylalanine and tyrosine (RPMI*). Migration was measured by the number of cells that move across the porous filter of a Boyden chamber in response to Matrigel that had been coated on the underside of the filter. Cells that had been cultured in RPMI* for several weeks exhibited elevated melanin levels and high migration levels, both of which are also known to occur with cells grown in DMEM (Table 1). These properties were consistent with the increased melanogenesis and aggressive metastasis of these cells *in vivo* (6, 17). Adhesion to Matrigel was

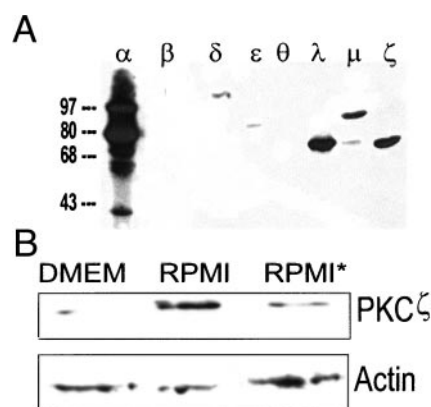


Fig. 1. Western blot analysis of B16 F1 cells cultured in different media. **A**, immunochemical analysis of several PKC isoforms in protein samples (25 $\mu\text{g}/\text{lane}$) isolated from cells grown in RPMI 1640. Size markers are given in kDa. In **B**, lysates (20 $\mu\text{g}/\text{lane}$) that had been isolated from cells cultured in DMEM, RPMI 1640, or RPMI* media were analyzed for PKC ζ or actin. The intensity of the actin signal in each sample served as the loading control.

significantly lower in these cells, thus reflecting the higher rate of movement. By contrast, cells that had been cultured in unsupplemented RPMI 1640 displayed characteristically decreased melanin content and migration behavior that also mirrored their previously established low metastatic activity in mice. The elevated cell movement induced by aa supplements to RPMI 1640 was sustained when cells were cultured continuously under these conditions.

In view of previous studies linking PKC to these phenotypes in murine melanoma cells (9–11, 16, 18), a mechanistic basis for the difference in migration behavior was sought in terms of PKC isoform expression in B16 F1 cells. Immunochemical analysis of several PKC isoforms was carried out in parallel on a single blot by using a 10-trough manifold (Pharmacia). In cells that were cultured in RPMI 1640 (weakly migratory), the expression profile (Fig. 1A) showed that PKC α (the 80-kDa band) was expressed most abundantly, whereas PKC λ , μ , and ζ were expressed at moderate levels, and the novel isoforms PKC δ and ϵ were expressed at exceedingly low levels. Neither PKC β nor θ were detected, however (Fig. 1A). Comparative analysis by Western blot carried out with B16 F1 cells cultured in DMEM, RPMI 1640, or RPMI* media revealed that PKC α was expressed in very high abundance in both RPMI 1640 and RPMI* conditions, whereas the absence of PKC β and the low expression of δ and ϵ remained unchanged in these conditions (not shown). However, a major difference in expression was found for the atypical isoform PKC ζ , as shown in Fig. 1B. Detected with PKC ζ -specific antisera as a 72 band, this isoform was expressed at a relatively high level in RPMI 1640 cells but was significantly decreased in cells grown in either RPMI* or DMEM and that are actively producing melanin (Table 1). The diminished signals reflected differences in PKC ζ protein since as an equivalent amount of total protein had been applied to all lanes (20 μg), as verified by actin staining (Fig. 1B). It is important to note that, although DMEM consists of high levels of tyrosine and phenylalanine as well as

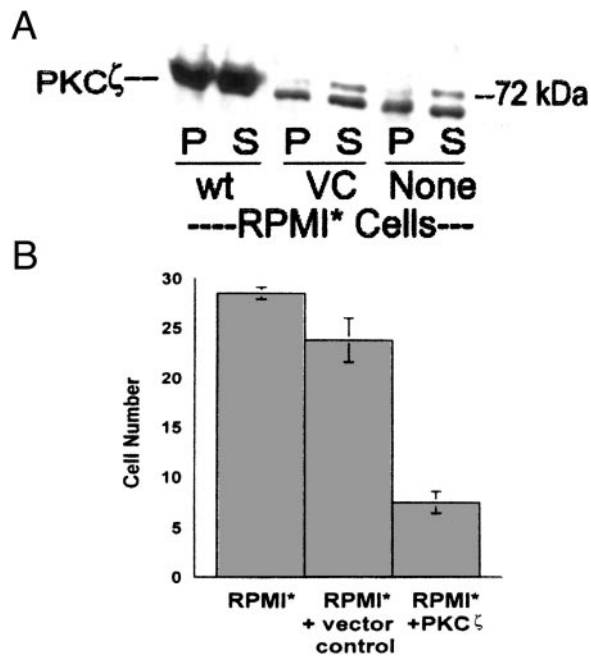


Fig. 2. PKC ζ expression and migration of transfected RPMI* cells. B16 F1 cells that had been cultured in RPMI* media cells were compared with cells that had been subjected to transient transfection with either wildtype PKC ζ cDNA (wt) or the vector control (VC), as described in "Materials and Methods." In A, the cells were lysed, fractionated, and analyzed by Western blot for PKC ζ expression in the particulate (P) and soluble (S) fractions (5 μ g protein/lane). In B, the migration behavior of cells in each condition was measured.

other ingredients that distinguish it from RPMI 1640, the persistent loss of expression of PKC ζ in RPMI*-grown cells was directly related to increased levels of only these aa in the RPMI 1640 culture medium (see "Materials and Methods").

