

Phosphoprotein Enriched in Astrocytes-15 kDa Expression Inhibits Astrocyte Migration by a Protein Kinase C δ -dependent Mechanism

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Phosphoprotein enriched in astrocytes-15 kDa (PEA-15), a phosphoprotein enriched in astrocytes, inhibits both apoptosis and proliferation in normal and cancerous cells. Here, analysis of PEA-15 expression in glioblastoma organotypic cultures revealed low levels of PEA-15 in tumor cells migrating away from the explants, regardless of the expression levels in the originating explants. Because glioblastomas are highly invasive primary brain tumors that can originate from astrocytes, we explored the involvement of PEA-15 in the control of astrocyte migration. PEA-15^{-/-} astrocytes presented an enhanced motility *in vitro* compared with their wild-type counterparts. Accordingly, NIH-3T3 cells transfected by green fluorescent protein-PEA-15 displayed a reduced migration. Reexpression of PEA-15 restored PEA-15^{-/-} astrocyte motility to wild-type levels. Pharmacological manipulations excluded a participation of extracellular signal-regulated kinase/mitogen-activated protein kinase, phosphatidylinositol 3-kinase/Akt, and calcium/calmodulin-dependent protein kinase II in this effect of PEA-15. In contrast, treatment by bisindolylmaleimide, Gö6976, and rottlerin, and chronic application of phorbol 12-myristate 13-acetate and/or bryostatin-1 indicated that PKC δ mediated PEA-15 inhibition of astrocyte migration. PEA-15^{-/-} astrocytes constitutively expressed a 40-kDa form of PKC δ that was down-regulated upon PEA-15 reexpression. Together, these data reveal a new function for PEA-15 in the inhibitory control of astrocyte motility through a PKC δ -dependent pathway involving the constitutive expression of a catalytic fragment of PKC δ .

INTRODUCTION

Astrocytes are one of the cell types from which gliomas, the main primitive brain tumors of adulthood originate (Kleihues *et al.*, 2002). In contrast to astrocytes, gliomal cells exhibit high motility. Their ability to infiltrate rapidly the normal brain parenchyma limits the efficacy of surgical resection and targeted radiotherapy. Thus, understanding the

intracellular mechanisms underlying cell migration within the CNS is of great importance for prognosis and development of therapeutics.

Control of cell motility has been shown to depend on protein kinase C (PKC) activity. PKCs form a family of at least 11 isoforms. Based on their structural and biochemical properties, these PKC isoforms can be divided into three major groups: 1) classical PKCs (α , β_1 , β_2 , and γ), which are activated by diacylglycerol (DAG) and are Ca²⁺ dependent; 2) novel PKCs (nPKC: δ , ϵ , η , θ , and μ), which are activated by DAG but are Ca²⁺ independent; and 3) the atypical PKCs (ζ , λ , and ι), which do not respond to either DAG or calcium. PKC isozymes have been implicated in the three independent but highly coordinated cellular processes involved during tumor cell migration: 1) cell attachment to extracellular matrix or basement membrane (Fagerholm *et al.*, 2002); 2) cell motility, which involves the reorganization of the actin cytoskeleton (Iwabu *et al.*, 2004); and 3) cell invasion, through extracellular matrix degradation by proteolytic enzymes (Woo *et al.*, 2004). Accordingly, inhibition of PKC has been shown to inhibit cell motility and invasiveness (Kermorgant *et al.*, 2001). Interestingly, phosphoprotein enriched in astrocytes-15 kDa (PEA-15), a major small cyto-

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Abbreviations used: CaMKII, calcium/calmodulin-dependent protein kinase II; CF-PKC δ , catalytic fragment of protein kinase C δ ; ERK, extracellular signal-regulated kinase; GST, glutathione S-transferase; MAPK, mitogen-activated protein kinase; MLC, myosin light chain; PEA-15, phosphoprotein enriched in astrocytes-15 kDa; PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PTD, protein transducer domain; TGF, transforming growth factor.

plasmic astrocytic phosphoprotein, is a PKC substrate. Furthermore, PEA-15-induced insulin resistance in type 2 diabetes results from a dysregulation of the balance between the activities of PKC α and ζ (Condorelli *et al.*, 2001).

PEA-15 was first identified as an abundant phosphoprotein in brain astrocytes (Araujo *et al.*, 1993); subsequently, it was shown to be widely expressed in different tissues and highly conserved among vertebrates (Danziger *et al.*, 1995; Estelles *et al.*, 1996). It is composed of an N-terminal death effector domain and a C-terminal tail of irregular structure that contains the serines phosphorylated by PKC (S104) or by Akt/calcium/calmodulin-dependent protein kinase II (CaMKII) (S116) (Renault *et al.*, 2003). PEA-15 inhibits apoptosis (Estelles *et al.*, 1999; Condorelli *et al.*, 1999; Kitsberg *et al.*, 1999; Hao *et al.*, 2001). In human malignant glioma cell lines, PEA-15 expression and phosphorylation on its PKC site are required for resistance to tumor necrosis factor-related apoptosis-inducing ligand-mediated apoptosis (Hao *et al.*, 2001). PEA-15 also modifies extracellular signal-regulated kinase (ERK) signaling by controlling ERK subcellular localization (Formstecher *et al.*, 2001; Whitehurst *et al.*, 2004) and thereby restricts cell proliferation. Recently, the phosphorylation of PEA-15 was reported to determine whether PEA-15 binds ERK or FADD (Krueger *et al.*, 2005; Renganathan *et al.*, 2005). Therefore, PEA-15 occurs as a regulator protein that controls cell apoptosis as well as proliferation, two cell functions dysregulated in cancer. PEA-15 involvement in cancer is complex. In transformed and metastatic murine squamous carcinoma cells, the *pea-15* gene is up-regulated (Dong *et al.*, 2001) and PEA-15 overexpression favors skin tumors (Formisano *et al.*, 2005). In contrast, a tumor suppressor function was recently reported for PEA-15 in cellular models of breast and ovary tumors (Gaumont-Leclerc *et al.*, 2004; Bartholomeusz *et al.*, 2006). This led us to investigate human tumors arising from astrocytes.

Here, examining human glioblastomas, we observed *ex vivo* that cells migrating away from the tumor core express low levels of PEA-15, regardless of the expression level in the original tumor. This prompted us to explore the role of PEA-15 in the motility of astrocytes. We report that loss of PEA-15 expression results in an increased astrocyte motility by a PKC δ -dependent mechanism related to the constitutive expression of a novel 40-kDa form of PKC δ .

MATERIALS AND METHODS

Reagents

Phorbol 12-myristate 13-acetate (PMA), KN-62, U0126, LY294002, Gö6976, ZVAD, and DEVD were from Calbiochem (San Diego, CA). Fibronectin from human plasma, leptomyacin B, aphidicolin from *Nigrospora sphaerica*, rottlerin, and bryostatin-1 were from Sigma (Lyon, France). Recombinant human transforming growth factor (TGF) α was obtained from AbCys (Paris, France). PEA-15 (Ab-7) rabbit polyclonal antibody has been characterized previously (Sharif *et al.*, 2004). PKC δ (C20), PKC ϵ (C15), PKC η (C15), PKC μ (C20), and PKC θ (C18) polyclonal antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). PKC ϵ mouse monoclonal antibody (clone 21, ref 610085) was from BD Biosciences Transduction Laboratories (Lille, France). PKC θ mouse antibody (2059), phospho-PKC δ (Thr505), myosin light chain (MLC) and phospho-MLC (serine 19) antibodies were from Cell Signaling Technology (Ozyme, St. Quentin-en-Yvelines, France). Mouse green fluorescent protein (GFP) antibody was from Roche Diagnostics (Mannheim, Germany). MIB (Ki-67) and glial fibrillary acidic protein antibodies were from Dako Denmark (Glostrup, Denmark).

Plasmids

GFP-PKC δ Construct. Rat PKC δ cDNA was provided by Prof. Peter Parker (CRUK Institute for Cancer Studies, London Research Institute, London, United Kingdom). Rat PKC δ open reading frame was amplified by polymerase chain reaction (PCR) by using 5'-CGGAATTCATGGCACCGTTCCTCCGCA-3' as forward primer and 5'-GGGGGTACCCTATTCAGGAATTGCTC-3' as reverse primer. The product of PCR was then digested with EcoRI and KpnI restriction enzymes and was cloned into pEGFP C2 vector (Clon-

tech, Mountain View, CA). GFP-PEA-15 (Kitsberg *et al.*, 1999) and GFP-CF-PKC δ (DeVries *et al.*, 2002) have been described previously.