The finding of PKC ζ down-regulation prompted the idea of restoring the level of PKC ζ to RPMI*-cultured cells in order to determine whether PKC ζ played a mechanistic role in cell migration. First, PKC ζ cDNA was transiently transfected into RPMI* cells, and cell extracts were prepared and separated into particulate and soluble fractions, with subsequent Western blot analysis. The results, shown in Fig. 2A, indicate that PKC ζ protein was substantially enriched in both the particulate (P) and soluble (S) fractions of transfected RPMI* cells. By contrast, the PKC ζ protein level in cells that had been transfected with the empty vector (VC) remained low and was equivalent to untreated RPMI* cells. An identical experiment was conducted with RPMI* cells in order to assay for an effect on migration behavior. As a result of elevating the content of PKC ζ protein in RPMI* cells in this manner, cell movement was suppressed to a large extent (Fig. 2B). By contrast, transfection of the control vector was only slightly suppressive when compared with nontransfected RPMI* cells.

A curious yet fortuitous outcome of the transfection experiment presented in Fig. 2 is that melanin stores were released from the cells by the transfection reagent, as evidenced by a very low level of intracellular melanin in both the PKC ζ -enriched and vector control cells (Table 1). This loss of

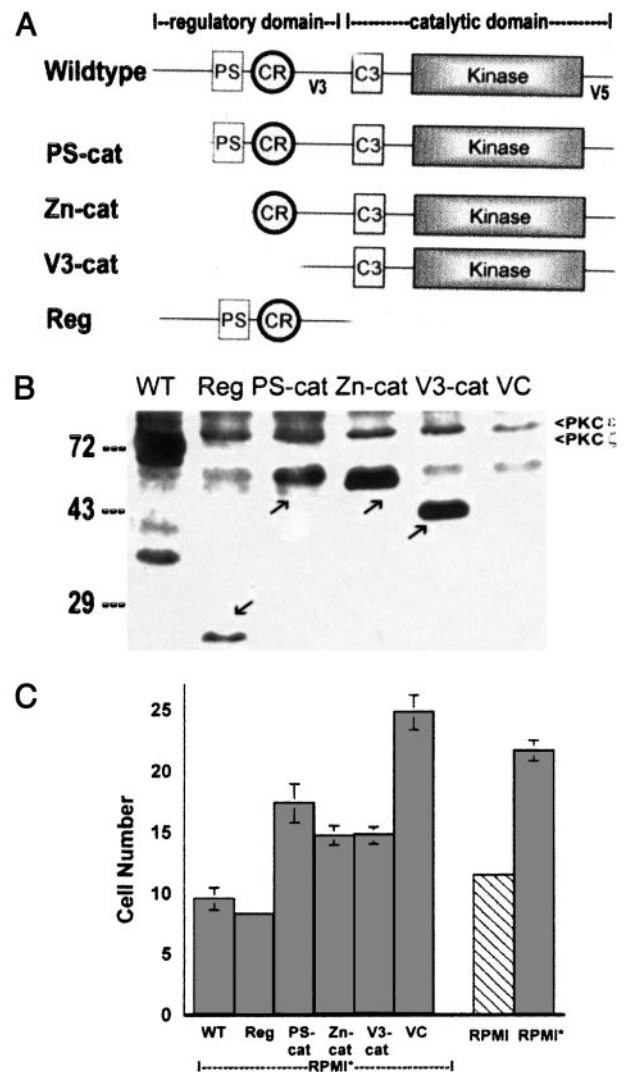


Fig. 3. Structure-function analysis of PKC ζ and migration of B16 F1 cells. A, the scheme highlights the structural elements retained by the resulting mutant PKC ζ proteins. The pseudosubstrate region (PS), cysteine-rich (zinc-finger) domain (CR), variable regions V3 and V5, and conserved region C3 are indicated. B, Western blot analysis of lysates (55 μ g protein/lane) prepared from transiently transfected cells cultured in RPMI*. Arrows, position of each mutant protein. The antibody used to detect the ϵ -tag of each protein also detects a small amount of endogenous PKC ϵ whose position is indicated on the blot. C, Cells that had been cultured in RPMI* media were transiently transfected with wild type PKC ζ (WT), PKC ζ mutant cDNA, or a control vector (VC), and the effect on migration was determined.

melanin neither diminished nor elevated the observed migration of vector control cells. However, transfection of PKC ζ into high-motility cells (RPMI* cells) decreased migration to a level that effectively reversed the motility-stimulating effect induced by tyrosine and phenylalanine in these cells. Importantly, the release of melanin in all cases by the transfection reagent was not itself responsible for inhibition of migration, because vector control-treated RPMI* cells continued to exhibit high migration activity comparable with that of untreated cells.

Structure-Function Analysis of PKC ζ in Melanoma Cells.

To characterize the structural requirements of PKC ζ in suppressing the migration activity of RPMI* cells, several mutant cDNA constructs were developed (Fig. 3A). The series of PKC ζ mutant proteins consisted of three NH₂-terminal deletion fragments representing aa truncations up to the pseudosubstrate region (*PS-cat*; aa 112–592), the cysteine-rich zinc finger region (*Zn-cat*; aa 131–592), and the V3 region (*V3-cat*; aa 181–592), and the Reg (aa 1–250). All pertinent structural elements are designated in the scheme given in Fig. 3A. Each mutant was expressed with the ϵ -epitope tag (ϵ -tag2) that had been tailored on a sequence in PKC ϵ (19). The use of a commercial ϵ -tag antibody distinguished the PKC ζ protein fragments from endogenous PKC ζ but also recorded the presence of endogenous PKC ϵ . The cDNA encoding each mutant protein was transiently transfected into RPMI* cells, as described in “Materials and Methods,” with subsequent Western blot analysis of cell lysates. The results, shown in Fig. 3B, document the expression level of each mutant PKC ζ (identified with arrows) whose mobility in SDS-PAGE was consistent with its predicted molecular mass. For most of the mutant proteins, PKC ζ -related signals were of equivalent intensity and therefore described closely similar levels of expression. However, the Reg was of significantly lower intensity, suggesting that its intracellular stability was somewhat lower.

Cells that had been transfected with PKC ζ mutant cDNA were compared for their inhibitory effects on RPMI* cell migration (Fig. 3C). Relative to the vector control (VC) or parental RPMI* cells, which represented the highest rates of migration, each mutant of PKC ζ was introduced by transfection and consequently found to produce some degree of suppression on cell migration. Importantly, a mutant consisting of the regulatory fragment alone (*Reg*) exhibited the greatest impact and was most like the suppressive effect on migration produced by the WT-PKC ζ . Deletion analysis showed that a mutant lacking the first NH₂-terminal 111 aa but retaining the rest of the PKC ζ polypeptide (*PS-cat*) produced a significant suppressive effect but was weaker than the regulatory fragment in suppressing migration. The suppressive effect observed with *PS-cat* was not dramatically altered by additional truncation to the V3 region (*Zn-cat* and *V3-cat* mutants). Overall, these findings indicate that, in order to observe a degree of suppression that is comparable with that of the WT PKC ζ , an intact regulatory domain is sufficient. It is notable that, despite its lower level of expression relative to the other mutant proteins (Fig. 3B), the intact regulatory domain of PKC ζ still had the strongest impact on migration, which suggests that this domain of PKC ζ could participate in a potent and specific interaction in the cell.

The transfection efficiency for the foregoing experiments was judged by comparing the transfection of cDNA that encodes GFP. For this experiment, identical conditions were employed that had been used for the PKC ζ constructs, *i.e.*, the GFP cDNA insert was subcloned into the same vector used for PKC ζ (pCR3), and the same amount of cDNA was employed for transfection. After transient transfection, the GFP protein product was visualized in quadruplicate by fluorescence microscopy. By comparing the number of

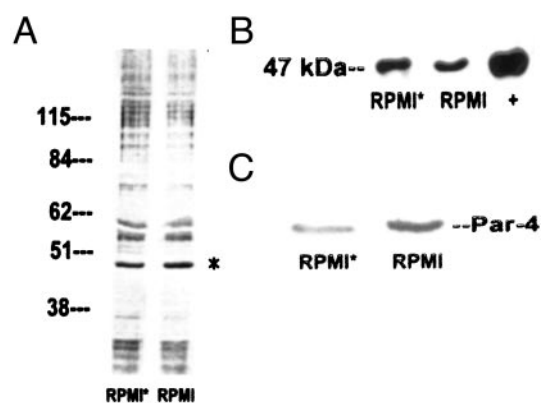


Fig. 4. Par-4 is expressed by B16 F1 cells and interacts with PKC ζ . A, coimmunoprecipitation with anti-PKC ζ (Upstate Biotechnology, Inc.) of lysates (0.35 mg) prepared from RPMI* and RPMI 1640 cells, followed by SDS-PAGE and detection by silver stain. The major band at 47 kDa is identified with an asterisk (*). B, Western blot analysis with anti-Par-4 of lysates (20 μ g protein/lane) prepared from RPMI* and RPMI 1640 cells. A lysate of AT-3 rat prostate cells was used as the positive control (+) for Par-4. C, immunoprecipitation of lysates with anti-PKC ζ was carried out as in A, except that it was followed by Western blot analysis with Par-4 antibody. These results are representative of two or more experiments.

fluorescent cells with the total cell number in a selected field of cells for several fields, the transfection efficiency was judged to be $96 \pm 3\%$ (data not shown). On the basis of this result, it was concluded that a similarly high percentage of B16 F1 cells had received the pCR3 plasmid after transient transfection with the PKC ζ -bearing constructs. The migration behavior shown in Fig. 3C, therefore, reflected the response of virtually all cells rather than a subpopulation of cells that had selectively received the plasmid.