Cell Culture

Glial cultures were prepared as described previously (Araujo *et al.*, 1993). Briefly, culture dishes were coated with 1.5 μ g/ml poly-L-ornithine (mol. wt. 40,000; Sigma). Striatal and cortical cells from 1-d-postnatal wild-type or PEA-15 $^{-/-}$ mice were dissociated and plated in culture defined minimal essential medium (MEM)-F-12 medium consisting of a 1:1 mixture of MEM and F-12 nutrient (Invitrogen, Cergy Pontoise, France) supplemented with 33 mM glucose, pH 7.4, 2 mM glutamine, 3 mM sodium bicarbonate, 5 mM HEPES, 50 IU/ml penicillin, 5 μ g/ml streptomycin (all from Invitrogen), and 10% fetal bovine serum (Perbio Science France, Brebieres, France). The culture medium was changed every 3 d. When cells were almost confluent, around day 7, cells were treated by 5 μ M cytosine arabinoside for 2 d.

Tumor Explants Collection and Culture

Glioblastomas (n = 11) were collected by an anatomopathologist in the surgical room and examined immediately by smears technique (Beuvon *et al.*, 2000) to make sure that sample contained a majority of tumor cells. These samples were either directly fixed by formalzinc solution (formaldehyde 2% with 8 g/l NaCl, 3 g/l ZnSO $_4$) for an histopathological control or cut by 1 cm 3 in a transport medium and then minced in 1-mm 3 fragments and maintained on surgical sponge of gelatin (Gelfoam; Pharmacia and Upjohn, Guyancourt, France; also available upon request) in RPMI 1640 medium (Sigma) containing 2 g/l sodium bicarbonate, 100 IU/ml penicillin, 100 IU/ml streptomycin supplemented with 6% fetal calf serum at 37°C, and 5% CO $_2$ from 10 to 30 d. The blocks of gelatin containing tumor explant were then fixed and paraffin embedded, before being cut into 4- μ m-thick sections by using a microtome (Microm HM340E; Electron Microscopy Sciences, Fort Washington, PA).

Incubation of the Ab-7 Antibody with Glutathione S-Transferase (GST) or GST-PEA-15 Proteins

PEA-15 Ab-7 antibody diluted 1/1000 in phosphate-buffered saline (PBS) buffer (without Ca $^{2+}$ or Mg $^{2+}$, pH 7.4; Sigma) was incubated 24 h at 4°C, with rotation, with 1.5 μ g of GST-PEA-15 or GST adsorbed on glutathione-Sepharose beads.

Immunohistochemistry

Tissue sections were first deparaffinized before being incubated in 0.3% H $_2$ O $_2$ in methanol for 5 min. Tissue sections were then washed twice in PBS before being immunostained for 1 h at room temperature with the indicated antibodies diluted in PBS containing 0.3% Triton X-100. Immunohistochemical detection was achieved using the avidin-biotin complex immunoperoxidase technique and the diaminobenzidine chromogen (Vector Laboratories, Burlingame, CA). Hematoxylin and eosin (Mayer hematoxylin; Merck, Darmstadt, Germany) staining was then performed to visualize cells nuclei and bodies, respectively.

Immunocytochemistry and Confocal Laser Scanning Microscopy

PEA-15 immunocytochemistry was performed as described previously (Sharif *et al.*, 2004). Actin staining was performed using phalloidin coupled to Alexa 488 (Invitrogen). Nuclei were stained with TO-PRO-3 iodide (Invitrogen) according to manufacturer's specifications. Cells were examined using a Leica TS2 (Leica, Wetzlar, Germany) confocal microscope with appropriate filters.

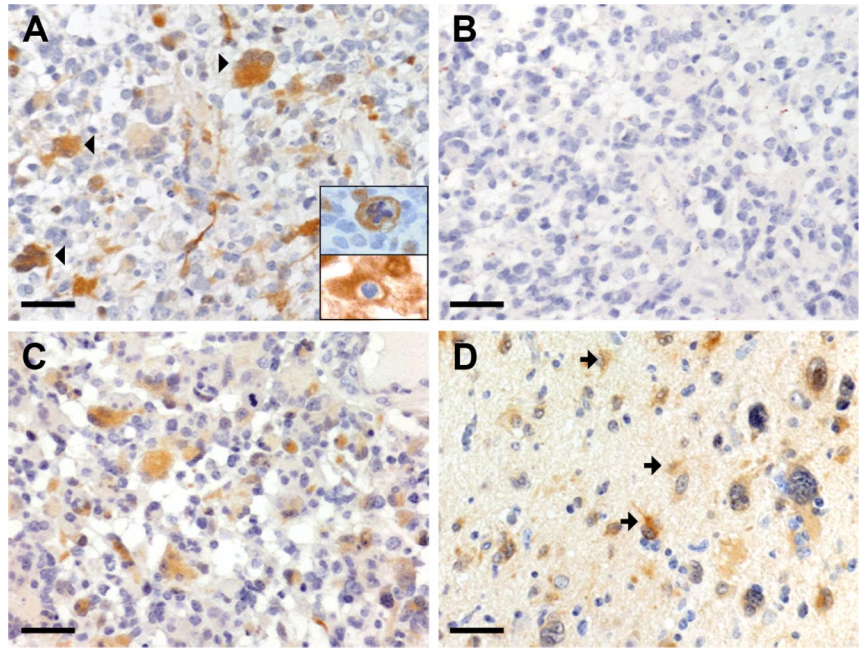
Wound Scratch Assay

Astrocytes were replated between day 11 and 13 *in vitro* on polyornithine-coated 12-well culture dishes (Falcon; BD Biosciences Discovery Labware, Bedford, MA). The next day, a scratch was done in the confluent monolayer by using a sterile pipette tip. Then, cells were washed three times with PBS buffer (without Ca $^{2+}$ or Mg $^{2+}$, pH 7.4; Sigma) and replaced in MEM-F-12 medium supplemented with 10% fetal calf serum or 50 ng/ml TGF α for the indicated times. Pictures of marked fields were taken using an inverted phase contrast microscope (Nikon Diaphot) at different time intervals. By using Lucia software (Laboratory Imaging, Hostiva, Czech Republic), areas of fields that were not yet colonized by cells were measured.

Astrocyte Adhesion Assay

Immulon-2 96-well plates were coated overnight (4°C) with 10 μ g/ml human fibronectin. Uncoated control wells were blocked with 2% heat-inactivated bovine serum albumin (BSA) (Sigma) for 4 h (4°C). Wild-type and knockout astrocytes were subsequently added (2×10^5 cells/well) in serum-free media and incubated for 60 min (37°C). Wells were washed twice with PBS before being fixed for 20 min with 2% glutaraldehyde (Electron Microscopy Service Laboratories, Westmont, NJ). The fixed cells were washed once with PBS and then stained with 0.1% crystal violet (Serva Biochemicals, Hauppauge, NY) for 45 min. Adherent astrocytes were washed three times with PBS, solubilized in

Figure 1. PEA-15-immunohistochemical staining in human glioblastomas (WHO grade IV). PEA-15-immunoreactivity is indicated in brown. Hematoxylin and eosin counterstaining was used to label the cell cytoplasm (pink) and nucleus (blue). (A) PEA-15-immunoreactive tumor cells are observed within the tumor mass (arrowheads). Top inset, PEA-15-immunopositive cell (brown) that exhibits the typical appearance of malignancy. Note the nuclear anomalies. Bottom inset, PEA-15-immunopositive cell (brown) that exhibits a positive nuclear immunolabeling for MIB (blue), without any counterstaining. (B) Immunohistochemical staining of a section adjacent to A with the PEA-15 antibody preincubated with GST-PEA-15. Note the total lack of immunoreactive signal demonstrating the specificity of the Ab-7 antibody in human. (C) Immunohistochemical staining of a section adjacent to A and C with the PEA-15 antibody preincubated with GST alone. Note the maintenance of the immunoreactive signal. (D) Reactive astrocytes (arrows) exhibiting PEA-15 immunolabeling of their cytoplasm in the peritumoral infiltration zone. Bar, 100 μ m.



0.1 N sodium citrate (Sigma) for 30 min, and absorbance at 595 nm was determined using an ELISA plate reader (Molecular Devices, Sunnyvale, CA). To determine 100% of cells for quantitation, a set of wells was fixed and stained without being washed. Data represent experiments done in triplicate.