A Role for Par-4 in PKC ζ -mediated Suppression of Migration. Because the regulatory domain had the strongest impact on the migration activity of RPMI* cells, its significance to the suppression phenotype was pursued. The possibility that binding interactions of PKC ζ would explain its role in the suppression phenotype was addressed. Immunoprecipitation of cell lysates with anti-PKC ζ was carried out, and the coimmunoprecipitated proteins were resolved by SDS-PAGE and detected by silver stain. Among several minor bands shown in Fig. 4A, there was a single major protein band at 47 kDa that coimmunoprecipitated with PKC ζ . The identity of this 47-kDa protein could be Par-4, a proapoptotic protein originally identified in prostate cells and shown previously to bind PKC ζ at the cysteine-rich zinc finger region of the regulatory domain (2). In Fig. 4B, it was observed that Par-4 is indeed expressed in B16 F1 melanoma cells. By Western blot analysis with anti-Par-4, this protein was observed as a 47-kDa band present at equivalent levels of expression in both RPMI 1640 and RPMI* cells. In addition, a 47-kDa protein that was immunoreactive with anti-Par-4 could be shown to coimmunoprecipitate with PKC ζ in lysates from RPMI 1640 and RPMI* cells (Fig. 4C), suggesting that the two proteins interact in B16 F1 cells.

To test the possibility that Par-4 participates in the migration phenotype, transient transfection of Par-4 into B16 F1 cells with subsequent analysis of migration was carried out.

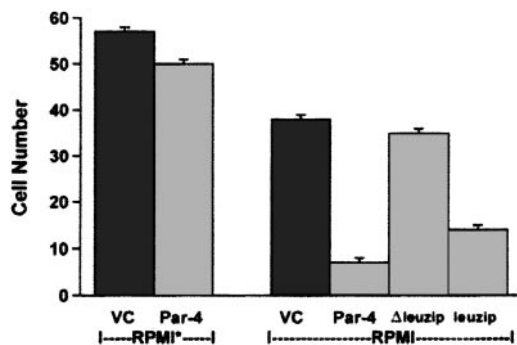


Fig. 5. Par-4 contributes to suppression of B16 F1 cell migration. Cells cultured in RPMI 1640 or RPMI* were transiently transfected with WT Par-4, Par-4 mutant cDNA, or the vector control (VC), and the effect on migration was evaluated. The results are the average of triplicate measurements and are representative of three independent experiments.

This experiment revealed that Par-4 further suppresses the already weakly migratory RPMI 1640 cells (Fig. 5) that express PKC ζ at a moderate level (Fig. 1B). By contrast, Par-4 overexpression in RPMI* cells, which express PKC ζ at a low level (Fig. 1B), had only a slight effect on migration, suggesting that the availability of PKC ζ is critical to suppression by Par-4. To evaluate further the effect of Par-4 on the migration of RPMI 1640 cells, transfection of Par-4 mutant constructs into RPMI 1640 cells and a subsequent assay of migration was carried out. Consistent with the fact that Par-4 binds to PKC ζ through its leucine zipper region (20), a mutant whose leucine zipper region had been deleted (Δ leu/Par-4) was ineffective in promoting suppression of RPMI 1640 migration (Fig. 5), thereby implicating the leucine zipper region of Par-4 as the site that participates in the suppression phenotype. Furthermore, transfection of cDNA encoding the leucine zipper element alone (leuzip) simulated the effect of the WT Par-4 and was thus sufficient to suppress migration.

Discussion

A new finding of the foregoing study is that the atypical PKC ζ isoform suppresses the migration of metastatic melanoma cells. Cell culture conditions that support the synthesis of melanin (RPMI*) were accompanied by down-regulation of PKC ζ and concomitant elevation of migration activity. Restoring PKC ζ levels by transient transfection produced a compensatory decrease in migration. The inhibitory action by PKC ζ distinguishes it from the metastasis-promoting role ascribed to certain conventional and novel PKC isoforms in murine and human melanoma cells (9, 11, 16, 21). PKC ζ exerts its suppressive effect primarily through interactions that involve its regulatory domain and Par-4, a PKC ζ -binding protein whose action was previously associated with apoptosis in NIH-3T3 cells (2, 22). In both high- and low-motility cells where Par-4 expression level is equivalent, suppression of migration is apparently determined by the availability of PKC ζ . This idea was borne out by the finding that no suppressive effect by Par-4 overexpression was observed in high-motility cells that have diminished PKC ζ levels (RPMI* cells; Fig. 5).

In view of the established role of Par-4 in apoptosis (2, 20, 22), our results suggest a model in which the PKC ζ /Par-4 complex suppresses cell movement while elevating apoptosis. Interaction of Par-4 with PKC ζ (a prosurvival enzyme) with subsequent cleavage (by caspases) of PKC ζ into regulatory and catalytic fragments are critical events in apoptosis (22, 23). Although the catalytic fragment was recently shown to undergo additional degradation by the ubiquitin-proteasome pathway (23), a consequence for the PKC ζ regulatory fragment has not been addressed. As shown by the lower expression level of the regulatory fragment (Fig. 3B), it is possible that this domain is also rapidly degraded in the cell. To the extent that WT PKC ζ is expressed by the cell, its potent inhibitory effect on the motility-signaling pathway is enabled by Par-4. In both apoptosis and motility, the leucine zipper element of Par-4 provides the critical site of interaction with the cysteine-rich region of the PKC ζ regulatory domain. Although the leucine zipper alone was not sufficient to reproduce the proapoptotic effect of Par-4 (20), it was able to simulate the suppressive effect of Par-4 on migration (Fig. 5). To explain this effect, we speculate that the leucine zipper element acts: (a) to block the action of another PKC ζ -binding protein; or (b) to stabilize the conformation of the PKC ζ regulatory domain, either of which might enable the interaction of PKC ζ with a downstream signaling component that drives cell movement. In this regard, PKC ζ (but neither PKC α nor δ) is known to inhibit protein kinase B/Akt, a serine/threonine protein kinase that can be activated by serum growth factors via phosphatidylinositol 3-kinase (24). Inhibition of PKB/Akt by PKC ζ /Par-4 would thereby oppose phosphatidylinositol 3-kinase, whose signaling pathway has been linked to adhesion and migration of diverse cell types such as breast (25) and melanoma cells (26).

These studies also examined PKC ζ in terms of the controversial relationship between melanogenesis and metastasis. Our findings suggest that PKC ζ down-regulation correlates with elevated melanin levels (Table 1; Fig. 1B). Because melanin is synthesized and remains sequestered in the melanosomes, and proteins involved in cell metastasis are localized in the cytoplasm, plasma membrane, and cytoskeleton, any model that seeks to link the two phenomena must take into account their physical compartmentation (27, 28). At present, only PKC β is known to be associated with the outer surface of melanosomes (14), but no information about the precise intracellular location of PKC ζ has been reported. PKC β has been shown to activate tyrosinase and melanogenesis by phosphorylating the cytoplasmic domain of this protein. The possibility that PKC ζ might oppose the action of PKC β by preventing the activation of tyrosinase provides an avenue for additional study. Importantly, the present study indicates that the loss of melanin caused by transfection (Table 1) does not impair the migration of high motility cells (RPMI*). We conclude from this observation that melanin does not drive cell motility but is coincidental to it. The mechanism by which the process of aa-induced melanization elicits down-regulation of PKC ζ remains to be determined.

The participation of Par-4 in the metastatic phenotype does not exclude potential contributions to suppression by other PKC ζ -binding proteins. Although the 47-kDa band in Fig. 4A (the presumptive Par-4 band) is the major protein band that coimmunoprecipitated with PKC ζ , other minor bands were evident. The relative effects produced by the regulatory fragment (Reg) and the PS-cat mutant (Fig. 3C) support the conclusion that the initial NH₂-terminal 111 aa are critical to the suppression phenotype. Furthermore, the significant suppression observed with the V3-cat mutant that lacks the Par-4 binding site, argues for additional binding interactions in the V3 region or catalytic domain. In this regard, caspase cleavage sites have been identified in the hinge region (V3) of PKC ζ (23), and recognition sites for PICK-1 and actin have been assigned to the COOH terminal (V5) region (1).

In summary, expression of PKC ζ is substantially decreased in metastatic melanoma cells, a consequence of aa supplements in the culture medium. Restoration of PKC ζ protein to these high-motility cells leads to a compensatory suppression of migration that apparently is assisted by formation of a high-affinity complex with Par-4. Identification of a means to elevate and maintain PKC ζ levels in metastatic melanoma cells *in vivo* or to prevent its down-regulation through dietary restriction of tyrosine and phenylalanine may therefore provide an effective approach to controlling the metastatic behavior of melanoma cells.

Materials and Methods

All cell culture media, serum, aa, and antibiotics were purchased from Life Technologies, Inc. (Gaithersburg, MD). Unless otherwise stated, all biochemicals were acquired from Sigma (St. Louis, MO). Matrigel was purchased from Collaborative Bioscience (Bedford, MA), silver stain was purchased from Pierce Co. (Rockford, IL), and PKC isoform-specific antibodies were obtained from Transduction Laboratories (Lexington, KY) or Upstate Biotechnology, Inc. (Lake Placid, NY). Secondary antisera and actin antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The Par-4 antibody was a gift from Dr. Vivek M. Rangnekar (University of Kentucky, Lexington, KY).

Mutagenesis. Deletion mutants were generated by PCR using the full-length PKC ζ mouse cDNA as a template (29). For each construct, 24-mer forward and reverse primers based on the PKC ζ sequence were synthesized. *Sall* and *MluI* sites were added in frame in the forward and reverse primers, respectively, to facilitate subcloning. The identity of each PCR product was confirmed by sequencing. The corresponding PCR products were subcloned into *Sall*-*MluI* sites in the mammalian expression vector pCR3 ϵ 2 (19), a modified version of pCR3 (Invitrogen, San Diego, CA), to generate the different mutants described in the text.