Transwell Assay

The underside of the polycarbonate membranes of transwells (6.5 mm in diameter, 10 μ m in thickness, 8- μ m pores; Corning Life Sciences, Acton, MA) were coated with 10 μ g/ml human fibronectin for 3 h at 37°C. Primary astrocytes were trypsinized at day 13 in vitro, stained with trypan blue, and counted using a hemocytometer. Cells (2×10^4) were plated onto the uncoated topside of the filter in MEM-F-12 medium in presence of drugs for 45 min (for 2 h with leptomycin B), before adding 50 ng/ml TGF α . The inferior well contained same concentrations of drugs and TGF α . After 12 h of migration at the incubator (37°C, 5% CO $_2$), cells were fixed 20 min at room temperature with paraformaldehyde (4% in PBS, pH 7.5). The topside of the transwell was washed, and each filter was swabbed with a cotton-tipped applicator to remove cells that did not migrate through the filter. Cells were stained using Hoechst dye (sanofi-aventis, Bridgewater, NJ) according to manufacturer's specifications. Filters were cut out and mounted on slides in Fluoromount-G medium (Southern Biotech, United Kingdom, obtained through Cliniscience, Maubeuge, France). By using Lucia software (Laboratory Imaging) connected to an epifluorescence microscope, 15 fields per membrane were counted. For PMA-induced PKC down-regulation experiments, astrocytes were treated 48 h by 1 μ M PMA before migration assay.

Purification of Protein Transducer Domain (PTD)-Fusion Proteins

PTD-PEA-15 and PTD-GFP constructs have been described previously (Caron *et al.*, 2001; Embury *et al.*, 2001). The isolation and purification were done as described previously (Embury *et al.*, 2001). Titration of proteins obtained was performed comparing increasing volumes of PTD-fusion protein with standards of BSA loaded on a 15% polyacrylamide gel and then stained with Coomassie blue. Astrocytes were treated with 4 μ M PTD-fusion proteins for 2 h before trypsinization and during the duration of migration.

Immunoblotting

After treatment, cells were rinsed twice with PBS and then scraped on ice in lysis buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% NP-40, 2 mM EDTA, 2 mM EGTA, and 2 mM sodium orthovanadate, supplemented with phosphatase inhibitor cocktails 1 and 2 (Sigma) and Mini Complete protease inhibitor cocktail without EDTA (Roche Diagnostics) following manufacturer's specifications. Cell lysates were passed thrice through a 26-gauge needle and then centrifuged at 13,000 \times g during 20 min at 4°C. After protein titration by microBCA protein assay (Pierce Chemical, Rockford, IL), supernatants were mixed with sample buffer (Laemli), boiled, and then separated on 10% polyacrylamide gels (8 and 13% for blotting PKC μ and PEA-15, respectively). After transfer to a polyvinylidene difluoride membrane (Immo-

bilon-P; Millipore, Billerica, MA) blots were probed with indicated primary antibodies before visualizing with horseradish peroxidase-conjugated secondary antibodies followed by development with an enhanced ECL kit (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom).

RESULTS

Cells Migrating Away from Tumor Explants Maintained in Organotypic Cultures Express Low Levels of PEA-15

Histochemical staining of human glioblastomas (World Health Organization [WHO] grade IV) was performed using a specific antibody against PEA-15 that we recently characterized (Sharif *et al.*, 2004). In the seven glioblastoma biopsies examined, a strong PEA-15-immunoreactive signal was observed in tumor cells of the tumor solid core (Figure 1A). However, in agreement with the well-known heterogeneity of glioblastomas, we also observed some areas of the tumor core that were not PEA-15 positive. The tumor cells were identified by a histopathologist with regard to their morphological characteristics and their positive MIB immunolabeling, the latter identifying proliferating cells (Figure 1A, inset). Specificity of this labeling was confirmed through the preabsorption of the PEA-15 antibody with a GST-PEA-15 fusion protein that suppressed any immunohistochemical staining (Figure 1B), whereas preabsorption with GST alone did not extinguish the signal (Figure 1C). In the parenchyma surrounding the tumor solid core, which corresponds to the infiltrative component of the tumor, both tumor cells and reactive astrocytes were positive for PEA-15 (Figure 1D). Whereas in reactive astrocytes the immunohistochemical staining was always localized in the cytoplasm, some tumoral cells exhibited in addition a nuclear localization of the signal. Because in these tumors only a few tumor cells exhibited a positive MIB immunolabeling, it remained difficult to identify every malignant cell inside the tumor infiltrative component, thus preventing the *in vivo* study of PEA-15 expression in tumor cells.

Further immunohistochemical analysis was thus performed on human glioma explants grown at the top of surgical sponges from 10 to 30 d. This model confirmed that

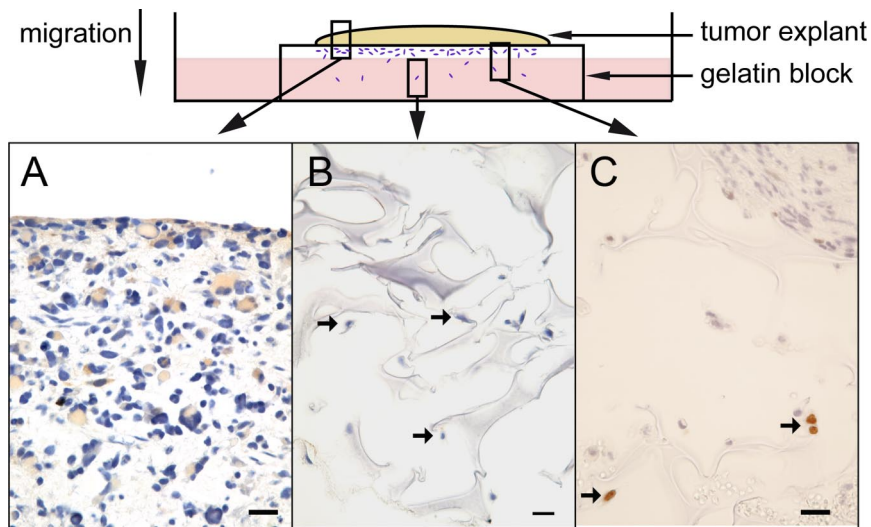


Figure 2. Migrating tumor cells express low levels of PEA-15, regardless of the expression levels in the originating tumors. Localization of A, B, and C is indicated by boxes in the schematic description of the experimental model. (A) Example of PEA15-immunoreactive tumoral cells (brown) in explants of glioblastomas maintained in organotypic culture for 30 d. (B) Lack of PEA-15 immunoreactivity in cells migrating away (arrows) from the explant depicted in A. (C) Tumoral nature of cells (arrows) migrating from glioblastoma explant, demonstrated according to their MIB immunolabeling (brown). Cell bodies are colored by eosin in pink, and nuclei by hematoxylin in blue. Bar, 100 μ m.

initial explants were largely composed of tumor cells. Moreover, such organotypic cultures have the advantage that they preserve the histological integrity of the tumor for several weeks (Rubinstein *et al.*, 1973), whereas they allow monitoring of the robust invasive capacities of the tumor cells that characterize high-grade gliomas (Sorour *et al.*, 1975). Accordingly, tumor cells, identified by their MIB-positive immunolabeling (Figure 2C), migrated away from the explant and infiltrated the depths of the sponges in 11 of the 12 samples studied. Macrophages, distinguished from tumor cells by their very different morphology (round and voluminous cytoplasm) as well as their CD68-immunohistochemical staining also infiltrated the depths of the sponges, as observed in tumors *in vivo* (our unpublished data). The level of the PEA-15-immunoreactive signal in the glioma explants varied from high to low, but it was always detectable (Figure 2A). Regardless of the intensity of the PEA-15-immunoreactive signal in the explant, all cells migrating away exhibited weak or no PEA-15 immunoreactivity (Figure 2B). These observations led us to test whether PEA-15 expression can alter cell motility.

The Lack of PEA-15 Expression Enhances Migration of Normal Astrocytes

Astrocytes constitute one of the cell types from which gliomas arise and are known to express high levels of PEA-15 protein (Araujo *et al.*, 1993). We thus compared motility of astrocytes grown in primary culture from either PEA-15 knockout or wild-type mice.

Cellular motility was first analyzed at the edge of a scratch wound made across an astrocyte monolayer in the presence of fetal calf serum (FCS). As expected, wounding induced migration of the remaining cell sheet into the gap (Figure 3A). Wild-type astrocytes extended lamellipodial protrusions in the direction of migration (Figure 3B, left). Wounding induced much more drastic morphological changes in PEA-15^{-/-} astrocytes, which emitted long oriented protrusions (Figure 3B, right). PEA-15^{-/-} astrocytes recolonized the wound faster than their wild-type counterparts (Figures 3A, right, and C) ($p < 0.05$ at 32 h and $p < 0.001$ at 48 h).

Because the difference between the migratory capabilities of wild-type and PEA-15^{-/-} astrocytes may result from changes in adhesion properties, adhesion of wild-type and PEA-15^{-/-} astrocytes on various concentrations of fibronectin was analyzed revealing no difference (Figure 3D).

Because some discrepancies concerning the effect of other proteins on migration, e.g., p27 (Assoian, 2004), were likely related to the experimental procedures used, we further confirmed the PEA-15 effect on astrocyte migration, by using another migration assay, the transwell assay. In the transwell assay, in agreement with the results obtained using the wound scratch assay, in the presence of 10% FCS PEA-15^{-/-} astrocytes presented a significant 1.6 ± 0.58 -fold enhanced migration compared with wild-type astrocytes (Figure 4A). On a second type of stimulation, in the presence of 50 ng/ml TGF α , which has been previously described as a strong stimulator of astrocyte motility (Faber-Elman *et al.*, 1996), PEA-15^{-/-} astrocytes migrated 1.9 ± 0.65 -fold more than their wild-type counterparts (Figure 4A). Considering the more robust effect of PEA-15 in the presence of TGF α , further studies were done using TGF α as a migration inducer. Whereas the wound assay mimics a directed migration of cells still contacting their neighboring cells, the transwell assay, which requires trypsinization of cells, measures haptokinesis toward fibronectin of independent cells. The influence of PEA-15 on cell migration thus seems to be relatively independent of the extracellular context. The transwell assay was used for further studies because this migration assay reveals a difference of migratory capability between both types of astrocytes in less time (12 h) compared with the wound scratch assay.

Migration is a highly integrated cellular process composed of multiple coordinated events involving a great number of molecules (Lauffenburger and Horwitz, 1996). It was therefore necessary to rule out the possibility that the enhanced migration of PEA-15^{-/-} astrocytes was related to an epiphenomenon, rather than to a direct effect of PEA-15. We thus analyzed PEA-15^{-/-} astrocyte motility by using the transwell assay after reintroducing PEA-15. The poor efficiency of cDNA transfection for astrocytes in primary cultures led us to restore PEA-15 expression by using a fusion protein composed of the PTD and PEA-15 (PTD-PEA-15) (Embury *et al.*, 2001). The 11 amino acids of the PTD sequence allow the transduction into living cells of fused proteins dissolved in aqueous solution (Schwarze *et al.*, 1999). Neither the fusion protein control PTD-GFP, at any concentration used, nor a low dose of 0.4 μ M PTD-PEA-15 had a significant effect on astrocyte motility. Treatment by 4 μ M PTD-PEA-15, although having no significant effect on wild-type astrocyte migration, led to a major reduction of

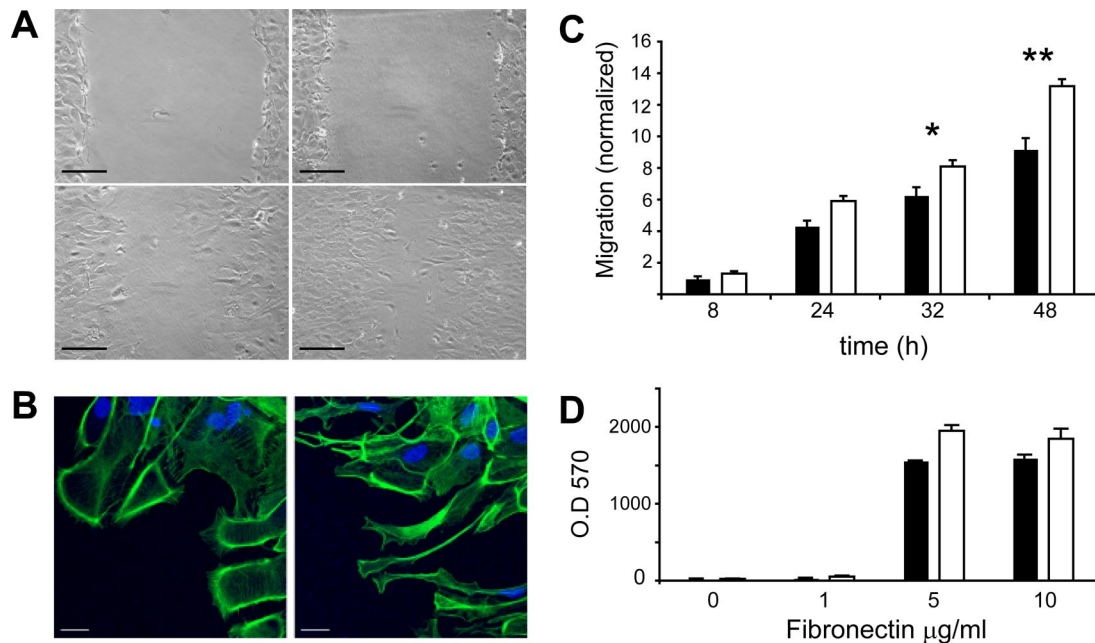
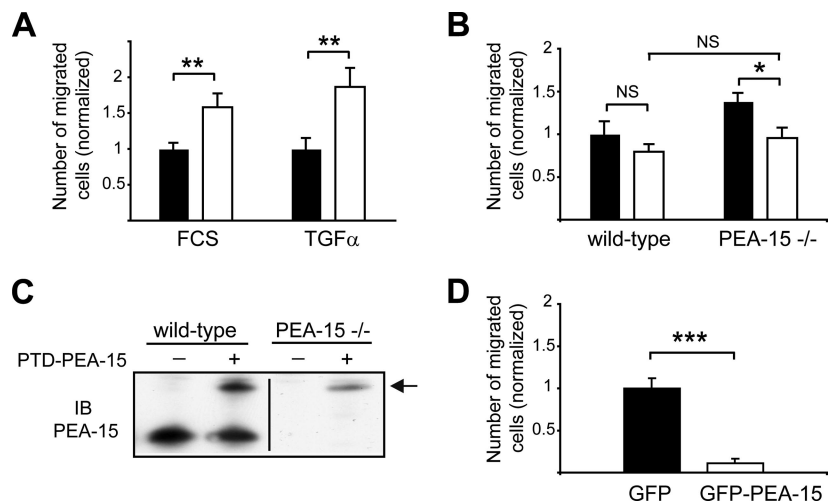


Figure 3. Accelerated wound healing in astrocyte monolayers lacking PEA-15 expression. (A) Confluent monolayers of wild-type (left column) or PEA-15^{-/-} astrocytes (right column) were scratched. The recolonization of wounded areas was studied using an inverted phase-contrast microscope from $t = 0$ (top) to 48 h (bottom). Images shown are representative of three independent experiments. Bars, 100 μm . (B) Actin staining (green) using phalloidin reveals that wounding induces lamellipodia extension in wild-type astrocytes (left), whereas the morphology of PEA-15^{-/-} astrocytes is distinct, with cells emitting long protrusions (right picture). Nuclei were stained (blue) with TO-PRO3 iodide. Bars, 20 μm . (C) Quantitation of mean distances moved by migrating cell leading edge at different intervals of time (black bars, wild-type astrocytes; white bars, PEA-15^{-/-} astrocytes). Normalization is based on the value of wild-type astrocyte migration at $t = 8$ h. Data shown are mean + SD of one experiment representative of three independent experiments ($n = 10$, * $p < 0.05$, ** $p < 0.01$). Two-way analysis of variance (ANOVA) followed by Bonferroni posttest). (D) Wild-type (black bars) and PEA-15^{-/-} astrocytes (white bars) adhesion on various concentrations of fibronectin is similar.

the extent of the migration of PEA-15^{-/-} astrocytes (Figure 4B). Western blotting experiments confirmed the efficiency of the transduction by PTD-PEA-15 (Figure 4C). It is noteworthy that although the theoretical weight of PTD-PEA-15 is 16 kDa, the apparent molecular weight on a polyacrylamide gel of the protein is 26 kDa, probably due to the presence of numerous charged amino acids in the PTD

sequence. Together these data demonstrate that expression of PEA-15 inhibits astrocyte motility. In addition, transient transfection of NIH-3T3 cells by GFP-PEA-15 cDNA drastically reduced migration on transwell assay, whereas transfection by GFP alone had no effect (Figure 4D), thus indicating that the control exerted by PEA-15 on motility is not restricted to astrocytes.

Figure 4. PEA-15 expression controls cell migration in a transwell assay. (A) PEA-15^{-/-} astrocytes (white bars) show an enhanced migration in comparison with wild-type astrocytes (black bars) in the transwell assay in the presence of either 10% FCS or 50 ng/ml human recombinant TGF α . (B) Wild-type or PEA-15^{-/-} astrocytes were treated with 4 μM PTD-PEA-15 (white bars) or PTD-GFP (black bars) assay. Note that PTD-PEA-15 treatment reduces significantly the migration of PEA-15^{-/-} astrocytes, whereas having a minor effect on wild-type astrocytes. (C) Transduction by PTD-PEA-15 led to PEA-15 reexpression in wild-type and PEA-15^{-/-} astrocytes. Note that the PTD-PEA-15 fusion protein (arrow) has an enhanced apparent molecular weight in comparison with endogenous PEA-15. The line on the image indicates where an irrelevant section has been deleted. (D) Transient transfection by GFP-PEA-15 drastically decreases migration of NIH-3T3 cells demonstrating that the control exerted by PEA-15 on cell migration is not restricted to astrocytes. Cell migration assays are done in the presence of 50 ng/ml TGF α , in triplicate and data shown are mean \pm SEM of three independent experiments ($n = 9$; NS, not statistically significant; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, two-way ANOVA analysis and Bonferroni posttest).