Cell Culture. B16 F1 cells were cultured in complete RPMI 1640 (10% FCS, 1% penicillin-streptomycin, and 0.05% fungizone) that normally contains 15 mg/liter L-phenylalanine and 29 mg/liter L-tyrosine. Cells were also cultured in DMEM containing 10% FCS, 1% penicillin-streptomycin, 0.05% fungizone, 1% nonessential aa, 1% sodium pyruvate, 1.5% MEM vitamin solution, and 1%

L-glutamine. DMEM contains 66 mg/liter L-phenylalanine and 104 mg/liter L-tyrosine. RPMI* consisted of RPMI 1640 supplemented with phenylalanine and tyrosine concentrations that corrected for their differences in RPMI 1640 and DMEM, *i.e.*, 51 mg/liter L-phenylalanine and 75 mg/liter L-tyrosine.

Adhesion Assay. Twenty-four-well tissue culture plates were coated with 200 μ l of Matrigel (43.5 μ g/cm²) and incubated at 37°C in 5% CO₂ for 1 h and then washed with PBS. Immediately before use, the coated wells were overlaid with 1% BSA for 30 min, washed five times with PBS, and dried for 30 min at room temperature in the tissue culture hood. Cells were applied to individual wells at 1.5×10^5 /well and incubated for 1 h at 37°C in 5% CO₂. Nonadherent cells were removed by aspiration and three additional washes with PBS. Adherent cells were counted visually using a Nikon Diaphot-TMD inverted microscope at $\times 400$. In each well, cells were counted in eight randomly chosen fields and numerically averaged. Each experimental group consisted of triplicate measurements.

Migration Assay. Costar transwells (Boyden chamber) containing a polycarbonate membrane with a 12- μ m pore size were used to measure cell movement. The bottom surface of the membrane was coated with 35 μ g of Matrigel and then the transwells were incubated at 37°C for 1 h. Cells (1.5×10^5) were seeded into the upper chamber, complete medium (DMEM, RPMI 1640, or RPMI* with 10% FCS) was added to the lower chamber, and the transwells were incubated at 37°C and 5% CO₂. After incubation for 3 h, the upper chamber was carefully wiped with a cotton swab in order to remove cells that remained on the upper membrane surface. Cells that had migrated to the lower membrane surface were fixed and stained by the H + E Method (Fisher Diagnostics Leukostat). By the use of a Nikon Diaphot-TMD inverted microscope at $\times 400$, eight fields of adherent cells were randomly counted in each well and numerically averaged. Each condition was conducted in triplicate and averaged.

Transfection. B16 F1 cells were transiently transfected using Lipofectamine Plus (Life Technologies, Inc.) with cDNA (4 μ g) corresponding to the WT PKC ζ or individual PKC ζ mutant cDNA constructs and incubated at 37°C in 5% CO₂. Identical experiments were carried out with WT Par-4 or Par-4 mutant constructs (gifts of Dr. Vivek M. Rangnekar, University of Kentucky, Lexington, KY). After 48 h incubation, cells were replated into transwells and analyzed for migration behavior. In some experiments, the cells were harvested, lysed, immunoprecipitated, and analyzed by Western blot. To determine transfection efficiency, B16 F1 cells were transfected with the cDNA for GFP (Promega) that had been subcloned into the pCR3 vector, and the cells were visualized by fluorescence microscopy (Leitz DMRB) equipped with a 470/509-nm filter set.

Cell Lysis and Fractionation. Cells were cultured in 15-cm Nunc plastic dishes to 50–80% confluency and collected in 1 ml of lysis buffer [50 mM Tris (pH 7.5), 5 mM EDTA, 5 mM EGTA, 15 mM 2-mercaptoethanol, 0.1% Triton X-100, 0.25 mM phenylmethylsulfonyl fluoride, 10 μ g/ml leupeptin,

and 10 $\mu\text{g/ml}$ soybean trypsin inhibitor]. Cell disruption was carried out on ice by Dounce homogenization (20 strokes) and sonication (30 sec). The lysate was centrifuged for 10 min at $550 \times g$, and the supernatant was saved as the cell extract. Where indicated, cell lysates were fractionated further by high-speed centrifugation at $100,000 \times g$. The supernatant (soluble fraction) was isolated and the pelleted material (particulate fraction) was resuspended in buffered medium containing protease inhibitors. All samples were evaluated for protein content by use of the Bio-Rad protein reagent and bovine serum albumin as standard. Sample buffer (5 \times) was added to a final concentration $1 \times [80 \text{ mM Tris (pH 6.8), 10\% glycerol, 0.004\% bromophenol blue, 2\% SDS, 100 mM dithiothreitol}]$, and the samples were heated to 95°C for 5 min. with subsequent SDS-PAGE (see "Western Blot Analysis," below).

IP. Cells were disrupted and extracted in 1-ml IP buffer [10 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 0.5% NP40, 0.2 mM sodium vanadate, 0.2 mM sodium phosphate, and protease inhibitors]. The extract was transferred to a new Eppendorf tube, 5 μg of anti-PKC ζ was added, and the mixture was incubated overnight at 4°C with constant rotation. Protein A/G-agarose (25 μl) was added to each tube, and incubation was continued for 1 h. The immunocomplexes were collected by centrifuging the tubes at $550 \times g$ for 10 min. The resulting pellets were washed three times with 0.5 ml of IP buffer, and the final pellet was mixed with an equal volume of 2 \times sample buffer, heated to 95°C for 5 min, and centrifuged at $550 \times g$ for 10 min to exclude the beads. The supernatant was used for Western blot analysis.