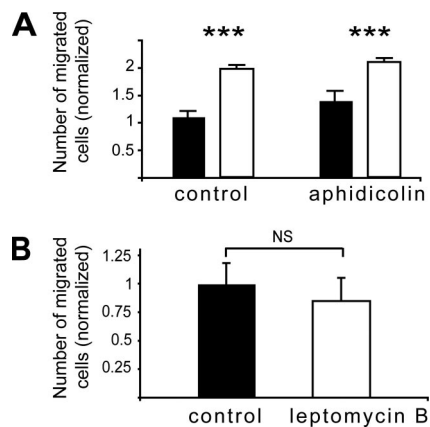


Figure 5. The control of astrocyte migration by PEA-15 is independent of its control of proliferation. (A) Migration of wild-type (black bars) and PEA-15^{-/-} astrocytes (white bars) in the presence of an antimetabolic, 10 μ g/ml aphidicolin, does not alter the enhanced migration of PEA-15^{-/-} astrocytes. (B) The effect of PEA-15 on migration is independent of the nuclear export machinery. The inhibition of the nuclear export protein CRM1, by 10 ng/ml leptomycin B, does not modify the migration of wild-type astrocytes. Cell migration assays are done in the presence of 50 ng/ml TGF α , in triplicates and data shown are mean + SEM of three independent experiments (n = 9; NS, not statistically significant; ***p < 0.001, two-way ANOVA analysis and Bonferroni posttest).

The Antiproliferative and Antimigratory Functions of PEA-15 Are Distinct

PEA-15 inhibits astrocyte proliferation (Formstecher *et al.*, 2001). The increased number of migrating PEA-15^{-/-} astrocytes could thus be a consequence of increased cell division. To investigate this hypothesis, migration assays were done in the presence of 10 μ g/ml aphidicholin, a mitotic inhibitor reported to have no effect on the motility of astrocytes stimulated by basic fibroblast growth factor (Milner *et al.*, 1999). 5-Bromo-2-deoxyuridine incorporation experiments confirmed that aphidicholin completely inhibited both wild-type and PEA-15^{-/-} astrocyte proliferation for at least 48 h (our unpublished data), a period of time longer than the duration of the transwell experiments (12 h). Inhibition of proliferation by aphidicholin did not alter the migration of either wild-type or PEA-15^{-/-} astrocytes (Figure 5A). This result demonstrates that the enhanced migration of PEA-15^{-/-} astrocytes is not a consequence of their increased proliferation.

The Effect of PEA-15 on Migration Is Independent of the Nuclear Export Machinery

PEA-15 blocks ERK-dependent proliferation by binding ERK1/2 and preventing their prolonged localization in the nucleus (Formstecher *et al.*, 2001). PEA-15 contains a nuclear export sequence required for its capacity to dynamically localize ERK in the cytoplasm (Formstecher *et al.*, 2001). As reported previously, treatment of wild-type astrocytes with 10 ng/ml leptomycin B, a potent inhibitor of the nuclear export machinery protein CRM1 (Nishi *et al.*, 1994; Ossareh-Nazari *et al.*, 1997) led to the relocalization of PEA-15 in the nucleus, as observed by confocal microscopy (our unpublished data). In the presence of leptomycin B, the motility of wild-type astrocytes was not modified (Figure 5B). It thus seems that the mechanisms whereby PEA-15 controls cell migration differ from those involved in its control of cell proliferation.

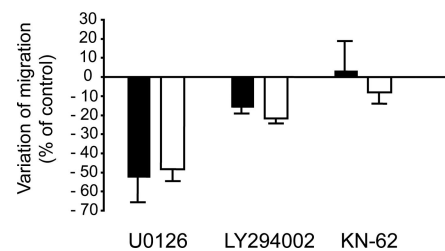


Figure 6. ERK, PI3K, and CaMKII activities are not required for PEA-15 inhibition of astrocyte migration. Inhibition of ERK/MAPK pathway (10 μ M U0126), or PI3K pathway (30 μ M LY294002) or CaMKII (10 μ M KN-62) have similar effects on wild-type (black bars) and PEA-15^{-/-} astrocytes (white bars), indicating that these pathways are not implicated in the effect of PEA-15 on astrocyte migration. Histograms represent variations relatives to respective controls. Cell migration assays are done in the presence of 50 ng/ml TGF α , in triplicates and data shown are mean + SEM of three independent experiments.

ERK/Mitogen-activated Protein Kinase (MAPK), Phosphatidylinositol 3-Kinase/Akt, and CaMKII Are Not Required for the PEA-15 Effect on Migration

Our cell motility assays use FCS or TGF α as inducers of motility. Both FCS and TGF α are known to activate MAPK/ERK and PI3K/Akt pathways. We therefore tested whether these pathways are involved in the effect of PEA-15 on cell migration. The presence of 10 μ M U0126, a specific inhibitor of mitogen-activated protein kinase kinase (Favata *et al.*, 1998) during migration experiments reduced similarly by ~50% the migration extent of both cell types (Figure 6) (52 ± 13.5 and $48 \pm 6.0\%$ for wild-type and PEA-15^{-/-} astrocytes, respectively). Therefore, the migration of astrocytes in presence of TGF α depends on ERK/MAPK activity, but this signaling pathway is not involved in the specific effect of PEA-15. PI3K inhibition by 30 μ M LY294002 decreased the migration extent of wild-type astrocytes by $15.4 \pm 3.6\%$ and did the same for PEA-15^{-/-} astrocytes ($-21.7\% \pm 2.5\%$) (Figure 6), demonstrating that Akt activity is also required for full astrocyte motility but that it does not interfere with the specific effect of PEA-15 on cell migration. Next, we tested the CaMKII, which can regulate cell migration (Pauly *et al.*, 1995). The inhibition of CaMKII by 10 μ M KN-62 did not significantly modify the migration of either wild-type or PEA-15^{-/-} astrocytes. (Figure 6), suggesting that CaMKII is not involved in astrocyte motility.

A Member of the Novel PKC Family Mediates PEA-15 Inhibition of Astrocyte Motility

Members of the PKC family are known to modulate cell migration (Gomez *et al.*, 1999). We thus investigated their role in the effect of PEA-15 on astrocyte migration. In the presence of 5 μ M bisindolylmaleimide (BIM), a broad-spectrum inhibitor of PKC, the migration of PEA-15^{-/-} astrocytes was reduced by $38.3 \pm 11.9\%$, matching the extent of migration of wild-type astrocytes, whereas the migration of these latter was not significantly affected (Figure 7A). This result implicates a PKC in the enhanced migration observed for PEA-15^{-/-} astrocytes. The isoforms of PKC are classified in three families based on their activation requirements. Long-term treatment (48 h) with 1 μ M PMA is known to desensitize PKCs belonging to the classical and novel PKC families, through down-regulation of PKC protein levels (Blackshear, 1988). PMA treatment inhibited dramatically the migration of PEA-15^{-/-} astrocytes by $45 \pm 4.9\%$ (Figure 7B), allowing PEA-15^{-/-} astrocytes to return to the

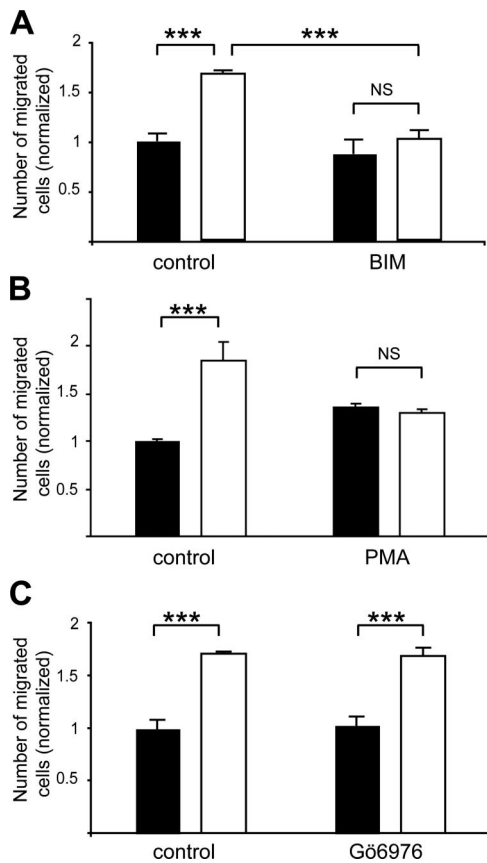


Figure 7. The control exerted by PEA-15 on astrocyte migration is mediated by an isoform of PKC belonging to the novel PKC family (black bars, wild-type; white bars, PEA-15^{-/-}). (A) Broad-spectrum inhibition of PKCs by 5 μ M BIM inhibits the enhanced migration of PEA-15^{-/-} astrocytes, whereas not affecting the migration of wild-type astrocytes. (B) Down-regulation of classical and novel PKCs by long-term exposure to 1 μ M PMA equalizes the migration of wild-type and PEA-15^{-/-} astrocytes. (C) Isoforms of the classical PKC family are not implicated in the effect of PEA-15 on migration because inhibition of these isoforms by 100 nM Gö6976 does not affect the migration of either PEA-15^{-/-} or wild-type astrocytes. Cell migration assays are done in the presence of 50 ng/ml TGF α , in triplicates, and data shown are mean \pm SEM of three independent experiments ($n = 9$; NS, not statistically significant; ** $p < 0.01$, *** $p < 0.001$, two-way ANOVA analysis and Bonferroni posttest).

same migration rate as their wild-type counterparts. This result indicates that the PKC isoform implicated belongs to either the classical or the novel PKC family. Treatment of astrocytes with 100 nM Gö6976, previously shown to inhibit selectively the family of classical PKCs (Martiny-Baron *et al.*, 1993), did not alter the migration of either PEA-15^{-/-} or wild-type cells (Figure 7C), therefore excluding the participation of these classical PKCs and thereby demonstrating that PEA-15 inhibition of astrocyte motility is mediated by a PKC belonging to the novel PKC family.

A 40-kDa Form of PKC δ Mediates PEA-15 Inhibition of Astrocyte Migration

The expression of nPKC isoforms was therefore investigated in wild-type and PEA-15^{-/-} astrocytes (Figure 8). Because cleavage of nPKC by various proteases can generate catalytic fragments of PKC around 40 kDa located in their C-

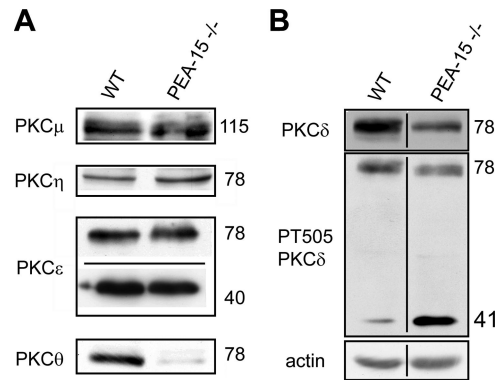


Figure 8. Expression patterns of novel PKCs display two major differences between wild-type and PEA-15^{-/-} astrocytes. (A) Contrary to PKC μ , η , and ϵ that display similar expression, PKC θ is strongly down-regulated in PEA-15^{-/-} astrocytes. The line on the image of PKC ϵ Western blot indicates where an irrelevant section has been deleted. (B) Whereas the holoenzyme PKC δ is weakly down-regulated in PEA-15^{-/-} astrocytes, please note the presence in PEA-15^{-/-} astrocytes of a 40-kDa PKC δ exhibiting a strong phosphorylation on its threonine 505. The line on the image indicates where an irrelevant section has been deleted. Eighty micrograms of astrocyte lysates was loaded onto polyacrylamide gels and immunoblotted with indicated antibodies. Shown are representative blots of at least three repeats.

terminal half (Schaap *et al.*, 1990; Emoto *et al.*, 1995; Datta *et al.*, 1997; Endo *et al.*, 2000), we therefore explored nPKC expression by using antibodies recognizing epitopes located in the C-terminal part of the enzymes. All nPKC isoforms were expressed in wild-type astrocytes. PKC ϵ , PKC μ , and PKC η were similarly expressed in wild-type and PEA-15^{-/-} astrocytes (Figure 8A). Western blot analysis revealed a weak down-regulation of endogenous 78-kDa PKC δ in PEA-15^{-/-} astrocytes in comparison with their wild-type counterparts (ratio knockout/wild type = $67 \pm 8\%$, $n = 3$; Figure 8B). In addition, we observed a strong down-regulation of PKC θ in PEA-15^{-/-} astrocytes that was confirmed using a second antibody recognizing an epitope located in the N-terminal domain of the enzyme (our unpublished data).

In both types of astrocytes, we noted the presence of two forms of PKC ϵ , one isoform corresponding to the holoenzyme, and the other isoform corresponding to the low-molecular-weight C-terminal fragment. Regarding PKC δ , use of the antibody directed against the PKC δ phospho-threonine 505, located in the catalytic moiety, revealed the presence only in PEA-15^{-/-} astrocytes of a 40-kDa form of PKC δ .

PKC δ and PKC θ have both been reported to control cell migration (Tang *et al.*, 1997, 1999; Kruger and Reddy, 2003; Li *et al.*, 2003; Iwabu *et al.*, 2004); therefore, we determined their implication in the control exerted by PEA-15 on astrocyte migration.

We first performed dose-response experiments in presence of rottlerin, initially described as a specific inhibitor of PKC δ (Gschwendt *et al.*, 1994) that was further shown to inhibit PKC θ (Villalba *et al.*, 1999). Very low doses of rottlerin, 0.1 and 0.3 μ M, had a significant inhibitory effect only on the migration of PEA-15^{-/-} astrocytes, and they abolished the difference between wild-type and PEA-15^{-/-} astrocyte motilities (Figure 9A). The down-regulation of PKC θ in PEA-15^{-/-} astrocytes implies that the enhanced motility of PEA-15^{-/-} astrocytes relies on an increased enzymatic activity associated to PKC δ . This was further confirmed by coapplication of 300 nM bryostatin-1, known to specifically protect PKC δ during PMA-induced down-