Western Blot Analysis. Cell lysates or immunocomplexes were subjected to 8% SDS-PAGE and subsequently transferred electrophoretically to a nitrocellulose filter (30). Immunochemical assay was carried out with primary antisera that were isoform-specific (Transduction Laboratories). Assays for PKC ζ were conducted with a primary antibody (Upstate Biotechnology, Inc.) that was not cross-reactive with atypical PKC λ . For all assays, detection was carried out with an appropriate secondary antibody that was conjugated to horseradish peroxidase (Santa Cruz Biotechnology, Inc.), and the blot was developed by chemiluminescence (Amersham). To establish equivalent protein loading, actin staining was carried out with a rabbit polyclonal antibody (Santa Cruz Biotechnology). To analyze a single blot with several different isoform-specific antibodies in parallel, a 10-trough manifold (Deca Probe) was employed (Pharmacia Bio-Tech, Piscataway, NJ).

Determination of Melanin Content. The concentration of melanin was calculated for 10^6 cells by a previously published method (6). Cells were extracted three times with trichloroacetic acid, twice with ethanol-ether (3:1), and once with ether alone. After the extracts were allowed to dry, they were dissolved in 1 ml of 0.85 N KOH, heated to 100°C for 10 min, and then assessed for absorbance at 400 nm. A calibration curve was constructed from commercially available melanin (Sigma).

Acknowledgments

The cDNA constructs encoding Par-4 and its related mutant forms, the Par-4 antibody, and the AT-3 cell lysate used in these studies were generous contributions of Prof. Vivek M. Rangnekar (University of Kentucky, Lexington, KY).

References

- Ron, D., and Kazanietz, M. G. New insights into the regulation of protein kinase C and novel phorbol ester receptors. *FASEB J.*, **13**: 1658–1676, 1999.
- Diaz-Meco, M. T., Municio, M. M., Frutos, S., Sanchez, P., Lozano, J., Sanz, L., and Moscat, J. The product of *par-4*, a gene induced during apoptosis, interacts selectively with the atypical isoforms of protein kinase C. *Cell*, **86**: 777–786, 1996.
- Konishi, H., Kuroda, S., and Kikkawa, U. The pleckstrin homology domain of RAC protein kinase associates with the regulatory domain of protein kinase C ζ . *Biochem. Biophys. Res. Commun.*, **205**: 1770–1775, 1994.
- Tokunaga, C., Kuroda, S., Tatematsu, K., Nakagawa, N., Ono, Y., and Kikkawa, U. Molecular cloning and characterization of a novel protein kinase C-interacting protein with structural motifs related to RBCC family proteins. *Biochem. Biophys. Res. Commun.*, **244**: 353–359, 1998.
- Fidler, I. J. Selection of successive tumour lines for metastasis. *Nature (Lond.)*, **242**: 148–149, 1973.
- Prezioso, J. A., Wang, N., Duty, L., Bloomer, W. D., and Gorelik, E. Enhancement of pulmonary metastasis formation and γ -glutamyl-transpeptidase activity in B16 melanoma induced by differentiation *in vitro*. *Clin. Exp. Metastasis*, **11**: 263–274, 1993.
- Paley, B. A., Fu, Y.-M., and Meadows, G. G. Inhibition of B16BL6 melanoma invasion by tyrosine and phenylalanine deprivation is associated with decreased secretion of plasminogen activators and increased plasminogen activator inhibitors. *Clin. Exp. Metastasis*, **17**: 841–848, 1999.
- Gopalakrishna, R., and Barsky, S. H. Tumor promoter-induced membrane-bound protein kinase C regulates hematogenous metastasis. *Proc. Natl. Acad. Sci. USA*, **85**: 612–616, 1988.
- LaPorta, C. A., and Comolli, R. Activation of protein kinase C- α isoform in murine melanoma cells with high metastatic potential. *Clin. Exp. Metastasis*, **15**: 568–579, 1997.
- Dennis, J. U., Dean, N. M., Bennett, C. F., Griffith, J. W., Lang, C. M., and Welch, D. R. Human melanoma metastasis is inhibited following *ex vivo* treatment with an antisense oligonucleotide to protein kinase C- α . *Cancer Lett.*, **128**: 65–70, 1998.
- Sullivan, R. M., Stone, M., Marshall, J. F., Uberall, F., and Rotenberg, S. A. Photo-induced inactivation of protein kinase C α by dequalinium inhibits motility of murine melanoma cells. *Mol. Pharmacol.*, **58**: 729–737, 2000.
- Timar, J., Liu, B., Bazaz, R., and Honn, K. V. Association of protein kinase C- α with cytoplasmic vesicles in melanoma cells. *J. Histochem. Cytochem.*, **44**: 177–182, 1996.
- Szalay, J., Bruno, P., Bhati, R., Adjodha, J., Schueler, D., Summerville, V., and Vazeos, R. Associations of PKC isoforms with the cytoskeleton of B16 F10 melanoma cells. *J. Histochem. Cytochem.*, **49**: 49–66, 2001.
- Park, H. Y., Perez, J. M., Laursen, R., Hara, M., and Gilchrist, B. A. Protein kinase C- β activates tyrosinase by phosphorylating serine residues in its cytoplasmic domain. *J. Biol. Chem.*, **274**: 16470–16478, 1999.
- Mahalingam, H., Vaughn, J., Novotny, J., Gruber, J. R., and Niles, R. M. Regulation of melanogenesis in B16 mouse melanoma cells by protein kinase C. *J. Cell. Physiol.*, **168**: 549–558, 1996.
- LaPorta, C. A., Di Dio, A., Porro, D., and Comolli, R. Overexpression of novel protein kinase C δ in BL6 murine melanoma cells inhibits the proliferative capacity *in vitro* but enhances the metastatic potential *in vivo*. *Melanoma Res.*, **10**: 93–102, 2000.
- Bennett, D. C., Dexter, T. J., Ormerod, E. J., and Hart, I. R. Increased experimental metastatic capacity of a murine melanoma following induction of differentiation. *Cancer Res.*, **46**: 3239–3244, 1986.