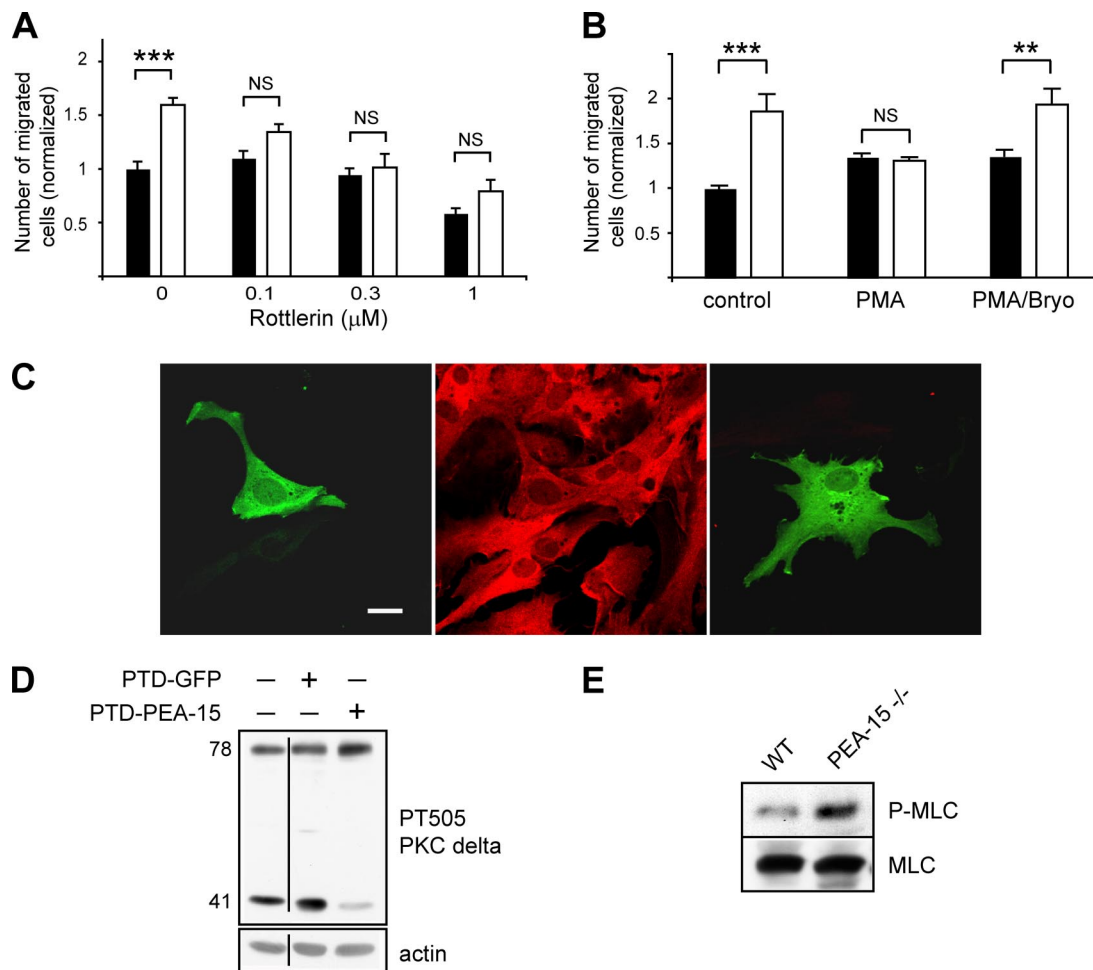


Figure 9. Evidence for an involvement of PKC δ in the inhibitory control exerted by PEA-15 on astrocyte motility. (A) Low doses of rottlerin decrease significantly the enhanced migration of PEA-15^{-/-} astrocytes (white bars), whereas not affecting significantly the motility of wild-type astrocytes (black bars), thus abrogating the difference in migration between PEA-15^{-/-} astrocytes and wild-type astrocytes. (B) Although PMA-induced down-regulation of novel and classical PKC isozymes equalizes the migration of wild-type and PEA-15^{-/-} astrocytes, note that specific protection of PKC δ down-regulation by coapplication of PMA and bryostatin-1 restores the enhanced migration of PEA-15^{-/-} astrocytes but did not affect their wild-type counterparts. Cell migration assays are done in the presence of 50 ng/ml TGF α , in triplicates, and data shown are mean \pm SEM of three independent experiments ($n = 9$; NS, not statistically significant; ** $p < 0.01$, *** $p < 0.001$, two-way ANOVA and Bonferroni posttest). (C) The localization of GFP-PKC δ in astrocytes is not affected by the expression of PEA-15. GFP-PKC δ is localized mainly in the cytoplasm of wild-type (left) and PEA-15^{-/-} astrocytes (right), similarly to PEA-15 in wild-type astrocytes (middle). Bar, 15 μm . (D) Reexpression of PEA-15 in PEA-15^{-/-} astrocytes by transduction of PTD-PEA-15 strongly down-regulates the 40-kDa form of PKC δ . The line on the image indicates where an irrelevant section has been deleted. (E) Phosphorylation of myosin light chain is strongly enhanced in PEA-15^{-/-} astrocytes. Eighty micrograms of astrocyte lysates was loaded onto polyacrylamide gels and immunoblotted with indicated antibodies. Shown are representative blots of at least three repeats.

regulation, contrary to all other PMA-sensitive PKC isoforms that are down-regulated (Szallasi *et al.*, 1994a, b; Lu *et al.*, 1997). In this condition, PEA-15^{-/-} astrocytes conserved their enhanced migration (Figure 9B). Together, these results indicate that PEA-15 inhibits astrocyte migration by inhibiting a PKC δ -dependent stimulatory pathway.

Because biological activity of a kinase results also from its intracellular localization, we verified the localization of PKC δ in wild-type and PEA-15^{-/-} astrocytes. Probably because of low expression levels of PKC δ in astrocytes, we were unable to detect endogenous PKC δ by immunocytochemistry. We thus transfected GFP-PKC δ into wild-type and PEA-15^{-/-} astrocytes and confocal analysis revealed a mainly cytoplasmic localization in both cases (Figure 9C). In addition, GFP-PKC δ did not colocalize with the cytoskeletal proteins actin and tubulin or with the focal adhesion protein vinculin (our unpublished data).

To more precisely test the involvement of the 40-kDa form of PKC δ observed in PEA-15^{-/-} astrocytes in their enhanced migration, we examined the effect of PTD-PEA-15 transduction of PEA-15^{-/-} astrocytes. Whereas treatment with PTD-GFP had no effect, reexpression of PEA-15 led to the total disappearance of the 40-kDa form of PKC δ in PEA-15^{-/-} astrocytes (Figure 9D).

Phosphorylation of the threonine 505 located in the activation loop is considered as a reliable marker of PKC δ activation (Stempka *et al.*, 1999; Rybin *et al.*, 2003). Lack of a specific immunoprecipitating antibody against the 40-kDa isoform prevented a direct kinase assay to precisely quantify the enzymatic activity of the 40-kDa-PKC δ in PEA-15^{-/-} astrocytes lysates. We next tried to quantify the total PKC δ enzymatic activity in astrocyte lysates to demonstrate a global increase of the enzymatic activity of PKC δ in PEA-15^{-/-} astrocytes. Unfortunately, we were unable to immu-

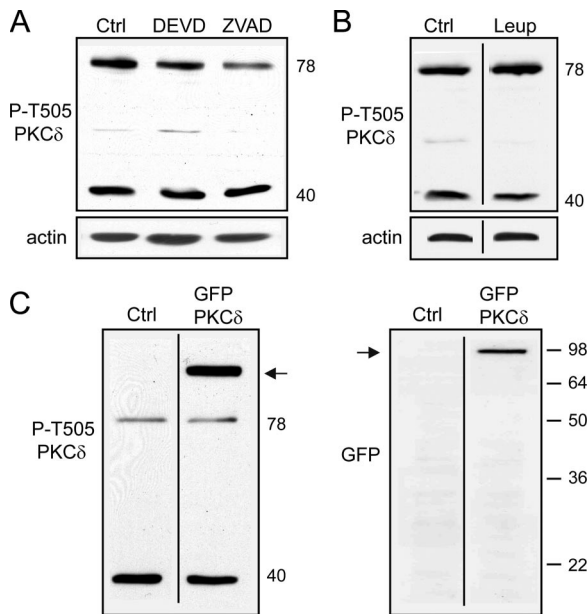


Figure 10. The 40-kDa form of PKC δ expressed in PEA-15 $^{-/-}$ astrocytes does not result from a proteolytic cleavage. (A) Specific inhibition of caspase 3 by 50 μ M DEVD, or pan-caspase inhibition by 50 μ M ZVAD, does not change the expression level of the 40-kDa form of PKC δ in PEA-15 $^{-/-}$ astrocytes. (B) Broad-spectrum inhibition of serine and thiol proteases by 100 μ M leupeptin had no effect on the expression level of the 40-kDa form of PKC δ in PEA-15 $^{-/-}$ astrocytes. (C) Forty-eight hours after transfection by cDNA encoding GFP-PKC δ , wherein GFP is fused to the amino-terminal part of the PKC δ , PEA-15 $^{-/-}$ astrocytes were lysed, and evidence for cleavage of the fusion protein was determined. Blotting using either an antibody recognizing an epitope located in the carboxy-terminal part of PKC δ (left blot), or an anti-GFP (right blot), did not evidence any short fragments of GFP-PKC δ (arrows). Eighty micrograms of astrocyte lysates was loaded onto polyacrylamide gels and immunoblotted with indicated antibodies. Shown are representative blots of at least three repeats. The lines in B and C indicate where irrelevant sections have been deleted.

nonprecipitate the protein from astrocyte lysates, thus preventing the determination of this enzymatic activity. This was probably due to the low endogenous expression level of PKC δ in astrocytes, since we were able to immunoprecipitate PKC δ from lysates of Chinese hamster ovary cells overexpressing PKC δ (our unpublished data), wherein the catalytic fragment of PKC δ is not present.

We thus searched for indirect evidence of modified PKC δ activity in PEA-15 $^{-/-}$ cells. PKC δ has been shown to mediate a signaling cascade during epidermal growth factor (EGF)-induced cell migration, leading to the phosphorylation of MLC on its serine 19 (Iwabu *et al.*, 2004). Accordingly, we observed by Western blotting an enhanced phosphorylation of the MLC on its serine 19 in PEA-15 $^{-/-}$ astrocytes in comparison with their wild-type counterparts (Figure 9E).

Together, these results strongly suggest that the presence of the 40-kDa form of PKC δ in PEA-15 $^{-/-}$ astrocytes is responsible for the enhanced migration of these cells.

A 41-kDa fragment of PKC δ (catalytic fragment [CF]-PKC δ) exhibiting a constitutive enzymatic activity has been reported after induction of apoptosis (Emoto *et al.*, 1995; Ghayur *et al.*, 1996; Steinberg, 2004). CF-PKC δ results from caspase 3-dependent cleavage of the holoenzyme and is proapoptotic. We transfected wild-type and PEA-15 $^{-/-}$ astrocytes with a cDNA encoding this CF-PKC δ fused to

GFP. Both types of astrocytes died after apoptosis (our unpublished data). Contrary to CF-PKC δ , the 40-kDa PKC δ observed is constitutively expressed in PEA-15 $^{-/-}$ astrocytes, and no sign of apoptosis was observed, suggesting they represent two different forms of the kinase.