18. Dumont, J. A., Jones, W. D., and Bitonti, A. J. Inhibition of experimental metastasis and cell adhesion of B16 F1 melanoma cells by inhibitors of protein kinase C. *Cancer Res.*, 52: 1195–1200, 1992.
19. Fernandez, N., Caloca, M. J., Prendergast, G. V., Meinkoth, J. L., and Kazanietz, M. G. Atypical protein kinase C- ζ stimulates thyrotropin-independent proliferation in rat thyroid cells. *Endocrinology*, 141: 146–152, 2000.
20. Sells, S. F., Han, S-S., Muthukkumar, S., Maddiwar, N., Johnstone, R., Boghaert, E., Gillis, D., Liu, G., Nair, P., Monnig, S., Collini, P., Mattson, M. P., Sukhatme, V. P., Zimmer, S. G., Wood, D. P., MacRoberts, W., Shi, Y., and Rangnekar, V. M. Expression and function of the leucine zipper protein Par-4 in apoptosis. *Mol. Cell. Biol.*, 17: 3823–3832, 1997.
21. Powell, M. B., Rosenberg, R. K., Graham, M. J., Birch, M. L., Yamanishi, D. T., Buckmeier, J. A., and Meyskens, F. L. Protein kinase C β expression in melanoma cells and melanocytes: differential expression correlates with biological responses to 12-O-tetradecanoylphorbol 13-acetate. *J. Cancer Res. Clin. Oncol.*, 119: 199–206, 1993.
22. Frutos, S., Moscat, J., and Diaz-Meco, M. T. Cleavage of ζ PKC but not λ/ι PKC by caspase-3 during UV-induced apoptosis. *J. Biol. Chem.*, 274: 10765–10770, 1999.
23. Smith, L., Chen, L., Reyland, M. E., DeVries, T. A., Talanian, R. V., Omura, S., and Smith, J. B. Activation of atypical protein kinase C ζ by caspase processing and degradation by the ubiquitin-proteasome system. *J. Biol. Chem.*, 275: 40620–40627, 2000.
24. Doornbos, R. P., Theelen, M., van der Hoeven, P. C. J., van Blitterswijk, W. J., Verkleij, A. J., and van Bergen en Henegouwen, P. M. P. Protein kinase C ζ is a negative regulator of protein kinase B activity. *J. Biol. Chem.*, 274: 8589–8596, 1999.
25. Mao, M., Fang, X., Lu, Y., LaPushin, R., Bast, R. C., and Mills, G. B. Inhibition of growth factor-induced phosphorylation and activation of protein kinase B/Akt by atypical protein kinase C in breast cancer cells. *Biochem. J.*, 352: 475–482, 2000.
26. Metzner, B., Barbisch, M., Bachmann, F., Czech, W., and Norgauer, J. Evidence of the involvement of phosphatidylinositol 3-kinase in the migration, actin stress fiber formation, and $\alpha v\beta_3$ -integrin-mediated adherence of human melanoma cells. *J. Invest. Dermatol.*, 107: 597–602, 1996.
27. Vancoillie, G., Lambert, J., and Naeyaert, J-M. Melanocyte biology and its implications for the clinician. *Eur. J. Dermatol.*, 9: 241–251, 1999.
28. Raposo, G., Tenza, D., Murphy, D. M., Berson, J. F., and Marks, M. S. Distinct protein sorting and localization to premelanosomes, melanosomes, and lysosomes in pigmented melanocytic cells. *J. Cell Biol.*, 152: 809–824, 2001.
29. Goodnight, J., Kazanietz, M. G., Blumberg, P. M., Mushinski, J. F., and Mischak, H. The cDNA sequence, expression pattern and protein characteristics of mouse protein kinase C- ζ . *Gene*, 122: 305–311, 1992.
30. Towbin, H., Staehelin, T., and Gordon, J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA*, 76: 4350–4354, 1979.