We envisaged that the 40-kDa PKC δ expressed in PEA-15 $^{-/-}$ astrocytes resulted from a cleavage by a caspase. PEA-15 $^{-/-}$ cells were treated by a caspase 3 inhibitor, DEVD (50 μ M), or a pan-caspase inhibitor, ZVAD (50 μ M), during 12 h, a time sufficient for the disappearance of this fragment upon treatment by PTD-PEA-15 (Figure 9D). Both specific caspase 3 and broad-spectrum caspase inhibition did not modify the expression level of the 40-kDa PKC δ in PEA-15 $^{-/-}$ astrocytes (Figure 10A), whereas the strong decrease of the 40-kDa fragment of PKC ϵ observed in both types of astrocytes was used as an internal control of inhibitors efficiency (our unpublished data). Furthermore, broad-spectrum inhibition of serine and thiol proteases by 100 μ M leupeptin had no effect on the expression level of the 40-kDa PKC δ in PEA-15 $^{-/-}$ astrocytes (Figure 10B). These results strongly suggested an absence of cleavage of PKC δ in PEA-15 $^{-/-}$ astrocytes. To further test this hypothesis, we transfected a cDNA encoding GFP-PKC δ , wherein the GFP is fused to the N-terminal extremity of PKC δ . No cleaved form of GFP-PKC δ could be detected up to 72 h posttransfection in PEA-15 $^{-/-}$ astrocytes (Figure 10C).

Altogether, these results strongly suggest that the 40-kDa fragment of PKC δ observed in PEA-15 $^{-/-}$ astrocytes does not result from a proteolytic cleavage.

DISCUSSION

Here, we describe a new role for PEA-15 as an inhibitor of astrocyte migration, one cell type thought to originate gliomas, highly invasive tumors. Reexpression of PEA-15 by using PTD-PEA-15 fusion protein restores the migration of PEA-15 $^{-/-}$ astrocytes to wild-type levels, demonstrating that the loss of PEA-15 is indeed responsible for the increased motility of PEA-15 $^{-/-}$ astrocytes. Moreover, transfecting NIH-3T3 fibroblasts with GFP-PEA-15, we demonstrated that the control exerted by PEA-15 on cell migration is not restricted to astrocytes.

Cell migration involves two sets of factors. Intracellular factors determine intrinsic cell properties, adhesion, and cell migration, whereas external factors, including extracellular matrix (ECM) and cell-cell interaction, control tissue permissivity to cell migration. A rich field of research revealed the complexity of brain ECM components as well as some of their remodeling during development or pathologies. For example, fibronectin deposited in the ECM of brain tumors strongly promotes the migration of glioma cells (Ohnishi *et al.*, 1998). Interestingly, PEA-15 has been shown to block H-Ras-initiated inhibition of integrin activation (Ramos *et al.*, 1998). PEA-15 binding to ERK is required for this modulation (Chou *et al.*, 2003). Increased integrin activity is known to inhibit migration and would be consistent with PEA-15 inhibition of cell migration. However, comparing wild-type and PEA-15 $^{-/-}$ astrocytes we have not seen any difference in integrin activation (Ramos, unpublished data), a result in agreement with the observation that both types of astrocytes have similar adhesion on fibronectin. Further studies should reveal if PEA-15 expression modulates cell migration differently according to the matrix composition.

Most of PKC isozymes have been implicated in cell migration. Western blotting experiments as well as pharmacological manipulations on PKC isozymes allowed us to demonstrate that PEA-15 inhibits astrocyte migration by a

PKC δ -dependent mechanism. PKC δ has been particularly implicated in cell migration (Kruger and Reddy, 2003; Li *et al.*, 2003; Iwabu *et al.*, 2004). PKC δ is indeed responsible for a major part of the EGF-induced fibroblast contractile force generation, because RNA interference-mediated PKC δ depletion prevents MLC phosphorylation, which in turn promotes motility (Iwabu *et al.*, 2004). Increased PKC δ has also been correlated with enhanced metastatic potential in mammary tumor cells (Kiley *et al.*, 1999).

The expression of a 40-kDa form of PKC δ (40-kDa-PKC δ) in PEA-15 $-/-$ astrocytes seems to account for their enhanced migration. Indeed, reexpression of PEA-15 in PEA-15 $-/-$ astrocytes upon transduction with PTD-PEA-15 simultaneously decreased astrocyte migration and expression of this 40-kDa-PKC δ . The strong phosphorylation of threonine 505 of 40-kDa-PKC δ in PEA-15 $-/-$ astrocytes suggests a constitutively active form, because this phosphorylation has been reported as a reliable marker of PKC δ activation (Stempka *et al.*, 1999; Rybin *et al.*, 2003). The enhanced phosphorylation of myosin light chain in PEA-15 $-/-$ astrocytes is in agreement with an increased PKC δ activity. However, the biological effect of this 40-kDa-PKC δ may also result from phosphorylation of specific substrates that are not affected by the holoenzyme.

Contrary to the proapoptotic CF-PKC δ previously observed upon apoptosis induction (Steinberg, 2004), the 40-kDa-PKC δ is constitutively expressed in PEA-15 $-/-$ astrocytes. Although PEA-15 $-/-$ astrocytes exhibit an increased sensitivity to various inducers of apoptosis (Kitsberg *et al.*, 1999; Renault *et al.*, 2003), no sign of apoptosis was observed in the cells in any of the culture conditions used in this study. Furthermore, apoptosis was induced after transfection of PEA-15 $-/-$ astrocytes with GFP-CF-PKC δ , indicating that they did not develop a resistance to apoptosis and further supporting that the 40-kDa-PKC δ expressed in these cells differs from CF-PKC δ . Accordingly, whereas the CF-PKC δ results from the proteolytic cleavage of the holoenzyme by caspase 3 (Emoto *et al.*, 1995; Ghayur *et al.*, 1996), the lack of effect of the inhibition of various proteases as well as the absence of cleavage of transfected GFP-PKC δ in PEA-15 $-/-$ astrocytes suggest that the 40-kDa-PKC δ does not result from proteolytic cleavage. The size and the enzymatic activity of the 40-kDa-PKC δ indicate that it is not encoded by the two alternative splicing variants of PKC δ that have already been described in mammals (Ueyama *et al.*, 2000; Sakurai *et al.*, 2001). The molecular mechanisms linking PEA-15 expression and 40-kDa-PKC δ generation remain to be elucidated.

In L6 skeletal muscle cells, PEA-15 action on glucose transport is mediated by an enhanced activation of the classical PKC α that in turn inhibits PKC ζ (Condorelli *et al.*, 2001). In astrocytes migration, the inefficiency of Gö6976, the inhibitor of classical PKC, to reverse the difference between wild-type and PEA-15 $-/-$ astrocyte motilities, rules out the involvement of PKC α . It is noteworthy that, contrary to the desensitization of PKC induced by PMA, which is actually a down-regulation, the inhibition of PKC activity by BIM had no significant effect on wild-type astrocyte migration. This suggests that down-regulation of PKC α by PMA abrogates its inhibition of PKC ζ , this latter isoform being known to stimulate astrocyte polarization (Etienne-Manneville and Hall, 2001, 2003) and cell migration (Crean *et al.*, 2004; Petit *et al.*, 2005). Contrary to PMA, BIM should inhibit both PKC α and PKC ζ , although less efficiently for this latter, thus possibly explaining the different effects exerted by PMA and BIM on wild-type astrocyte migration. Whether PEA-15 expression modulates PKC signaling, i.e., PKC δ or PKC α ,

through a common molecular mechanism or whether it is specific of each isoform needs further investigation.

It remains also to be determined whether phosphorylation of PEA-15 can modulate its control of cell migration. The observation that CaMKII or PI3K/Akt inhibition does not interfere with PEA-15 effect on astrocyte migration suggests that phosphorylation of PEA-15 on its serine 116 is not involved. Because PEA-15 contains a serine substrate of PKC (serine 104) (Kubes *et al.*, 1998), a cross-regulation between PKC and PEA-15 may tune astrocytes motility.

In consideration of the different known cellular functions of PEA-15 in different chronic disorders, including cancer (Eramo *et al.*, 2005; Stassi *et al.*, 2005) and diabetes (Condorelli *et al.*, 1998; Condorelli *et al.*, 2001; Vigliotta *et al.*, 2004), the novel observation that PEA-15 inhibits cellular motility may have a very significant impact on future investigation of key areas such as metastasis and diabetes complications.

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